DEVELOPMENT OF TECHNIQUES FOR THE ISOLATION OF A GRANULOVIRUS FROM POTATO TUBER MOTH, *PHTHORIMAEA OPERCULELLA* (ZELLER)

Submitted in fulfilment of the requirements for the degree of

MASTER OF SCIENCE

of

RHODES UNIVERSITY

by

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December 2010

ABSTRACT

Phthorimaea operculella, commonly known as the Potato Tuber Moth, is an economically important agricultural pest worldwide. The baculovirus, *Phthorimaea operculella* granulovirus (PhoGV) has been considered as a means of control alternative to chemical control because of its host specificity and harmless impact on other organisms and ecosystems. An isolate of PhoGV obtained from a South African PTM population would be beneficial in the production of a biopesticide, which is not yet available. An efficient and cost-effective rearing method would be advantageous for potential commercial production.

Commercial table and seed potato plantations and storage facilities located in Patensie, Bathurst, Howick and Ivanhoe were surveyed for PTM infestations. Patensie was the only site where milky discoloured larvae were found, a potential symptom of PhoGV infection. TEM analysis revealed no virus in these samples. Since no virus was found in the field-collected samples, PTM insects were collected to initiate rearing in the laboratory.

PTM was raised by three different methods in the laboratory. A cost/benefit analysis, survival rate, fertility and sex ratio were recorded for each rearing method. Rearing method one was deemed unsuccessful for efficient commercial rearing, as survival percentage and fertility were low. Rearing methods two and three had high survival rates and high fertility, and were efficient and less labour intensive than rearing method one. Rearing method three was the most productive technique, but for commercial production rearing method two was considered the most manageable and efficient. The sex ratio was 1:1 for all three cultures. The cost analysis revealed that rearing methods two and three were less expensive than rearing method one because less labour was required to monitor insects. The success of rearing PTM for 19 months will enable these cultures to be up-scaled to a large production facility for mass rearing.

Virus was not found in the field surveys or in laboratory cultures, therefore chemical, temperature, humidity and carbon dioxide stressors were used in an attempt to initiate a baculoviral infection. Symptoms were exhibited in larvae subjected to chemical, temperature and humidity treatments, but these were confirmed by TEM analysis not to be a result of PhoGV infection.

The success of rearing PTM in the laboratory suggests that the method could be used in the commercial rearing of the insects in a large mass-rearing facility. The data obtained from induction protocols have allowed for better understanding for future induction for PhoGV and other

baculoviruses in other insect species. The failure to isolate a South African PhoGV strain for developing a biopesticide against PTM has motivated further studies in obtaining a baculovirus in order for South Africa to develop a commercial product against this pest.

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as shown.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 INTRODUCTION

Phthorimaea operculella (Zeller) (Gelechiidae), commonly known as the Potato Tuber Moth (PTM), is an important insect pest of potatoes (*Solanum tuberosum* L.) as it causes severe economic losses in potato production worldwide, predominantly in countries with warm temperate to tropical climates (Radcliffe, 1982; Fenemore, 1988; Vickers *et al.*, 1991; Hamilton, 2003). The larvae of this pest mine the plant foliage and tuber, both in the field and in storage, thereby increasing the difficulty of controlling infestations (Vickers *et al.*, 1991). Several different management strategies including insecticides, cultural and biological control have been attempted to control outbreaks within this crop (Hanafi, 1999). The aim of the investigation is to identify and isolate a granulovirus in South Africa for the potential biological control of PTM in South Africa.

1.2 THE HOST PLANT: *SOLANUM TUBEROSUM* L

1.2.1 Description

Potatoes are an annual crop belonging to the Solanaceae, first cultivated in South America in the Andes near Lake Titicaca near the Peru and Bolivia border (Horton, 1987; Visser, 2009). The plant foliage can grow to a height of approximately 60cm, with white flowers and yellow stamens (Figure 1.1 A, B and D). The potato is a modified swollen underground stem, commonly known as a tuber (Winch, 2006) (Figure 1.1 C). Potatoes need to be grown in fairly cool climates but not in regions where frost might occur or in high rainfall regions (Visser, 2009).

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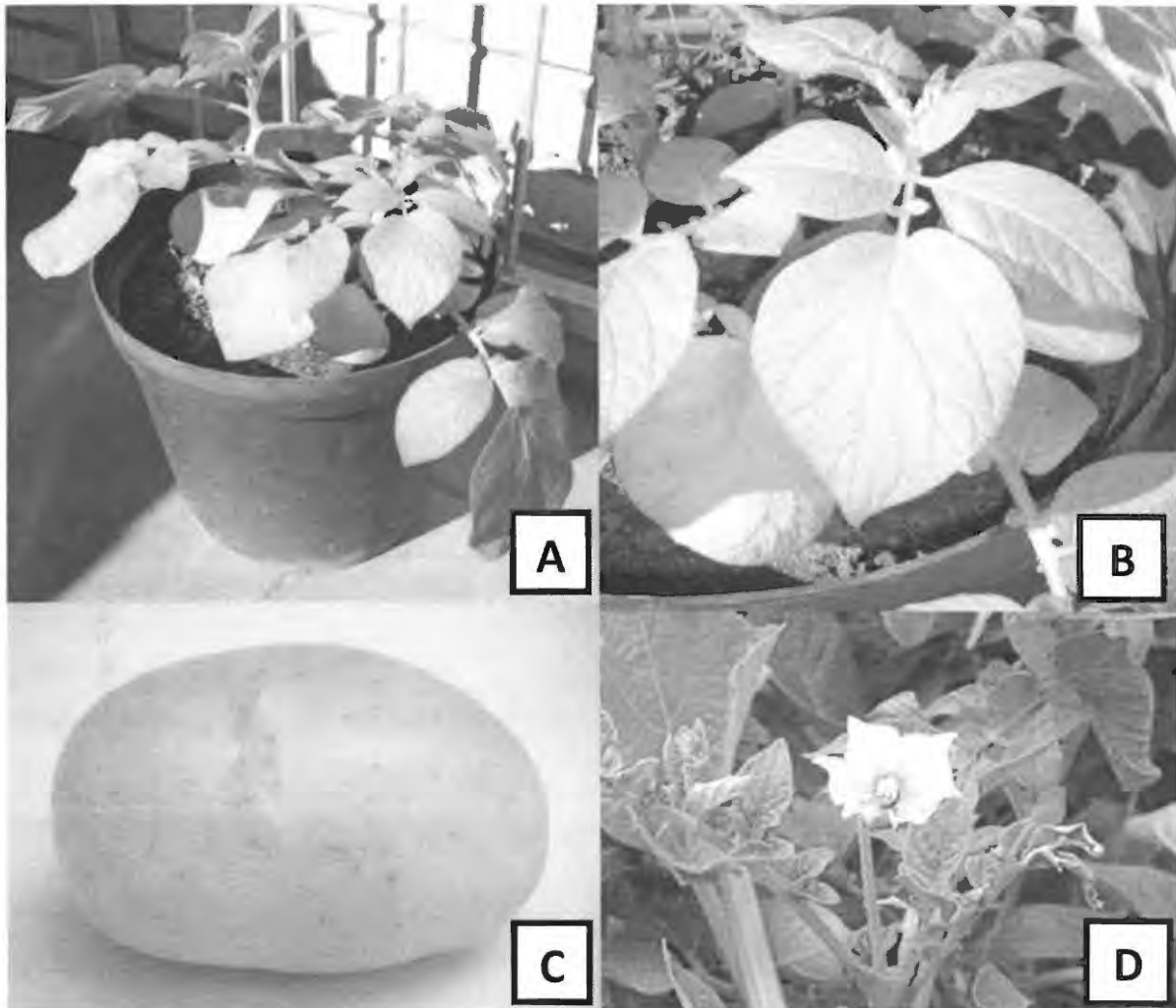
Figure 1.1: *Solanum tuberosum* L leaves (A and B), single tuber (C) and white flowers (D).

1.2.2 Potato as a crop worldwide

Potatoes are considered the fourth most important crop globally after rice, wheat and maize (Ross, 1986). Potato production occurs in 149 out of the 227 countries worldwide (Figure 1.2). The global production of potatoes as a food crop differs between developed and developing countries in terms of production. From 1950 until 1999, there was a steady decline of approximately 92 000 hectares per year of potatoes in developed countries, while developing countries have had increases of 30 million tonnes per year over the same period (Hijmans, 2001). This trend is because potatoes are a cheap, low maintenance and easy crop to grow, with high nutritional value. Therefore it is a good food source for developing nations (Wade, 2008). More than half the world's potatoes are produced in Europe and just over a third are produced in Asia (Hijmans, 2001).

The distribution of potato growing areas is also determined by latitude. Only 6.9% of potatoes are grown annually in the southern hemisphere, and only 4 out of the 26 countries in the southern

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hemisphere have potato producing areas exceeding 100 000 hectares. Between latitudes 22°N and 59°N, 90% of the world's potatoes are grown, mainly in Europe and Asia. Potato production near the equator is limited, as they do not grow well at high temperatures or high humidities. If grown in the tropics, the crop is found in the cool highlands. In the subtropics of both hemispheres, potatoes are grown best in the cool season (winter, autumn or spring). The global potato distribution pattern can also be related to human demographics. In the northern hemisphere, specifically the European and Asian regions, there are high population densities and potatoes are an easy and cheap food to produce for a staple diet to satisfy hunger needs (Hijmans, 2001).

1.2.3 Potato as a crop in South Africa

Potatoes were brought to South Africa from the Netherlands in order to provide food for the mariners visiting the Cape in the 18th century. Since then the potato industry in South Africa has developed so successfully that it is one of the country's most important food crops. The potato industry currently accounts for 43% of the gross value of the vegetables produced in the country, and makes up 4% of the total agriculture production in South Africa. A total of 1700 farmers contribute to the production of potatoes, of which 400 are seed potato farmers. The total value of harvested potatoes is R1.6 billion per annum. The potato industry has also resulted in job opportunities for 66 600 workers. Globally South Africa is ranked 31st in potato production and supplies 0.5% of the world's total (Department of Agriculture, 2003). There is no African country that produces more than 100 000 hectares of potatoes. Egypt is the largest producer with 82 561 hectares of potato land, with South Africa being the second largest producer with 61 000 hectares (Hijmans, 2001).

Potatoes are grown throughout the year in South Africa, and croplands are found in all nine provinces, with the exception of large areas of the Northern Cape where climatic conditions are too dry. Most potato crops are planted in the cooler seasons, winter, autumn and spring. The months of December and November are mostly avoided due to the high temperatures (Department of Agriculture, 2003). Roughly 80% of potato crops in South Africa are irrigated and produce an average yield of 40 tonnes per hectare, but when considering all plantations, irrigated or not, the average yield is 30 tonnes per hectare (Visser, 2004). The temperate regions with reliable summer rainfall (Mpumalanga highlands and Eastern Free State) are able to plant in spring or early summer (Department of Agriculture, 2003).

Of the 157.8 million bags of potatoes produced annually in South Africa, 7% is exported (10.3 million bags), 13% is seed potatoes (21.1 million bags) and the remaining 80% is for consumption. Even though South Africa exports predominantly to four countries (Zimbabwe, Angola, Mozambique and Mauritius) there is a need for an increased production as more African countries are requesting potatoes. Out of the 80% of potato production consumed in South Africa, 65% of the table potatoes are sold through domestic fresh produce markets, while the remaining 35% is sold directly from the farmer to the consumer (Department of Agriculture, 2003). Seed potatoes need to be certified disease and virus free when planted, but only 70% of plantations abide by this regulation (Steyn, 1999).

1.2.4 Potato Pests in South Africa

In South Africa many potato pests have been identified, representing various insect and other invertebrate families (Table 1.1). The status of these pests has been established in South Africa, however they may differ depending on the circumstances and various environmental conditions, such as the areas in which the potatoes are grown and climatic conditions (Visser, 2009).

Table 1.1: Potato pests found in South Africa (Visser, 2009)

Order	Common Name	Species Names	Type of Pest	Pest Status
Coleoptera	Black maize beetle	*Heteronychus arator*	K	Low to Medium
	Plant-eating lady beetle	*Epilachna canina* (Fabricius) *Epilachna dregei* Mulsant *Epliachna paykullii* Mulsant *Henosepilachna hirta* (Thunberg)	C	Low to Medium
	Spotted maize beetle	*Astylus astomaculatus*	F	Low
	Blister beetle	*Epicauta velata* (Gerstaecker)	Oc/ F	Low to Medium
	Potato weevil	*Sciobius horni*	Oc	High when present in large numbers in fields at planting time
	White grubs	*Anomala* cf. *transvaalensis* Arrow *Hypopholis sommeri* Burmeister *Temnorhynchus retusus* (Fabricius)	K	Low for most white grubs, except it is high for the large wattle chafer
	Other leaf beetles	*Monolepta cruciata*	Oc	Low
	Other weevils and snout beetles	*Listroderes costirostris* *Naupactus leucoloma* *Protostrophus amplicollis*	Oc	Low to Medium
Diptera	*Liromyza* and other leaf miners	*Liriomyza huidobrensis* (Blanchard) *Liriomyza trifolii* (Burgess)	K	Medium to High
Hemiptera	Aphids	*Macrosiphum euphorbiae* (Thomas) *Aulacorthum solani* (Kaltenbach) *Rhopalosiphoninus latysiphon* (Davidson) *Smynthurodes betae* Westwood *Aphis nasturtii* Kaltenbach *Myzus ornatus* Laing.	C	Low to Medium
	False chinch bugs	*Nysius ericae*	Oc	Low
	Green vegetable bug	*Nezara viridula* (Linnaeus)	Oc	Low to Medium
	Mealybugs	*Ferrisia malvastra* (McDaniel)	Oc	Low
	Milkweed bugs	*Haemobaphus concinnus* (Dallas) *Spilostehus revularis* (Germar)	Oc	Low
	Tip wilter	*Anoplocnemis curvipes* (Fabricius) *Elasmopoda valga* (Linnaeus)	Oc	Low to Medium

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	African bollworm	*Helicoverpa armigera* (Hubner)	C	Medium
	Cutworm	*Agrotis biconicus* *Mentaxya albifrons* *Mentaxya ignicollis*	Oc	Low Medium
	Death's Hawk moth	*Acherontia atropos*	Oc	Low
Lepidoptera	Lesser armyworm	*Spodoptera exigua*	C	Low to Medium
	Potato Tuber Moth	*Phthorimaea operculella*	K	High
	Semi – loopers	*Thysanopulsia orichalcea* *Chrysodeixis acuta* *Ctenopulsia limirena* *Cornutiplusia circumflexa*	C	Low to Medium
	Tomato moth	*Spodoptera littoralis*	Oc	Low
Nematode	Nematodes	*Meloidogyne* spp. * *Pratylenchus* spp. * *Globodera rostochiensis*	K	Medium to High
Orthoptera	Mole cricket	*Gryllotalpa africana*	Oc	Low to Medium
Thysanoptera	Thrips	*Frankliniella shultzei*	C	Low to High
Other pests	Millipedes	*Chaleponcus* spp.* *Doratogonus* spp. * *Ommatoiulus* spp. * *Oxidus* spp. * *Spinotarsus* spp. * *Spirostrptus* spp. * *Zinophora* spp. *	Oc	Low to Medium
	Mites	*Aculops lycopersici* *Polyphagotrasonemus latus* *Tetranychus evansi* *Rhizoglyphus* spp. *	C	Low to High

* =   Confusion exists as to which species exactly cause vegetable damage, the species are not mentioned
K =   Key Pest (A well established, frequent and important pest of the crop. Serious yield losses may be expected under optimum conditions)
C =   Common Pest (Does not commonly result in serious crop damage)
F =   Flower Pest (The pest consumes pollen or flowers from the potato plant)
Oc =   Occasional Pest (Only occasionally attacks the crop)

1.3 THE HOST INSECT: *PHTHORIMAEA OPERCULELLA*

1.3.1 Taxonomy

Potato Tuber Moth, *Phthorimaea operculella* (Zeller, 1873) (Gelechiidae), commonly abbreviated PTM, is an exotic pest which originated from South America, and has been present in South Africa for over 100 years. PTM first established in South Africa in 1895 in Cape Town, possibly as a result of importing potatoes from South America, but it was only later that it was recognised as a pest (Visser, 2007). In other countries, such as United States of America and China, PTM is not only a potato pest, it is also an important pest of tobacco. It is also referred to as a tobacco split worm (Lawrence, 2009).

1.3.2 Distribution

The Potato Tuber Moth originated in tropical mountainous regions of South and Central America and was first recorded as a pest of potatoes in 1917 by Graf (Sporleder *et al.*, 2008b). The species is distributed along equatorial and tropical climatic belts worldwide and has been reported in most potato production zones within these climatic areas (Kroschel and Sporleder, 2006; Chumakov and Kuzentsova, 2009). PTM occurs in over 90 countries and all continents except Antarctica (Chumakov and Kuzentsova, 2009) (Table 1.2). In potato producing countries there is considerable yield and crop loss, especially in developing countries, due to PTM infestation (Radcliffe, 1982; Sporleder *et al.*, 2008b).

Table 1.2: Countries that have recorded Potato Tuber Moth as a pest (Chumakov and Kuzentsova, 2009)

Continent	Countries with PTM infestation
Europe	Italy, Spain, Portugal, Greece, Albania, Bulgaria, Yugoslavia, Ukraine, Moldova, Georgia and France
North and South American	United States, Mexico, Brazil, Peru, and Argentina
Asia	Japan, Vietnam, Korea, China, India, Pakistan, Iran, Iraq, Afghanistan, Syria, Turkey, Cyprus and southern regions of Russia
Africa	Algeria, Kenya, Congo, Morocco, Sierra Leone and South Africa
Australia	Australia, New Zealand and surrounding Islands of Oceania

The life cycle of PTM is limited by the 10°C annual isotherm in both the northern and southern hemispheres (Sporleder *et al.*, 2008b). PTM has the ability to adapt to a wide range of climatic conditions in different agro-ecologies, which is a possible result of its origins in the Eastern Andes where there is an extreme daily and seasonal fluctuation in temperature (Trivedi *et al.*, 1994; Sporleder *et al.*, 2008b). The host tolerates a specific temperature range, which indirectly influences the pest distribution (Sporleder *et al.*, 2008b).

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A predicative model of PTM distribution has been designed, based on extensive studies in various regions of the world in both the field and laboratory (Fenemore, 1977; Briese, 1980; Broodryk and Pretorius, 1974; Chi, 1988; Chi and Getz, 1988; Kroschel and Koch, 1994, Trivedi *et al.*, 1994; Keller, 2003). PTM's rate of development is directly related to temperature which influences the moths ability to populate a region. Predication can therefore be made about potential establishment, which is ecologically and economically important for pest establishment and pest management (Sporleder *et al.*, 2004). All the countries that have reported PTM infestations (Figure 1.2) correspond to the predictive model to some degree.

Figure 1.2: Potato Tuber Moth distribution worldwide (Dots indicate the countries in which PTM has been reported) (Adapted from Rondon, 2010 and Internet Resource 1).

In South Africa, PTM infestations have increased and have resulted in this insect being considered the most important potato pest in South Africa, as it can cause up to 30% yield loss annually, equating to R40 million loss per annum (Maredia *et al.*, 2003). Therefore monitoring its distribution and population growth is of economic importance to farmers in South Africa (Visser, 2007). PTM has established in both commercial and subsistence potato crops in nine provinces in South Africa (Broodryk, 1971; Visser, 2007). Therefore PTM exists wherever potatoes are grown in South Africa and it is able to survive various climatic conditions (Broodryk, 1971). Potatoes grown in dry climates in the warmer seasons are impacted most by infestation due to the high rate of PTM reproduction (Sporleder *et al.*, 2008b).

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1.3.3 Biology and life history

The PTM as an adult is brownish-grey with dark markings on the forewing. The length of the moth is approximately 10mm, with a wing span of 12 - 16mm (Figure 1.3). Adult moths can be observed hiding in foliage or on the ground during the day. They become active at night when the insect flies around and females lay their eggs (Hamilton, 2003).

The adult moth lays oval, pearly-white eggs on the foliage or on the soil under plants, either singularly or in a group. The eggs change to a yellowish colour as they develop until they turn black just before hatching (Figure 1.3 A). When conditions are optimal, the eggs hatch within 4 to 5 days. The neonate larvae (less than 1mm in length) emerge and actively search for a food source (leaves, stem or tubers of potato plant) (Figure 1.3 B). Once the larva mines into the plant, it remains there for two weeks while it feeds and undergoes four larval stages of development (Hamilton, 2003). The larvae emerge from their feeding site as fifth instars, and move to the ground to spin a silk cocoon to begin pupation (Figure 1.3 C). The larvae secrete silk from the silk glands situated in the mouth parts. Small sand particles and debris are incorporated into the cocoon, in order to camouflage it, but a complete silk cocoon can be spun when no sand or debris is present (Visser, 2005).

The larvae undergo metamorphosis from pre-pupae to pupae inside the cocoon over 2 days, and within 7 days the moths eclose (Hamilton, 2003) (Figure 1.3 D). The moths take 2 - 3 days to mate and lay eggs (Figure 1.3 A). The adults survive for approximately one week but, if supplied with water, can live for several more days (Visser, 2005) (Figure 1.3 E). Duration of development from egg to adult life stage in the field can take from 4 weeks in warm temperatures to several months in cooler temperatures (Hamilton, 2003). All life stages of PTM are described in Table 1.3.
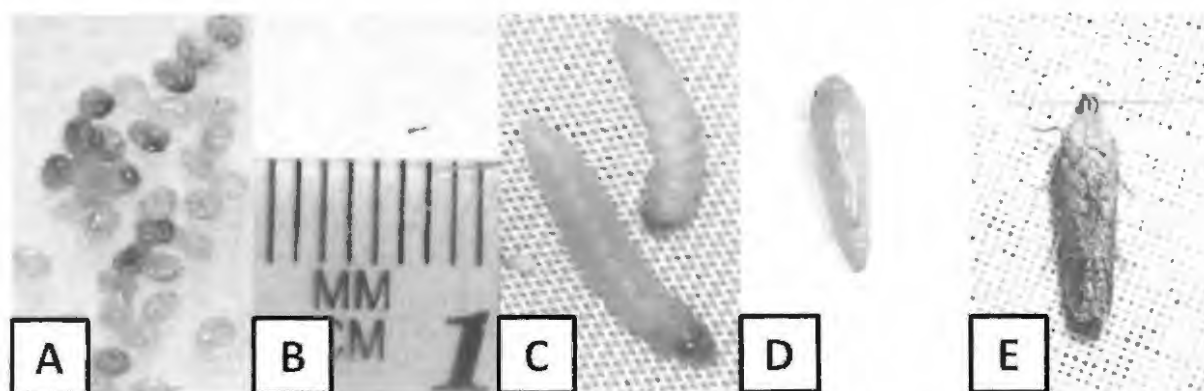
Figure 1.3: Potato Tuber Moth eggs, laid on a filter disc (Left). Once the neonates emerge from the eggs they undergo four stages (Centre) and then become pupae which eclose into adults (Right).

Table 1.3: Description and period of development for each development stage of PTM (Sinha, 1997).

Stage of Development	Period of development	Description
Egg	5 - 7 Days	• Creamy white which changes yellow and finally becomes black just before hatching • 1mm in diameter
1st Instar		• Grey/ yellowish white with dark brown head • 1-2mm in length
2nd Instar - 4th Instar	15 - 25 Days	• Creamy white or pinkish with a black head (Tubers) • Green with a black head (foliage) • Grows from 2mm-20mm in length
Pupae	5 - 10 Days	• Greyish silken cocoons covered with soil particles and debris for camouflage • Pupae light brown in colour and become dark brown as they develop • 10mm in length
Adult Moth	5 - 8 Days	• Brownish grey moth • 12-16mm with tiny dark scattered marks on the forewings • Lays eggs after 2 days • 1-2mm in length

1.3.4 Economic importance

1.3.4.1 Host range

Potatoes are the major solanaceous crop with the most impact of infestation from Potato Tuber Moth worldwide. However, PTM is not restricted to potato plants; PTM also attacks other solanaceous crops such as tobacco, tomatoes, eggplant, cape gooseberry and certain broad leaf weeds (Das and Raman, 1994; Visser, 2005). A total of 52 other solanaceous plant species exist, which are both cultivated and wild and are alternative hosts for PTM. In addition two species from Scrophulariaceae and one species of each from Boraginaceae, Rosaceae, Typhaceae, Compositae, Amaranthaceae and Chenopodiaceae have also been found to be alternative hosts for PTM (Das and Raman, 1994). Three alternative hosts for PTM have been identified in South Africa. Two of the alternative hosts belong to the solanaceous plant family (*S. incanum* L [Bitter Apple] and *S. sisymbriifolium* Lam. [Wild Tomato]) and one species of the Compositae family (*Xanthium strumarium* L. [Cocklebur]) (Broodryk, 1971; Das and Raman, 1994).

1.3.4.2 Pest status on potatoes

PTM infestation in potato tubers is important because the larval stages are able to pass through sorting, packaging and shipping without being detected. This can result in rejection of the exported product after several weeks. So it is extremely important to ensure that PTM infestations are

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controlled before the harvesting of the tubers. Samples of PTM infestation are illustrated in Figure 1.4.
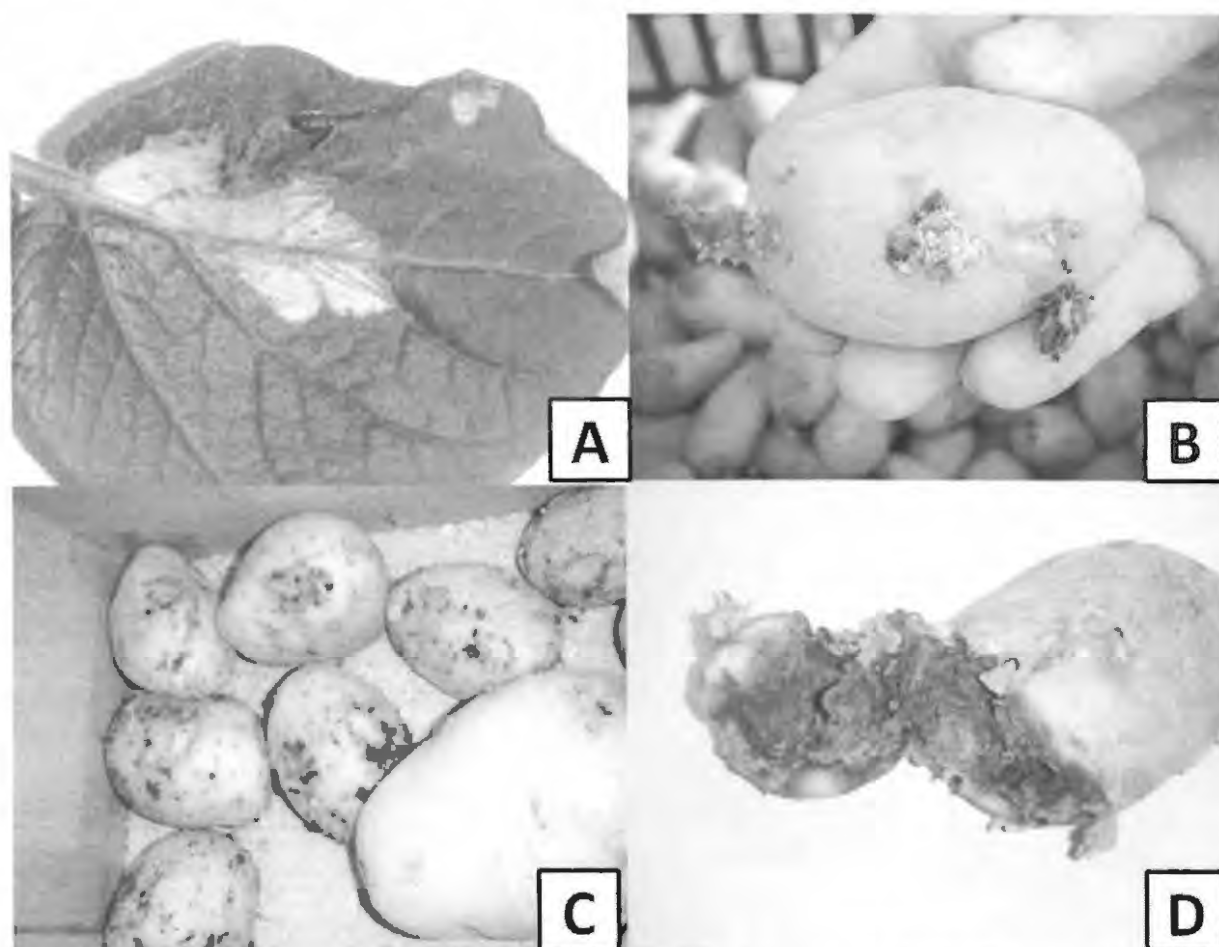
Figure 1.4: Potato Tuber Moth infestation causes 'windows' to occur in the leaf tissue of the potato foliage (A). Evidence of PTM frass on the outer surface of the potato tubers (B and C). The potato quality is spoiled, as tunnels are burrowed through the tuber by larval stages of PTM (D).

1.4   THE CONTROL OF *PHTHORIMAEA OPERCULELLA*

There are several pre- and post- harvest strategies in place to attempt to minimise the impacts of PTM on crops. Pre-harvest measures include traps to monitor PTM activity, application of several microorganisms (*Bacillus thuringiensis* Berliner and Granulovirus) to foliage and soil and cultural techniques. In the field, chemical, cultural and biological strategies are important to limit the PTM infestation. The most effective measure used in the field is chemical control; however chemical pesticides which are overused have serious negative effects. Therefore cultural and biological strategies have been attempted in order to replace the excessive use of pesticides, in an integrated pest management (IPM) strategy.

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Post-harvest strategies are instituted to protect potato tubers from PTM once placed into storage facilities. Stores should be kept at less than 10°C to prevent egg hatching and larvae feeding on the tubers (Hanafi, 1999). Refrigerated stores in developing countries are costly therefore rarely used. Therefore PTM infestation needs to be minimised through other means such as sorting infested potatoes from clean non-damaged tubers and treating with insecticides before placing the vegetables into storage. The potato sacks and storage rooms should be cleaned to ensure no PTM infestation (Hanafi, 2005). If the tubers in storage are intended for seed potatoes, chemicals such as pyrethroids are suggested to control PTM, as well as biological agents such as *Bacillus thuringiensis* and *Phthorimaea operculella* granulovirus (Ben-Salah and Aalbu, 1992; Hanafi, 1998). Storage facilities should have screens in place to prevent any insect entrance to the tubers, thereby reducing the likelihood of females laying eggs on the stored tubers (Hanafi, 1999). Regularly monitoring for PTM presence will ensure that any undetected tubers with PTM infestation are controlled before damage to other tubers can occur. Monitoring can be done through visual observations or pheromone traps to determine adult male populations within the stores (Raman, 1988; Hanafi, 1999).

Integrated pest management is a strategy whereby an effective and economical approach to pest control is established. This strategy incorporates the use of chemicals and less hazardous means of control, by identifying and correcting pest status levels through various cultural and biological controls. IPM favours cultural, mechanical, physical and biological control over chemical control as it has fewer negative effects on human and animal health, and the surrounding ecosystem (Illinois State Board of Education, 2000). Several IPM elements have been established, and are used in conjunction with each other, and interchangeably, during different levels of infestation and growing seasons (Cornell University, 2003) (Figure 1.5).
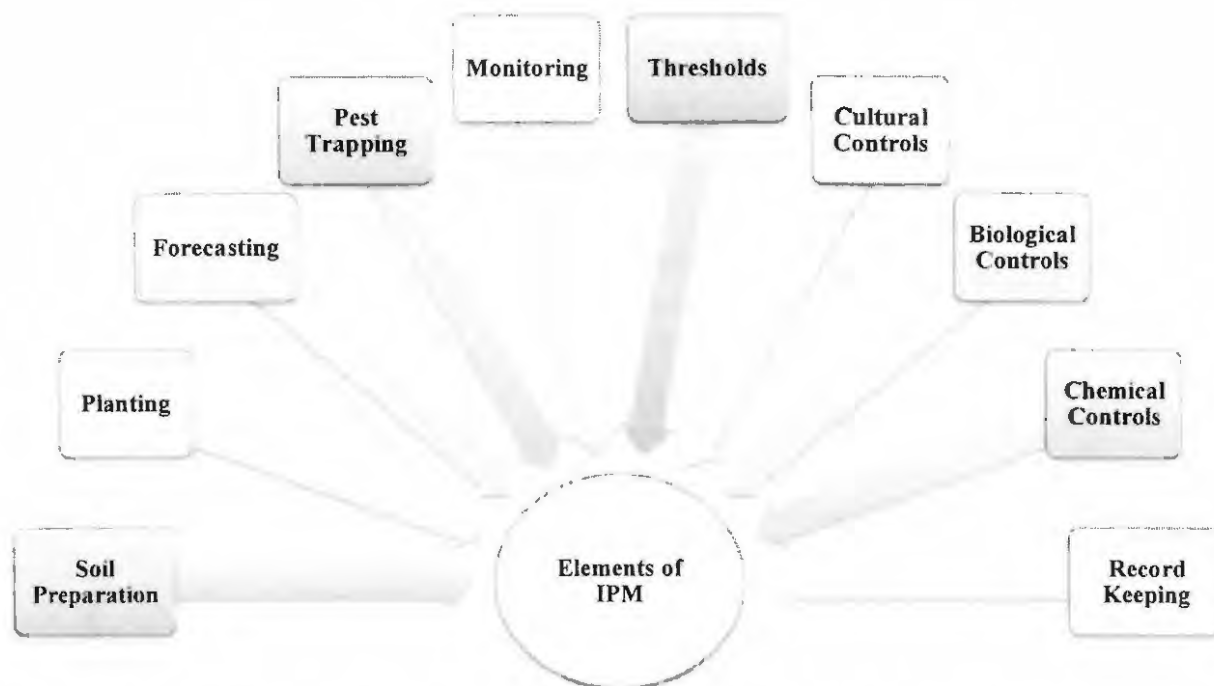
Figure 1.5: Elements of integrated pest management which can be used in conjunction with each other to maintain and control pest level infestations (Adapted from Cornell University, 2003).

1.4.1 Types of control

1.4.1.1 Chemical control

1.4.1.1.1 Pesticides

The conventional method of chemical control of PTM is the use of broad spectrum insecticides, but the problem associated with this is that PTM has developed resistance to many of the insecticides used (Lacey and Kroschel, 2009). In some cases 12 to 20 different insecticides have to be used in a growing season in order to control PTM infestations (Madkour, 1999). Common examples of pesticides used in South Africa regularly to control PTM are presented in Table 1.4.

Table 1.4: Pesticides used on potato crops to control PTM infestations in South Africa (Nel *et al.*, 2002).

Pesticide (common name)	Formulation		Dosage	Pre-harvest Application intervals (in days)
	Type	Active ingredient (g)	Per 100L water or as indicated	
Acephate	Water soluble powder	750g/kg	500g/ha	14
Alpha-cypermethrin	Emulsifiable concentrate	100g/L	100ml/ha	21
	Suspension concentrate	100g/L	100ml/ha	21
Azinphos-methyl	Suspension concentrate	350g/L	700ml/ha	21
		200g/L	1.25L/ha	21
	Wettable powder	350g/kg	700g/ha	21
Beta-cyfluthrin	Emulsifiable concentrate	50g/L	200ml/ha	14
Bifenthrin	Emulsifiable concentrate	100g/L	300ml/ha	21
	Tablet	0.18g/tablet	1 tablet/10L water	21
Cartrap hydrochloride	Water soluble powder	500g/kg	Max 2kg/ha	-
Chlorphenapyr	Suspension concentrate	360g/L	400ml/ha	14
Deltamethrin	Emulsifiable concentrate	25g/L	60 – 300ml/ha	2
	Tablet	5g/tablet	6-7 tablets/ha	8
Esfenvalerate	Emulsifiable concentrate	50g/L	450ml/ha	3
		200g/L	115ml/ha	3
Fenvalerate	Emulsifiable concentrate	200g/L	375ml/ha	3
Indoxacarb	Water disperable granule	300g/kg	150g/ha	-
Lamba-cyhalothrin	Capsule suspension	50g/L	120ml/ha	3
	Emulsifiable concentrate	50g/L	132ml/ha	3
Lufenuron	Emulsifiable concentrate	50g/L	800ml/ha	-
Methamidophos	Soluble concentrate	580g/L	500ml/ha	6
Methidathion	Emulsifiable concentrate	420g/L	500g/ha	6
	Wettable powder	400g/L	500g/ha	6
Methomyl	Water soluble powder	200g/L	300 – 500g/ha	3
	Soluble concentrate	900g/kg	1350 – 2250ml/ha	3
Monocrotophos	Soluble concentrate	400g/L	750ml/ha	21
Novaluron	Emulsifiable concentrate	100g/L	350ml/ha	-
Permrthrin/ Pheromone	Grease	60g/kg	100 -200g/ha	-
Phenthoate	Emulsifiable concentrate	500g/l	200ml	3
Profenofos	Emulsifiable concentrate	500g/L	1L/ha	14
Spinosada	Suspension concentrate	480g/L	30ml + 500ml light narrow range mineral oil	7
Tralomethrin	Emulsifiable concentrate	36g/L	120ml	14

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These pesticides pose serious health and environmental problems, which promote the search for alternative means (Lacey and Kroschel, 2009). Negative impacts of pesticides include damage to natural ecosystems, pests are able to become resistant to previously effective pesticides and pesticide residues on food crops after harvest are consumed by humans, impacting on health (Jiménez and Poveda, 2009). Ideally, markets need residue-free foods. This is becoming an increasing demand, but it also increases the risk of PTM infestation (Magnusson and Cranfield, 2005).

1.4.1.1.2   Sex pheromones

The sex pheromones released by female moths have been identified and used in both pre- and post-harvest monitoring and control of PTM (Persoons *et al.*, 1976). In pre-harvest monitoring, sex pheromones are used to determine flight activity of males and dynamic and seasonal population trends. The sex pheromones in the traps are at high concentrations to ensure that they overpower the female pheromones that are released naturally, in order to attract the males (Kroschel and Zegarra, 2008). They can also be used to monitor PTM populations, because a threshold has been established for pre-harvest conditions in potato fields. If 15 - 20 moths are trapped at any one time, it is an indication that treatment for the pest infestation should begin (Ferro and Boiteau, 1993; Rondon *et al.*, 2007; 2010). However, if the activity of PTM does not reach the action threshold value before the point of vine kill during harvesting, treatment is not cost effective (Hanafi, 1999).

Potato Tuber Moth sex pheromones have been developed into commercial products which are predominantly used post-harvest in the storage facility through an attract-and-kill method. By removing the males from the population, mating disruption occurs, resulting in fewer fertilised eggs. This method has been effective (Kroschel and Zegarra, 2008).

1.4.1.2 Cultural control

There are several cultural practices which have been implemented as preventative measures to reduce PTM infestation. These are planting seeds deeper, hilling seed potato rows, constant drip irrigation, delayed vine kill and early harvest (Langford and Cory, 1932; Langford, 1933; Shelton and Wyman 1979a; Hanafi, 2005; Jensen *et al.*, 2005). Consistent hilling can inhibit PTM access to tubers, thereby reducing infestation. Soil should be kept moist, not excessively damp, as this may affect the tubers in the soil, but moist enough to reduce cracks in the soil allowing access to the tubers by PTM larvae. If seed potatoes were to be planted deeper, it would reduce PTM access to the tubers, but there is an economic disadvantage as deeper tubers do not grow as large as tubers close to the surface. Vine kill is performed in order to collect soil tubers easily; however this leaves

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tubers prone to PTM infestation as they search for new food. Delaying vine kill reduces the infestation of PTM greatly (Langford and Cory, 1932; Langford, 1933; Shelton and Wyman 1979a; Hanafi, 2005; Jensen *et al.*, 2005).

The best cultural method for monitoring foliage infestation by PTM is to physically scout through the potato plants and make note of how many plants exhibit signs of infestation. This practice has been found to be very successful in New Zealand (Foot, 1979) and in another study by Von Arx *et al.* (1987) in Tunisia.

1.4.1.3 Biological control

Many entomological pathogens are responsible for infecting and killing economically important insect pests. These organisms include bacteria, fungi, viruses, protozoa and nematodes. Several microbial biopesticides have been developed using these entomopathogens, in order to reduce the economic impacts of PTM on agriculture (Von Arx *et al.*, 1987; Hamilton and Macdonald, 1990; Raman, 1994; Kroschel *et al.*, 1996b; Lacey *et al.*, 1999; Lacey *et al.*, 2001; Sporleder, 2003; Wraight *et al.*, 2007). Further research is being done worldwide to develop a more effective biopesticide or microbial cocktail to control infestations (Lacey and Arthurs, 2008).

Three biological control methods have been used to control PTM: classical, augmentation and conservation (Lacey and Kroschel, 2009). Classical biological control involves the introduction of exotic parasitoids of PTM to establish in the infested region and potentially become a long-term means of control (Lacey and Kroschel, 2009). To date 18 species of parasitoids have been identified globally. They belong to the families Braconidae (example: *Apanteles subandinus* Blanchard and *Orgilus lepidus* Muesebeck) and Encyrtidae (*Copidosoma koehleri* Blanchard) (Lacey and Kroschel, 2009). In Zimbabwe, when PTM parasitoids were introduced there was a positive impact: PTM infestations were reduced so successfully that the insect was no longer considered a significant potato pest (Mitchell, 1978).

In South Africa, two parasitoids originating from South America have been successfully established. *Copidosoma uruguayyensis* Tachikawa and *Apanteles subandinus* Blanchard have been found several kilometres from where the species were initially released. In the northern part of South Africa, these two parasitoids have achieved better control of PTM than any indigenous predators (Watmough *et al.*, 1973).

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Augmentative biocontrol is a practice whereby natural enemies are mass-reared and repeatedly introduced into pest populations. Here, the combination of both parasitoids and pathogens as biocontrol agents results in a fast and effective means of control. In the case of PTM, the application of natural agents such as viruses, bacteria and parasitoids ensures the pest is restricted (Lacey and Kroschel, 2009).

Conservation biological control is an agricultural technique whereby the impacts of natural enemies are optimised through cultural practices. Cultural practices include habitat management, decreased use of insecticides or ensuring that application of the pesticides is optimised (Lacey and Kroschel, 2009). In Australia PTM was controlled successfully by an indigenous parasitoid. The impact of this control became apparent only after IPM strategy had been put into place to monitor all potato pests (Horne and Page, 2008).

1.4.1.3.1 Nematodes

Steinernema (Steinernematidae) and *Heterorhabditis* (Heterorhabditidae) are two genera of nematodes that have been used against PTM. These two genera have a symbiotic relationship with bacteria, *Xenorhabdis* and *Photorhabdis*, which rapidly kill the host insect (Lacey and Arthurs, 2008). The use of entomopathogenic nematodes has only recently been applied for controlling insect pests commercially (Grewal *et al.*, 2005). Research has shown that nematodes have resulted in high levels of mortality in PTM larvae, whilst a few of the pupae died from nematode infection (Lacey and Arthurs, 2008).

1.4.1.3.2 Bacteria

Bacillus thuringiensis Berliner (*Bt*) is the only bacterium that has been evaluated and used in the control of PTM populations (Lacey and Arthurs, 2008). This bacterium occurs naturally in the environment and produces crystalline toxins which result in the fatal rupturing of the epithelial cells in the midgut of the insect (Garczynski and Siegel, 2007). Insecticides that are produced using the *Bt* toxin are the most widely used microbial pesticides. These pesticides are produced commercially for a broad spectrum of entomological pests such as beetles (Coleoptera), flies (Diptera) and caterpillars (Lepidoptera), and with specific reference to those that attack potatoes (Lacey *et al.*, 1999; Arthurs *et al.*, 2008). *Bt* has been developed for insect pest management because it is highly effective, is safe to humans and has no effect on the environment. There are several types of *Bt* formulations including wettable powders, liquid concentrates, dusts, and baits, which are traded under Acrobe, Bactospeine, Certan, Dipel, Javelin, Leptox, Thuricide and Victory (Lacey and Arthurs, 2008).

In many countries the application of *Bt* has been attempted in order to reduce the infestation of PTM post-harvest in storage conditions. This application is particularly important to potatoes that are not kept under refrigeration (Lacey and Arthurs, 2008). When *Bt* is applied to harvested tubers in storage, it was found that 96% of larvae which were already present in the tubers were killed, and larvae hatching from eggs laid after the application did not develop (Kroschel and Koch, 1994). *Bt* used in conjunction with low risk pesticides is very effective in controlling PTM (Farag, 1998). If *Bt* is applied at the initial point of storage, with the combination of cultural control methods, there is a decreased need to rely on parathion pesticide during storage (Von Arx *et al.*, 1987).

For *Bt* to be effective, the larval stage of the insect must ingest the toxin, as it is ineffective against the adult stage. The insect can die within a few hours to days depending on the insect and its stage of development, as well as the concentration of *Bt* consumed. There are several *Bt* strains, each having its own specific toxin for a specific insect pest. For example, *Btk* is specific and efficient at controlling Lepidoptera (Lacey and Arthurs, 2008). Native PTM populations have been naturally controlled by *Bt*, as has been observed in Bolivia (Hernanndez *et al.*, 2005). However, many strains have been isolated from soil, tubers and other environments which are more toxic against PTM, suggesting that there is a future in developing a more potent *Bt* strain which can be indigenous (Lacey and Arthurs, 2008).

The application of *Bt* to PTM populations that have infested potato crops has been tested in the laboratory, greenhouse, field and storage conditions. Two crystalline toxins have been identified as effective against PTM: Cry1A (b) and Cry1B. In greenhouse and laboratory conditions, *Bt* can remain lethal to PTM for up to 60 days after application in the soil and tubers (Amonkar *et al.*, 1979). *Bt* has been effective in field trials, but the product needs to be applied several times because *Bt* is degraded by UV light and rain washes the bacteria off the plant or out of the soil (Salama *et al.*, 1995b).

Recent studies indicate that *Bt* toxins could be a potentially good method of controlling PTM, by using genetically modified potato crops. Research has shown that PTM infestation was reduced on potato foliage and tubers when in the field and in storage (Davidson *et al.*, 2004). However the use of genetically modified crops is not always accepted by the general public, and this might be the main deciding factor as to how widely these transgenic *Bt* potato plants would be utilized (Douches *et al.*, 2008).

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1.4.1.3.3   Fungi

Fungi are effective against several insect species which are important economic agricultural pests (Lacey *et al.*, 1999; Goettel *et al.*, 2005). Studies have shown the effectiveness of *Beauveria bassiana* and *Metarhizium anisopliae* fungi as microbial control agents for PTM (Sewify *et al.*, 2000). *Beauveria bassiana* has been found to be effective not only against larvae, but also the pupae and adults, whereas *M. anisopliae* is lethal only to the larvae. The combination of *B. bassiana* in high concentration with the baculovirus PhoGV, in low concentration, is highly efficient at controlling PTM larvae (Sewify *et al.*, 2000).

1.4.1.3.4   Viruses

Phthorimaea operculella Granulovirus (PhoGV) has been isolated in several countries worldwide where PTM populations have established (Figure 1.2), (Lacey and Arthurs, 2008). PhoGV was first isolated in Sri Lanka by Steinhaus and Marsh (1967). The virus has been characterised in South America (Alcázar *et al.*, 1991; Alcázar *et al.*, 1992b), North America (Hunter *et al.*, 1975), Africa (Broodryk and Pretorius, 1974; Laarif *et al.*, 2003), the Middle East (Kroschel and Koch, 1994), Asia (Zeddam *et al.*, 1999), and Australia (Reed, 1969; Briese, 1981).

1.5   THE PATHOGEN *PHTHORIMAEA OPERCULELLA GRANULOVIRUS*

1.5.1   Baculoviruses

1.5.1.1 Classification

Baculoviridae is a virus family comprising of two genera, Granuloviruses (GV) and Nucleopolyhedroviruses (NPV). The nucleopolyhedroviruses can be divided into single- and multiple- nucleopolyhedroviruses, the name referring to the number of virion particles found in each protein coat is known as an occlusion body. GV possess only a single virion particle within each occlusion body (Kalmakoff and Ward, 2003) (Figure 1.6). There is a morphological difference between the genera as well, the GV have a granular appearance, whereas the NPV have a crystalline structure (Kalmakoff and Ward, 2003).
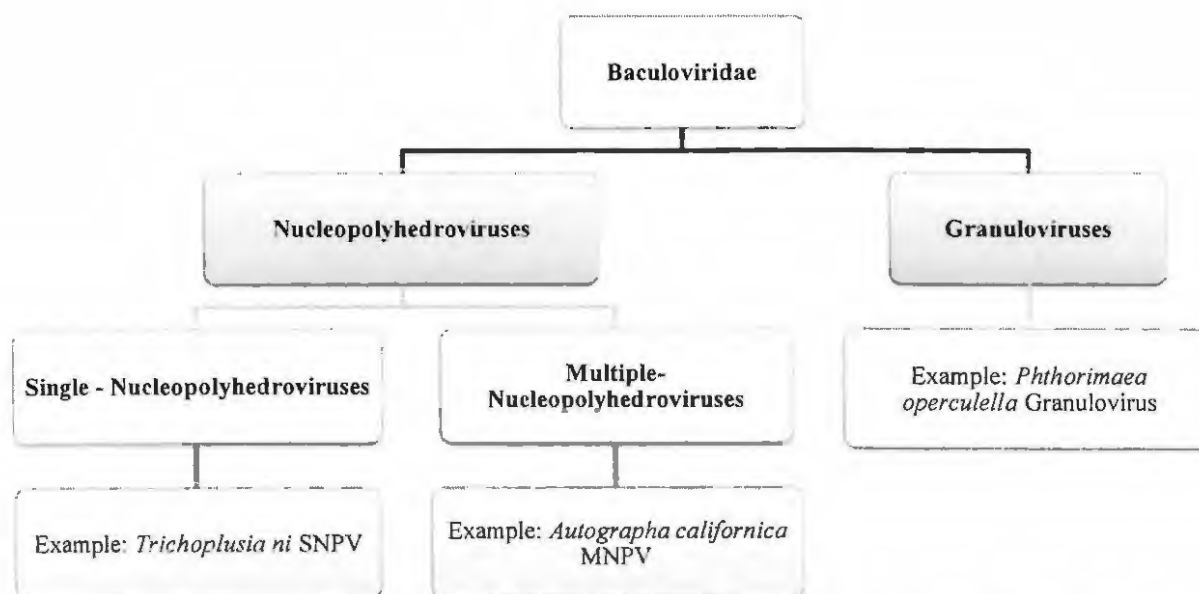
Figure 1.6: The classification of *Baculoviridae* (Adapted from Kalmakoff and Ward, 2003)

1.5.1.2 Genome

Baculoviruses have a circular double-stranded DNA genome, which varies in size from 80 - 180 kbp (Kalmakoff and Ward, 2003). The open reading frame encodes between 120 - 160 proteins. The genome contains several repeated sequences referred to as homologous regions, which are found throughout the genome sequence. These repeated sequences are important for enhancing early gene transcription as well as acting as origins of replication (Kalmakoff and Ward, 2003).

1.5.1.3 Structure

All baculoviruses have a distinct rod-shaped nucleocapsid ranging in diameter from 30 to 60nm, and in length from 250 to 300nm. Granuloviruses are biochemically and structurally similar to nucleopolyhedroviruses, except that the virions are occluded individually in small ovicylindrical occlusion bodies composed of granulin and referred to as granules (Figure 1.7). NPVs are occluded in a polyhedral occlusion body composed of polyhedrin. Granuloviruses are approximately 150nm in diameter and 400 - 600nm in length, whereas nucleopolyhedroviruses vary in occlusion body size from 150 - 1500nm (Kalmakoff and Ward, 2003). Granuloviruses have been isolated predominantly from lepidopteran species, unlike nucleopolyhedroviruses which have been found in crustacean and lepidopteran species (Federici, 1997).

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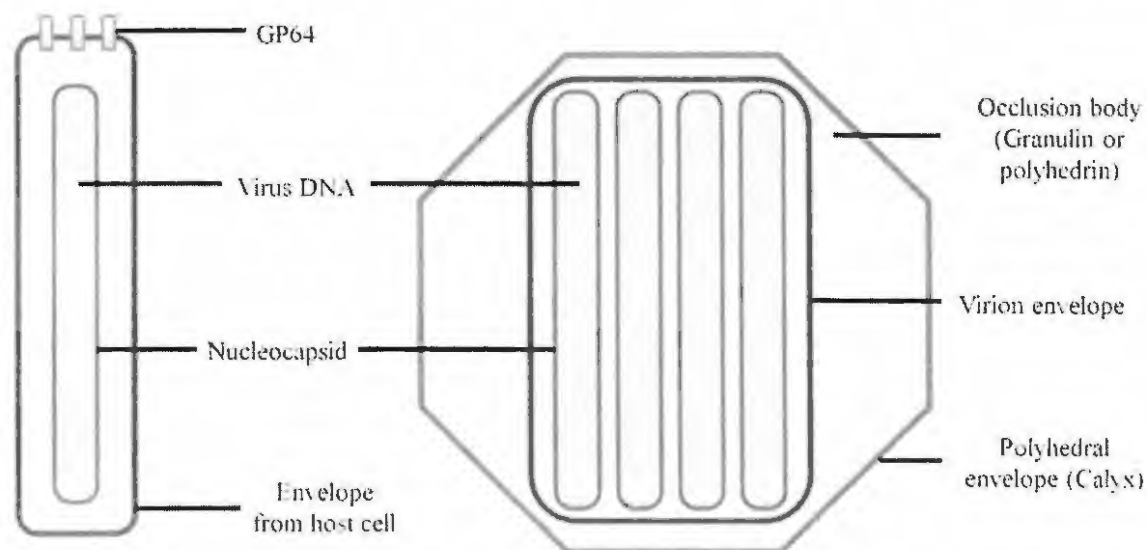
Budded Virus   **Polyhedron derived Virus**

Figure 1.7: Baculovirus structure (Adapted from Kalmakoff and Ward, 2003).

Another difference between the two genera of baculoviruses is the protein present in the occlusion bodies (Jehle *et al.*, 2006). Nucleopolyhedroviruses have polyhedrin present in their occlusion bodies, whereas granuloviruses have granulin (ICTVdB management, 2006; Rohrmann, 2008). The occlusion bodies formed are highly stable and can resist harsh environmental conditions (Rohrmann, 2008). The occlusion body varies between 0.13 - 0.5µm in size in granuloviruses and between 0.15 - 3µm in nucleopolyhedroviruses (ICTVdB management, 2006; Jehle *et al.*, 2006).

1.5.1.4 Host range

Baculoviruses are highly host specific to the insect species they infect. These viruses infect several entomological orders: Coleoptera, Diptera, Hymenoptera, Lepidoptera, Neuroptera, Siphonaptera, Thysanura and Trichoptera. Infection occurs predominantly at larval stages and in some rare instances the pupae and adults can be infected (Evans and Shapiro, 1997)

Phthorimaea operculella Granulovirus (PhoGV) is a natural occurring virus which infects Potato Tuber Moth and has accompanied the moth worldwide (Reed, 1969; Broodryk and Pretorius, 1974; Hunter *et al.*, 1975; Alcázar *et al.*, 1991; Kroschel *et al.*, 1996b; Zeddam *et al.*, 1999). PhoGV has been shown not only to be symptomatic but can also be lethal to PTM, as well as the Guatemalan PTM, *Tecia solanivora* (Sporleder and Kroschel, 2008). There is an Andean PTM, *Symmetrischema tangolias*, which is becoming a serious economic problem in the Andean region because it is resistant to PhoGV infection (Sporleder and Kroschel, 2008).

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1.5.1.5 Life cycle

Baculoviruses undergo two phenotypic events during their life cycles, when virions enter the gut epithelium (phenotype I, also known as occlusion-derived bodies) and when the nucleocapsids emerge from the plasma membrane of infected cell surfaces (phenotype II, also known as budded virions). The budded virions (BV) are a result of initial infection, where the virus particles have budded through the plasma membrane of the infected cells. Occlusion-derived bodies (OB) can have a single or multiple nucleocapsid within the envelope which has different origins and composition to BV envelopes. These two phenotypic forms are required for transmission of the virus to occur. The BV is used for cell-to-cell infection, whereas the OB is used for insect-to-insect transmission (Jehle *et al.*, 2006).

Following ingestion by the host larvae, the virus particles and the protein occlusion body disintegrate in the alkaline conditions. The virion particles are released from the occlusion bodies and enter the midgut epithelial cells through cell receptors. The virions enter the nucleus of the insect midgut cells through the nuclear pores and begin replicating the genome and producing the necessary proteins to assemble more virus particles. In the nucleus, the nuclear material is moved to the periphery and a virogenic stroma is produced which aids the encoding and replication of the virus DNA (Rohrmann, 2008) (Figure 1.8).

Budded virus particles can move from the epithelial midgut cells through the basal lamina to other surrounding tissue cells such as fat body cells, tracheal matrix and hemolymph. When the virions begin to replicate and assemble in surrounding cells, this is known as early infection and this stage is not necessarily symptomatic. As cells become infected with virus particles and they are destroyed, resulting in late infection which is when symptoms are presented. At this stage, the nucleus is ruptured, resulting in the nucleoplasm and cytoplasm merging. In late infection, virions become enclosed within occlusion bodies, allowing for the transmission of the virus from the infected larva to another larva or surrounding environment, known as horizontal transmission (Moscardi, 1999; Rohrmann, 2008) (Figure 1.8).

Uncoated virions released from midgut cells

Virions enter surrounding cells (fat cells and basal lamina cells)

Uncoated virions enter the midgut Cell

VS

N

Early Infection
Nucleus enlarges and nuclear material concentrates near the periphery. At this stage virions are produced in the nuclei, not occluded virus particles.

Virions uncoated in the alkaline midgut conditions

VS

N

VS = Virogenic Stroma
N = Nuclear Material

Occluded virus particles

Late Infection
Nuclear membrane and material disintegrates resulting in nucleoplasm and cytoplasm to merge. At this stage infectious occluded virus particles are released.

Figure 1.8: Life cycle of a Granulovirus in its insect host (Adapted from Rohrmann, 2008)

1.5.1.6 Gross pathology and symptomatology

Once virus infection has occurred within a larva, death occurs within 10 – 14 days. Healthy larvae have a brown colouration, however within 4 days of infection, the larvae will begin to exhibit colour change, from a brown to a creamy yellow or white. As infection progresses, the larvae become swollen. The discolouration and swelling observed is a result of the accumulation of many infected hemocysts in the haemolymph (Federici, 1997).

If the virus is ingested in the first or second instar larval stages, death can occur within 24 to 72 hours, with very few symptoms (Federici, 1997). Once the colouration has become apparent, and the larvae die, the cadavers liquefy, releasing the contents of the larvae which possess the baculovirus occlusion bodies. Just prior to the death of a larva infected with a baculovirus, the insect will crawl up the vegetation or the container, and then die. In most cases, the larvae will be hanging from their proleg crochets, and hanging head down, within a few hours liquefaction occurs which is a result of loss of turgor (Federici, 1997).

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1.5.2 *Phthorimaea operculella* Granulovirus (PhoGV)

The virus is thought to have co-evolved with the insect in South America, and when PTM was accidently transported to other potato producing countries, the virus accompanied the insect (Alcázar *et al.*, 1991).

The virus PhoGV forms part of the rod-shaped Granulovirus genus, which are virions consisting of both an envelope and nucleocapsid (ICTVdB Management, 2006). PhoGV has a genome length of 119kbp which encodes for 130 genes (ICTVdB Management, 2006). PhoGV needs to be extensively studied in the laboratory, for pathogenicity, efficacy, safety and the development of commercial production protocols, before the virus can be effectively used as a competitive biological control agent for the pest (Vickers *et al.*, 1991).

The replication cycle of PhoGV (viral replication, protein production and nucleic acid synthesis) and the development time of *P. operculella* larvae (synthesis of cell components) are both regulated by temperature (Sporleder *et al.*, 2008a). However, this relationship between larvae and virus is very complex (Sporleder *et al.*, 2008a). Virus replication is easily inhibited when rearing hosts at high temperatures for a constant period of time. The reason for this is either that the virus is not thermostable or that there is a temperature – induced host resistance mechanism (Tanada and Tanabe, 1965).

1.5.2.1 Microbial control using PhoGV

The potential success of PhoGV as an agent for biological control of PTM has resulted in the virus being incorporated into integrated pest management (IPM) systems, especially in developing countries (Reed, 1969; Sporleder and Kroschel, 2008). Due to the narrow host range, and the fact that the virus occurs naturally, it can be applied to both stored and field potatoes without having any harmful impacts on animals, humans or the environment (Sporleder and Kroschel, 2008).

1.5.2.1.1 Virus production

A dust formulation has been developed for the potatoes in storage in order to reduce infestation of PTM (Fuglie *et al.*, 1993; Winters and Fano, 1997). Larvae infected with PhoGV are ground and mixed with talc, at a concentration of 20 larvae per 1kg talc in 1 litre of water. The dried powder product is then applied to stored potatoes at a rate of 5kg per tonne of potatoes (Alcázar *et al.*, 1991; Das *et al.*, 1998).

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1.5.2.1.2 Biological activity

PhoGV has been found to have a low stability in the field when exposed to solar radiation (Sporleder and Kroschel, 2008). Direct sunlight can inactivate the virus within one day. Therefore several applications of the virus are required in the field for effective survival of the virus (Kroschel *et al.*, 1996b). However virus particles that are shaded by foliage or found in cavities of the plant have increased half lives and it has been suggested that adding antioxidants to the value of 1% of the final virus solution can result in 2 or 3 fold increased virus stability (Sporleder and Kroschel, 2008).

There is a decreasing susceptibility to PhoGV as PTM larvae mature. Therefore it is suggested that when the virus is applied to fields and storage potatoes, it is directed at infecting and killing neonate to 2nd instar larvae. The increased resistance with age, indicates that it is difficult to control entire populations of PTM as some stages of development are unaffected by the virus (Sporleder and Kroschel, 2008).

Most baculoviruses have a very narrow host range, and the same applies to PhoGV. PhoGV is highly host specific to PTM, but can also infect *T. solanivora*. This ability of PhoGV to be highly host specific ensures that other fauna and flora are not affected (Sporleder and Kroschel, 2008).

1.5.2.1.3 Field application

In several countries field trials using PhoGV have been attempted and the application of the virus has been successful in suppressing PTM. In some cases, success rates were similar to those of chemical pesticide applications. The virus, once applied, not only protected potato crops, but also spread extensively to other untreated crops and surrounding areas (Kroschel *et al.*, 1996b; Reed, 1971). However the limiting factor for field applications of virus is that large numbers of virus-infected larvae are required (Sporleder and Kroschel, 2008).

1.5.2.1.4 Storage application

The use of PhoGV on potatoes in storage post-harvest has shown to be successful in controlling infestation of PTM (Alcázar *et al.*, 1992b; Setiawati *et al.*, 1999). The virus has been introduced as a potential alternative to chemical pesticides in global IPM programmes (Raman *et al.*, 1987; Alcázar *et al.*, 1992b). Several enterprises have been launched in Peru, Bolivia, Colombia, Egypt and Tunisia for mass production of PhoGV into a dust formulation, specific for potatoes in storage (Fuglie *et al*, 1993; Winters and Fano, 1997). This technique has shown results of up to 95% reduced infestation in stored potatoes (Alcázar *et al*, 1992b).

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1.6 RESEARCH AIMS

1.6.1 Motivation

Potato Tuber Moth is the most damaging and economically important potato pest in South Africa. Potato farmers in South Africa use various pesticides to control the levels of infestation by this pest (Table 1.1). However, as effective as these chemicals are in controlling this potato pest, there are environmental and health consequences of using them.

PTM can be controlled fairly successfully using chemical control, however in some cases it has been found that PTM can develop a resistance to the insecticide. PTM has been known to have population explosions when conditions are optimal, which results in no pesticide being effective against infestation, as their reproductive rate is so rapid. The increase in organic farming in South Africa and the pressure of exporting to discerning first world markets, has increased the demand for alternative pest control methods.

There is currently no registered biological pesticide which can be used to control PTM in South Africa. PhoGV has been commercially produced in Peru, Bolivia, Egypt and Tunisia, where there have been successful results (Lacey and Arthurs, 2008). Therefore if the strain of virus isolated from the laboratory colony is effective for the control of PTM, there are good prospects for developing an alternative option to the commercial insecticides in South Africa.

The overall objective of this study is to isolate, identify and characterise PhoGV from PTM in South Africa for potential development as a biological control agent for this pest.

1.6.2 Objectives

The specific aims of this study were to conduct field surveys to collect larvae that showed symptoms of viral infection (chapter 2), develop a cost-effective rearing protocol that would allow *in vivo* production of any virus (chapter 3), and develop induction protocols for effective expression of PhoGV (chapter 4).

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CHAPTER 2

BIOPROSPECTING FOR POTATO TUBER MOTH AND *PTHORIMAEA OPERCULELLA* GRANULOVIRUS

2.1 INTRODUCTION

Bioprospecting in the field and storage facilities was conducted to attempt to isolate larvae of Potato Tuber Moth (PTM), which expressed symptoms of a granulovirus infection. Four sites were identified, as high population numbers of PTM had been recorded in these cropping lands. The investigation for infected PTM larvae ranged from searching seed and table potatoes to foliage in both storage and field environments.

It has been noted that wherever potatoes are grown in South Africa, PTM has established in the field (Broodryk, 1971). Potatoes are grown in all provinces in South Africa (as indicated in Figure 2.1), offering ample sampling areas (Department of Agriculture, 2003). PTM does not only persist within the field, destroying the potato foliage and tubers, but can also be a devastating problem if infested tubers are placed into a storage facility for a long time and not maintained properly (Arnone *et al.*, 1998; Rondon, 2010).
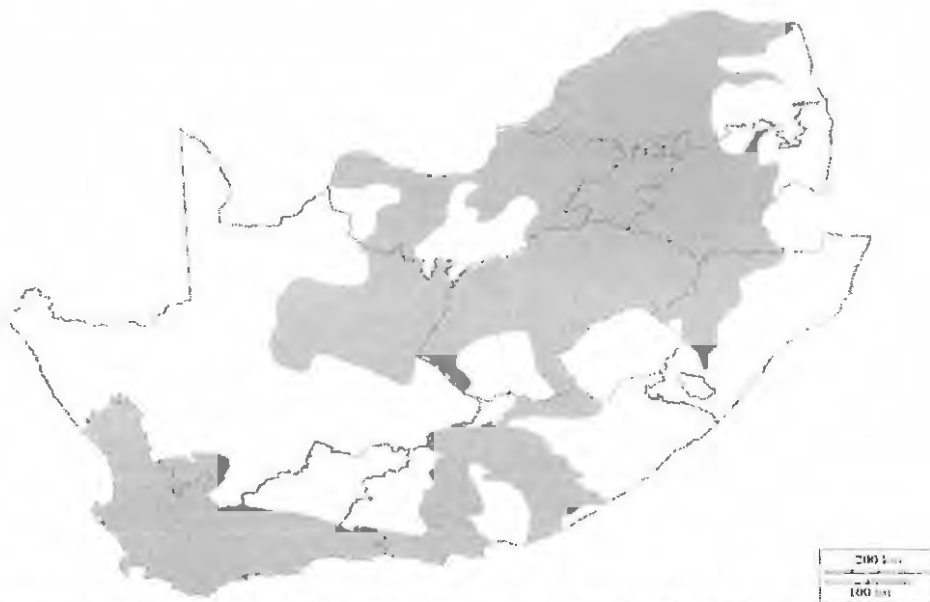
Figure 2.1: Areas where seed and table potatoes are grown in South Africa are indicated by green shading (Adapted from Department of Agriculture, 2003)

PTM is regulated by climatic conditions, predominantly by temperature (Trivedi *et al.*, 1994; Sporleder *et al.*, 2004). In warmer climates and higher temperatures during spring and summer months there is a greater chance of finding PTM infestation, as conditions are favourable for rapid growth and development of all of the insect stages (Raman, 1988). In storage facilities, if the tubers are not stored below 10°C, infested tubers with PTM could result in rapid infestation resulting in a large loss of yield (Ferro and Boiteau, 1993; Trivedi *et al.*, 1994; Arnone *et al.*, 1998; Sporleder *et al.*, 2004), while potatoes kept in refrigerated stores at 2 - 4°C tend to have low PTM infestations (Raman *et al.*, 1987; Hanafi, 1999). PTM infestation rates are influenced by environmental factors such as warm temperatures ranging from 20 - 25°C (Hanafi, 1999). A crop field or storage unit with little or no pesticide use, will have an increased chance of high levels of infestation of PTM (Foot, 1974b; Rondon, 2010). Farmers who do not monitor the presence of PTM thresholds regularly and respond to action thresholds, could discover high PTM infestations in their crops (Attia and Mattar, 1939; Broodryk, 1970b; Sporleder *et al.*, 2004; 2008b). The lack of good sanitation and cultural practices increases the impact of PTM on pre- or post- harvested tubers (Foot 1974b; Shelton and Wyman, 1979b; Clough *et al.*, 2008).

Identification of PTM in the field can be done using visual recognition of the insect and observation of development stages. In the field, an indication of PTM infestation is the appearance of windows in the foliage (Rondon, 2010). PTM mine into the dermal tissue of the leaf, and consume the plant tissue, creating clear windows in the leaf between the upper and lower epidermis (Rondon, 2010). In most cases, the larvae can be observed inside these windows. They damage the foliage by forming necrotic areas. Damaged leaves are usually found in the upper third of the foliage (Rondon *et al.*, 2007). Larvae can also be found crawling on the stem of the potato plant as well as on the soil (Ferro and Boiteau, 1993). In rare instances, pupae can be found in the soil.

PTM can be found infesting tubers once the insects are in the later larval stages, as they produce frass on the outside of the tunnel they have burrowed (Ferro and Boiteau, 1993). If eggs or first instar larvae gain access to tubers, they may go unnoticed for a few days (Kroschel and Koch, 1994). The larvae also crawl on the outside of the tuber, which makes for easy collecting of the specimen. The pupae can be found to be embedded in the tuber skin, crevices, or attached by a silk covering between two tubers, but they are rarely found inside tubers (Rondon, 2007).

Symptoms of a baculoviral infection are identified by morphological changes and discolouring of the larva. A commonly seen change is that of the brown-green PTM larva to a swollen milky larva

(Matthiessen *et al.*, 1978; Evans and Shapiro, 1997; Federici, 1997; Laarif *et al.*, 2006). The colour is accompanied by progressive weakening and decreased motility and flaccidity. In the final stage of viral infection, the insect tegument ruptures, releasing the virions. Death is normally observed in the larval stages of PTM, however it can be delayed to prepupal and pupal stages (Laarif *et al.*, 2006).

The work reported in this chapter consisted of surveying several sites in South Africa such as Patensie, Bathurst, Howick and Ivanhoe in order to eollect PTM to generate a laboratory culture, and then to identify potential virus-infected larvae from the field.

2.2   MATERIALS AND METHODS

2.2.1   Collection sites

PTM was collected on a commercial potato plantation in Patensie in the Eastern Cape, South Africa (33° 45` 20``S; 24° 49` 6``E) (Figure 2.2). Adults and larvae were collected from the site by sorting through seed potatoes in crates (Figure 2.3). Potato plants were surveyed and collected from Bathurst (33°30`13``; 26°51`52``) in the Eastern Cape. KwaZulu-Natal field collections were conducted at two sites, Ivanhoe (29°31`41``S; 29°51`38``E) and Howick (29°29`16``S; 30°10`23``E) (Figure 2.2). PTM adults were collected from potato foliage on a commercial seed potato plantation in the Ivanhoe district (Figure 2.3), whereas adults collected from Howick were collected from potato foliage and tubers from a commercial table potato field. The tubers from Howick were also collected to determine infestation rates and to initiate the laboratory insect culture. The collection sites and all their information are summarised in Table 2.1.

Table 2.1: A summary of each collection site

Collection sites		Time of collection	Type of potatoes	What was collected
Eastern Cape	Patensie	April 2009 May 2009 November 2009 February 2010	Seed	Adults, larvae and potentially infested potatoes were collected from seed potato crates
	Bathurst	August 2010	Table	Infested potato plants
KwaZulu-Natal	Ivanhoe	January 2010	Seed	Adults collected from foliage
	Howick	January 2010	Table	Adults and potentially infested potatoes were collected from the field
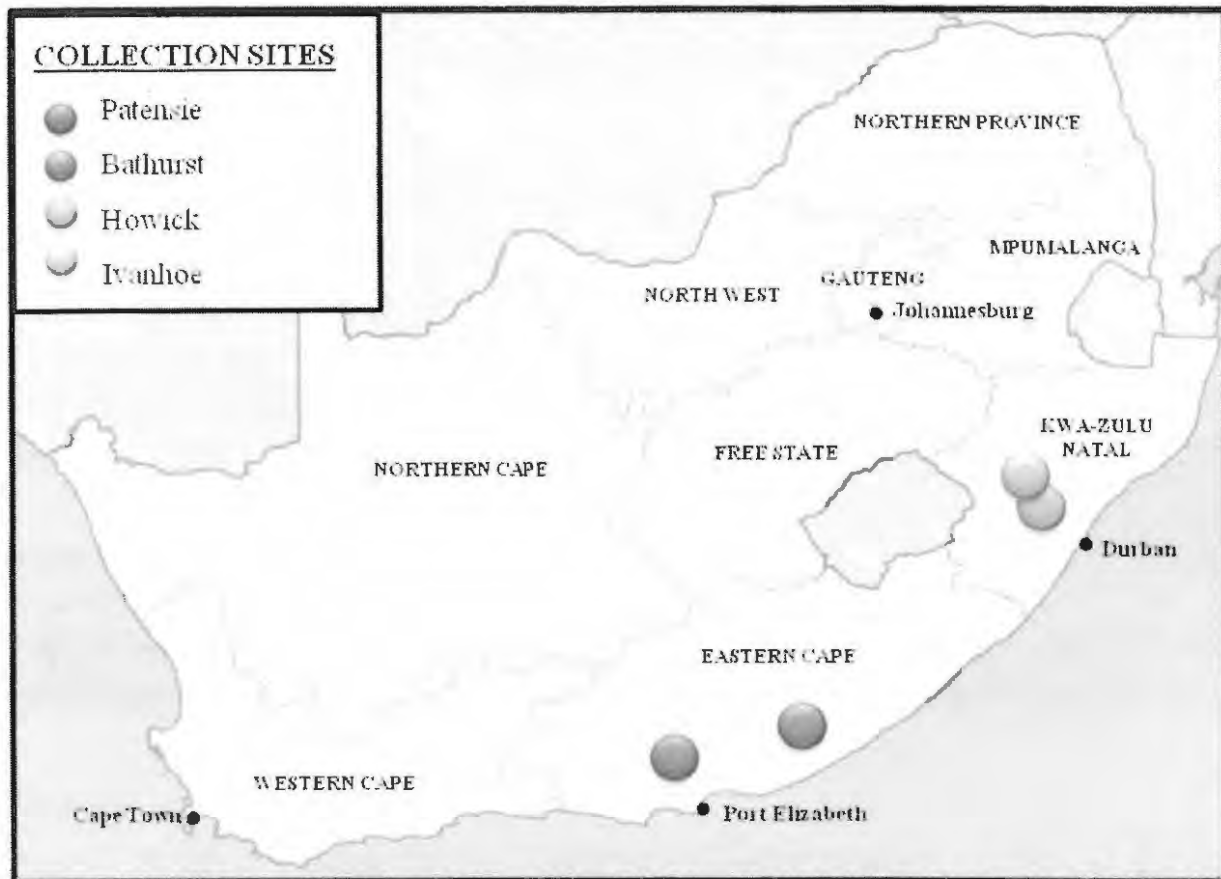
Figure 2.2: Collection sites for PTM for this study (Patensie (33° 45` 20``; 24° 49``E) and Bathurst (33°30`1``; 26°51`52``) in the Eastern Cape and Howick (29°29`16``S; 30°10`23``E) and Ivanhoe (29°31`41``S; 29°51`38``E) in KwaZulu-Natal).
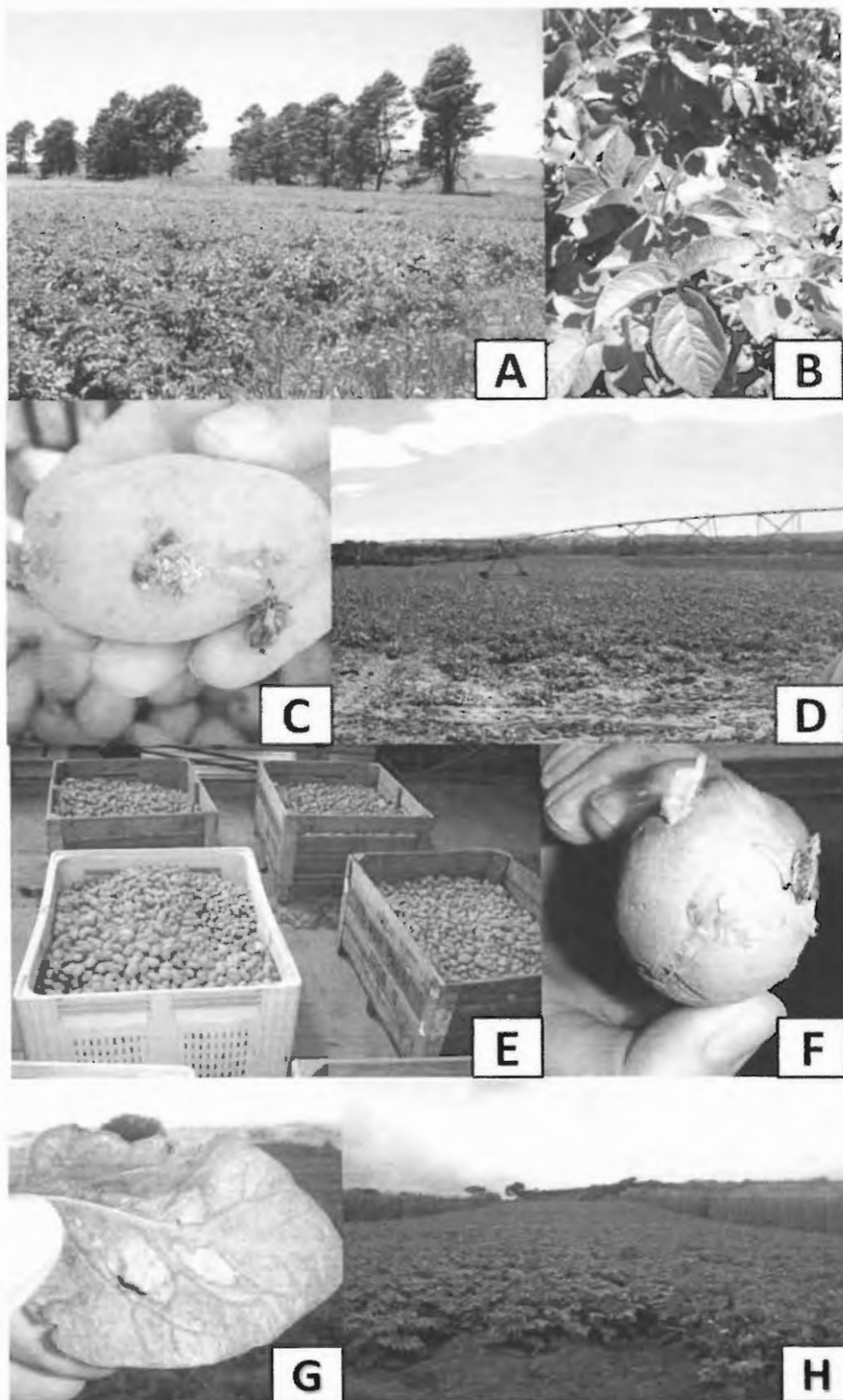
Figure 2.3: PTM was collected at Ivanhoe site (A) by sifting through seed potato foliage (B). The Howick site (D) required searching commercial table tubers and foliage for PTM infestation (C). Seed potatoes were sorted at Patensie from crates (E), for adult and larval stages of PTM (F). The PTM infestation was indentified in foliage (G) at the Bathurst site (H).

2.2.2 Collection of samples

Larvae from Patensie which were found on foliage or potato tubers were collected and placed onto uninfested potato tubers (Figure 2.4). These containers were kept on ice during transportation, in order to reduce stress. The larvae were then maintained in a constant environment room at 27°C and left to develop.

Figure 2.4: Healthy larvae collected in the field placed on uninfested potato tubers to be monitored for symptoms of viral infection.

Dead larvae or larvae showing symptoms that were possibly due to baculovirus infection were collected individually in 2ml microcentrifuge tubes, using a pair of fine forceps (Figure 2.5). All microcentrifuge tubes were labelled and kept in cryoboxes on ice, until transported to the laboratory, where they were stored at -20°C until required.

Figure 2.5: Dead larvae and symptomatic larvae were collected individually into microcentrifuge tubes.

2.2.3   Virus identification from larvae collected in the field

2.2.3.1 Symptomatology

Larvae collected from the field and storage crates were analysed for symptoms characteristic of baculoviral infections, including flaccidity or liquefaction of the insect, colour changes of the cuticle (specifically any larvae exhibiting a milky swollen appearance) and larval cadavers hanging from substrates.

2.2.3.2 Preliminary identification by light microscope

Larvae were prepared for light microscopy using a protocol adapted from Evans and Shapiro (1997). A fifth of the midsection of the whole insect body suspected of a baculovirus infection was smeared onto a microscope slide. Gut and internal organs were included in each smear. The smear of tissue was spread thinly across the slide which was then left to air dry. The slide was immersed in a staining rack containing Buffalo Black solution heated to 40°C on a hot plate, for 5 minutes. The slide was then washed under running tap water for 15 seconds, left to air dry and examined using a light microscope at 1000X magnification under oil immersion.

2.2.3.3 Crude extraction of granuloviruses

Crude extractions were performed according to the method described by Hunter-Fujita (1998). Symptomatic fifth instar larvae were macerated using a micro pestle in 1ml of 0.1% SDS, in a 2ml microcentrifuge tube. The tubes were centrifuged at 400xg for 30 seconds. The supernatant was removed and pipetted into a JA-20 tube (Beckman Coulter). The pellet was re-suspended in 500µl of 0.1% SDS and further centrifuged for 30 seconds at 400xg. The supernatant was removed and transferred to the JA-20 centrifuge tube. This step was repeated four times. The final pellet was discarded and the combined supernatant fractions were centrifuged at 10 000xg for 30 minutes at 4°C. The supernatant was removed and the pellet re-suspended in 100µl of sterile distilled water.

2.2.3.4 Transmission electron microscopy (TEM)

Crude suspensions were examined by adapting the technique described by Horne and Rochetti (1974). A carbon grid was securely fastened between the points of fine forceps. A drop of the crude extract was placed directly onto the carbon grid for 1 minute. Filter paper (Whatman, Lasec) was used to gently touch the edge of the grid. A drop of 5% uranyl acetate was placed onto the carbon grid and left for 1 minute. The excess uranyl acetate was removed from the grid using filter paper. The carbon grid was left to air-dry for 20 minutes and examined using a JEOL JEM 1210 transmission electron microscope operated at 100kV. Magnification ranged from 10 000X to 120 000X and particle sizes were measured using the TEM computational software (ANALYSIS).

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2.2.4 Collection of potato tuber samples

Potato tubers found in the field and storage units which showed signs of infestation because of the presence of frass were collected and stored in containers lined with perlite. Any pupae found attached to the tubers or larvae beginning to pupate on tubers were undisturbed and left to develop in the containers (Figure 2.6). All tubers collected were transported to a constant environment room at 24°C in order to observe infestation. Single tubers were placed into ventilated containers and observed daily to remove symptomatic larvae and PTM adults that may have eclosed. All moths that emerged from the potatoes were transferred to the relevant laboratory culture being reared. (Refer to Chapter 3 for information on rearing of the laboratory cultures.) The containers with moths were noted and the tuber was considered infested.

Figure 2.6: Infested potato tubers found in storage crates in Patensie (A) and from the field in Howick (B), with pupae attached to the surface of the tuber (A) or developing in its crevices (B).

2.2.5 Collection of adult moths in the field

Adult moths were collected during midday to late afternoon from Patensie and Howick sites using an insect net. A modified malaise trap with a black UV light was used to collect moths from the Ivanhoe site at dusk until dark (Figure 2.7). Moths were transferred from the net and malaise trap to ventilated containers to allow them to mate and lay eggs on filter discs situated on the mesh of the containers. Eggs were used to generate laboratory cultures from each collection site. See Chapter 3 for rearing.

Figure 2.7: A modified malaise trap with a black UV light set up at the Ivanhoe site to collect adult moths at dusk

2.2.6 Collection of potato plants from the field

Commercial fields near Bathurst, Eastern Cape were scouted for tuber PTM infestation but no infestation was visible, only the foliage was damaged. A survey for moths was carried out on fallow land on which no pesticides or other chemicals had been used in one year (Figure 2.8 A and B). Potato plants with evidence of PTM infestation were excavated from the soil and transplanted into 25cm diameter plastic pots (Figure 2.8 C) using soil from the field, supplemented with potting soil and fertilizer (ammonium sulphate) (Figure 2.8 C).
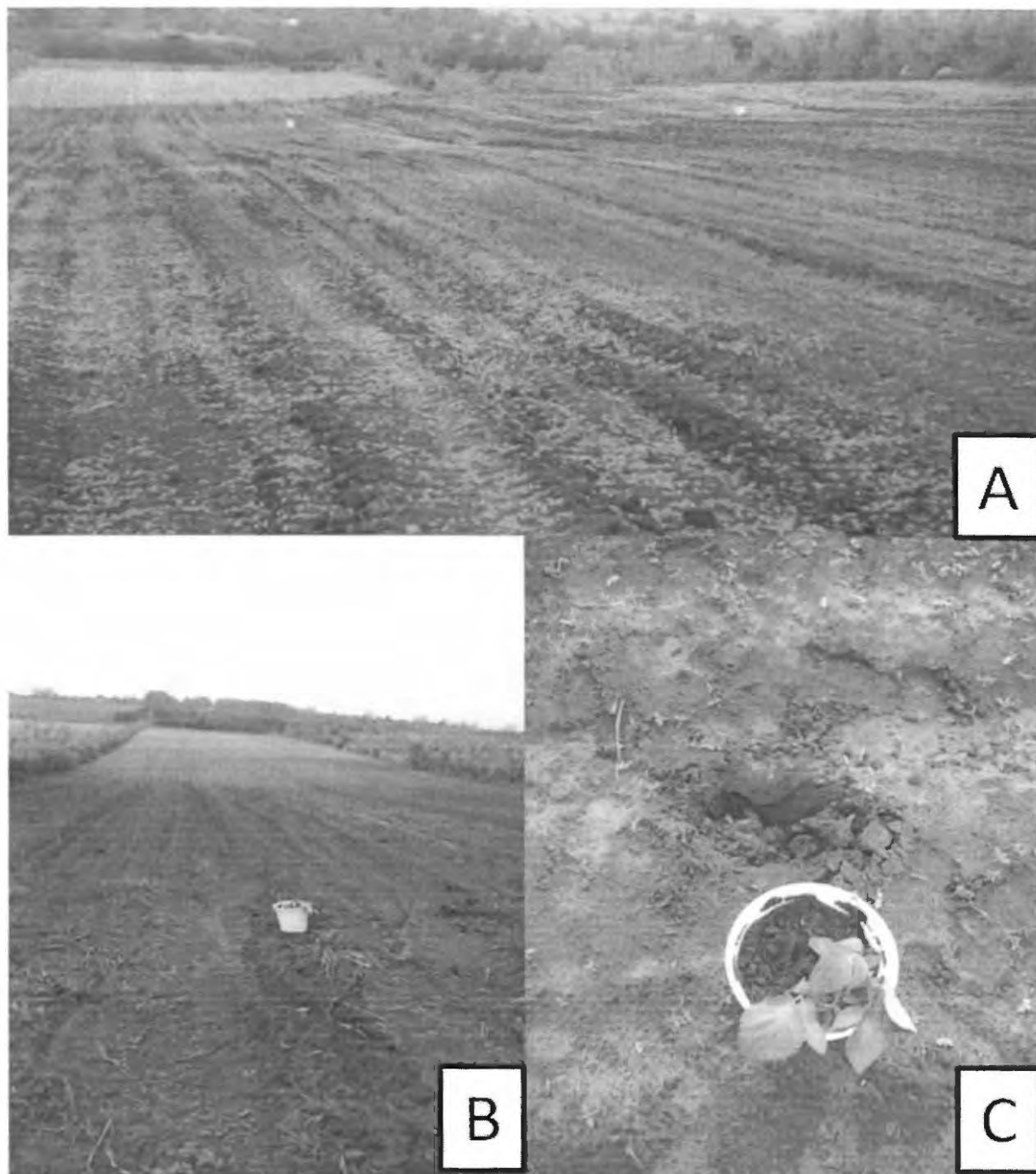
Figure 2.8: A fallow potato field in Bathurst (A). Potato plants emerged from previously planted tubers (B). Infested potato plants were transplanted into plastic pots (C).

Twenty five plants were collected and transported to a greenhouse tunnel where the plants were covered with mesh (Figure 2.9). The foliage was analysed daily for PTM larvae that were symptomatic for virus infection. All larvae were separately stored in 1.5ml microcentrifuge tubes and kept at -20°C for further analysis using techniques described in section 2.2.3.

Figure 2.9: Infested potato plants collected in Bathurst were grown in a greenhouse tunnel

2.3   RESULTS

2.3.1   Collection sites

Two sites in the Eastern Cape (Patensie and Bathurst) and two sites in KwaZulu-Natal (Howick and Ivanhoe) were explored, as these sites were reported to have a very high level of infestation of PTM (S. Moore, personal communication; J. Berriman, personal communication,) (Table 2.1).

Symptomatic larvae were found only at the Patensie site, and any larvae which emerged from the tubers and foliage collected from Bathurst and Howick were removed and analysed (Table 2.1). In KwaZulu-Natal, PTM was found only in the adult stage at both sites. The Patensie site was the most successful in yielding various stages of PTM infestation. The site was re-visited four times over a period of 18 months in an attempt to find sick larvae in the field (Figure 2.10).
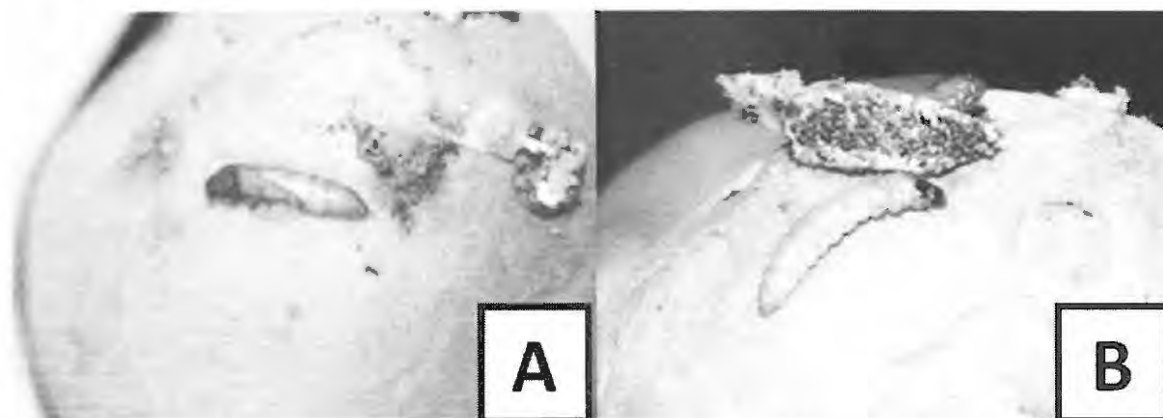
Figure 2.10: Two symptoms were observed in larvae collected from seed potatoes in storage in Patensie, flaccid creamy brown larvae (A) and milky coloured larvae (B).

2.3.2 Infestation of tubers collected in the field

The infested tubers collected from Patensie and Howick exhibited a 100% infestation, but varying rates of adult emergence. A total of 372 seed tubers were collected from Patensie and 51 table potatoes from Howick, which led to the emergence of 1158 moths from Patensie and 668 moths from Howick. Tubers collected in Patensie had a mean of 3.11(±2.29) moths emerge per tuber, whereas those collected at Howick produced an average of 12.71(±4.00) moths. The potatoes collected at the Howick site indicated higher levels of infestation of PTM larvae. The tubers were badly damaged and large amounts of frass were present on the surface of the tuber. Several pupae were also observed in the frass. No symptomatic larvae were observed in containers of infested tubers collected from both Patensie and Howick. Moths that did emerge were collected and transferred into their relevant cultures to contribute to the rearing of the cultures.

2.3.3 Collection of adult moths from the field

All moths collected in the field at Patensie, Ivanhoe and Howick were transported to the laboratory to be reared under laboratory conditions. The moths were integrated into a host rearing method (chapter 3). Any offspring that hatched and developed were closely monitored for potentially diseased larvae because PTM adults are able to transmit the baculovirus vertically to their offspring which can result in virus-infected larvae without the offspring ingesting any virus particles. This phenomenon is known as a latent viral infection (Cory *et al.,*1997; Fuxa *et al.*, 1999; Alberts *et al.*, 1994; Boots *et al.*, 2003). It has been reported that PTM can possess a latent baculovirus infection until a stimulus causes induction of an active infection (Il'inykh and Ul'yanova, 2005).

2.3.4 Larvae collected from potato foliage

When the potato plants were inspected in the field in Bathurst, there was clear evidence of PTM in both the commercial and fallow fields. Several leaves were collected with windows that were suspected to be a result of PTM (Figure 2.11 A and B). The pots collected with infested foliage were analysed daily for PTM larvae even if symptoms were not present (Figure 2.12).

Figure 2.11: PTM infestation of potato foliage in a commercial plantation (A). The foliage damage collected from a fallow field by PTM larvae (B).

Figure 2.12: Leaf damage of a potato plant by a fifth instar PTM larva collected from a fallow field in Bathurst.

All larvae collected from the potato foliage were stored at -20°C. These samples were exposed to a crude differential centrifugation extraction, in order to obtain a suspension sample of each to view using a transmission electron microscope to confirm the presence of any viral particles All samples were analysed and virus particles were not observed (data not shown). Occlusion bodies of an expected size of 425 - 450nm in length and 225 - 250nm in width were not observed, suggesting that PhoGV was not present in the samples.

2.3.5 Virus identification of larvae collected from seed potato crates

Potentially symptomatic larvae were collected from the Patensie site, while searching for seed potato tubers in storage crates. Two observations were made. One was that of dead, flaccid creamy-brown larvae lying on the surface of the potato tubers (Figure 2.10 A). The other observation was the presence of live larvae which were slow in movement and milky in appearance (Figure 2.10 B).

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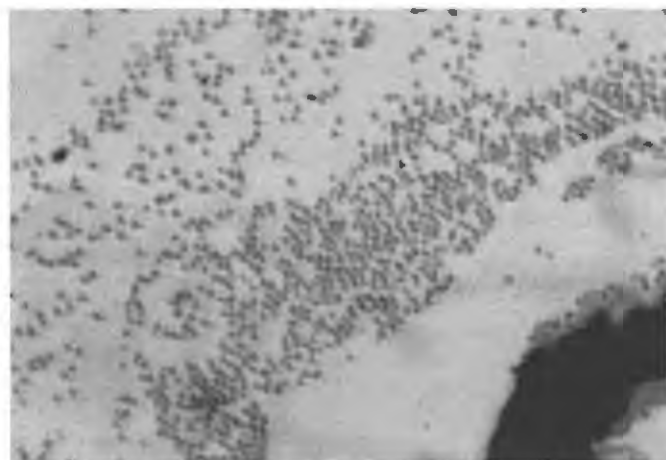
Figure 2.13: Larval smear viewed by light microscopy. Particles of approximately 650 - 700nm were observed.

An extract of symptomatic larvae shown in Figure 2.10 was viewed by light microscopy. Crude extract from the milky larvae observed (Figure 2.10 B) contained small particles of the same shape and size of approximately 650 - 700nm, suggesting the presence of a virus. The suspected virus-infected larval samples were viewed by TEM (Figures 2.13 and 2.14). Particles of approximately 650 - 700nm were observed but were too large in size to be PhoGV as the virus is 425 - 450nm in length and 225 - 250nm in width. The morphology of the particles in Figure 2.14 did not resemble that of a virus, when compared with the results of a study described by Laarif *et al*. (2006).
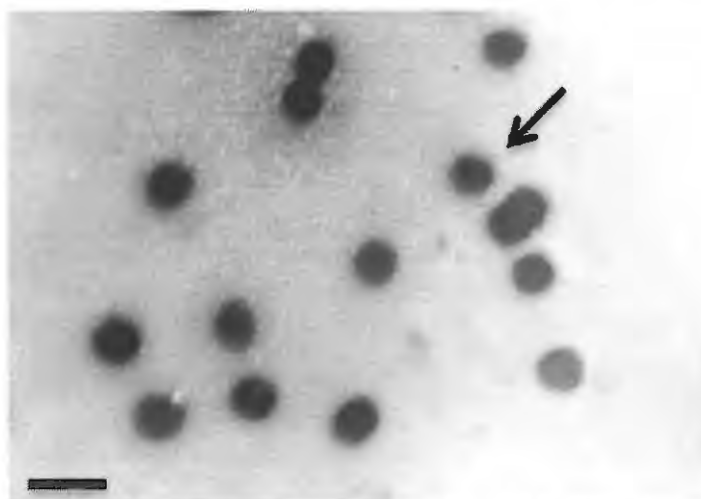
Figure 2.14: Crude extract of PTM field larvae viewed by TEM. Particles of approximately 650 - 700nm were observed (Scale bar represents 1000nm).

2.4 DISCUSSION

All four sites surveyed for PTM presented evidence of PTM infestation, with Patensie and Howick displaying high levels of infestation. Infested tubers collected from Patensie and Howick had 100%

infestation of all the tubers collected, but the average number of moths which eclosed from the tubers varied.

An explanation for the difference in adult emergence from infested tubers could be a result of the type of potatoes the insects were found on. Patensie tubers were obtained from stored seed potatoes, whereas Howick tubers were collected from table potatoes in the field. As the Howick tubers are table potatoes, they are sold to the market for consumption, unlike seed potatoes which are reused to plant the following year's crop. As table potatoes are grown for human consumption, residue restrictions lead to usage of fewer and safer pesticides than is the case on seed potatoes. With seed potatoes, the amount of pesticide applied is not limited or monitored extensively as the farmer's intention is to ensure that there are seed tubers to continue the crop (J. Berriman, personal communication). The lower usage of pesticides on seed potatoes could explain why more PTM emerge from the Howick samples.

Collection time may also be a factor explaining the difference in emergence between Patensie and Howick. Patensie tubers were collected in April 2009 and Howick tubers were collected in January 2010. April in South Africa is the start of autumn season when potato foliage dies and potatoes are harvested. In Howick, the potato foliage was still alive and it was approximately 2 - 3 weeks before the foliage would start dying. The tubers in Patensie were entering into cooler temperatures but Howick was still experiencing summer-like temperatures of above 20°C, therefore the development of the larvae in storage would not be comparable to those in the field in warmer temperatures, such as Howick.

Adults were collected at Patensie, Ivanhoe and Howick sites, but adults were the only life stage collected in the field at the KwaZulu-Natal sites, which can be explained as it was towards the end of the growing season and the plants were about to undergo vine-kill for tuber harvesting to occur. At the end of the growing season, the larvae begin to move into the soil to infest the tubers (Radcliffe, 1982; Hamilton, 2003), which would explain the lack of larvae on the foliage.

Larvae were collected from potato foliage that had been obtained in Bathurst, but no baculoviral symptoms were evident. Patensie was the only site where larvae displayed discolouration, but this symptom contrasted with known symptoms of PhoGV infection. None of the larvae were completely milky, some displayed immobility and some larvae were found flaccid, with their bodies still intact. The latter observation was evidently not indicative of a baculovirus infection, and the light microscope and TEM results indicated that no pathogen was present. The milky coloured

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larvae from Patensie contained circular particles of 650 - 700nm in diameter, whereas PhoGV has been described as oval, ranging from 400 to 425nm in length and 225 to 250nm in width (Laarif *et al.*, 2006). The observations of dead larvae may be a result of a pesticide or other chemicals that had been applied to the seed potatoes. The farmer claims not to have applied any pesticide to the seed potatoes within the previous 4 months, yet when the seed potatoes were placed in the unrefrigerated storage facility a small amount of pesticide powder (endosulfan) was sprinkled over crates as a precaution. This pesticide may have been present in a small enough concentration to affect the larvae slowly over the period of development.

There are two purposes for surveying PTM infestation in the field: (1) to attempt to find PTM larvae which display symptoms of PhoGV infection, and (2) to collect insects in order to rear them in the laboratory. Locating PTM in the field has been considered easy because PTM populations have established in potatoes fields and the insects have been collected for laboratory purposes (Fenemore, 1977; Briese, 1980; Broodryk and Pretorius, 1974; Chi, 1988; Chi and Getz, 1988; Kroschel and Koch, 1994, Trivedi *et al.*, 1994; Keller, 2003). PTM has been reported in most of the potato production zones found within the equatorial and tropical climate belts worldwide (Kroschel and Sporleder, 2006; Chumakov and Kuzentsova, 2009). Over 90 countries and all continents except Antarctica have reported PTM infestations (Chumakov and Kuzentsova, 2009). In South Africa, PTM populations are established wherever potatoes are grown, throughout all provinces (Broodryk, 1971). In this study, the identification of sites with PTM infestation was successful.

Successful isolation of PhoGV from PTM in the field has been reported in several studies (Lacey and Authurs, 2008). PhoGV has been characterised and found in South America (Alcázar *et al.*, 1991; Alcázar *et al.*, 1992b), North America (Hunter *et al.*, 1975), Africa (Broodryk and Pretorius, 1974; Laarif *et al.*, 2003; Visser, 2004), the Middle East (Kroschel and Koch, 1994), Asia (Zeddam *et al.*, 1999), and Australia (Reed, 1969; Briese, 1981). Broodryk and Pretorius (1974) isolated the first infection of PhoGV in South Africa, and only in 2004 was PhoGV recorded to be isolated from field-collected insects (Visser, 2004).

The successful isolation of PhoGV in South Africa, and the extensive presence of the virus worldwide in PTM populations, suggests that isolation of PhoGV would be unproblematic. All four sites in this study were examined for disease-infected larvae. The symptoms associated with PhoGV infection are a milky-white discolouration, compared to healthy larvae which are a translucent green or brown (Matthiessen *et al.*, 1978; Evans and Shapiro, 1997; Federici, 1997; Laarif *et al.*, 2006). A behavioural symptom, not always noted, is that fully grown diseased larvae are not able to orientate

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themselves for pupation and that the insects are found clustered around food sources such as leaves, stems and tubers (Reed and Springett, 1971).

Several attempts have failed to find baculovirus-infected larvae in the field; therefore rearing of the insects in the laboratory was implemented in order to induce an infection (Broodryk, 1971; Broodryk and Pretorius, 1974; Visser, 2004; Mascarin *et al.*, 2010). Although PhoGV has been found in PTM worldwide (Briese and Mende, 1981) and baculoviruses are known to be abundant in the environment (Black, 1999), the incidence of viral infection in the field is naturally low (Reed, 1971), which may explain why diseased larvae were not successfully identified at the collection sites. Broodryk and Pretorius (1974) is the only published report of PhoGV in South Africa, but there is a comprehensive unpublished dissertation on PTM and PhoGV (Visser, 2004). Furthermore, Broodryk and Pretorius (1974) and Visser (2004) isolated virus from laboratory cultures, suggesting that rearing the insects could stress them sufficiently to stimulate induction.

In conclusion, surveys were carried out in both seed potatoes and table potato fields, as well as in an unrefrigerated storage facility, at sites in the Eastern Cape and KwaZulu-Natal provinces. Potentially symptomatic larvae were collected only from the Patensie site, but on analysis these proved negative for PhoGV. Adult moths and healthy larvae were collected in hope that vertical transmission would result in a baculoviral outbreak within the PTM colonies in the laboratory, as reported by Broodryk and Pretorius (1974) and Visser (2004). The following chapter describes the bioprospecting for PhoGV in the field and the collection of live insects to be used in laboratory rearing of the insects, with the objective of inducing virus.

CHAPTER 3

THE REARING OF *PHTHORIMAEA OPERCULELLA* IN THE LABORATORY

3.1 INTRODUCTION

Attempts to isolate virus from Potato Tuber Moth (PTM) larvae in the field (Chapter 2) were unsuccessful, therefore laboratory cultures of *Phthorimaea operculella* were required in an attempt to induce the virus (Chapter 4). This chapter describes efforts to determine the most efficient and cost effective laboratory rearing technique for PTM.

The first rearing method for *Phthorimaea operculella* was published by Finney *et al.* (1944). This method was further refined by Finney *et al.*, (1947) and this formed the basis for rearing these insects in the laboratory. Rearing of potato tuber moth in the laboratory for research purposes has been adapted and studied extensively (Bartlett and Martin, 1945; Platner and Oatman, 1968; Reed, 1971, Broodryk, 1971; Cardona and Oatman, 1975; Meisner *et al.*, 1974; Griffith *et al.*, 1979; Singh and Charles, 1977; Etzel, 1985; Rahalkar *et al.*, 1985; Musmeci *et al.*, 1997).

Briese (1980) attempted to rear PTM on potato tubers rather than the potato plants, which reduced excessive maintenance, such as watering on a regular basis, or ensuring that the plants were completely sterilized. *Phthorimaea operculella* was kept at room temperature on tubers in a ventilated container with a 12 hour photoperiod. Tubers were punctured to ensure that the neonates could enter the root efficiently. Once PTM had pupated, they were transferred to a separate oviposition cage. The cages were supplied with 10% honey solution, and wax paper for oviposition (Briese, 1980). Das *et al.*, (2007) used a similar rearing technique, except that the adult PTM were transferred to glass containers to reduce the number of eggs being laid on the sides of the container but rather on the roof of the container onto wax paper.

Many artificial diets have been tested, however all have resulted in a lower larvae survival rates than tuber diets. The first attempt to rear *P. operculella* on an artificial diet had success rate of 81.9%, at a temperature of 30°C (±2°C) with a 16 hour photoperiod, and neonate larvae were transferred onto the diet (Singh and Charles, 1977). A few decades later, Kashyap *et al.*, (2008) developed an artificial diet and compared survival rates with larvae grown on potatoes. Survival on tubers was significantly higher than on the artificial diet. It has been generally accepted that to rear

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P. operculella on an artificial diet is difficult, and largely unsuccessful (Singh, 1974; Singh and Charles, 1977; Gomaa *et al.*, 1978). Consequently, it is easier to rear PTM on tubers than using an artificial diet (Griffith *et al.*, 1979), however tubers are difficult to sterilise and can result in contamination if not washed and maintained correctly.

The major objective for laboratory rearing is to produce large numbers of healthy individuals, but there are many problems associated with rearing live insect cultures. Such problems include contamination by fungi, viruses and bacteria, or infestations of other organisms (Finney *et al.*, 1947). An easy means of sterilising a colony is to surface-sterilise the egg sheets using sodium hypochlorite, as it ensures that all virus and other surface contaminations are all removed (Ludewig, 2003). Overcrowding of insects and poorly ventilated containers create ideal micro-environments for organisms to grow, which can be harmful to workers (Findlay, 1975). Lepidoptera produce scales which are known allergens and are a health hazard when inhaled (Davis and Jenkins, 1995), therefore care needs to be taken when working with cultures, masks should be worn or cultures handled in a room with an extractor fan. Another problem is preventing adults and larvae escaping from cultures (Etzel 1985; Rahalkar *et al.* 1985).

An understanding of the production costs for laboratory rearing is an important issue. Cost analysis refers to a process whereby the economic costs of a project are analyzed in order to determine whether the project is viable for investment purposes (DEAT, 2004). The expenses per rearing technique will differ depending on the size of the culture. Small-scale rearing is considered to be a lab culture of approximately 500 moths, whereas a medium culture consists of 10 000 moths (Visser, 2004). The expenses and requirements for a large rearing facility are described by Fisher and Lappla (1985) and Goodenough and Parnell (1985).

There are important factors to consider for optimal rearing of PTM under laboratory conditions. Moths tend to lay their eggs from the second day of emergence, as they copulate on the first day (Fenemore, 1977; Visser, 2004), and the females prefer to lay eggs on rough surfaces rather than smooth (Finney *et al.*, 1947). The number of moths per oviposition container does not influence female fertility (Traynier, 1983). Moths will postpone egg laying when exposed to bright light (Broodryk, 1971), when adults do not receive nutrition (Labeyrie, 1957) or when there is poor larval nutrition (Meisner *et al.*, 1974). If reared at temperatures between 25°C and 30°C, PTM development from egg to adult takes approximately 17 - 24 days (Briese, 1980; Visser, 2004). However, oviposition will occur between 11°C to 29°C and over a wide humidity range, suggesting the moths are highly adaptable to various climatic conditions (Broodryk, 1971). Fertility can vary

within PTM populations, where 2 - 232 eggs can be laid per female (Broodryk, 1971; Al-Ali *et al*, 1975; Visser, 2004). Adults fed water or honey solution survive longer and lay more eggs (Fenemore, 1977; Visser, 2004). The sex ratio for laboratory cultures has been found to be approximately 1:1 (Al-Ali *et al.*, 1975; Cardona and Oatman, 1975; Briese, 1980). In some PTM populations, development is faster and the life span longer in females than in males (Koizumi, 1955; Briese, 1980) while in others the reverse is true (Cardona and Oatman, 1975; Fenemore, 1977; Singh and Charles, 1977).

The aim of the work reported in this chapter was to establish PTM successfully in the laboratory by the most efficient, cost effective and least labour intensive method. The laboratory cultures were monitored for viral outbreaks and used to determine an effective production protocol for rearing the insects.

3.2   MATERIALS AND METHODS

3.2.1   Source of PTM

PTM larvae used in all rearing methods were obtained from the field and storage collections done in Patensie, Howick and Ivanhoe areas (chapter 2). Larvae and adults collected from the sites were used as the parental generation (P1) for initiating the laboratory rearing of each PTM culture.

3.2.2   Rearing of PTM

Three insect cultures of fewer than 1000 adult moths were reared, separately in two constant environment (CE) rooms. The Patensie culture was raised in one CE room, while the two KwaZulu-Natal cultures were raised in another. The Ivanhoe and Howick cultures were kept on separate sides of the CE room and confined to cages, to ensure that insects from the one culture did not contaminate the other. During the rearing process, containers were handled as little as possible in order to ensure minimal disruption to the insects. For the three rearing techniques used, cultures and generations within cultures, the number of eggs laid and number of adults emerged were recorded. All dead moths were collected from the containers and sexed.

3.2.2.1 Rearing method one

Phthorimaea operculella was first reared in the laboratory by adapting the method described by Briese (1980) and Visser (2004) (Figure 3.1). The culture was kept at a constant temperature of 27°C and a photoperiod of 12hours: 12 hours. Humidity was not controlled in the rearing room.
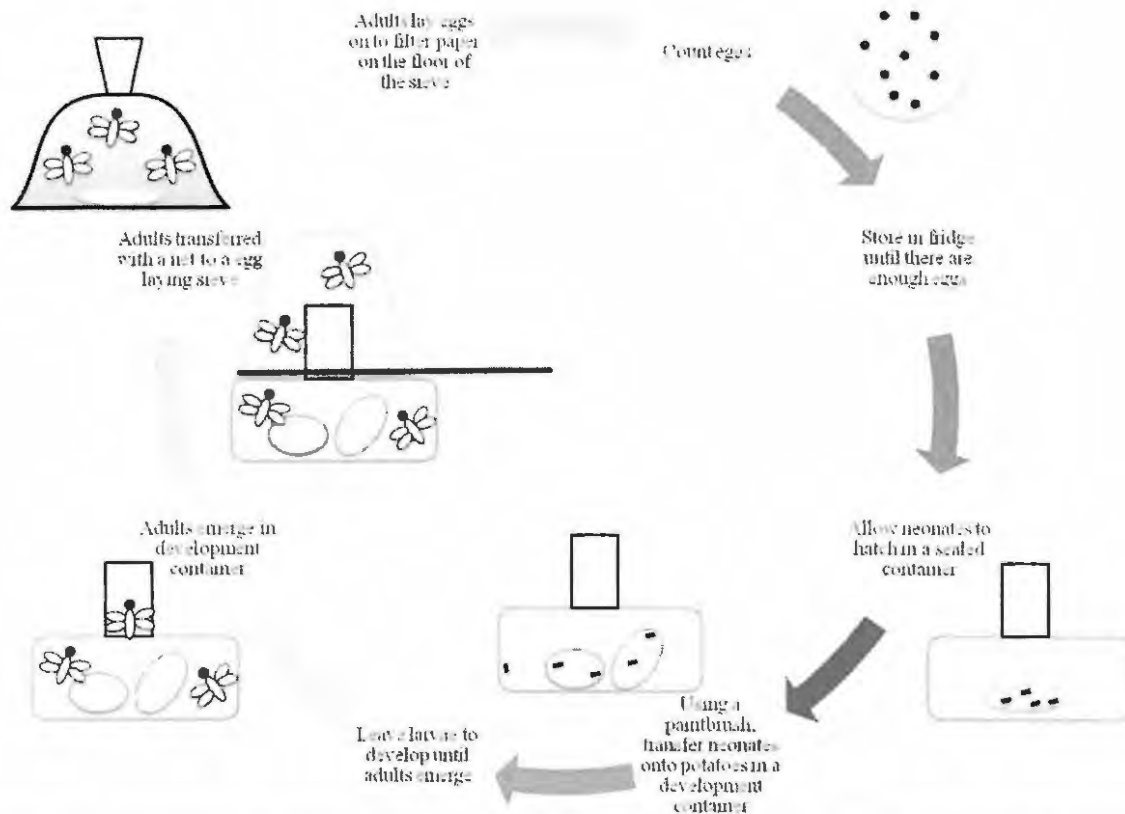
Figure 3.1: Summary of rearing method one, for *in vivo* rearing of Potato Tuber Moth (adapted from Briese, 1980; Visser 2004).

Infested potatoes collected from Patensie, were placed into containers lined with autoclaved river sand (Figure 3.2). Ventilation was achieved by fixing a container with mesh-covered opening to the lid of the development container (Figure 3.2). The ventilated upper container also provided an easy means of collecting the adults once they emerged because the moths fly upwards.

Figure 3.2: Development container in which infested potatoes were stored until adults eclosed from pupae which were present in the river sand.

Once the PTM adults had eclosed, they were transferred to an oviposition cage through a funnel fixed to the top of the sieve (Figure 3.3). Oviposition cages consisted of a metal sieve placed on top of wax paper, allowing for easy removal of eggs (Figure 3.3). Eggs were collected and stored in a fridge at 4°C until there were sufficient eggs for the hatchling container (Visser, 2004). The funnel on top of the sieve was blocked with sterile cotton wool which was soaked in 10% honey solution and replaced daily (Figure 3.3).

Figure 3.3: Oviposition cage where adult PTM were placed in order to lay eggs on the wax paper floor of the containers.

Wax sheets covered with eggs were placed in separate airtight containers and left to hatch (Visser, 2004). Once hatched, larvae were counted and transferred onto potatoes in a clean sterile development container, using a fine paintbrush. Potato tubers were punctured to allow access to the potato tissue. Approximately 2g of potato tuber was used per larva in a development container, as recommended by Singh and Charles (1977) and Briese (1980).

3.2.2.2 Rearing method two
After 3 months of not being able to achieve better than a 40% survival rate, a second rearing method adapted by Visser (2004) and Headrick and Jones (2007) was implemented and adjusted to suit the rearing facility (Figure 3.4). The insects were maintained at 24°C, humidity was not controlled and insects developed under a 12hour:12hour photoperiod.

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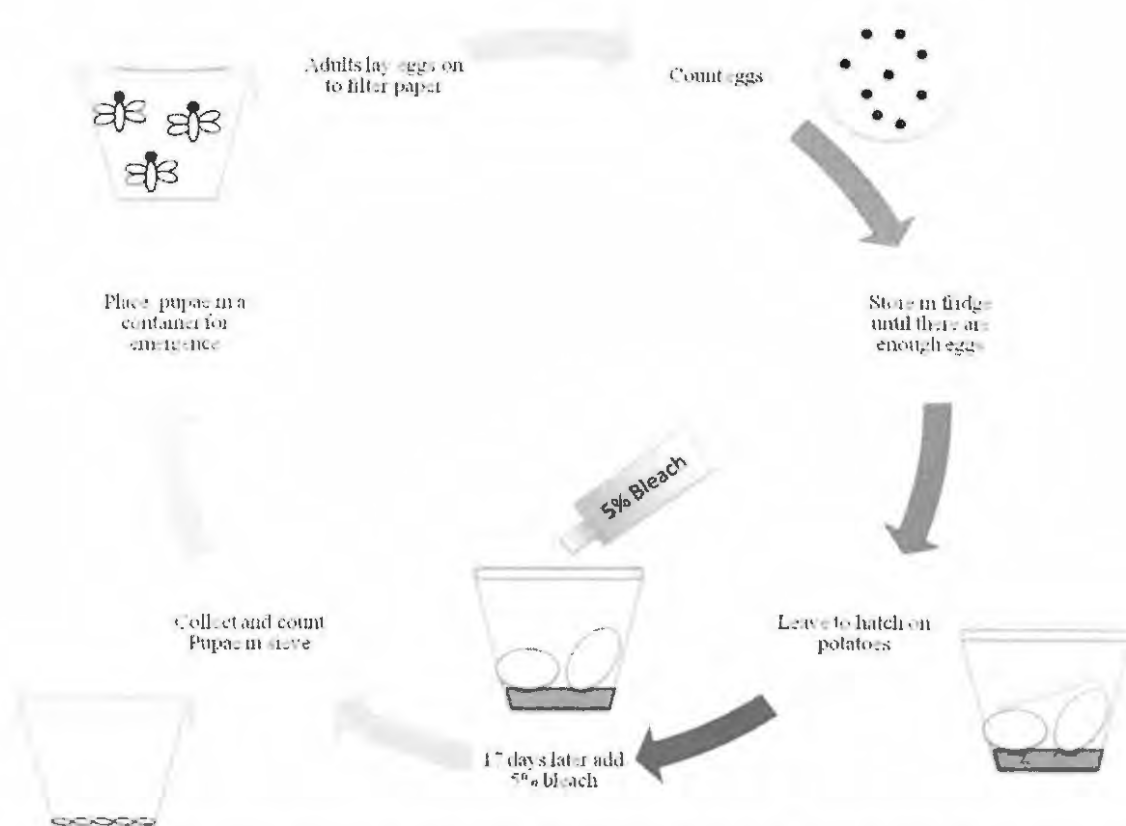
Figure 3.4: Summary of rearing method two for Potato Tuber Moth (adapted from Visser 2004; Headrick and Jones, 2007).

Eggs laid and counted on Whatman filter discs were placed in development containers lined with perlite or vermiculite. Potatoes were pierced with a needle in order to have entry points for the neonate larvae. Approximately 2g of potato is a sufficient amount of food per larva (Singh and Charles, 1977; Briese, 1980). Emergence containers were covered with mesh and left for 18 days at 24°C (±2°C) in a controlled environment (Figure 3.5).

Figure 3.5: Hatching container used in the host rearing method of Potato Tuber Moth. The pierced potatoes are infested by neonates of PTM upon hatching from egg sheets. The floor of the container is lined with perlite which acts as a medium in which the 5th instar larvae pupate.

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On the 17th day after hatching, the potatoes were removed. Any larvae still present within or on the potato were transferred to a new container and left to pupate. The perlite and pupae lining the development container were then subjected to 5% bleach (NaOCl) for 2 minutes, while stirring slowly. The content of the container was poured through a sieve, on which all perlite and pupae were retained. The perlite and pupae were rinsed twice with tap water. The content was then sifted to remove all pupae present. A total of 200 pupae were placed into a single emergence container, which was covered with mesh (Figure 3.6).

Figure 3.6: Adult emergence containers with pupae. The adults eclosed from the pupae and lay eggs on the egg sheets placed above the mesh.

After approximately 4 - 5 days at 24°C (±2°C) in a controlled environment room, adults emerged from the pupae (Figure 3.7) and Whatman filter discs were placed onto the top of the mesh. A petri dish was placed on top to force the filter disc downward (Figure 3.6). Water was given to the adults daily by wetting a string of wick.

Figure 3.7: Adult emergence containers for host rearing of Potato Tuber Moth. The adults emerge from the pupae in the container and lay eggs on the egg sheets placed above the mesh.

3.2.2.3 Rearing method three

A third variation of rearing PTM was attempted after 12 months once the colony was stable and well established. This method arose as a combination of the first and second rearing methods adapted from Briese (1980), Visser (2004) and Headrick and Jones (2007). The cultures were reared at 24°C with the 12hour: 12 hour photoperiod, and humidity not controlled (Figure 3.8).
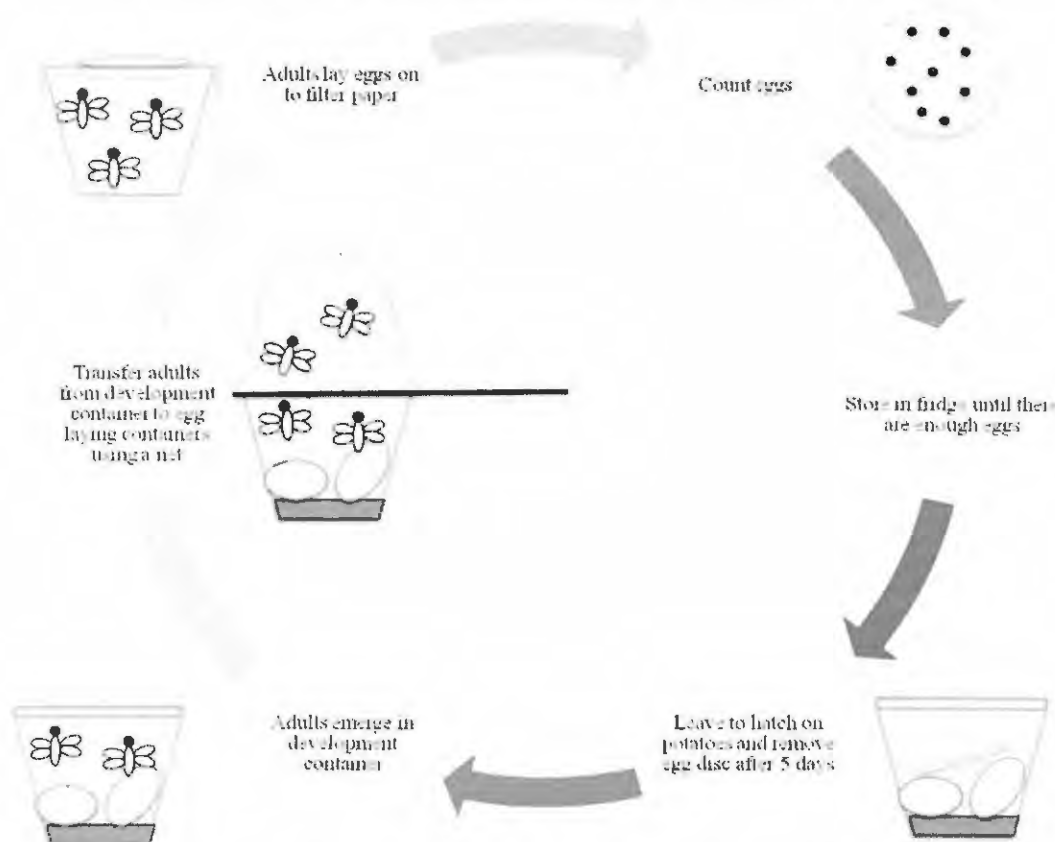
Figure 3.8: Summary of rearing method three, for *in vivo* rearing of Potato Tuber Moth

Eggs laid on the Whatman filter discs were counted and placed into the fridge until there were sufficient numbers to set up a development container. The egg sheets were placed, with eggs on the lower surface, onto pierced potato tubers. The discs were left in the containers for 5 days, and then removed. The containers remained undisturbed for the full development of PTM from larvae to adult at 24°C.

Once adults began to emerge from development containers after approximately 23 – 27 days, they were transferred to emergence containers using a net. Adults were caught using a clear sealable container (Figure 3.9 A) and transferred into the emergence container (Figure 3.9 B). The adults laid eggs on the filter discs placed onto the mesh of the container (Figure 3.7), and were fed water

daily through a dropper onto the mesh of the container (Figure 3.9 C). Every 3 days, eggs sheets were collected and stored in a fridge at 4°C.
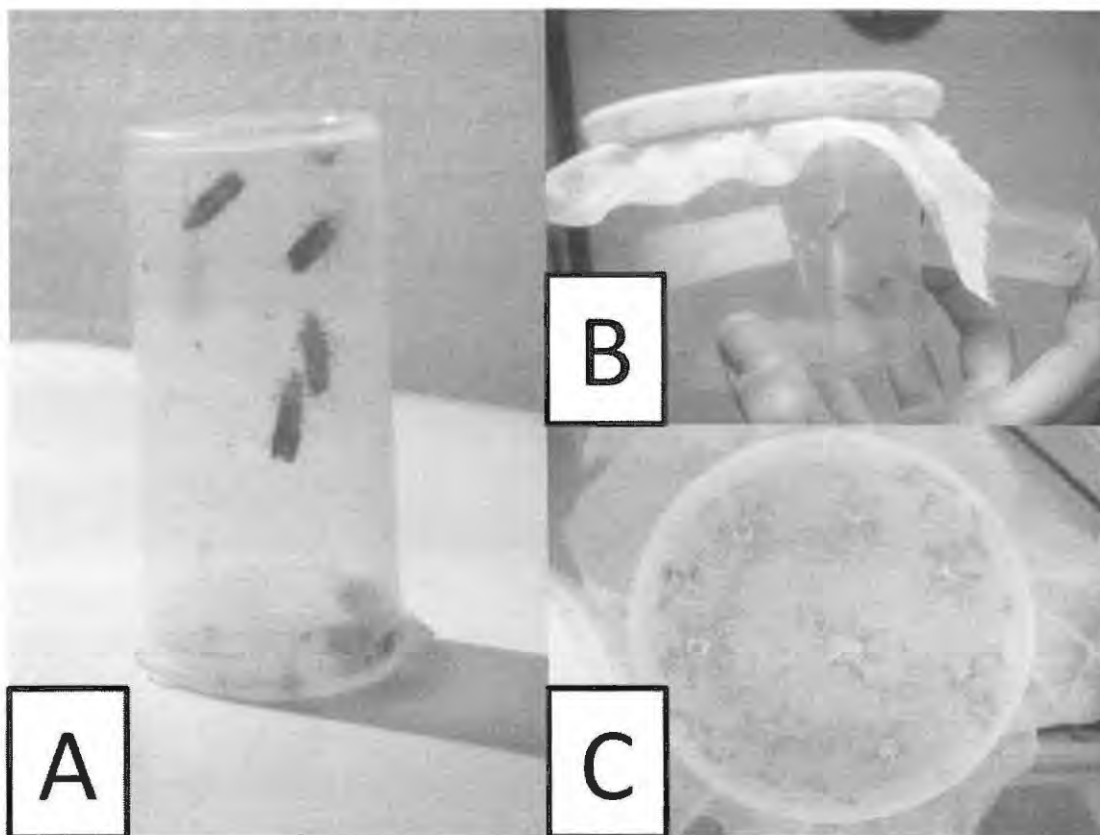
Figure 3.9: Adult moths are collected from the transfer net using a clear sealable container (A). The containers are placed alongside the emergence container where there is a hole allowing the moths to pass through (B). When droplet feeding the moths daily with water, they all converge around the droplet to drink (C).

To ensure sterility, all containers were washed in 15% bleach. All counters and surfaces were wiped down daily with 70% ethanol and 5% Virkon® S (DuPont) in order to minimise viral and bacterial contamination.

3.2.3 Fitness of laboratory cultures

3.2.3.1 Survival rates of the laboratory cultures

The survival rates of the three laboratory cultures were determined as shown in the equation below. The survival rate indicates the success of the rearing technique.

$$\text{Survival rate (\%)} = \frac{\text{Number of adults emerged}}{\text{Number of eggs laid}} \times 100$$

3.2.3.2 Fertility

Fertility was determined by the number of eggs laid per female. This indicates the average number of eggs that one female had laid in their lifecycle. The following equation was used:

$$\text{Fertility} = \frac{\text{Number of eggs laid}}{\text{Number of females}}$$

3.2.3.3 Sex ratio

Dead adults were collected from the emergence containers and sexed, allowing for the determination of a ratio between the genders. Two morphological differences were used to distinguish between males and females. At the end of the abdomen of males is a brush-like structure whereas in females the abdomen has a conical shape with no brush like structure (Figure 3.10 A). The second morphological difference between genders is on the wings of the insect. Males have 2 to 3 dots on each forewing, whereas the females have a cross on the forewings (Chauhan and Verma, 1991; Rondon *et al.*, 2007; Rondon and Xue, 2010) (Figure 3.10 B). The adults were sorted using abdominal structures, because it was difficult to distinguish between the differences in forewing markings once the insects had died. Numbers of males and females were recorded for each laboratory culture.

Figure 3.10: The female PTM moth has a conical abdomen (Left) whereas the male moth has a brush-like structure on its abdomen (Right) (A). On the forewings, the females have a cross (Left) and the males have 2-3 dots (Right) (B).

3.2.4 Budget analysis for rearing PTM in the laboratory

Expenses involved in the rearing of PTM were documented for each rearing method. Equipment, labour and consumables were decided upon for each rearing technique based on previous studies (Briese, 1980; Moore, 2002; Visser, 2004; Headrick and Jones, 2007). A labour expense of R77 per

hour was based on the current salary rate for graduates at Rhodes University for 2010. The number of hours required to complete daily monitoring and maintenance was recorded. Costs of major equipment and consumables for rearing included plastic containers, mesh, elastic bands, potato tubers, bleach and perlite.

3.3   RESULTS

3.3.1   Fitness of laboratory cultures

3.3.1.1 Survival rates of laboratory cultures

Survival rates of PTM varied between rearing techniques. In the Patensie culture, F1 and F2 generations were raised according to rearing method one (Briese, 1980; Visser 2004); F3 to F8 generations were reared according to rearing method two (Visser, 2004; Headrick and Jones, 2007) and generations F9 to F11 were raised using rearing method three (Figure 3.11).
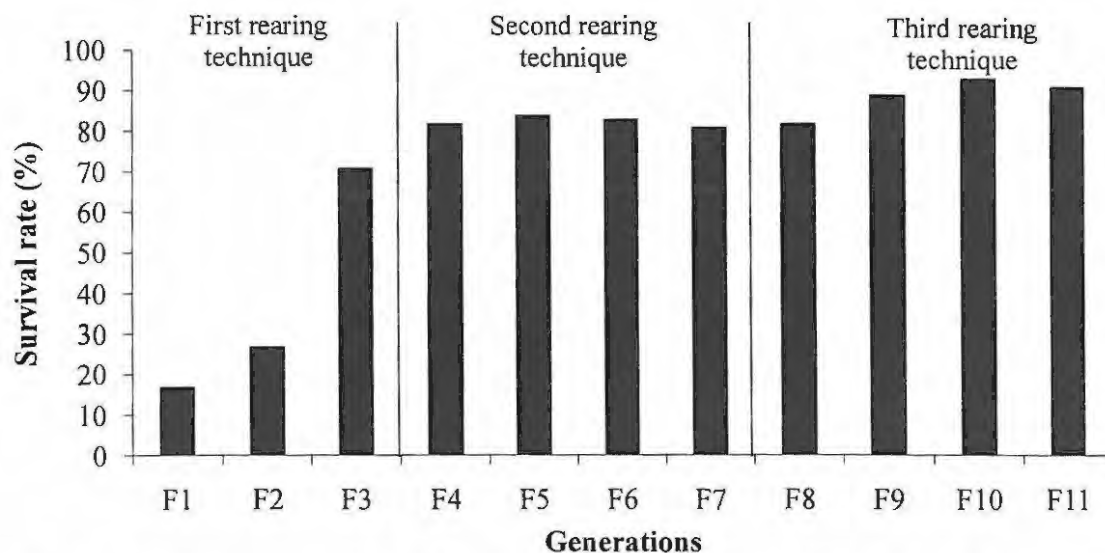
Figure 3.11: The percentage survival of Potato Tuber Moth collected from Patensie

Rearing method one had a low survival rate, with the F2 generation showing only 27% survival rate (Figure 3.11). When the rearing technique was altered, the survival rates increased to a minimum of 82% by the F4 generation. The rates became stable over the F4 - F7 generations, varying between 81 - 84%. Rearing method three was initiated from the F8 generation which had a minimum survival rate of 82%.

Ivanhoe and Howick laboratory cultures were raised according to rearing technique two and three, as they were collected in January 2010, approximately 8 months after the Patensie culture. Generations F1 and F2 were reared using the second rearing technique, whereas F3 to F5 generations were reared using rearing method three (Figure 3.12).

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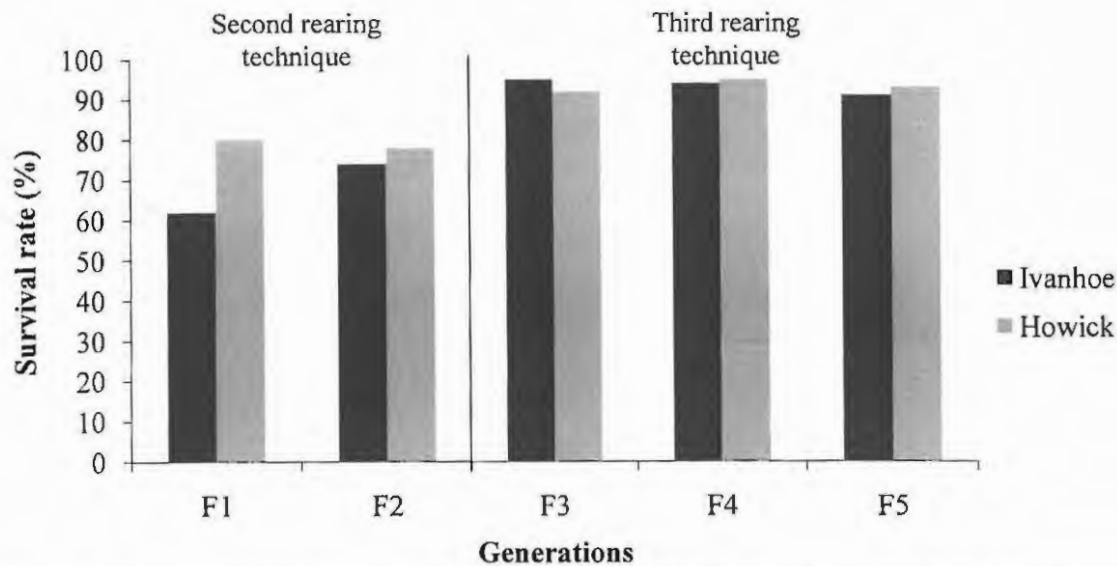
Figure 3.12: The percentage survival of Potato Tuber Moth collected from Ivanhoe and Howick.

The initial generation of the Ivanhoe culture was low, with a 62% survival rate, whereas the Howick culture had 80% survival rate (Figure 3.12). However, the Ivanhoe culture increased in its survival rate in the second generation to 74%, and the Howick culture decreased slightly to 78%. When the rearing technique was amended to method three, survival rates of both cultures increased. The Patensie (Figure 3.11), Ivanhoe and Howick cultures indicated an increase from rearing method two to rearing method three (Figure 3.13).

In order to determine the impacts of the various rearing techniques on survival of the insects, the average survival rate was determined for each rearing method (Figure 3.13).
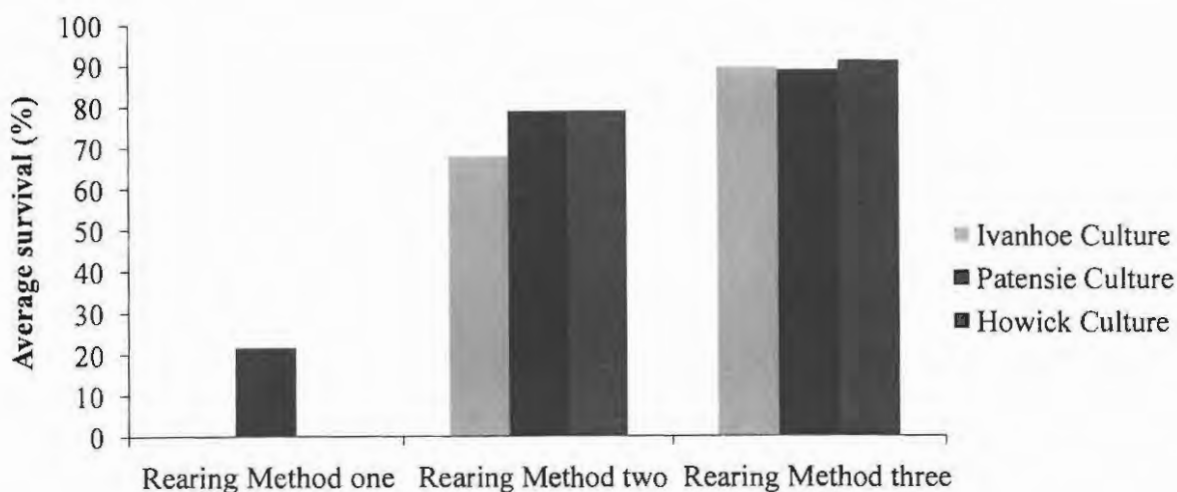
Figure 3.13: The average survival percentage of each culture for all three rearing techniques.

The average survival rate per rearing method for each culture showed an increase from the first to the third rearing technique (Figure 3.13). Patensie was the only culture subjected to rearing method one, with a survival rate of 22%. Ivanhoe and Howick cultures were collected within 4 days of each other in KwaZulu-Natal, and therefore were subjected to laboratory rearing at the same time. These cultures were introduced into the laboratory after rearing method two had been established. The survival rates for the two cultures using rearing method two, indicates that the Howick culture established with the same average survival as the Patensie culture of 79% (Figure 3.13), but the Ivanhoe culture had an average survival of only 68% (Figure 3.13). The Howick culture increased to 91% and the Ivanhoe culture to 90%. The Howick culture adjusted to the laboratory conditions as successfully as did the Patensie culture (Figure 3.12). The Ivanhoe culture did not establish well in the laboratory especially in the F1 and F2 generations (Figure 3.12), however once the insects had been reared according to rearing method three, the survival rates of generations started to resemble those of the Howick and Patensie cultures (Figure 3.12).

3.3.1.2 Fertility of the PTM females

Fertility of female moths was determined according to the equation indicated in 3.2.2.5. Fertility was calculated for each rearing technique separately and for each laboratory culture (Figure 3.14).
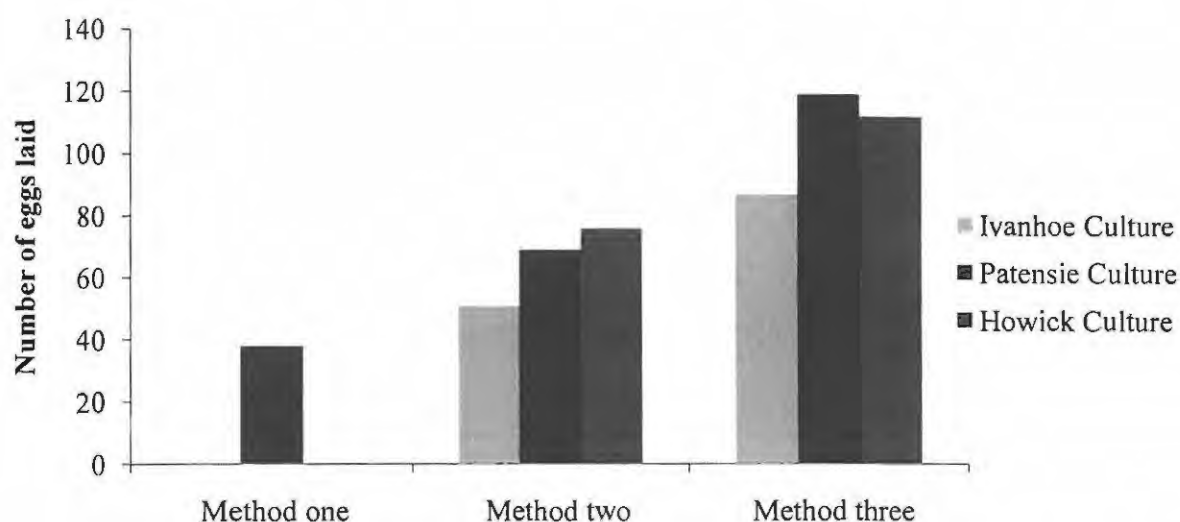
Figure 3.14: The average number of eggs laid by a single female moth during its life cycle using three different rearing methods.

The Patensie culture increased from 38 eggs to 69 eggs and then 119 eggs laid per female (Figure 3.14). In the second rearing technique, Howick females laid 76 eggs while Ivanhoe females laid only 51 eggs. Ivanhoe and Howick females increased their egg laying capabilities in the third rearing technique to 87 and 112 eggs per female, respectively.

3.3.1.3 Sex ratio

Using a dissecting microscope, dead adults were sexed according the abdominal morphological features illustrated in 3.2.2.6 and Figure 3.15.
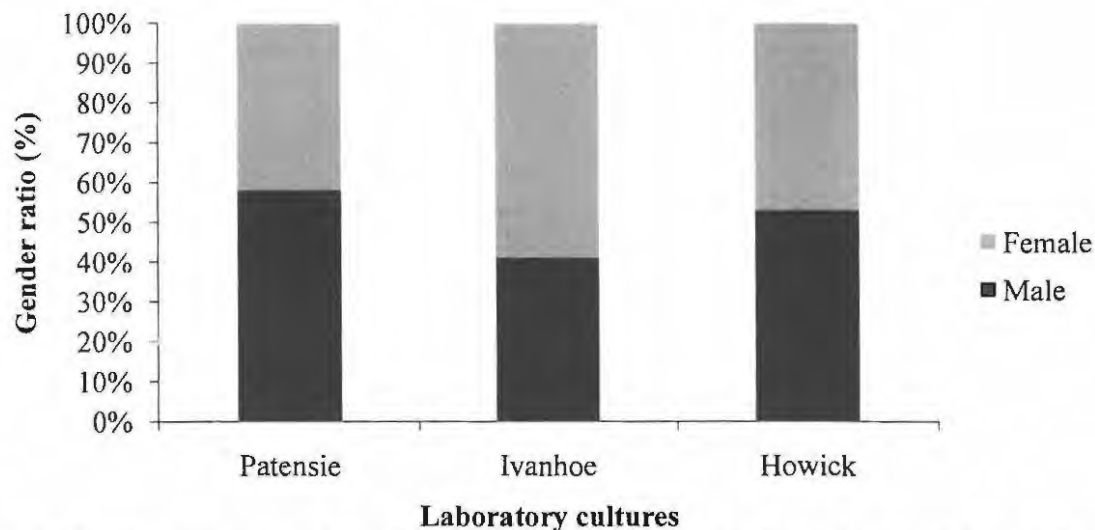
Figure 3.15: The sex ratio of the Potato Tuber Moths in the Patensie, Howick and Ivanhoe laboratory cultures.

There were more males than females in the Patensie and Howick cultures: Patensie had 58% males and 42% females and Howick had 53% males and 47% females (Figure 3.15). The Patensie culture had a 1:0.73 male to female ratio, and Howick had 1:0.89 male to female ratio. In the Ivanhoe culture there were more females than males (59% females and 41% males). The sex ratio was 1:1.44 male to female.

3.3.2   Budget analysis of PTM host rearing

In order to raise an insect culture in the laboratory for research or commercial purposes, there are costs involved. There are once-off costs which incorporate initial set up in the laboratory, equipment and collection of insects from the field or a previously established culture. Ongoing or monthly costs include the diet for the insects to survive, and labour. All expenses were recorded for each rearing method and are tabulated in Table 3.1.

Table 3.1: Expense budget for the rearing of PTM and production of PhoGV

Once-off cost	Quantity	Rearing method one	Rearing method two	Rearing method three
Plastic containers	R33 x 25 containers		R 66.00	R 66.00
Mesh	R11.95 per metre		R 35.85	R 35.85
Elastic bands	R19.99 per 100 bands	R 19.99	R 19.99	R 19.99
Large containers (24L)	R24.99 per container	R 299.88		
Labels	R38.99 per box	R 38.99	R 38.99	R 38.99
Insect net		R 11.98		R 11.98
Plastic tablet container	R3.00 per container	R 15.00		R 15.00
Wooden roller	R90.00 each	R 270.00		
Sieves	R 35.99 per sieve	R 107.97	R 35.99	
Bleach (2L)	R17.99 per litre		R 35.98	
TOTAL		**R 763.81**	**R 232.80**	**R 187.81**

Monthly cost	Quantity	Rearing method one	Rearing method two	Rearing method three
Labour	R77 per hour	100 hours = R7700.00	60 hours = R4020.00	30 hours = R2310.00
Diet	R10.99 per kg	R 43.96	R 43.96	R 43.96
Perlite	R24.99 per 500g	R 24.99	R 24.99	R 24.99
Wax paper	R16.49 per Roll	R 49.47		
Masking tape	R7.99 per Roll	R 15.98		
TOTAL		**R 7834.40**	**R 4088.95**	**R 2378.95**

*All this data is for a maximum 1000 moths and 10 hatching containers per generation

Rearing method one was the most expensive for both once-off (R763.81) and monthly costs (R7834.40) (Table 3.1). The expenses involved in rearing method two are much lower than rearing method one, and higher than rearing method three (Table 3.1). The monthly expenses resemble the same pattern seen in the once-off costs, the rearing method two cost R4088.95, while rearing method three cost R2378.95 (Table 3.1). The expenses described for this study have been developed and recorded for the period of the investigation. The labour expense was based on the number of hours and frequency of required maintenance.

3.3.3 Budget analysis and characteristics of false codling moth host

In order to determine the efficacy of the three rearing protocols, the rearing of PTM was compared to *Cryptophlebia leucotreta* Meyrick, False Codling Moth (FCM). The expenses of rearing the FCM culture were obtained from Chambers (personal communication) (Table 3.2). This insectary is well established and is now being used to commercially produce a baculovirus product, called Cryptogran®. Cryptogran® contains a naturally occurring baculovirus, *Cryptophlebia leucotreta*

granulovirus, which is highly specific and an effective biological control agent for FCM. The biopesticide has been registered in South Africa and is available in suspension concentrations to be applied on citrus (Moore, 2002). The purpose of this information is to compare the rearing costs of PTM to FCM for commercial production.

Table 3.2: Expense budget for the rearing of FCM (Chambers, personal communication).

	Once off expenses	Monthly expenses	Egg to adult (days)	Fecundity	Eggs laid per month	Larvae per jar	Survival rate (%)	Sex ratio	Diet
FCM	R 550 000	R 20 790	23	540 eggs per female	10.6 million	247.8	>95%	1:1	Artificial

In order to develop commercial production of PTM, the expenses involved need to be scrutinised to determine a cost-effective rearing means. Monthly costs and once-off costs were calculated for all three rearing methods. Rearing method one was the most expensive, both once-off and monthly. Expenses of methods two and three decreased for both the once-off and monthly costs, while rearing method three had the lowest expenses for both. The monthly costs are extremely high due to the number of hours required to perform maintenance for rearing method one, and as the hours of labour decrease so the expenses decrease. Comparing these expenses to that of an active commercial production of FCM, which is large-scale compared to the small-scale production of PTM, the FCM initial set up cost was R 550 000 and its monthly cost R 20 790 (Table 3.2). The expenses for FCM are more than the PTM, however the sizes of the cultures differ.

A comparison was carried out between FCM and PTM, and the small-scale laboratory Patensie culture was scaled up to a production scale such as FCM, in order to determine how successful PTM would be for commercial use (Table 3.3). Patensie culture characteristics were used from the second and third rearing technique, as the Patensie culture was the most stable culture and had the highest fertility and survival. The 1000 moths maintained for the PTM laboratory culture, were scaled up to the same number of moths and potential females as the FCM culture.

Table 3.3: Comparison between FCM and PTM rearing techniques (Chambers, personal communication).

Characteristics of cultures	FCM production scale	PTM			
		Rearing method two (Patensie culture)		Rearing method three (Patensie culture)	
		Laboratory culture	Production scale	Laboratory culture	Production scale
Neonate to adult longevity	23 days	23 days	23 days	23 days	23 days
Sex ratio	1:1	1:1	1:1	1:1	1:1
Eggs laid per female	540 eggs	69 eggs		119 eggs	
Number of moths	39260 moths	1000 moths	39260 moths	1000 moths	39260 moths
Number of females	19630 moths	500 moths	19630 moths	500 moths	19630 moths
Total eggs laid	10.6 million	34 500	1.36 million*	59 500	2.33 million*
Diet	Artificial	Tubers	Tubers	Tubers	Tubers

*Values were estimated based on current number of eggs laid per female

The production of FCM on large scale is 10.6 million eggs per 23 days, unlike the small scale Patensie PTM culture which varied between 34500 - 59500 eggs in 23 days. When PTM was scaled up to production scale, 1.36 million eggs would be produced by rearing method two and 2.33 million eggs by rearing method three. PTM therefore would produce fewer eggs than FCM when scaled up, but this can be optimised once an effective production method has been established and the culture has adapted to the rearing protocol. FCM is optimally reared on an artificial diet, whereas PTM is raised on the natural diet of potato tubers which are easier to maintain than artificial diet. The survival rate of PTM reached a maximum of 91% (Table 3.5), whereas FCM experiences 95% and greater survival per generation. However, when scaling PTM production, survival and other characteristics may decrease until the technique has been optimised for the insects.

3.4 DISCUSSION

In this study, several observations were made of the three rearing methods for the establishment of PTM cultures in the laboratory (Table 3.4).

Table 3.4: Benefits and problems associated with various rearing techniques

	Benefits	Problems
Rearing method one	• Able to monitor survival percentage and rates of most life stages	• Temperature of 27°C and poorly ventilated containers contributed to high levels of humidity and fungal contamination • Neonates and adults were stressed when moved from the one container to another • The process required daily attention and between 4-5 hours of work daily • Difficult to transfer adults into the sieve placed on wax paper • Honey solution for adults attracted ants • Large tubers were used because of the size of the development container, however not all the tuber was consumed leading to wastage
Rearing method two	• Cultures were attended to every 2 - 3 days • Neonates and adults did not need to be handled – so less stressed insects • Minimal fungal contamination and potato rot before adults emerged because of better ventilation • Eggs can be removed without opening the container • Water solution for adults did not attract ants • Small potatoes (size of an egg) were used to decrease diet wastage • Temperature of 24°C reduced the build up of humidity in containers	• Pupae removal step was time consuming and was detrimental to the pupae when carried out for too long or when the bleach concentration was too strong • Important that all pupae were at the same stage so that moths would not emerge at difference times and lay eggs later than others
Rearing method three	• Least labour intensive method, as containers only needed to be monitored every 4 days • Cultures required attention only when setting up eggs on potatoes and when collecting adults from development containers (±25days) • No contamination was noted and potatoes did not rot before adults emerged • Eggs can be removed without opening the container • Water solution for adults did not attract ants • Small potatoes (size of a egg) were used which ensured that most the tuber was consumed during rearing • Temperature of 24°C reduced the build up of humidity in containers	• Transporting insects from development to emergence container increases adult stress

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Each of the rearing methods described in this study had several benefits and problems associated with it (Table 3.4). Rearing method one was found to be beneficial for monitoring the various life stages, the method allowed for eggs, neonates and adults to be counted accurately for survival analysis. However, the insects were constantly stressed because of continual handling. There were several problems associated with rearing method one. The rearing temperature of 27°C, and the limited air movement within the rearing containers, stimulated high levels of humidity when the potato tubers respired. The condensation droplets formed by humidity became a problem as larvae drowned in the droplets. The increase in humidity created optimal micro-environments for the development of fungal contamination and soft rot of the tubers. These combining factors may explain the low survival of the Patensie culture in rearing method one.

The containers used in the first rearing technique were large and large, tubers were used as they were more cost effective, yet the diet was wasted as the larvae did not burrow deep into the tuber. The large tubers also contributed to humidity as they respired more than smaller tubers. The movement of insects from one container to another was considered to be stressful for the insects. The addition of moths to oviposition cages was problematic, as moths could easily escape when new moths were being added. Collecting the eggs from the ovipositing cage resulted in the cage being opened and releasing moths. The use of honey solution to feed adults was problematic as it attracted ants and this led to constant monitoring daily of cages to decrease moths being consumed by the ants. The same problem was encountered by Visser (2004).

Problems encountered in rearing method one led to the development of rearing method two. The first parameter changed was the temperature; it was altered from 27°C to 24°C (Table 3.4 and Table 3.5). Briese (1980) suggested that if the temperature was lowered to 23.5°C, fertility was optimal, although the study also indicted that insects developed within 20.7 days from egg to adult when reared at 27°C. The purpose was to establish a culture to optimally produce as many offspring as possible without significantly altering the development time. It was considered better to have a higher fertility and a slight delay of 1 to 2 days in development.

Lowering the temperature and sealing the development containers with perforated mesh, reduced the level of humidity. Visser (2004) suggested that if potatoes were spread out, humidity would be reduced. The decrease in humidity resulted in no fungal contamination or soft rot of the tubers. The use of small tubers instead of large tubers decreased respiration and humidity within containers. The honey solution was replaced with sterilised water, which decreased the presence of ants. All the above parameter changes decreased the need to monitor and observe the cultures. It was also found

that eggs could be removed from ovipositing cages without releasing adults. The eggs would then be placed into new development containers without any disturbance until pupation, which decreases any stress on the insects during development.

The handling of pupae instead of adults was easier, however there were problems associated with this technique. The removal of pupae was time-consuming and if the process took too long, or an incorrect concentration of NaOCl was used, the pupae turned black and did not develop. A second problem was that the pupae were at various development stages and therefore adults emerged at different times and the ovipositing containers were not consistent in egg production. Pupal age could be determined by the eye colour; white pigment is found in young pupae, red pigment for slightly older pupae and black eyes suggesting the oldest pupae (Brits, 1980; 1982).

Rearing method three differed slightly from rearing method two. This method ensured eggs were placed onto punched tubers, and left undisturbed until adult emergence, instead of removing pupae. This decreased handling stress except when adults were transferred. The cultures required egg removal and water daily, and other maintenance was carried out every 4 days, thus lowering labour intensity.

The three rearing protocols used in this investigation were compared to other known and published rearing techniques, in order to determine efficacy of the protocols. The previously described protocols and findings are presented in Table 3.5.

Table 3.5: Summary of various aspects of PTM rearing techniques (Adapted from Visser, 2004).

Rearing Protocols and References			Method Diagram	Eggs laid per female	Sex Ratio	Survival Rate (%)	Egg to adult (days)	Tempe (°C) and RH (%)	Day: Night (hours)	Food for Moths	Eggs substrate	Larval Substrate	Pupation substrate	Larval yield
Finney et al. (1947)				80 – 200			24*	26.7°C			Muslin sheet	Punched tubers	Sand/ paraffin waxed plates	
Broodryk (1967)								26.5°C	12:12		Filter paper	Punched tubers	Corrugated biscuit rolls	0.2 per 1 g tuber
Platner and Oatman (1968)								26.7°C		Water	Muslin sheet	Punched tubers	Sand / hardboard	75 per tuber
Broodryk (1970b)							22-23*	26.5°C						
Broodryk (1971)				0 - 232			24*	11°C; 25°C; 32C		Honey solution				
Wearne (1971)								26°C 45%			Tissue Paper	Punched tubers	Corrugated paper strips	
Meisner et al. (1974)								26.5°C		5% sugar on cotton wads	Potatoes & cheese cloth	Punched tubers	Sawdust	
Cordona and Oatman (1975)				178	1:1		22*	26.7°C 50%		Nothing provided				
Singh and Charles (1977) Initial rearing on tubers				169		100	22*	30°C	16:8	5% sucrose	Surgical gauze	Tubers		1 per 2g tuber
Singh and Charles (1977) Artificial Diet				100		81.9	22*	30°C	16:8	5% sucrose	Surgical gauze	Artificial Diet	Within or next to diet	4 larvae per tube
Fenemore (1977)				46 - 236				25°C		Sugar solution				
Matthiessen et al. (1978)								30-34°C			Tissue paper		Sand	0.2 per 1g tuber
Foot (1979)								20-25°C	8:16	5% sugar solution	Filter paper	Punched tubers	Double layer tissue paper	
Briese (1980)				102-130	1:1	85-94 (26°C)	17-24	Ranged from 16.5°C – 34°C	12:12	10% honey solution	Black organdie	Punched tubers	Tissue paper	1 per 2g tuber
Powers and Oatman (1984)								27°C	12:12		Muslin cloth	Punched tubers	White sand	
Etzel (1985)				85				22°C 70%	0:24	Nothing provided	Muslin cloth	Punched tubers	Sand/ wax paper	1 per 1g tuber
Rahalker et al. (1985)				155			17-22	29°C 65%	12:12	10% sugar	Cotton cloth	Potato slices	Sand/ aluminium sheet	2.5 per 1g tuber
Visser (2004)				77			23	26°C	0:24	Nothing provided	Tissue paper	Punched tubers	White sand/ wax paper	30 – 50 per tuber
Headrick and Jones (2007)							25*	27°C		15% Honey Solution	Filter Paper	Tubers	Sand	
This study	RT 1	P*	Figure 2.1	38	1:0.73	22	25	27°C	12:12	5% honey solution	Wax paper	Punched tubers	River sand	1 per 2g tuber
		I*			1:1.44									
		H*			1:0.89									
	RT 2	P*	Figure 3.4	69	1:0.73	79	23	24°C	12:12	Water	Filter paper	Punched tubers	Perlite/ vermiculite	1 per 2g tuber
		I*		51	1:1.44	68								
		H*		76	1:0.89	79								
	RT 3	P*	Figure 3.8	119	1:0.73	89	20-23	24°C	12:12	Water	Filter paper	Punched tubers	Perlite/ vermiculite	1 per 2g tuber
		I*		87	1:1.44	90								
		H*		112	1:0.89	91								

*not mentioned by author, but estimated using the times given for each development stage

P* = Patensie; H* = Howick; I* = Ivanhoe

Comparing the results of the three laboratory cultures (Patensie, Howick and Ivanhoe) to other known studies reveal similarities and differences (Table 3.5). The fertility of the laboratory cultures in this study, ranging from 38 to 119 eggs per female, was similar to that reported in other studies (Finney *et al.*, 1947; Broodryk, 1971; Cordona and Oatman, 1975; Singh and Charles, 1977; Fenemore, 1977; Briese, 1980; Etzel, 1985; Rahalker *et al.*, 1985). The sex ratio has been identified as 1:1 by Cordona and Oatman (1975) and Briese (1980), which corresponds to the findings in the cultures reared in the laboratory. The second and third rearing techniques used in this investigation had survival rates ranging from 68-91%, which were similar to survival rates recorded by Singh and Charles (1977) and Briese (1980). The first rearing technique had a low survival rate of 22%, which is below that reported in other studies. The Patensie culture was reared according to rearing method one which was carried out at 27°C, this corresponds to the temperatures used by most rearing studies (Table 3.2). The latter two rearing methods used 24°C, which is lower than most studies, with the exception of that by Etzel (1985), also reared the insects at 22°C. The use of 24°C was prompted by Briese (1980) discovering that PTM had a higher fertility at 24°C. The photoperiods for the laboratory cultures differed greatly between the various studies analyzed in Table 3.3. The rearing methods in this study used 12:12 hour intervals of light and darkness.

Humidity was an important factor to consider, and several changes were implemented with rearing method two to reduce humidity. The increase in humidity led to condensation on the walls of the containers, which caused drowning of larvae and soft rot of the tubers. Kleinkopf and Oslen (2001) found that soft rot was directly related to an increase in humidity. The use of mesh instead of lids increased ventilation in the containers which reduced humidity, but the size of the tubers was also changed. Visser (2004) reported that smaller tubers respire less than large tubers, therefore decreasing humidity.

Honey, sugar and water solutions were fed to adults in the cultures described in Table 3.4. It has been reported by Labeyrie (1957) that there was no difference in fecundity with females which had been fed water or 5% solutions of sugar or honey. Fenemore (1977) discovered that more eggs matured in females that had fed, suggesting that food and water are important for fecundity. Three studies reported that if the moths were not given food, there was little or no difference in egg to adult survival or longevity and fertility (Cordona and Oatman, 1975; Etzel, 1985; Visser, 2004). Visser (2004) did not provide food to the adults because ants were attracted to the honey solutions, as was observed in this study. PTM do seem to prefer to lay eggs on rough textured surfaces (Das *et al.*, 2007) (Table 3.4).

Reed (1971) reared insects on potato foliage, but Meisner *et al.* (1974) discovered that female PTM lay nearly double the number of eggs when reared on tubers than foliage. Potato slices (Rahalker *et al.*, 1985) and artificial diets (Singh and Charles, 1977) have been used for rearing PTM, but both were unsuccessful. Potato tubers require less maintenance than potato slices used by Rahalker *et al.* (1985), as the slices need to be replaced daily because of dehydration. An effective artificial diet has not been developed for PTM, therefore punched tubers have been established as an optimum means of rearing larvae because the neonates can easily penetrate the tuber, and potatoes are easier to maintain than any other type of diet. Punching tubers assists the neonates in penetrating the tuber and ensures that the larvae penetrate deeper into the tuber thereby limiting diet wastage (Finney *et al.*, 1947). A tuber should possess holes equating to a third of the maximum number of larvae that the tuber can sustain (Finney *et al.*, 1947). Gomaa *et al.* (1978) established that PTM raised on tubers developed better and faster in all the life cycle stages. Larval yield per tuber has been extensively examined, and in most cases approximately 1 larva is allocated to 1 or 2 grams of tuber. This amount of diet per larva has been found to be optimum for PTM survival and consistent development (Singh and Charles, 1977). Broodryk (1971) indicated that there was a rapid reduction in survival and pupal mass if PTM densities were greater than 1g of tuber per larva. From the previously mentioned studies 2g of tuber per larva was considered optimal in this study.

Several media have been established for pupation to occur. These include sand, paper, sawdust, artificial diet, and tissue paper. Pupation will occur in or on most media which are aerated and easy for the larvae to move in and around on. Perlite and vermiculite were established as an ideal substrate for pupae to develop, as this medium is aerated and does not hold moisture. It is very light and will not cause any mechanical damage to the pupae.

In this study, the adults were handled which resulted in moths escaping from the oviposition cage. Etzel (1985) and Rahalkar *et al.* (1985) prevented moths from escaping, however Visser (2004) found that it was not worth attempting to recover the moths that had escaped. The handling of pupae during rearing method two in this study sometimes resulted in the pupae turning black, indicating that they had died. Visser (2004) observed that if pupae were washed using running tap water, they were injured and would die, and therefore it was suggested that the pupae be submerged in water to rinse off the NaOCl.

The average survival rates observed for Patensie culture in rearing method one was lower than those observed by Von Arx and Gebhardt (1990), which were 34 - 39%. As rearing methods were adapted to improve the population numbers, so the neonate to adult survival rate increased to above 80%.

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Survival rates exceeding 80% have been reported in several studies (Charles and Singh, 1977; Briese, 1980).

The low number of eggs laid by the Patensie culture in rearing method one may be due to poor development conditions in immature stages, which resulted in low survival rates. It has been reported that females with poor larval nutrition have reduced egg-laying ability (Trehan and Bagal, 1944; Labeyrie, 1957; Meisner *et al.*, 1974; Briese, 1980; Traynier, 1983). By ensuring that tubers are edible by larvae, it can be expected that females will lay more eggs due to better nutrition. The eggs may have had a high hatching rate, but the high humidity levels indicated by the large amounts of condensation present in the containers were lethal to the newly emerged juveniles, as they could have drowned. Broodryk (1971) stated that humidity had no effect on egg, pupal or adult development and did not inhibit neonate penetration of tubers. Therefore it can be said that survival rates would have been higher for rearing method one if larval stages had not drowned in the condensation droplets. The low survival rate may be due to the humidity exceeding 90%, stimulating soft rot of the potato which decreased diet availability for the larvae to feed on (Kleinkopf and Olsen, 2001).

The fertility observed in the three cultures is similar to that observed by other studies. The cultures experienced fertility of 38 to 119 eggs per female, which falls within the fertility range reported by Finney *et al.* (1947), Broodryk (1971), Cordona and Oatman (1975), Fenemore (1977), Singh and Charles (1977), Briese (1980), Etzel (1985), Rahalker *et al.* (1985). Al-Ali *et al.* (1975) however reported that laboratory cultures do not produce more than 20 eggs per female.

Patensie culture had a sex ratio of 1: 0.73, Ivanhoe culture had a 1: 1.44 sex ratio and Howick culture had a 1: 0.89 sex ratio which is consistent with the literature (Al-Ali *et al.*, 1975; Cardona and Oatman, 1975; Briese, 1980). It has also been established that the sex ratio of PTM is not altered by temperature changes (Foot, 1979).

The rearing methods used in this study resulted in different culture sizes. Rearing method one had disadvantages described earlier. Rearing method two would be better for any scale of rearing, as the pupae are easier to work with and manage. The technique of removing the pupae using NaOCl was discussed and identified as the method for rearing PTM on a small and medium scale (Visser, 2004; Headrick and Jones, 2007). The slight differences in fertility and survival can be optimized to suit rearing method two rather than rearing method three for production purposes. Rearing method three

has not been described previously and was found to be successful in respect of the survival rate and egg production per female under the small scale laboratory conditions.

The expenses per rearing method varied for the initial set up and monthly costs. Rearing method one was considered too costly to be upgraded from a small scale culture to production scale. Rearing methods two and three were considerably less expensive for monthly and once-off expenses. Even though the expenses of rearing method three are less, and rearing method two's are slightly higher, the minor financial sacrifice for an effective, manageable and efficient colony may be acceptable in commercial production.

The small scale rearing characteristics of PTM were compared to characteristics of a production scale FCM culture. Using the FCM data provided by Chambers (personal communication), estimated values were determined for the Patensie PTM culture in order to compare the two different insect species for production. The Patensie culture was used for comparison, as the insect culture was well established and had been reared longer in the laboratory. When scaling up the egg production of PTM from small to production scale, the egg production per female for FCM versus PTM was significantly greater. The production scale of FCM was optimized to ensure easy maintenance for the labourers and high insect production from the colony, whereas PTM was limited to only 1000 moths and was adapted and suited for the maintenance by one laboratory worker. Visser (2004) stated that nearly every step in rearing PTM can be manipulated and adapted to purpose for rearing the moths.

FCM is reared on an artificial diet which is optimized for growth and development, whereas an artificial diet has not been established for PTM (Singh, 1974; Singh and Charles, 1977). Consequently, tubers are the easiest way to rear PTM (Griffith et al., 1979). With tubers being a simple and accessible diet, there is no mixing of reagents to form a diet and the tubers can easily be grown for the intended purpose of rearing. It has been reported that tubers are difficult to sterilise and maintain free of disease and organisms (Finney et al., 1947), which necessitates the search for an artificial diet. An artificial diet could be more convenient and valuable when controlled bioassays are performed and the precise concentration of a chemical or reagent has to be incorporated into the diet of the larvae (Musmeci et al., 1999).

The work reported in this chapter had the purpose of establishing PTM in the laboratory, using an effective rearing protocol. PTM was successfully reared for a period of 19 months and no population declines were experienced, even with the rearing technique transitions. The success of

the various rearing techniques, and the newly developed rearing method three, will allow for expansion into a production-scale rearing facility. From the three rearing techniques used in this study, the last two techniques can be manipulated to suit the purpose for rearing the insects continually and the expense analysis can be optimized for the most cost effective rearing. In this study, the insects were reared in hope that, without induction, a natural outbreak of a granulovirus would occur, although no symptomatic larvae were observed. The absence of diseased larvae in the field surveys (Chapter 2) and in laboratory rearing (Chapter 3), led to the initiation of induction methods in an attempt to stimulate a potential latent infection (Chapter 4).

CHAPTER 4

THE INDUCTION OF *PHTHORIMAEA*

OPERCULELLA GRANULOVIRUS

4.1 INTRODUCTION

Bioprospecting produced very few symptomatic larvae and even those larvae that appeared infected did not contain a virus (Chapter 2), thus a laboratory culture was established (Chapter 3) with the intention of conducting a series of virus induction experiments. This chapter describes various induction protocols which attempted to induce an active viral infection within the three PTM cultures.

Baculoviruses such as CpGV and PhoGV are widespread and predominantly infect insect populations (Lacey *et al.*, 2008; Federici, 1997; Visser, 2004). These viruses are commonly found to be in a latent phase within hosts (Eastwell *et al.*, 1999; Burden *et al.*, 2002; Il'inykh *et al.*, 2004) but can become an active infection, in which symptoms are observed if the insect is stressed (Fuxa *et al.*, 2002) leading to disease or death of the host (Alberts *et al.*, 1994; Boots *et al.*, 2003). Natural stressors such as population explosions, which occur in the field, can initiate an acute infection (Il'inykh *et al.*, 1995; Milks and Myers, 2001). In the laboratory, overcrowding, alternative diet, non-optimal environmental temperatures, changes in humidity, mechanical stress and the co-infection of another virus with a baculovirus have been used to induce an acute infection (Smith, 1963; Tanada, 1965; Longworth and Cunningham, 1968; Fuxa *et al.*, 2002; Ludewig, 2003). Stilbene chemicals used in the manufacturing of optical brighteners and dyes, have also been incorporated with virus suspensions or have been administrated on their own in order to induce virus (Black, 1999). Tinopal®, a stilbene chemical, inhibits sloughing of virus-infected cells in the peritrophic membrane lining the gut (Monobrullah, 2003; Martinez *et al*, 2004). A high density of insects increases the chance of a viral epizootic outbreak, because frequent interaction with individual insects of the population in high densities leads to a greater chance of viral transmission and creates stress (Steinhaus, 1958; Jacques 1962; Tanada, 1965). The co-infection of two viruses can promote the activation of the latent virus, therefore acting as a stressor for viral induction (Steinhaus, 1957; Vago, 1968). However, viruses can co-exist in the host without being induced (Tanada, 1965).

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Latent baculoviral infections are difficult to detect experimentally (McKinley *et al.*, 1981). If the latent phase is activated into an infectious state, the number of virus particles present for detection increases and symptoms of a viral infection become apparent. It is easier to detect baculoviral infections in the larval stages of development, especially the early instar (Evans, 1983; Elam *et al.*, 1990; Tanada and Kaya, 1993; Engelhard and Volkman, 1995). Sporleder and Kroschel (2008) reported that the *Phthorimaea operculella* granulovirus (PhoGV) is vertically and horizontally transmitted in a latent state. Moths are considered problematic for extracting virus, because they have scales and a hard exoskeleton which can contaminate the samples. Larvae have a soft body which is easier to homogenise in order to extract virus (Ludewig, 2003; Il'inykh and Ul'yanova, 2005).

This chapter describes various induction protocols carried out on the laboratory cultures developed as described in Chapter 3 to induce an active infection.

4.2   MATERIALS AND METHODS
4.2.1   Source of larvae
PTM larvae from Patensie, Ivanhoe and Howick were reared in the laboratory at the optimal temperature of 24°C as described in Chapter 3, and used in all the induction procedures.

4.2.2   Induction procedures on potato tubers
Potato tubers were washed three times before being used. All potatoes were pierced with a pair of fine forceps to allow entry points for the neonate larvae. Approximately 2g of potato tuber was allocated to each larva in the containers. All experiments were carried out in a constant environment room at 24°C and a humidity of 15%. Treatments within each induction experiment were replicated 30 times with 50 neonates. If any larvae were identified as symptomatic or dead, they were collected and placed into 1.5ml microcentrifuge tubes and stored at -20°C for further analysis. If larvae were not recovered as adults or collected because they were displaying symptoms, it was assumed that had either escaped from the containers or died as neonates inside the tubers.

4.2.2.1 Heat shock treatment

Neonates were placed on the pierced potatoes using a fine paintbrush and all containers were covered with mesh and left to develop undisturbed for 10 days at 24°C (Figure 4.1). Ten containers per site were subjected to temperatures of 24°C, 30°C, 40°C and 50°C for a period of 20 minutes. All containers were then returned to 24°C and observed daily for symptomatic larvae. Larvae which were asymptomatic and had pupated were transferred to their respective laboratory moth cultures.

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4.2.2.2 Chemical treatment

Potatoes were immersed for 20 seconds in various concentrations of Tinopal® (1%, 0.1%, and 0.01%) in double-distilled water. Ten containers per concentration and site were set up (Figure 4.1). Fifty neonates per container were used from each site. The control treatment was pierced potatoes which had been immersed in sterile double-distilled water. All treatments were left undisturbed at 24°C in a constant environment room. All containers were inspected daily for symptomatic larvae.

Figure 4.1: Chemical treatments, heat shock treatments and control humidity treatments were set up in containers lined with perlite, and covered with mesh to allow for sufficient ventilation.

4.2.2.3 Humidity treatment

Thirty containers per site with 50 neonates were used for each humidity treatment. Although not measured, two extremes of humidity were set up: a high humidity treatment whereby there were droplets of water on the side of the rearing containers and a low humidity treatment with adequate ventilation (Figure 4.1). The high humidity treatment containers were sealed with a plastic lid to increase the humidity. Holes were made in the lid to allow limited air flow, to ensure that the environment was not anaerobic (Figure 4.2). Although humidity was not measured, the development of water droplets against the sides of the containers as a result of the potatoes respiring and limited air flow was used as an indication that the humidity level had exceeded 80%. No condensation was observed in the containers with mesh covering, as there was adequate airflow, therefore humidity was considered to be less than 80%. All containers were examined for dead or symptomatic larvae daily.

Figure 4.2: Humidity experiments were set up in plastic containers which had sealing lids.

4.2.2.4 Carbon dioxide treatment created by ethanol fermentation

Containers with potatoes infested with 50 neonates were set up with plastic lids which were attached to a carbon dioxide dispenser. The carbon dioxide dispenser was set up as follows: 50g of baker's yeast, 250ml of white sugar and 1L of water were mixed together in a 2L bottle. The 2L bottle was sealed with a lid and a 5mm plastic tube from the bottle was attached to the lid of the potato container (Figure 4.3). A further tube was placed in the lid to allow excess gas to escape so that the container did not build up excessive pressure. Carbon dioxide is denser than air, so it was concentrated on the floor of the container, leading to oxygen depletion in the environment. Carbon dioxide levels were not measured, but two extremes were created. The one treatment had no additional carbon dioxide added, and the other treatment had carbon dioxide added. All containers were kept at 24°C in a constant environment room and left undisturbed but monitored daily.

Figure 4.3: Carbon dioxide treatments were set up with a 2 litre bottle containing yeast, sugar and water. Carbon dioxide was fed into the container with the larvae and potatoes as shown.

4.2.3 Induction procedures on potato foliage

Potato plants used in the induction treatments were grown in a greenhouse tunnel from commercial potato tubers which had been washed three times with sterile water before planting. Leaves were removed from the plants when needed. Individual leaves were placed in containers which were lined with damp Whatman filter paper (Lasec) and sealed with a lid punctured with holes. New leaves were placed in the containers every 2 days, and moisture content of the filter paper was monitored to ensure that it remained damp. All experiments were carried out in a constant environment room of 24°C. All treatments were replicated thirty times, and neonates from all three laboratory cultures (Patensie, Ivanhoe and Howick) were used in all induction protocols. Symptomatic or dead larvae were collected and placed in 1.5ml microcentrifuge tubes and stored at -20°C until needed for further studies.

4.2.3.1 Heat shock treatment

Larvae were reared on potato leaves for 10 days at 24°C in separate containers for each heat treatment and insect culture. Containers were then exposed to one of four heat treatments (24°C, 30°C, 40°C and 50°C) for a period of 20 minutes and returned to the constant environment room at 24°C.

4.2.3.2 Chemical treatment

Leaves cut from potato plants reared in the greenhouse tunnel were placed in separate sealable containers. Egg sheets were dipped into three concentrations of Tinopal® (1%, 0.1% and 0.01%) or sterile water (control). The eggs were placed on top of the foliage and containers were incubated at 24°C.

4.2.4 Microscopical and culture techniques

The symptomatology of PhoGV-infected larvae and the preparation of samples to view crude extracts for TEM was described in Chapter 2 (section 2.2.3).

4.2.4.1 Transmission electron microscopy (TEM)

Larvae were fixed in embedding medium and viewed using TEM, according to a protocol developed by Glauert (1974). Larvae that were symptomatic were cut into thirds to ensure that the preparative solutions effectively penetrated the samples. Segments were placed in separate glass containers (Polytop Corporation, USA) containing 2.5% glutaraldehyde, in 0.1M phosphate buffer and fixed for 24 hours at 4°C. The glutaraldehyde was decanted and segments washed twice for 10 minutes with 0.1M phosphate buffer, before fixation in 1% osmium tetroxide made up in phosphate buffer for 90 minutes. A further 2 washes with phosphate buffer were used to remove excess fixative. Samples were dehydrated using increasing concentrations of ethanol solutions (30% - 100%) for 5 minutes. Dehydration with 100% ethanol was repeated twice. Propylene oxide was applied for 15 minutes twice after dehydration. To ensure that the propylene oxide had infiltrated the samples successfully, the larvae were subjected to successive mixtures of unpolymerised embedding mediums and propylene oxide for 1 hour intervals. The first mixture was 75% propylene oxide and 25% embedding medium, the second 50% and 50% of each medium and the third was 25% propylene oxide and embedding medium. The final mixture of pure embedding medium was left to infiltrate the samples for 12 hours. Dehydrated segments were then transferred to embedding moulds containing pure embedding resin and left to polymerise over 36 hours at 60°C. Following polymerisation, the blocks were trimmed for ultramicroscopy. Ultrathin (70-80 nm) sections of the blocks were cut using an LKB UM III ultramicrotome, mounted on carbon grids and stained with 5% uranyl acetate. The grids were viewed using a JEOL JEM 1210 transmission electron microscope operated at 100kV. Magnification ranged from 10 000X to 120 000X and particle sizes could be measured using the TEM computational tool (ANALYSIS).

4.2.4.2 Bacterial isolation

The crude larvae extract obtained from the Patensie larvae that were turning red, was aseptically streaked onto nutrient and Luria agar. Samples were incubated at 27°C and 30°C for 24 hours, after which the plates were observed for bacterial growth.

4.2.5 Statistical analysis

Data obtained from each of the induction protocols were subjected to factorial analysis of variance (ANOVA) and Tukey post hoc test. The cultures and treatment types were considered as the independent variables, while the survival rate was the dependent variable. The level of significance was set at 0.05. All statistical analyses were carried out using STATISTICA Version 6 for Windows.

4.3 RESULTS

4.3.1 Heat shock treatment on tubers and foliage

Larvae were reared on potato tubers for 10 days and subjected to one of four heat treatments (24°, 30°C, 40°C and 50°C) for a period of 20 minutes. Insects were left to develop at the control temperature of 24°C. Larvae were observed daily for any baculoviral symptoms. The number of neonates and adults present was recorded. Any dead or symptomatic larvae were collected for further analysis.

4.3.1.1 Percentage survival of larvae

Mean survival rates indicated a significant decrease in survival with an increase in temperature per treatment (Figure 4.4: $F = 4.2770$; $p = 0.0003$). Percentage survival did not differ between 24°C and 30°C, but 40°C and 50°C were significantly different from the 24°C and 30°C treatments. Within cultures for each heat treatment, the Patensie culture had the greatest survival rate compared to the Howick and Ivanhoe cultures at 30°C, 40°C and 50°C, but this was only significantly different at 40°C.

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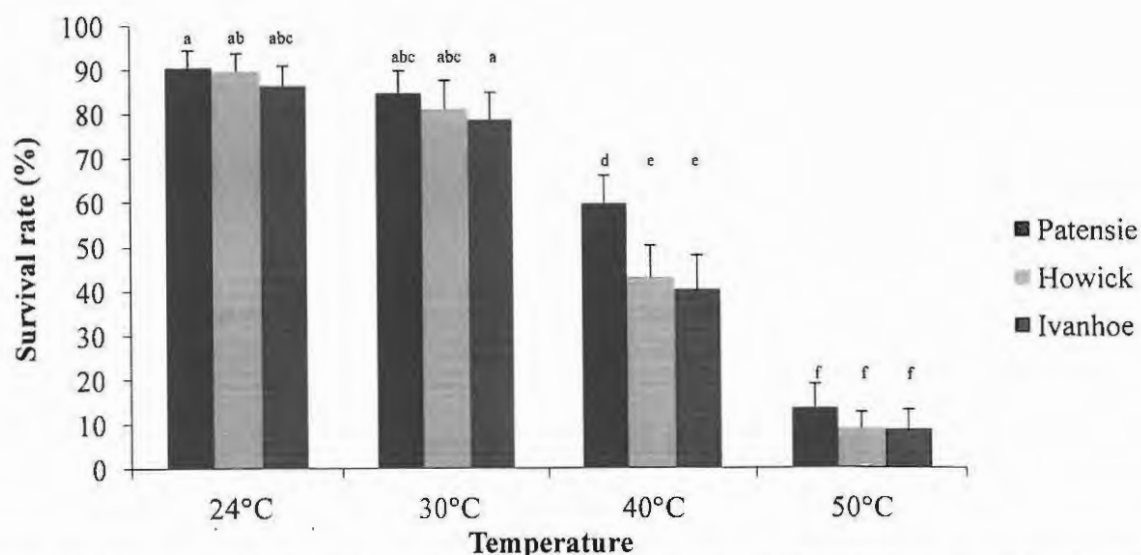
Figure 4.4: The mean (±standard error) survival of neonates to adults with heat shock treatment on potato tubers (F = 4.2770; p = 0.0003) (Means not followed by the same letter indicate significant differences).

On foliage, PTM mean survival decreased significantly with an increase in temperature, (F = 2.0642; p = 0.05). There was no significant difference between the 24°C and 30°C temperature treatments, yet these treatments were significantly different from the 40°C and 50°C heat treatments. Patensie had the highest mean survival compared to the other cultures for the 40°C and 50°C heat treatments, which is similar to the results of the PTM feeding on tubers that were heat shocked (Figure 4.5).
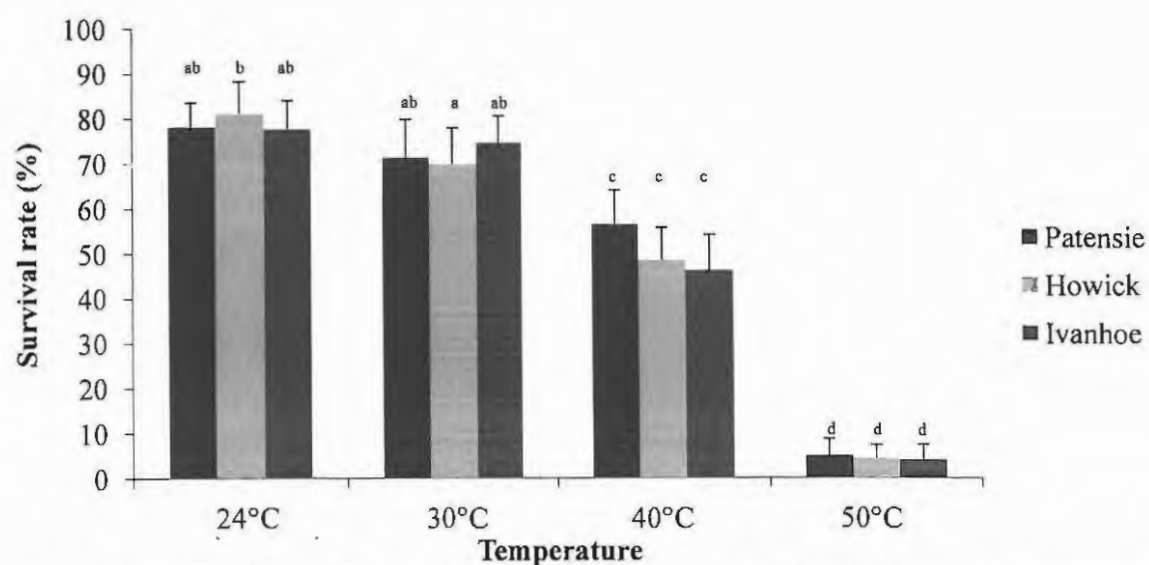
Figure 4.5: The mean (±standard error) survival of neonates to adults with heat shock treatment on potato foliage (F = 2.0642; p = 0.05) (Means not followed by the same letter indicate significant differences).

4.3.1.2 Condition of larvae

White milky discoloured larvae were observed only in the heat treatments of 40°C and 50°C. Symptoms observed for heat treatments on foliage and tubers were similar (Figure 4.6). The milky larvae were much smaller than the healthy larvae (Figure 4.6 A). Symptomatic larvae were not found at 30°C and 40°C.
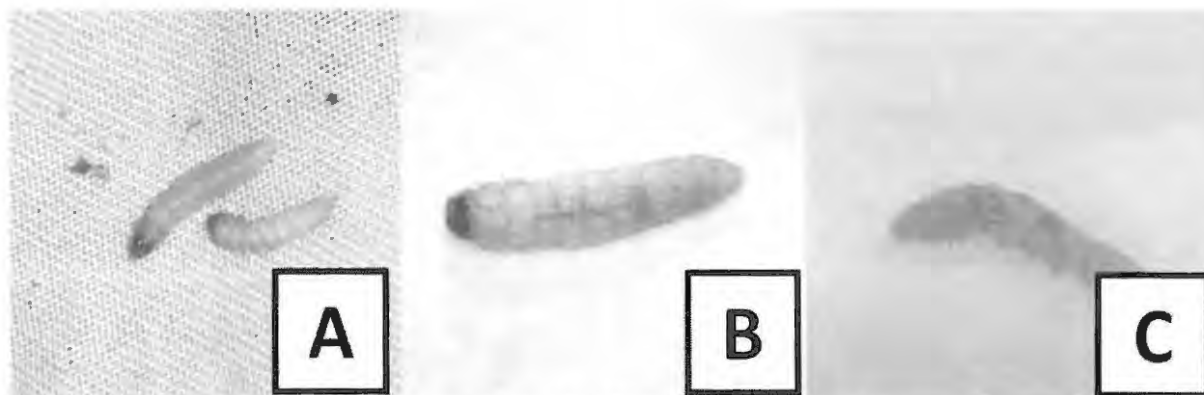
Figure 4.6: Appearance of the larvae from the 40°C and 50°C heat shock treatments on the three cultures. Patensie (A), Ivanhoe (B) and Howick (C).

4.3.1.3 Transmission electron microscopy

All insects collected from all the cultures and temperature treatments, were analysed, but none were positive for any virus particles when viewed under TEM (data not shown).

4.3.2 Chemical treatment on tubers and foliage

Neonates were placed on tubers that had been soaked in varying concentrations of Tinopal® and monitored daily for the symptoms of a granulovirus infection. Dead larvae were collected and survival was recorded (Figure 4.7).

4.3.2.1 Percentage survival of larvae

There was a significant increase in mortality with an increase in Tinopal® concentration ($F = 4.3554$; $p = 0.0003$) (Figure 4.7). A significant difference was observed between treatments. The relationships between insect cultures within each treatment were not significantly different from each other. One exception was noted at 0.01% Tinopal® where the Howick culture was significantly different from the Patensie and Ivanhoe cultures. Patensie and Ivanhoe cultures had mean survival rates greater than Howick culture for chemical treatments of 0.01%, 0.1% and 1% Tinopal®.
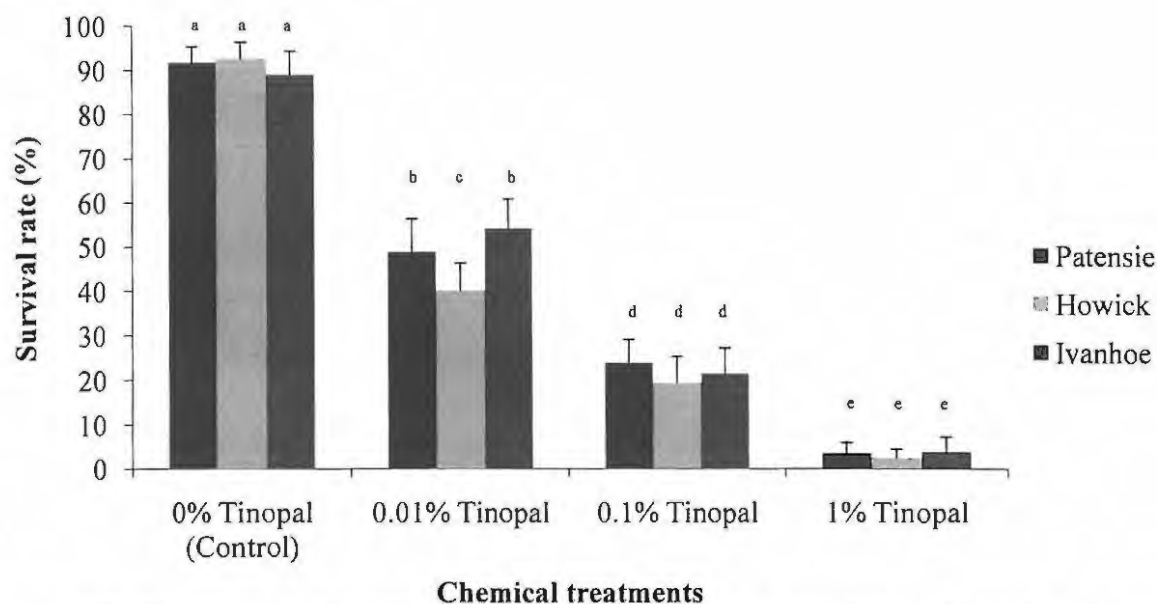
Figure 4.7: The mean (±standard error) survival of neonates to adults subjected to chemical treatment (Tinopal®) on potato tubers (F = 4.3554; p = 0.0003) (Means not followed by the same letter indicate significant differences).

A significant difference was shown between each chemical treatment, as well as between cultures per chemical treatment on foliage (Figure 4.8: F= 2.177; p= 0.04). The Howick culture was significantly different from the Patensie and Ivanhoe cultures, unlike the results of the chemical treatment on tubers (Figure 4.7), where the Howick culture had the highest mean survival for the 0.1% and 1% Tinopal.
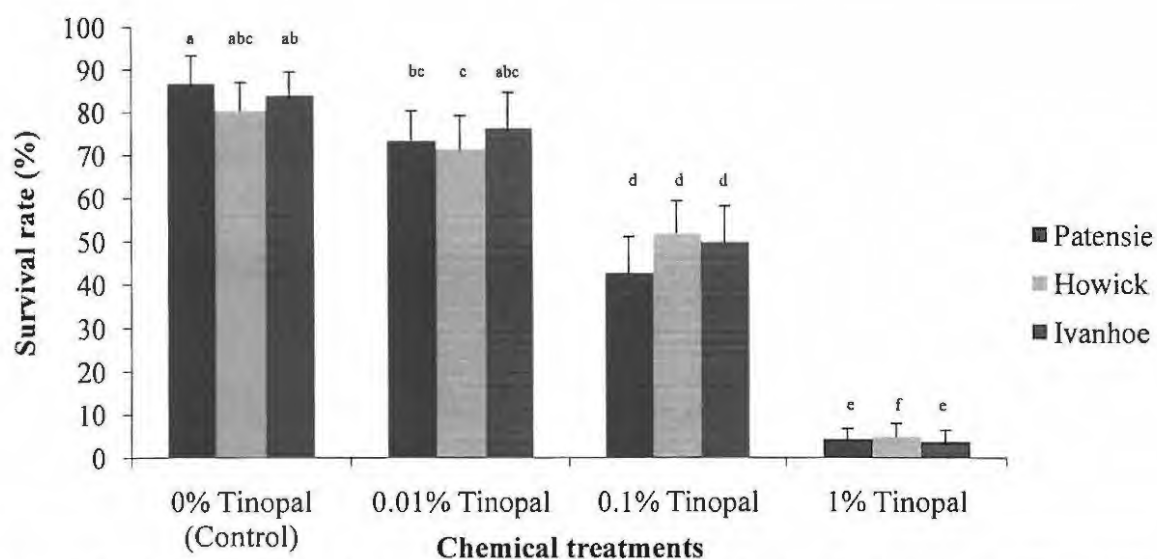
Figure 4.8: The mean (±standard error) survival of PTM with chemical treatment (Tinopal®) on potato foliage (F= 2.177; p= 0.04) (Means not followed by the same letter indicate significant differences).

4.3.2.2 Condition of larvae

Symptoms identified for the Tinopal® treatments were similar for all three cultures, and the same symptoms were observed for both foliage and tuber diets. All three cultures displayed a distinct blackening and hardening of the larval body for both larvae reared on tubers and eggs soaked in 0.1% and 1% Tinopal® (Figure 4.9 A). Dead larvae were collected for further analysis and stored at -20°C.
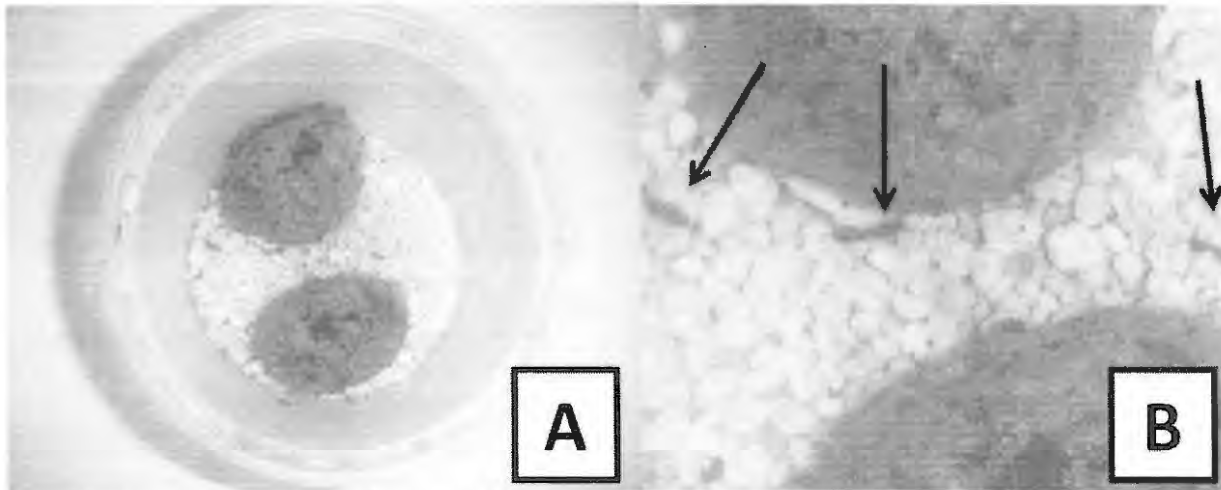
Figure 4.9.: The containers filled with black hardened larval carcasses (A). Black hardened larval carcasses indicated by arrows (B).

4.3.2.3 Transmission electron microscopy

Samples collected from the various chemical treatments were viewed by TEM, however no virus particles were observed (data not shown).

4.3.3 Humidity treatment on tubers

Two extreme treatments were established, a high humidity treatment exceeding 80%, and another treatment with less than 35% humidity. The larvae were monitored daily for baculoviral infections.

4.3.3.1 Percentage survival of larvae

A significant difference between mean percentage survival was observed between the two humidity extremes, but this was not the result of a viral infection (Figure 4.10: $F = 2.2211$; $p = 0.01$). The high humidity treatment showed significant differences between cultures. The Patensie culture had the lowest mean survival.
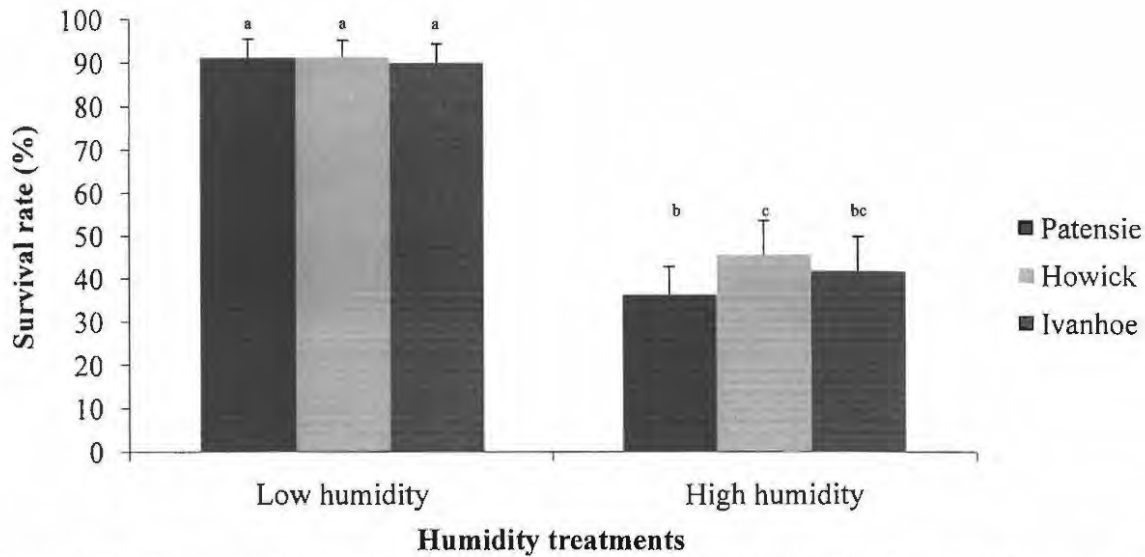
Figure 4.10.: The mean (±standard error) neonate to adult survival in the humidity treatments (F = 2.2211; p = 0.01) (Means not followed by the same letter indicate significant differences).

4.3.3.2 Condition of larvae

Symptomatic larvae from the humidity experiments were collected and analysed for viral infection. An interesting symptom appeared in the Patensie culture during the humidity inductions. Some larvae turned bright red and were less mobile (Figure 4.11 A, B and C). After these larvae had died, the cuticle and body of the larvae began to liquefy (Figure 4.11 D and E). After 24 - 36 hours, the entire insect was a liquid pool in the container (Figure 4.11 F and G).
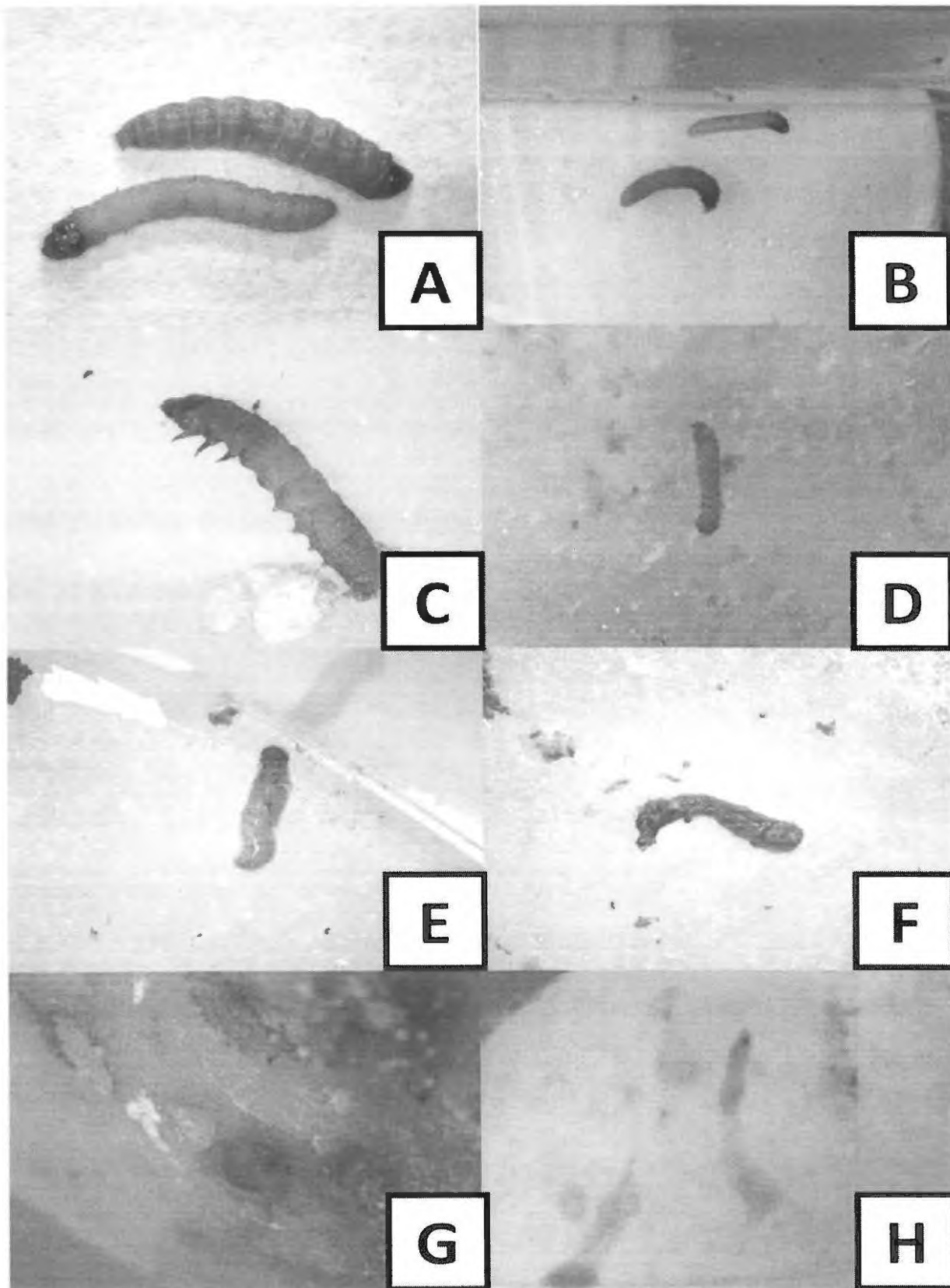
Figure 4.11: Healthy (below) and symptomatic larva (above) (A). The symptomatic larva below a healthy larvae during a humidity induction experiment (B). An immobilised symptomatic larva (C and D). The larva carcass begins to deteriorate and cause liquification (E and F). After 24 - 36 hours the entire larva carcass is a liquid pool (G and H).

The humidity treatments carried out using the Howick and Ivanhoe cultures resulted in larval cadavers that were dark brown in colour (Figure 4.12). These larvae were found in droplets of condensation formed from the humidity developed in the container. It was suspected that the cadavers were a result of the larvae drowning in the water droplets and not that of a baculoviral infection.

Figure 4.12: A dead brown coloured and soft bodied symptomatic larva, which was observed in humidity treatments carried out on Ivanhoe and Howick cultures.

4.3.3.3 Transmission electron microscopy

The red larvae were examined further with the use of TEM. A suspended crude extract was viewed on the carbon grid and a larva was fixed in embedding resin. The particles were approximately 850 - 900nm in size. The particles were oval shaped in both the suspended and fixed larva samples (Figure 4.13).
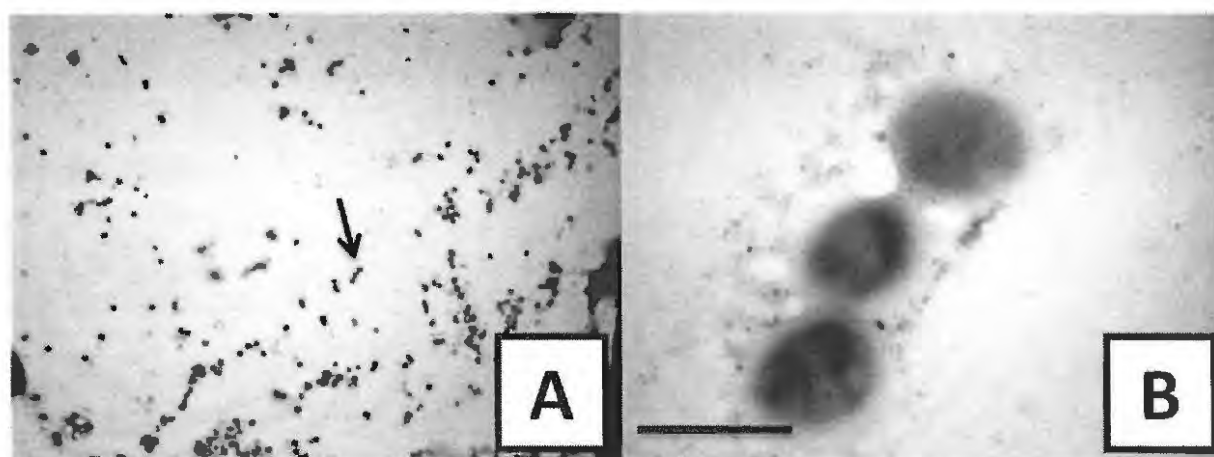
Figure 4.13: Transmission electron microscope images of particles in suspension on a carbon grid extracted from the red coloured larvae observed in the Patensie culture (A) (x 12 000). Particles of 850 – 900nm highlighted by the arrow in (A) were magnified (B) (Scale bar represents 1000nm).
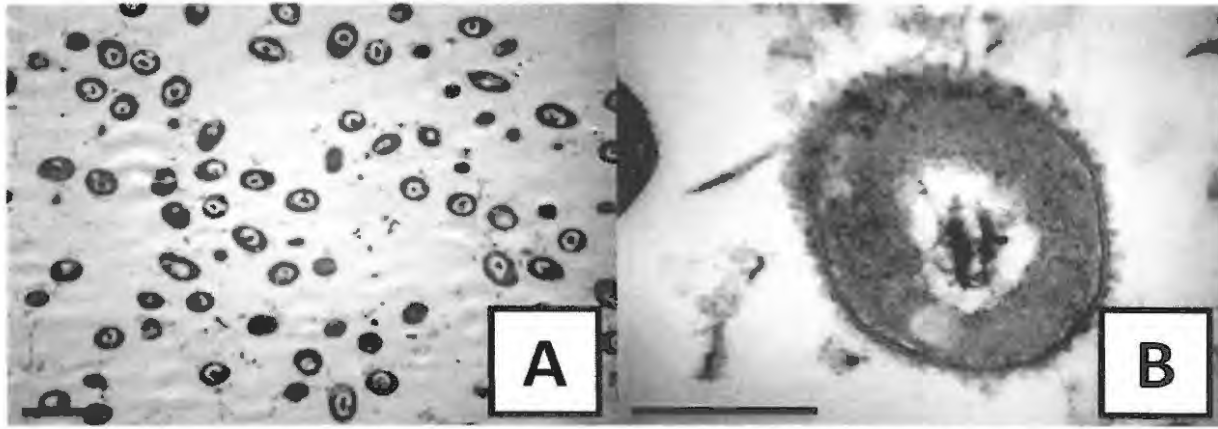
Figure 4.14: Transmission electron microscope images showing the pathogen causing the red symptomatic larvae which has embedded in resin (A) (Scale bar represents 2000nm). An individual transected particle from a larva fixed in resin indicating the internal structure of the pathogen (B) (Scale bar represents 500nm).

The TEM revealed that the particles observed were not virus (Figure 4.14). It was assumed from the symptoms and the structure of the pathogen, that it might be a species of *Serratia* (Sporleder, personal communication). Nutrient and luria agar plates were inoculated with the pathogen, but no colonies developed.

The TEM images (Figure 4.15A and B) indicate that the pathogen infecting both the Howick and Ivanhoe samples during humidity induction was probably bacterial, as binary fission was observed in many of the organisms (Figure 4.15 B). The organisms were approximately 1000nm in size (Figure 4.15)
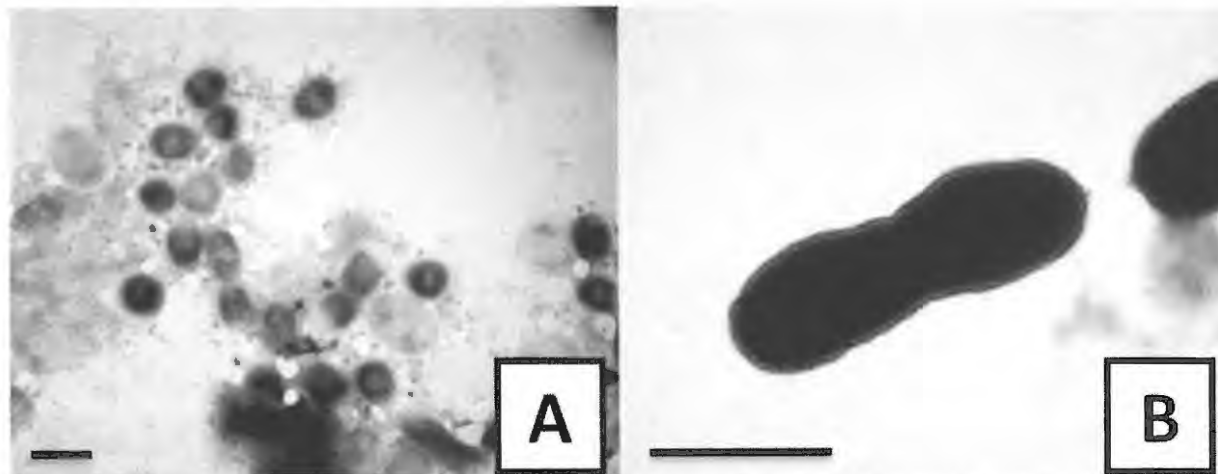
Figure 4.15: Transmission Electron Microscope photographs of Ivanhoe crude suspension samples (Howick data not presented). Particles ranging from 800 – 1000 nm were observed (A) (Scale bar represents 1500nm). These pathogens were bacteria, as binary fission was observed with several of the organisms (B) (Scale bar represents 1000nm).

4.3.4 Carbon dioxide treatment

Patensie, Howick and Ivanhoe cultures were exposed to an increase in carbon dioxide produced from the fermentation of sugar by yeast (Figure 4.3). The containers were observed daily for symptomatic larvae and the adult emergence was recorded (4.19).

4.3.4.1 Percentage survival

There was no significant difference in the mean survival rates between the increased carbon dioxide treatments and the controls (Figure 4.16: $F = 0.40808$; $p = 0.66556$). Ivanhoe had the greatest mean survival for both treatments. There were no significant differences observed between cultures for each treatment.
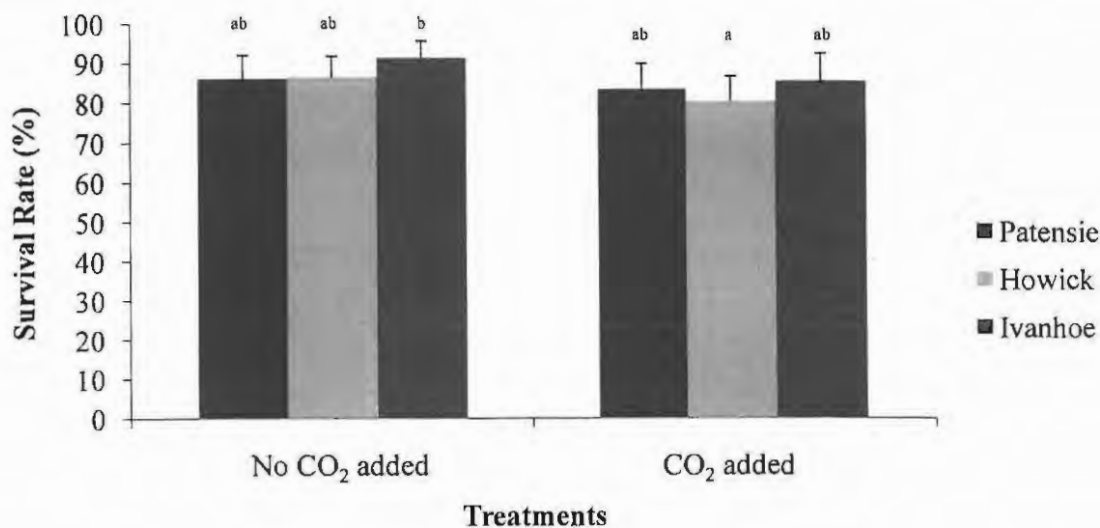
Figure 4.16.: The mean ($\pm$standard error) neonate to adult survival of PTM subjected to increased carbon dioxide ($F = 0.40808$; $p = 0.66556$) (Means not followed by the same letter indicate significant differences).

4.3.4.2 Condition of larvae

No symptomatic larvae or dead larvae were found in any of the treatment containers pertaining to the carbon dioxide experiment; therefore no further analysis was carried out.

4.4   DISCUSSION

Various induction protocols were used in an attempt to induce a baculoviral infection within PTM. Elevated temperature, humidity and carbon dioxide and chemical induction were used as stressors for the induction protocols. The humidity, temperature and chemical inductions produced symptomatic larvae, but no confirmation of baculovirus.

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The mean survival rates of the insects for the temperature treatments showed that the Patensie culture had the greatest survival percentage at temperatures of 40°C and 50°C. It can be argued that Patensie has a warmer climate than Howick and Ivanhoe, therefore the moths are adapted to higher temperatures (Broodryk, 1971).

The differences in mean survival of the temperature treatments between the tuber and foliage experiments may have been caused by the various diets. Tubers are able to act as an insulator and therefore they take much longer to adjust to the ambient temperature than does a thin leaf with a large surface area which would heat faster. Therefore larvae feeding on foliage were exposed to the temperature of the treatment longer than the larvae that burrowed into the tubers. It could also be that potato tubers provide a better source of nutrition for the immature stages of PTM, than the potato foliage. Nutritional quality of the diet can play a significant role in the rate of infection (Duffey *et al.*, 1995). Insect larvae reared on nutrient-poor diets are less susceptible to virus infections, even though they do die first if infected (McVean *et al.*, 2002). However, Rondon *et al.* (2007) observed that PTM larvae prefer to feed on foliage, which led them to assume that PTM probably would first establish on foliage as it is an immediate nutrient source. However, as the plant dies back and loses nutritional value and the tubers become exposed, so the larvae shift to the tubers.

The temperature treatments resulted in larvae having a milky appearance; however further analysis of these symptoms indicated that no pathogen was present. The induction treatments for heat were carried out on larvae which were 9 - 10 days old. It has been shown that older larvae develop immunity through cell sloughing (Tanada, 1965; Evans, 1983; Elam *et al.*, 1990; Engelhard and Volkman, 1995). It has also been proposed that as PTM undergoes metamorphosis the insects are able to overcome the viral disease because of histolysis of infection-sensitive cells (Stairs, 1965). Also, as larvae mature, the midgut pH changes because of changes in the digestive proteases activity and this can result in the insect overcoming a viral infection (Elam *et al.*, 1990; Milks and Myers, 2001).

The use of Tinopal® as a chemical inducer was not successful at expressing a baculoviral infection. The mean survival rates did indicate significant increases in mortality as the concentration of Tinopal® increased. It is suspected that the increased mortality was a result of chemical poisoning of the PTM larvae. Chemical inductions are usually effective at stimulating viral infections within insects (Black, 1999). Stilbene chemicals, such as Tinopal®, have been found to have viral enhancing capabilities in the laboratory (Hamm and Shapiro, 1992; Shapiro and Dougherty, 1994;

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Dougherty *et al.*, 1996; Okuno *et al.*, 2002; Shapiro *et al.*, 2002) and in the field (Hamm *et al.*, 1994, Webb *et al.*, 1994a; 1996; 1999). It is suggested that the mode of action of stilbene chemicals is to enhance viral activity (Black, 1999), but it is also believed that the chemical damages the integrity of the peritrophic membrane decreasing sloughing, which assists more midgut cells to become infected (Okuno *et al.*, 2002; Monobrullah, 2003; Martinez *et al*, 2004). Optical brighteners are considered valuable for improving the efficacy of baculoviruses (Martinez *et al.*, 2000). Tinopal® is a common stilbene chemical which has been used in conjunction with PhoGV or on its own at various concentrations, ranging from 0.01% to 1% ($^v/_w$) to assist with baculoviral expression (Sporleder, 2003; Martinez *et al.*, 2000; Okuno *et al.*, 2002).

The mean survival rates for the humidity induction indicated that the Howick and Ivanhoe cultures had higher survival values than the Patensie culture. This could be a result of the geographic area of origin in KwaZulu-Natal. This province has a more humid climate than the Eastern Cape. Therefore, the insects are perhaps adapted to higher levels of humidity which could explain their higher mean survival rates. The Patensie cultures displayed larvae turning red and liquefying when stressed with humidity. Extracts of the red larvae contained particles of approximately 850 - 900nm, which is much greater than the expected size of PhoGV (425 - 450nm in length and 225 - 250nm in width) and more likely to be bacteria of a *Serratia* species (Sporleder, personal communication). The pathogen was never conclusively identified, as the focus of this study was to identify a granulovirus. Larvae from the Howick and Ivanhoe cultures developed possible bacterial infections but no baculovirus particles were observed.

Carbon dioxide as an inducer was unsuccessful in stimulating a viral induction within the immature stages of PTM. There was no significant difference between the mean survival rates of the two treatments of carbon dioxide, suggesting that carbon dioxide may not have an impact on the larvae. It must be noted that PTM are continuously exposed to carbon dioxide when feeding on tubers, because when potato tubers respire they generate heat, water and carbon dioxide (Ghazavi and Houshmand, 2010) and the insects are most likely adapted to carbon dioxide in their environment. Carbon dioxide has been used as a viral stressor on healthy tissue cultures of insects to test whether any latent viruses were present within the cell lines, as well as on *Drosophila* insects in search of a latent viral infection (L'lleritier, 1958; Grace, 1958; 1962). *Cydia pomonella* granulovirus has also been induced in codling moth when an increase in carbon dioxide existed (Chambers, personal communication).

The only recorded study of induction of PhoGV in South Africa used high humidity and temperature as a combined stressor (Visser, 2004). High population densities, humidity and temperature changes are responsible for PhoGV outbreaks within PTM (Visser, 2004). However there are conflicting reports surrounding the effectiveness of humidity in increasing the susceptibility of insects to baculoviruses, and the impact of humidity as a stressor varies (Tanada, 1965). The red cadavers observed in the Patensie culture from humidity induction resembled symptoms described by Broodryk (1967) in the laboratory, who described the pathogen as *Serratia*. However, Broodryk (1967) showed that the pathogen was not virulent, as re-infection using cadavers did not occur, as was also observed in the Patensie culture. An increase in temperature is known to increase virulence and pathogenicity of viruses, and shortens the period of lethal infection once the host has ingested the virus. Yet, at high temperatures, some insects can develop a resistance to virus infections (Bird, 1955; Thompson, 1959; Tanada, 1953). Temperature as a stressor has been shown to induce baculoviruses in insect species other than PTM (Visser, 2004). *Cryptophlebia leucotreta* granulovirus has been induced through temperature in false codling moth (Ludewig, 2003).

The impacts and cause of a natural stressor, as well as the impact the stressor will have on a virus is not always well understood (Cory and Myers, 2003). The most common means of ensuring a viral outbreak in the field is to have sufficient pathogen levels and susceptible insect hosts (Cory and Myers, 2003). Since no active PhoGV was found in the field (chapter 3), it is possible that virus was not present within any of the cultures. It has been established that baculoviruses persist in low incidence levels in the field. As none of these cultures in the wild had come into contact with the virus as a biopesticide and no recorded spontaneous outbreaks of diseased insects had occurred this suggests that no virus was ever present in the insects.

Viral infections do not always result in the death of the insect. Some insects are able to complete their development and produce offspring while passing on the virus through vertical transmission (Cory *et al.*, 1997; Fuxa *et al.*, 1999; Alberts *et al.*, 1994; Boots *et al.*, 2003). Three main factors influence the susceptibility of virus infection in an insect: (1) the genetics of the insect, (2) the induction stimuli, and (3) the physiological condition of the larva at the point of induction. Stress factors without a doubt affect insect susceptibility to viral infections, but not all stressors are effective on all insect groups or individual insects. For a stressor to be effective a latent or chronic viral state should be present within the insect, and the stressor may have to be specific for different insect species.

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From the induction methods attempted, the probability of a virus being present in a latent phase is minimal. Testing for latency experimentally is difficult, and the reason for this is not well understood (McKinley *et al.*, 1981). However, PhoGV has been found previously without any induction in the laboratory (Broodryk and Pretorius, 1974; Mascarin, 2010).

CHAPTER 5

GENERAL DISCUSSION AND FUTURE STUDIES

The main objective of this study was to identify a South African isolate of PhoGV for the biological control of *Phthorimaea operculella* Zeller (Potato Tuber Moth). Several other objectives were defined, including identification and location of wild Potato Tuber Moth (PTM) populations, rearing PTM *in vivo*, induction of latent granulovirus, purification of the virus from symptomatic larvae and virulence bioassays to characterize the efficacy of the virus. The first three objectives were successfully carried out. However since PhoGV was not found in the field or in laboratory-reared insects, the latter three objectives were not achieved.

In South Africa, a few studies have been conducted on isolation and characterisation of PhoGV. Broodryk and Pretorius (1974) were the first to report PhoGV infecting PTM in South Africa. Subsequently Visser (2004) isolated a PhoGV as well, but never characterised the virus. Neither of these studies managed to obtain PhoGV from diseased larvae in the field. Broodryk and Pretorius (1974) isolated PhoGV from diseased larvae in a laboratory culture. Visser (2004) resorted to a combination of humidity and temperature to induce a PhoGV infection. Therefore bioprospecting for PhoGV in South Africa has not proved successful, as PhoGV has only ever been isolated from laboratory insects. Worldwide, PhoGV has been found in South America (Alcázar *et al.*, 1991; Alcázar *et al.*, 1992b; Mascarin *et al.*, 2010), North America (Hunter *et al.*, 1975), Africa (Broodryk and Pretorius, 1974; Laarif *et al.*, 2003), the Middle East (Kroschel and Koch, 1994), Asia (Zeddam *et al.*, 1999), and Australia (Reed, 1969; Briese, 1981). Yet, even worldwide, PhoGV has been identified from various sources. Mascarin *et al.* (2010) isolated PhoGV from laboratory cultures collected in Brazil, which is similar to the means in which Broodryk and Pretorius (1974) isolated PhoGV. Briese (1981) found diseased larvae in the field while surveying for PTM in Australia, suggesting that bioprospecting can be very successful for isolating PhoGV although this has not been the case in South Africa.

There are several possible reasons for the failure to isolate PhoGV from PTM in this study. The three laboratory cultures of PTM isolated from Patensie, Ivanhoe and Howick were predominantly generated from adult moths. Often larvae that develop into moths do not have a viral infection as they have survived, but in some instances the virus may be latent and therefore vertically transmitted to the adults. If laboratory cultures were initiated from larvae, as in the work by

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Broodryk and Pretorius (1974) and Visser (2004), there may have been an increased probability of identifying PhoGV. Collection time of the insects may also have affected finding a virus, as collections were always made near the end of the foliage growing season or once tubers were in storage. Collection of PTM during peak reproduction and high population numbers of PTM may have increased the likelihood of finding a virus. Also, since adults were predominantly collected for initiating the laboratory cultures, induction should have taken place on the first set of eggs laid. In this study, the cultures were subjected to induction only after several months, by which time the virus may have been eliminated.

In order to produce a commercial biopesticide for PTM, several steps need to be carried out. These (below) were adapted from a study used to develop a biopesticide for FCM (Moore, 2002). The proposed steps for the development of a PhoGV microbial product for pest control from identification to implementation of the biological agent are:

Step 1	Bioprospecting	Field surveys are carried out to isolate diseased larvae or identify PTM populations for isolating a granulovirus in the laboratory
Step 2	Laboratory rearing	A rearing protocol for high numbers of healthy insects is developed
Step 3	Induction of the virus	This step is only required if virus is not present in the field, or does not express during passive rearing
Step 4	Identification of the virus	When symptoms are expressed, identification needs to confirm that the pathogen is a granulovirus
Step 5	Characterisation of the virus	Once the pathogen is confirmed as a granulovirus, the strain needs to be characterised
Step 6	Bioassays	Biological activity and virulence of the granulovirus need to be determined in the laboratory
Step 7	Formulation	The granulovirus is developed into a formulation to be used in field trials
Step 8	Application of virus in the field	After the virus has been formulated, field trials need to be conducted in order to determine efficacy of the virus
Step 9	Commercial production and registration	After successful field trials, the product needs to be registered and commercial production needs to be initiated

Biopesticides have been developed commercially using steps similar to those described above. *Cryptophlebia leucotreta* granulovirus has been isolated from a South African false codling moth population, and has been effectively characterized and analyzed in both the laboratory and the field as a biological control agent by Moore (2002). The virus has recently been developed into a commercial biopesticide which is registered and traded as Cryptogran®. Therefore CrleGV has gone through all nine proposed steps and is used routinely in the citrus industry. Currently codling moth (*Cydia pomonella*) is undergoing the same steps in order to eventually produce a viral biopesticide containing *Cydia pomonella* granulovirus. CpGv has been used successfully worldwide as a effective biopesticide (Lacey *et al.*, 2008). The granulovirus has been isolated, it was found accidently when an air tight container was sealed overnight (Chambers, personal communication). The project requires a large amount of research before a product will be released, as this project has reached only step 5 of the proposed protocol for microbial commercial production. The PTM project has reached stage 3 only and therefore future studies are required to successfully produce a microbial product for PTM.

Future studies will include further bioprospecting in other PTM infested areas for potentially diseased PhoGV larvae, and attempts to improve induction protocols for stimulating latent baculoviral infections within asymptomatic PTM larvae. Should a virus be found research needs to be conducted from step 4 onwards of the proposed steps for manufacturing successful biopesticide for PTM commercially.

In this study, although field surveys and induction protocols were unsuccessful in isolating a granulovirus for a potential biopesticide, a successful, cost effective and efficient method has been developed for rearing PTM. The knowledge gained has ensured that PTM can be successfully reared on a production scale. The induction protocols were not successful, but they do open the way for further studies into identifying stressors which stimulate viral expression. According to the proposed steps for the production of a microbial biopesticide, PTM has reached stage 3.

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