APPENDIX 1

Protocols for tasks to be performed during the Bolivia visit in May 2002

Storage trial protocol

The volumes of spray liquid to apply to potato tubers for storage are unclear. Therefore, initial calibration of the application systems will have to be performed for ULV and water-based sprays. For water-based sprays there is some information available that the calibration can be based on. The literature available indicates that a spray volume of between 5 and 10 ml per Kg of tubers is required for effective coverage. However, for ULV oil-based spraying there's no information at all and a more broad ranging calibration will be necessary.

Calibration of water based spray (assuming application will be by knapsack sprayers)

Application rates of 5.00, 7.50, 10.00, 12.50 and 15.00 ml per Kg of tubers will be assessed. Volumes applied will be for 250Kg of potatoes although that weight will not actually be sprayed, just the area *covered* by that weight.

Table of spray volumes required

Application rate per Kg (mls)	Volume required per sprayer
5.00	1.25 litres
7.50	1.875 litres
10.00	2.50 litres
12.50	3.125 litres
15.00	3.75 litres

1. Place out 25Kg of tubers as they would be in a 'normal' treatment situation.
2. Measure the area covered by the tubers and make a note of it then multiply that area by 10 to obtain the area covered by 250Kg of tubers
3. Mark out that area on the ground and place 25Kg of tubers in the middle of it.
4. Calibrate the sprayer to obtain the flow-rate per minute.
5. Calculate the walking speed required to spray the area with only HALF of the particular spray volume to be tested.
6. Measure out the volume of liquid required to spray 250 Kg of tubers and fill the spray tank with that volume.
7. Spray the entire area including the actual tubers making sure just half the liquid is used.
8. Turn the tubers over and repeat the spray.

9. Make observations of the spray coverage taking particular notice of coverage to the sides of each tuber.
10. Repeat this for all spray volumes to be tested. If a satisfactory coverage is not achieved with any of the volumes tested then continue to increase the volume until an adequate coverage is achieved.

Calibration of ULV sprayer

There is no information to go by for ULV spraying. Suggested spray volumes to try are 0.5, 1.0, 2.0, 4.0, 5.0 and 7.5 ml per Kg of potatoes.

Table of spray volumes required

Application rate per Kg (mls)	**Volume required per sprayer**
0.5	125 mls
1.0	250 mls
2.0	500mls
4.0	1 litre
5.0	1.25 litres
7.5	1.875 litres

1. Place out 25Kg of tubers as they would be in a 'normal' treatment situation.
2. Measure the area covered by the tubers and make a note of it then multiply that area by 10 to obtain the area covered by 250Kg of tubers
3. Mark out that area on the ground and place 25Kg of tubers in the middle of it.
4. Calibrate the sprayer to obtain the flow-rate per minute.
5. Calculate the walking speed required to spray the area with only HALF of the particular spray volume to be tested.
6. Measure out the volume of liquid required to spray 250 Kg of tubers and fill the spray tank with that volume.
7. Spray the entire area including the actual tubers making sure just half the liquid is used.
8. Turn the tubers over and repeat the spray.
9. Make observations of the spray coverage taking particular notice of coverage to the sides of each tuber.
10. Repeat this for all spray volumes to be tested. If a satisfactory coverage is not achieved with any of the volumes tested then continue to increase the volume until an adequate coverage is achieved.

Storage trials of *Phthorimaea operculella* GV (PoGV) treated potatoes

The storage trials will consist of spraying potatoes laid out, as they would be in a usual treatment situation. Once sprayed they will be bagged and stored. Tubers will

be sprayed with the different formulations at three different concentrations that will consist of one that is equal to the dose already applied as Matapol powder, one at 10 times lower and one that is 10 times higher.

From the counts of virus I have done to obtain numbers of OBs per larva, the current dose rate of PoGV per 25Kg of potatoes is $8.00x10^{10}$ OBs. That equates to $3.20x10^{9}$ OBs/Kg. So, concentrations to test are $3.20x10^{8}$, $3.20x10^{9}$ and $3.20x10^{10}$ OBs per Kg. For ease of reading the doses will now be called low, medium and high.

Trial design

Treatments

Treatment 1a	Control (No treatment)
Treatment 2	CIP silica powder formulation or PROINPA clay powder formulation
Treatment 3	Crude aqueous suspension LOW (+0.02% TritonX100)
Treatment 4	Crude aqueous suspension MEDIUM (+0.02% TritonX100)
Treatment 5	Crude aqueous suspension HIGH (+0.02% TritonX100)
Treatment 6	Wettable powder LOW
Treatment 7	Wettable powder MEDIUM
Treatment 8	Wettable powder HIGH
Treatment 9	ULV formulation LOW
Treatment 10	ULV formulation MEDIUM
Treatment 11	ULV formulation HIGH
Treatment 12	Insecticide Control? MVP Bt

Replicates

Each treatment will be replicated five times with each replicate consisting of a 25Kg bag of potatoes.

Experimental details

The experiment will be set up with the aim of it being a long-term trial assessing the efficacy of each formulation in controlling *P. operculella* over a usual potato storage period. However, to obtain an early indication of the difference in efficacy of the formulations, sample tubers will be taken from each treatment immediately after spray to be bioassayed in the lab. Three tubers from three of the five replicates of each treatment should be taken and lab-assayed as soon as possible, placing 15 PTM larvae on each tuber. This will result in three replicates of each treatment with each replicate utilising 45 larvae. The incubation procedure will follow that of the bioassays of standard PoGV already established at PROINPA.

The storage of the treated potatoes for the long-term trial also needs advice.

Control no treatment

- Just prepare 5 bags of 25Kg of potatoes for storage.
- Ensure full labels are easily visible and won't become illegible over time.

Controls Silica and clay only

- Place 25Kg of potatoes in a sack and add 50 g of the silica or clay.
- Shake the bag to evenly distribute the powder.
- Place the bag for storage.
- Repeat four more time to obtain 5 replicates of each of the powders.
- Ensure full labels are easily visible and wont become illegible over time.

Control Triton

- Place 25Kg of potatoes on the ground in a single layer.
- Fill the sprayer with the volume of TritonX100 determined during calibration and spray the potatoes with half the liquid.
- Turn the potatoes and repeat the spray emptying the tank.
- Allow the spray to dry then place the treated potatoes into a single sack.
- Repeat this procedure four more times to obtain 5 replicates of the treatment.
- Place the bagged potatoes into store.
- Ensure full labels are easily visible and wont become illegible over time.

PROINPA Silica formulation

- Place 25Kg of potatoes in a sack and add 50 g of the PoGV silica-formulation at the Low-dose rate.
- Shake the bag to evenly distribute the formulation.
- Place the bag for storage.
- Repeat four more time to obtain 5 replicates of the low dose.
- Repeat the entire process for the Medium-dose.
- Repeat the entire process for the High-dose.
- Ensure full labels are easily visible and wont become illegible over time.

PROINPA Clay formulation

- Place 25Kg of potatoes in a sack and add 50g of the PoGV clay-formulation at the Low-dose rate.
- Shake the bag to evenly distribute the formulation.
- Place the bag for storage.
- Repeat four more time to obtain 5 replicates of the low dose.
- Repeat the entire process for the Medium-dose.
- Repeat the entire process for the High-dose.
- Ensure full labels are easily visible and wont become illegible over time.

Crude aqueous suspension

- Place 25Kg of potatoes on the ground in a single layer.
- Mix the Low-dose, crude PoGV freeze-dried powder in a small amount of 0.02% Triton.

- Add the mixture to the relevant volume of Triton as determined during sprayer calibration ensuring the mixing container is well rinsed.
- Spray the potatoes with half the tank volume
- Turn the potatoes and spray the other side, emptying the contents of the sprayer.
- Allow the spray to dry then bag the potatoes for storage.
- Repeat the process four more time to obtain 5 replicates of the Low-dose
- Repeat the entire process for the Medium-dose crude aqueous.
- Repeat the entire process for the High-dose crude aqueous.
- Ensure full labels are easily visible and wont become illegible over time.

Wettable powder formulation

- Place 25Kg of potatoes on the ground in a single layer.
- Mix the Low-dose, wettable powder formulation in a small amount of 0.02% Triton.
- Add the mixture to the relevant volume of Triton as determined during sprayer calibration ensuring the mixing container is well rinsed.
- Spray the potatoes with half the tank volume
- Turn the potatoes and spray the other side, emptying the contents of the sprayer.
- Allow the spray to dry then bag the potatoes for storage.
- Repeat the process four more time to obtain 5 replicates of the Low-dose.
- Repeat the entire process for the Medium-dose wettable powder formulation.
- Repeat the entire process for the High-dose wettable powder formulation.
- Ensure full labels are easily visible and wont become illegible over time.

ULV Oil formulation

- Place 25Kg of potatoes on the ground in a single layer.
- Mix the Low-dose ULV oil-formulation in a volume of vegetable oil equivalent to 30% of the final volume to be sprayed (as worked out from the calibration done previously).
- Add the mixture to the relevant volume of kerosene that will make up the remaining 70% of the final spray volume.
- Spray the potatoes with half the tank volume
- Turn the potatoes and spray the other side, emptying the contents of the sprayer.
- Allow the spray to dry then bag the potatoes for storage.
- Repeat the process four more time to obtain 5 replicates of the Low-dose.
- Repeat the entire process for the Medium-dose ULV oil formulation.
- Repeat the entire process for the High-dose ULV oil formulation.
- Ensure full labels are easily visible and wont become illegible over time.

Counting GV

Method of counting granulosis virus

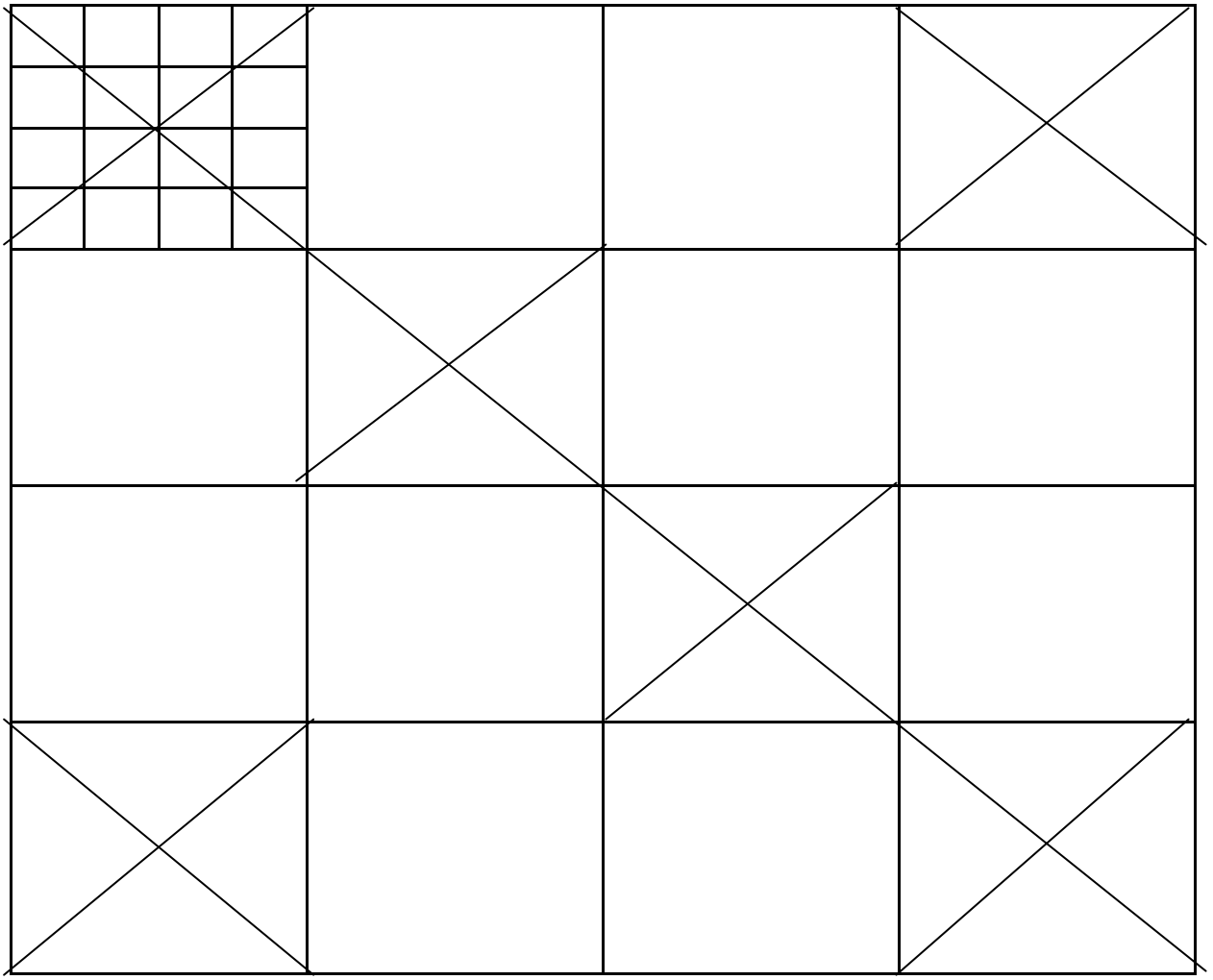
To use the chamber:

1. A small volume of test suspension is introduced to the slide chamber from a pipette, usually 5 - 10µl, though the precise amount is not important and an appropriately sized drop from a pasteur pipette will do. Make sure the sample is well mixed by vortexing or agitating before you draw off the sample to ensure you get a valid count. Failure to mix the GV sample before sub-sampling is a very common source of error.

2. The cover slip is placed over the drop and pressed down until fixed by capillary attraction of the drop. To tell that the coverslip is fixed correctly watch for the interference pattern of Newton's Rings seen under the part of the coverslip directly in contact with the counting chamber slide. The rings should be seen on either side of the actual counting area.

3. Only the specially thickened coverslips designed for use with haemocytometers should be used. If normal thin microscope coverslips are used these are distorted by the pressure of the air and capillary forces and so the volume of liquid over the grid is not exactly $0.00005mm^3$ intended (if using a standard Neubauer chamber) and the counts will be inaccurate.

4. Look at the slide under a microscope and use either dark field illumination or an optical light guide as a light source. (The optical light guide should be directed across the surface of the counting chamber from the side of the microscope stage.) Below is a diagram of what you will see. Count six large squares (96 small squares) on each grid in the standard pattern of all corner squares plus those in the diagonal cutting from top left to bottom right of the grid. Thus:



X indicates the squares to count GV in. Each "large" square is split into 16 "small squares" as in the top right example.

5. It is usual to make three separate counts on three sub-samples from the GV sample you wish to count then to average these to get the final count. Therefore to calculate the number of GV OB's per ml:

$$\text{Number of GV OB's per ml} = \frac{D \times X}{N \times K}$$

where:

D = dilution factor
X = (average of 3) total number of GV particles counted
N = number of small squares counted
K = volume above one small square in cm^3.

6. Area of each small square is 1/400 mm^2 = 0.0025 mm^2. Depth of chamber is 0.02mm. Volume of liquid above a single small square is 0.0025 mm^2 x 0.02mm = 0.00005 mm^3. To convert to cm^3 multiply by 1/1000 to get a volume of 5.0 x 10^{-8} cm^3.

Worked example:
Suppose in a sample diluted by a factor of 1000:
The number of small squares used for the count is 96 (6 large squares)
The number of GV OB's counted are 355--390--320 (three separate sub-samples)
Then:

D = 1000
X = 355
N = 96
K = 5.0×10^{-8} cm^3

Thus: $\frac{1000 \times 355}{96 \times 5.0 \times 10^{-8}} =$

$\frac{3.55 \times 10^{5}}{4.80 \times 10^{-6}} =$

7.395×10^{10} GV occlusion bodies/ml

Note:
When counting GV the number of OB's observed in any one sub-sample always varies greatly so the more counts made the more accurate the estimation of sample concentration.

Bioassay of GV strains

This bioassay will be set as a standard method to compare all new strains on GV that may be collected during the course of the project. It is based on the bioassay method already used at PROINPA but instead of using LEs we will count the GV suspension and use actual concentrations.

From literature it appears that LC_{50} values are around 1.0×10^5 OB/ml. The bioassay dose series should therefore run from 4.0×10^6 OB/ml down to 6.4×10^3 OB/ml.

Dose series preparation

Depending on the size of tubers prepare 50 to 100ml of each dose.

Serial dilution preparation

2. The concentration of the sample to be assayed must be known, therefore GV counts of the stock solution must be made using a counting chamber. An appropriate dose series of 6 different concentrations of GV suspended in 0.01% Triton is used.

3. When dealing with GV samples a 5-fold dilution series should be prepared for bioassay that will produce a dose response from 100 to 0 percent with gradual reduction in mortality from the top dose to the lowest. The dose series required to produce a dose response is a follows:

4.00×10^6 OB/ml
8.00×10^5 OB/ml
1.66×10^5 OB/ml
3.20×10^4 OB/ml
6.40×10^3 OB/ml
1.28×10^3 OB/ml

Method

4. Calculate the volume of stock GV sample required to achieve the top concentration in 100 ml of 0.01% Triton in the following way:

$$\frac{\text{Top conc. required}}{\text{Conc. of stock}} = \text{Volume of stock needed}$$

Example

Stock = 2.5×10^9 OB/ml
Top concentration required = 4.0×10^6 OB/ml (x100ml = 4.0×10^8 OB)

$$\frac{4.0 \times 10^8}{2.5 \times 10^9} = 0.160$$

5. This means 0.160 ml of stock is required in order to achieve 4.0×10^8 OB's. If the samples are made up in 100ml to get the correct concentration take 0.160 ml of stock and mix it with 0.840 ml of 0.01% Triton X100.

6. This will result in a 100ml suspension of your top concentration of 4.0×10^6 OB/ml. Label the tub "1" . From this the serial dilutions of the dose series can be made in the following way:

- Set out 7 tubs and fill each one with 80ml of 0.01% Triton X100, label them 2 to 8.
- From tub 1 above, remove 20ml of sample and add it to tub 2. Mix the resulting suspension very well.
- From tub 2, remove 20ml of the suspension and add it to tub 3, again mix well.
- Repeat this procedure for all the tubs and when reaching tube 8 remove 20ml and discard it.
- Your fivre-fold dilution series is now ready for use.

Bioassay protocol

1. The controls are always treated first before any GV suspension has been worked with. Take a tuber and dip it in 0.01% Triton.

2. Allow the solution to dry by suspending it from string held between two clamp stands then place the tuber in a suitable tub.

3. Place 25 neonate larvae on the tuber and close the tub to prevent escapees. Repeat this for a second tuber for the control to make 50 insect in the treatment.

4. Take another tuber and dip it in the lowest concentration of GV. Allow the suspension to dry then place the tuber in another tub as above. Place 25 larvae on the tuber and repeat the process to provide 50 larvae for the treatment.

5. Continue this process up the dose series for all concentrations. Once the bioassay has been set up, incubate it and make routine assessments. (Days of assessments to be advised by PROINPA staff).

6. The incubation of the assay will follow the protocol already established at PROINPA.

APPENDIX 2

Project Report. Establishment and preliminary results of trials with Phthorimaea operculella GV formulations in Bolivia to control the potato tuber moth.

Mark Parnell

Project C1498, R8044, ZA0485. "IPM of Major Insect Pests of Potatoes in Hillside Systems in the Cochabamba Region of Bolivia".

BACKGROUND

This project is funded by the CPP 1 September 2001 - 31 August 2004. It is managed by the Bolivian *Fundación para Promoción e Investigación de Productos Andinas* (PROINPA) with inputs by CIP and NRI as collaborators. An initial visit carried out in January identified that the first fieldwork conducted should begin trials of different formulations of GV and provide training to local scientists in identification of GV infection in potato tuber moth (PTM) larvae.

Andean potato is the principal staple food for Bolivians and also a major cash crop, grown nationally by 400,000 small-farm families. Most potato farmers are poor, and yields are low due to a complex of biotic factors, particularly insect pests. The most important of these are potato tuber moths, *Phthorimaea operculella* and *Symmetrischema tangolias*, and Andean potato weevils, particularly *Premnotrypes latithorax* and *Rhigopsidius tucumanus*, which can each cause losses of up to 500 US$/ha/year. In response to these pests farmers have been using increasing amounts of pesticides to improve potato productivity resulting in serious abuse and overuse. This project will aim to produce new biocontrol systems for these pests based on natural pathogens and attractants and to develop the technologies appropriately for use by poor farmers. Project outputs will be taken up in Bolivia by PROINPA and regionally through CIP as these pests are widespread in South America.

PROINPA have developed a powder formulation of the *P. operculella* granulosis virus (PoGV), marketed as "Matapol". NRI will help improve production and quality control techniques and develop a liquid formulation of the virus. *S. tangolias* appeared in Bolivia two years ago and is spreading rapidly displacing *P. operculella* as the more important pest, such that the virus approach must incorporate a virus for *S. tangolias* to be of any value. NRI and CIP will help PROINPA discover and develop an entomopathogenic virus for *S. tangolias.*

OBJECTIVES

The field visit was carried out from May 13th to 31st 2002 in order to coincide with the harvest season of the Andean potato. Three main activities were performed during the visit which were

1. Train local scientists in the identification and quantification of GV infecting *P. operculella* larvae.
2. Train Scientists in techniques for detecting new virus for *S. tangolias*

3. Establishment of a potato storage trial to investigate the efficacy of different PoGV formulations.
4. Establishment of a laboratory bioassay of samples from the potato storage trial in order to have a fast system for determining the efficacy of different formulations.

ACHIEVEMENTS

1. Training of local scientists in the identification and quantification of GV infecting *P. operculella* larvae

Training was provided in techniques to count GV particles using a microscope. The system available for counting GV is very poor and makes the task much more difficult. The main research microscope available to PROINPA (Olympus BH-2 microscope) does not have phase contrast or dark-field illumination. It would be very beneficial to the project for a dark-field system to be fitted to the BH-2 as without it accurate bioassay of new strains and QA of the GV production system are compromised. This could possibly jeopardise the success of this component of the project.

Training in the purification procedure provided PROINPA with a quantity of purified PoGV for use in production of Matapol. A problem of microsporidia contaminating Matapol preparations had been identified during the previous visit. It was originally suspected that the cause of contamination was poor quality seed inoculum for GV production, but the more likely cause was from the PTM colony, which was recently discarded. Having discarded the previous colony due to microsporidia contamination, three new colonies were established using pupae collected from different locations in Bolivia. No obvious signs of microsporidia were present but on microscopic investigation they were identified in all three colonies. A count was made of the current level and at present, the level of infestation is in the order of 10^6 per pupa. Currently no detrimental effects are obvious but over generations the levels will increase and begin to affect the GV production. I have suggested that they try two approaches to avoid collapse of the colony. They can either have a rolling system of colonies of new and old material brought in from the field or amalgamate all the colonies and continuously refresh it with new stock from the field every couple of generations to dilute the level of microsporidia building up.

The remainder of this report will concentrate on the potato storage trial and laboratory bioassay of the PoGV formulations.

2. Train Scientists in techniques for detecting new virus for *S. tangolias*

During the training an *S. tangolias* larva was identified as being GV infected. At present we have identified GV infection in only one larva but it's definitely GV. The project states that the identification and production of *S. tangolias* GV (StGV) should be performed by CIP but it was originally envisaged that an StGV would be found in Peru. There is uncertainty of the length of time it will take to obtain relevant permissions for export of Bolivian GV to Peru. Therefore, it has been proposed that a sample of the new GV is sent to the UK for DNA analysis to get a quick ID of

whether it is an St specific GV or if it's cross infection. This is being checked with CIP for their approval and if granted the GV will be bulked-up and an aliquot sent to NRI for DNA analysis. It was made clear that this would be a very basic study to get an immediate answer and the responsibility of CIP for the set objectives would not be compromised.

2. Potato Storage trial of PoGV formulations

The formulation trial was set up at the Toralapa Research Station 70km outside Cochabamba. The trial consisted of 22 treatments and the different formulations tested were Dry-Powder formulation (DP), Crude Aqueous formulation (CA), Wettable Powder formulation (WP) and ULV-Oil formulation (ULV). The WP and CA formulations were applied as sprays and as "dips". The PoGV used in all formulations was prepared at NRI from GV infected *P. operculella* larvae brought back to the UK from labs at Toralapa. It consisted of freeze-dried crude-extract that had been prepared by crushing the infected larvae in water, filtering the resulting slurry then freeze drying it to obtain a powder. A full list of treatments can be seen in Appendix I.

The application techniques for the spray treatments had not been worked on before and were therefore not known so before the storage trial could be set up, sprayer calibration had to be performed. Three different types of sprayer were calibrated (lever operated knapsack sprayer (LOK), hand-held (H-H) sprayer and ULV sprayer) using the technique described in Appendix II.

Once the calibration for each spray type had been performed the trial was begun. The storage trials consisted of spraying 6Kg of potato tubers for each of five replicates of the 22 different treatments. Tubers were laid out in a single layer and sprayed on both sides allowing the spray liquid to dry in between turning. Once sprayed, each replicate of 6Kg's was bagged and placed for storage. The precise locations are to be confirmed but will be areas in Bolivia where *S. tangolias* are not a problem as yet.

From the counts of virus performed at NRI prior to the visit, numbers of OBs per larva were obtained. From those calculations, the dose rate of PoGV per 25Kg of potatoes used in the Matapol formulations was calculated to be 8.00×10^{10} OBs (or 3.20×10^{9} OBs/Kg). Tubers were sprayed with the different formulations at three different concentrations of PoGV. The concentrations consisted of one that was equal to the dose already applied as Matapol powder, one at 10 times lower that dose and one that is 10 times higher that dose. Therefore, the concentrations tested were 3.20×10^{8}, 3.20×10^{9} and 3.20×10^{10} OBs per Kg. For ease of reading, the doses will now be called low, medium and high for the duration of this report.

From each replicate of each treatment a sub-sample of 5 treated tubers (c. 250g in weight) was taken for laboratory bioassay. The five tubers from each replicate were held collectively in plastic pudding bowls and 50 *P. operculella* eggs were placed in every bowl. Eggs were held on "egg-papers" from the lab colony and counted out using a binocular microscope. Once it was set up, the bioassay was held in a temperature-controlled room and assessed on a single occasion approximately six weeks from the start date. Assessments were made of PTM present in each replicate of the bioassay at the end of storage, level of infection present in larvae remaining and

level of damage caused to tubers by PTM larvae. Damage level was scored in every tuber by cutting each one in half to enable visual assessment of the level of damage within. A ranking of 0 to 4 was used whereby:

0 = No damage
1 = 1-25% damage
2 = 26-50% damage
3 = 51-75% damage
4 = 76-100% damage

Results, in terms of mean virus counts and damage scores for the respective data-sets, were analysed using a 1-way analysis of variance routine within the Sigma-Stat package.

Below are a series of photos showing various components of the field activities.

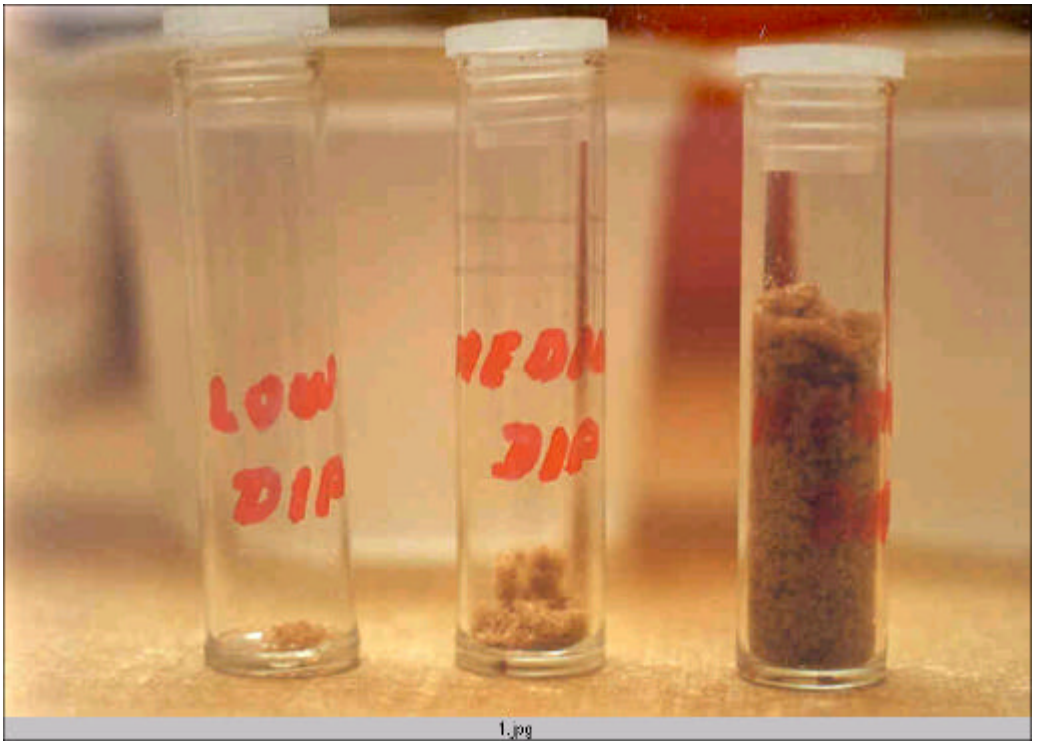


Plate 1. Crude, freeze-dried PoGV at the three different doses

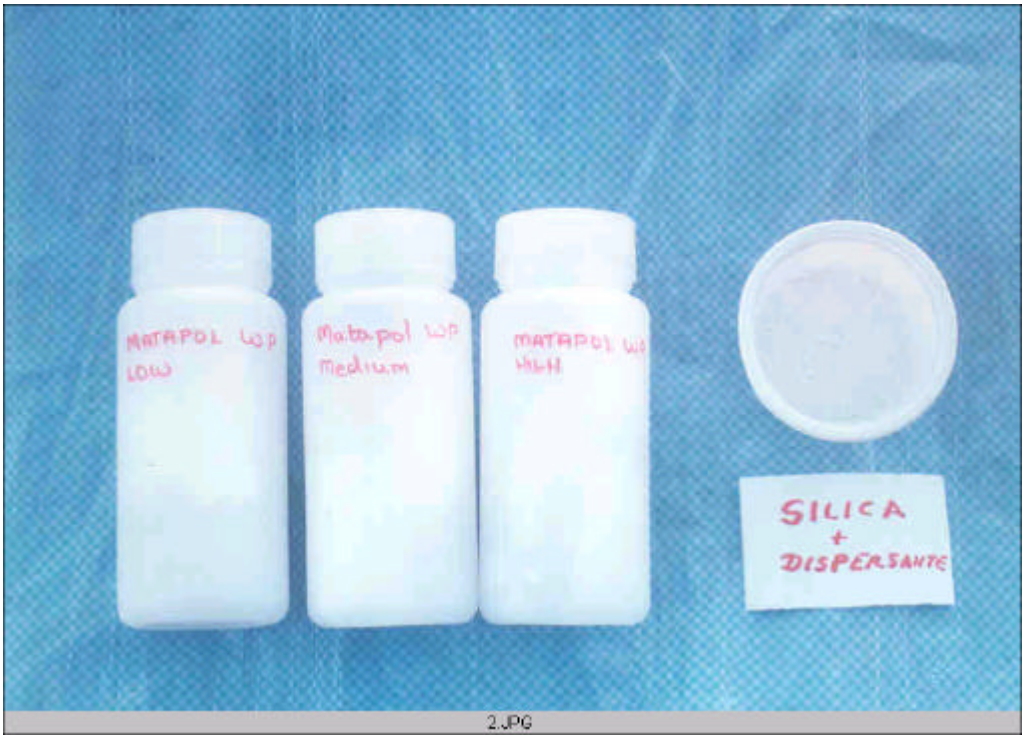


Plate 2. Wettable Powder formulation of the PoGV at three different doses

Plate 3. Dipping-Treatment

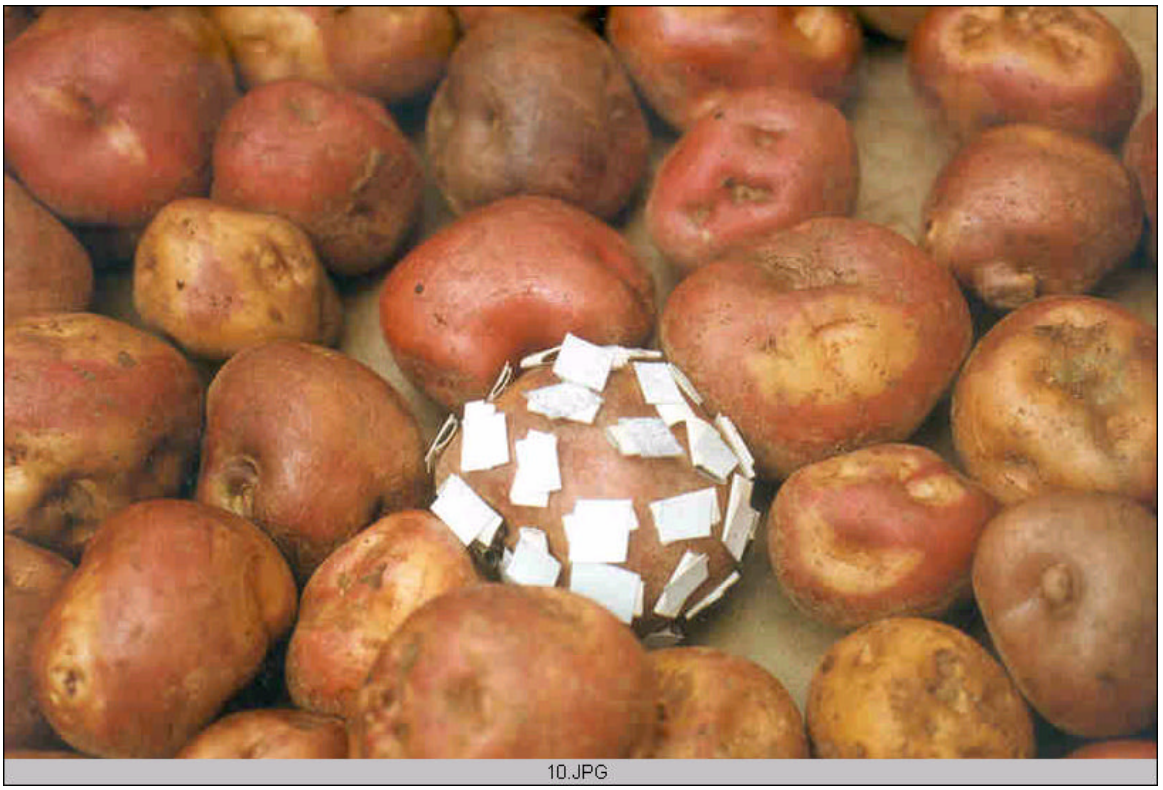


Plate 4. Detection of spray coverage using oil-sensitive paper

RESULTS OF LABORATORY BIOASSAY

Tuber damage

As can be seen in Figures 1 and 2, the Matapol+ formulation provided the most effective protection against tuber damage of all the treatments. With an average damage score of zero, it provided significantly lower damage scores than the no-treatment control (mean of 1.2) or any of the liquid-formulations but was not significantly better than the Matapol or Kaolin-only treatments ($P < 0.0001$, $df=21$, $F=10.49$). The Matapol and Kaolin-only treatments were also significantly better than the no-treatment control and liquid formulations with an average damage score of 0.1 compared to 0.7 to 2.0 for liquid formulations ($P < 0.0001$, $df = 21$, $F= 10.49$) but were not significantly better than the Matapol+. Within all the Dry formulations there were no significant differences in damage level.

Damage levels in tubers from any of the water or oil-based treatments were not significantly lower than the no-treatment control but some of the Dip treatments did have significantly higher levels of damage than the no-treatment control and spray treatments ($P < 0.001$, $df =21$, $F = 4.49$) (Figure 1). Within the spray treatments (oil and water-based, including those with no GV content) there were no significant differences in level of damage to tubers.

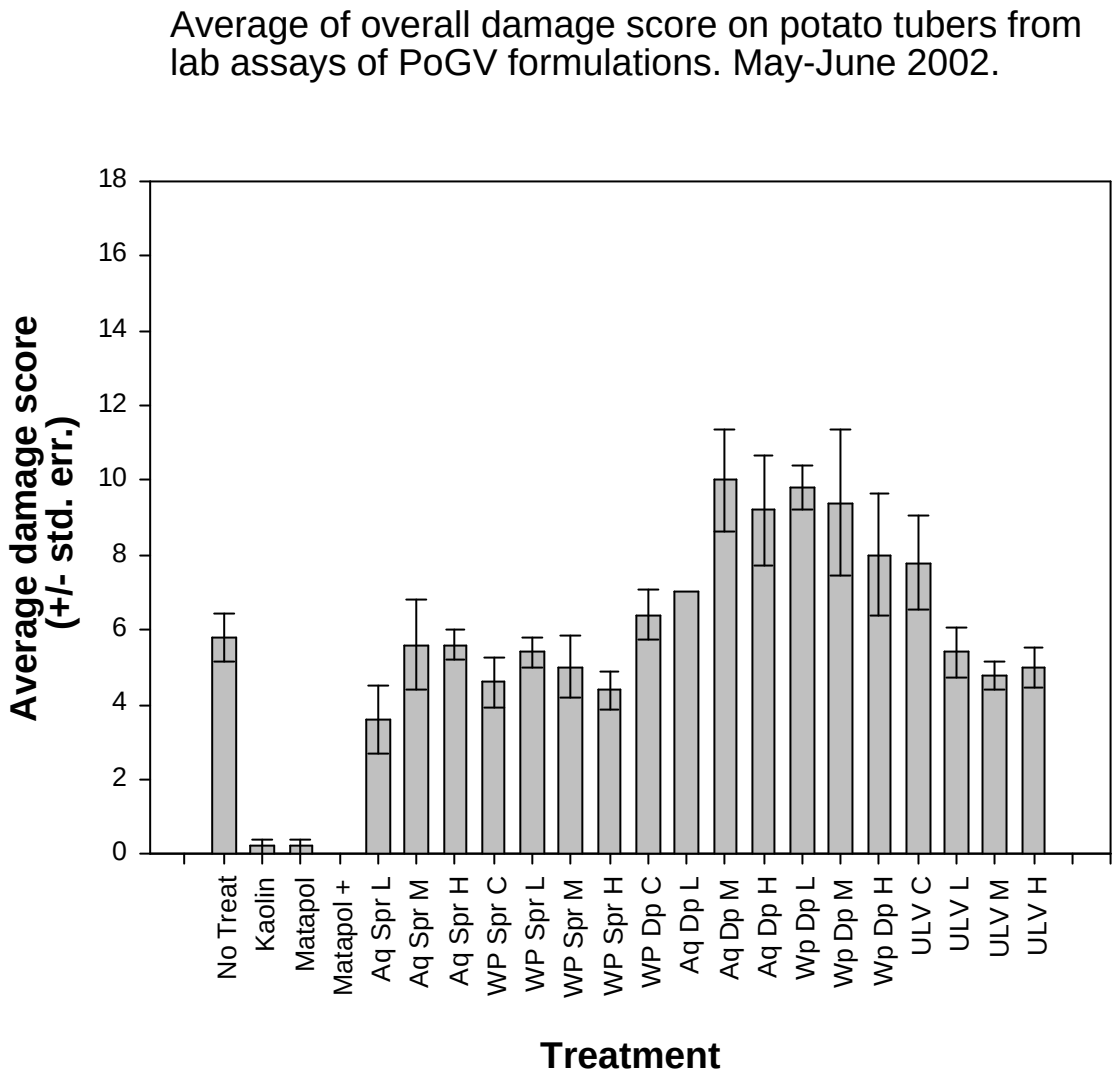


Figure 1. Bar chart of average damage scores to tubers in lab assays.

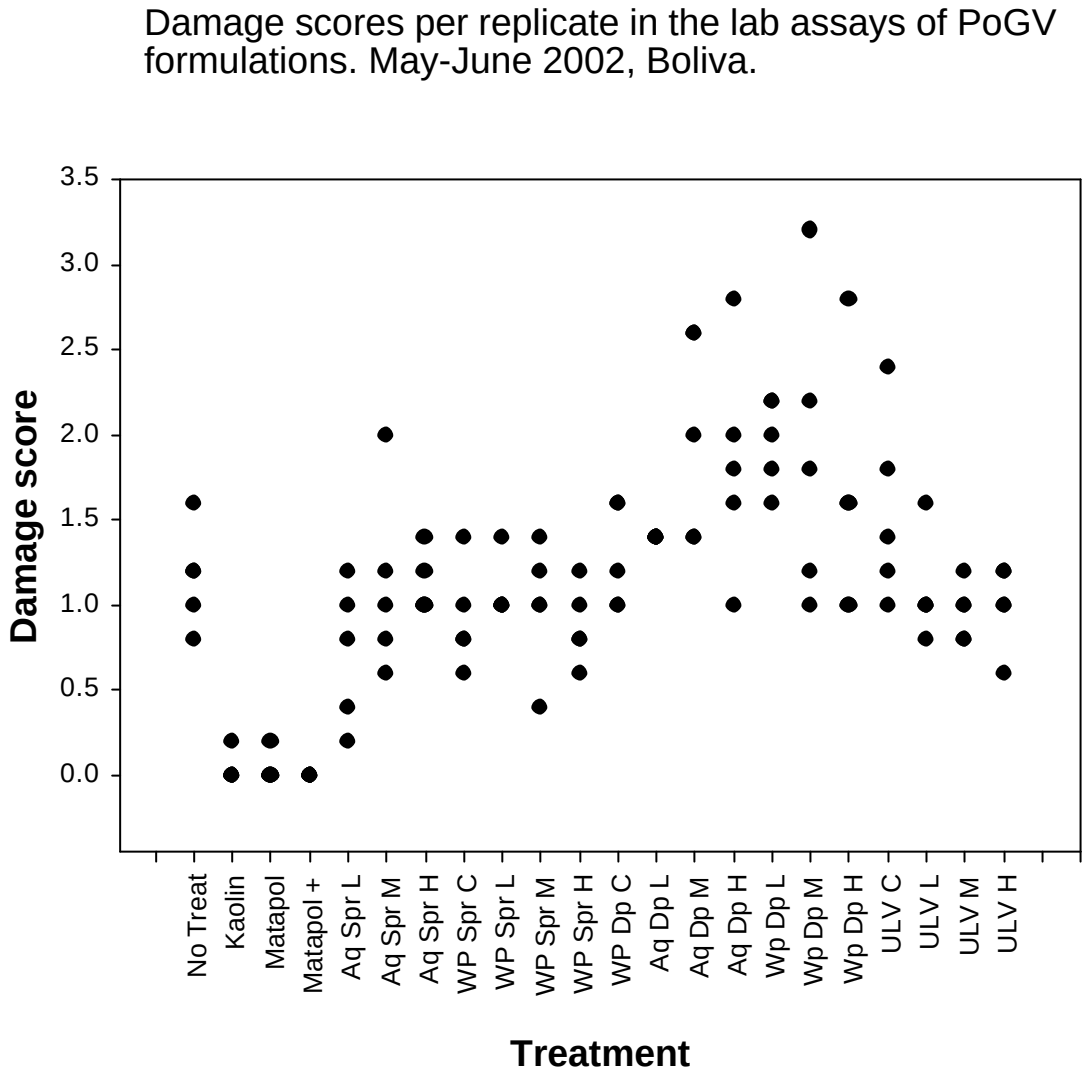


Figure 2. Point plots of damage scores to tubers in each replicate of treatments in the lab assays.

Number of PTM present

Potato tubers treated with the Matapol+ had the least number of PTM present of all the treatments. There were zero PTM present in any tuber, which was significantly less than the no-treatment control or any of the liquid-formulations but was not significantly less than the Matapol or Kaolin-only treatments. The Matapol and Kaolin-only treatments each had an average of 1 larva per 25 tubers which were significantly lower levels of PTM infestation than the no-treatment control and liquid formulations but were not significantly lower than the Matapol+. Within all the Dry formulations there were no significant differences in levels of PTM infestation (Figures 3 and 4).

PTM infestation levels in tubers from the CA-spray low-dose, WP-control and WP-medium and high-dose spray treatments were significantly lower than the no-treatment control, all of the Dip treatments and all of the ULV treatments ($P < 0.001$,

df = 21, F= 4.26) (Figure 3). Overall, the Dip treatments had highest levels of PTM infestation levels of all treatments with levels that were significantly higher than any water or oil-based spray treatment (P<0.001, df = 21, F= 4.26) (Figure 3).

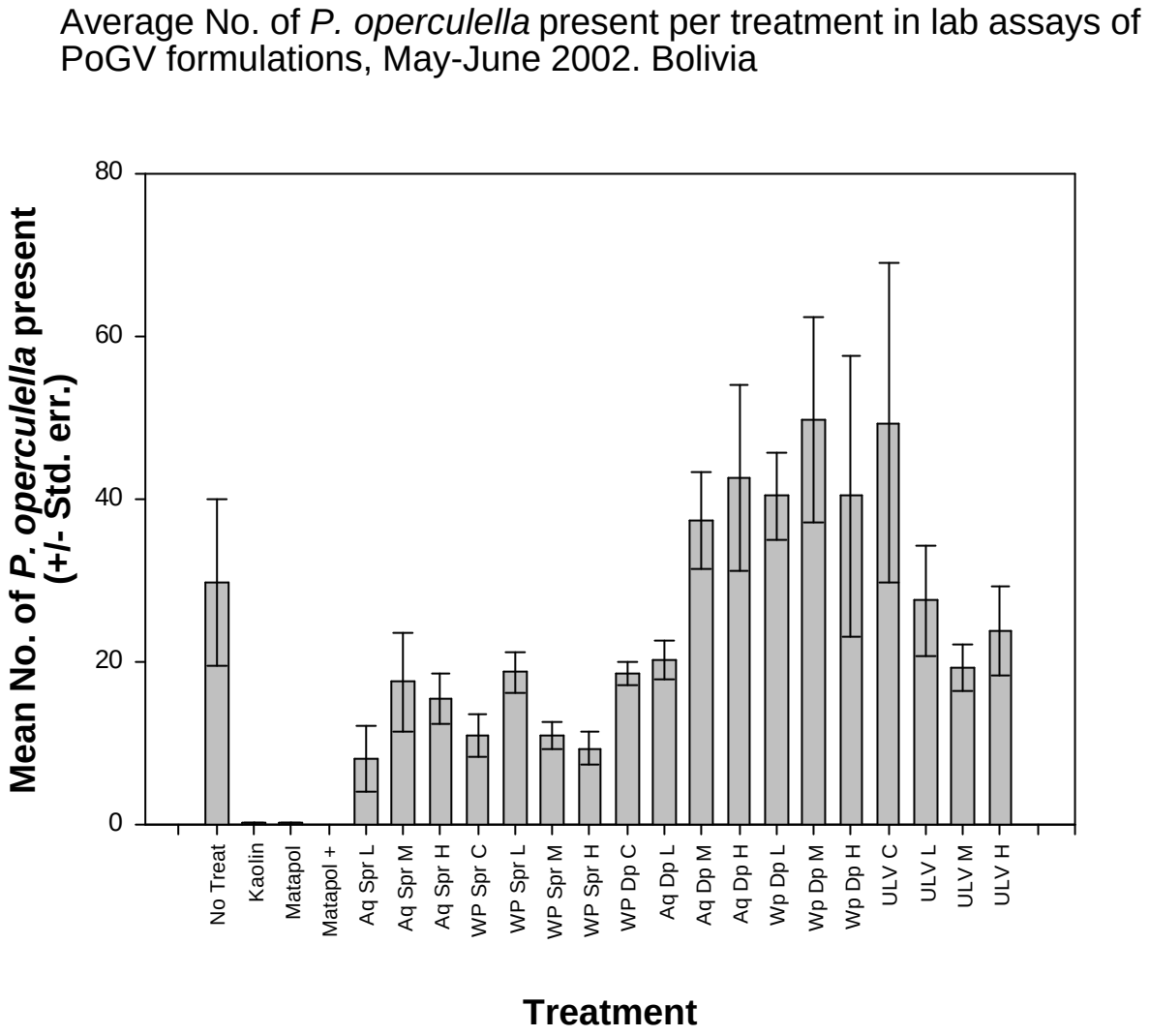


Figure 3. Bar chart of average number of PTM larvae present in lab assays of PoGV formulations.

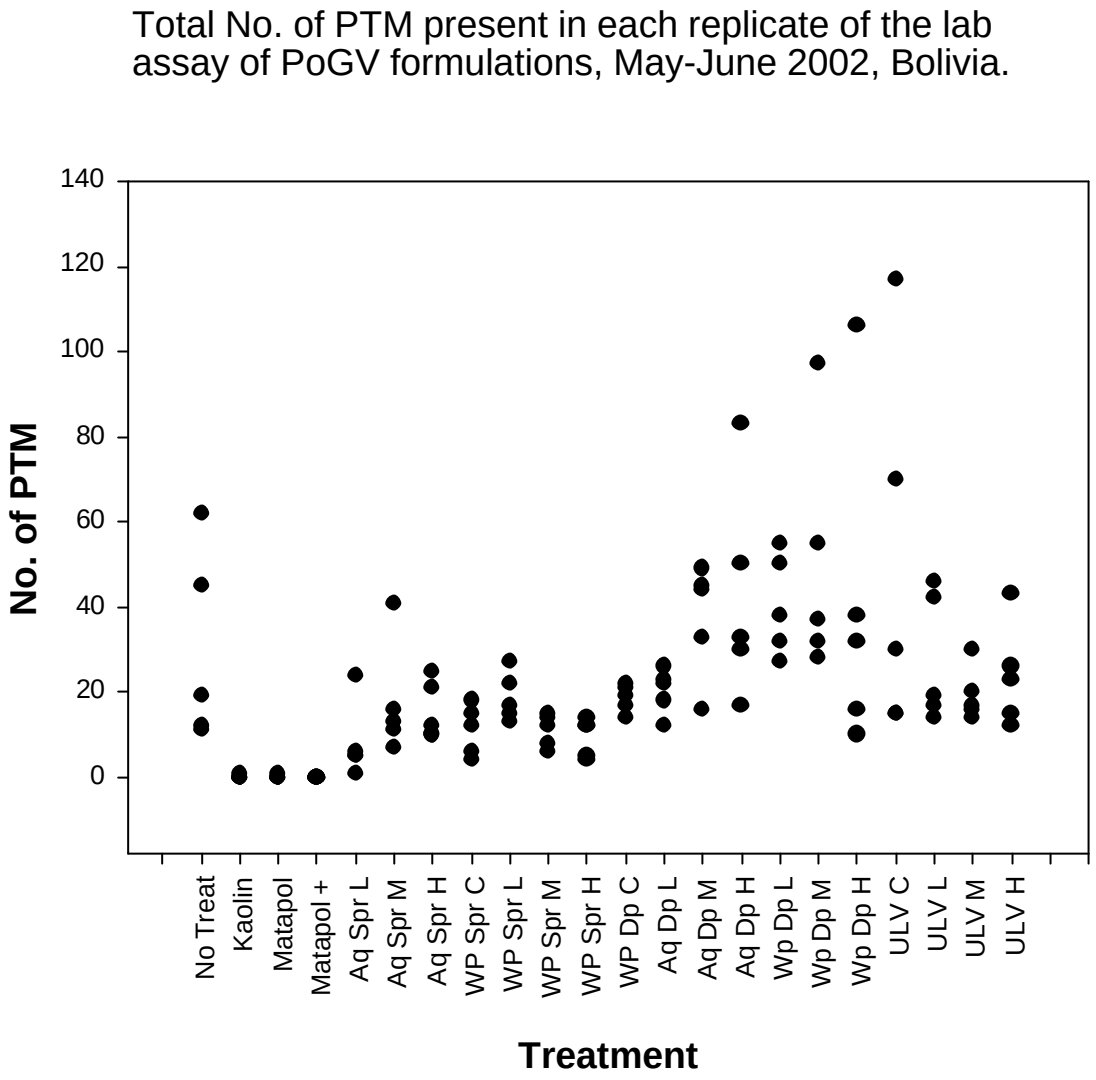


Figure 4. Point plots of damage scores to tubers in each replicate of treatments in the lab assays.

Infection rate in PTM larvae

In general the PoGV infection rate was on the low side with an average that did not reach any higher than 55%. Matapol and Matapol+ produced an average infection rate no higher than 5% and only the ULV doses and CA high-dose Spray produced infection levels that exceeded 25% (Figure 5).

A dose response was observed in the CA and WP Spray treatments and the ULV treatments but not in the Dip treatments of either CA or WP formulations. In general the ULV treatments produced the highest infection levels of all treatments giving rise to significantly higher infection rates than all treatments except CA-Spray high-dose, WP-Spray med & high-doses and the CA-Dip high-dose ($P<0.001$, $df = 21$, $F = 4.99$). The Dip treatments produced the lowest levels of infection in general although they were only significantly different to those from the ULV treatments and the CA Spray high-dose (Figure 5).

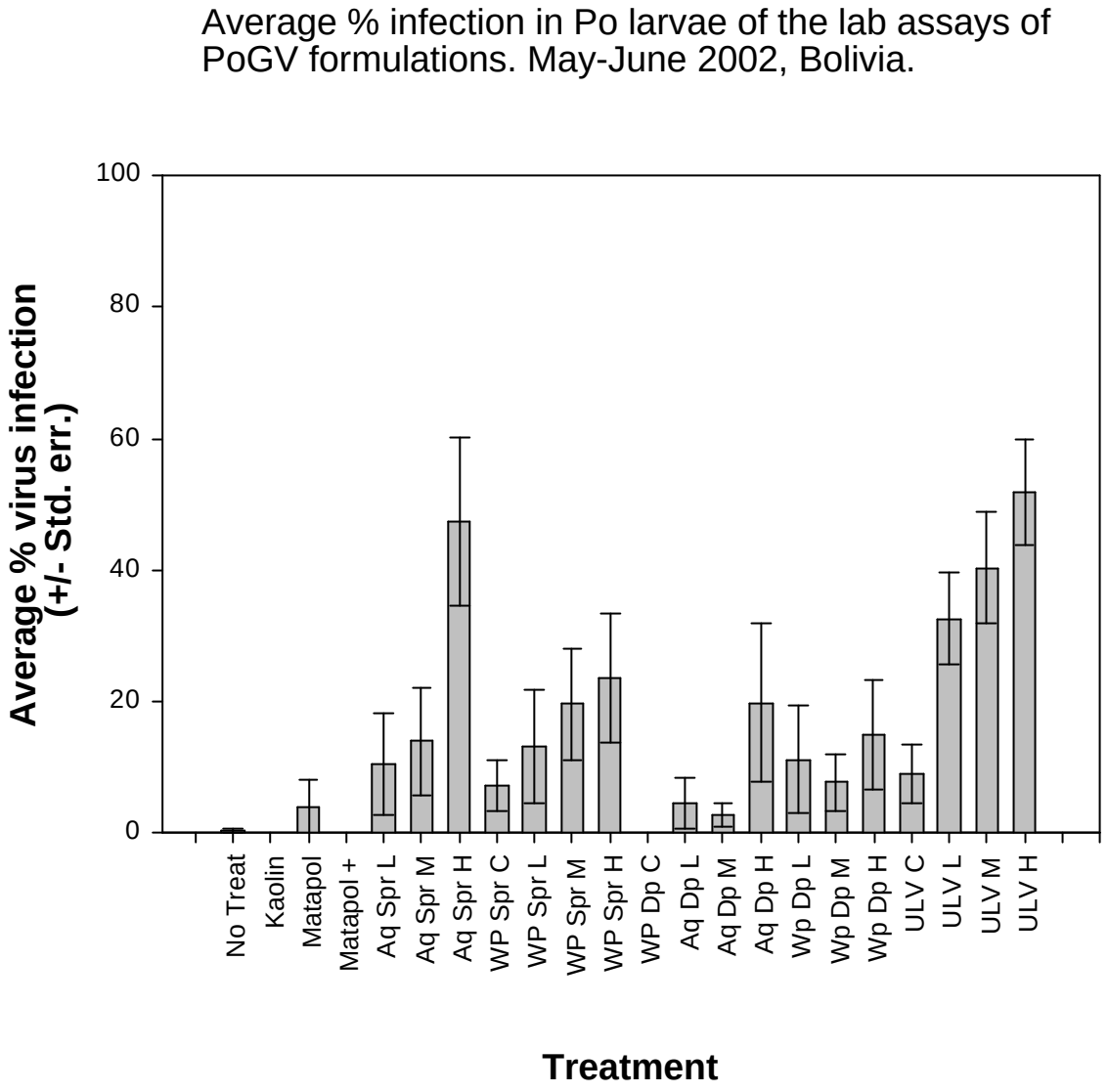


Figure 5. Average rates of infection in PTM larvae from the lab bioassay of PoGV formulations.

Discussion

The results have raised several important issues for discussion and consideration during planning of future trials due under this project. The results strongly indicate that both the oil and water-based formulations were not as effective as the dry-powder formulations currently used and from the damage data it would appear that none have prevented less damage being caused to tubers than the untreated control. This indicates that the PoGV dose rates applied were too low to have a significant effect on the PTM larvae. The current application rate of PoGV in the Matapol formulations is one larval equivalent per 25Kg bag of potatoes and in the trial dose rates equivalent to that and ten times higher than that were tested in each treatment but still, only a maximum average of 55% infection was recorded. For this reason future trials should include dose rates of 30 and 100 times higher than the current Matapol formulations.

The Dip treatments appear to have performed the least effectively out of all the treatments giving rise to the highest levels of damage and PTM infestation and the

lowest levels of PoGV infection. These treatments have even performed significantly worse than the no-treatment control in all aspects but level of infection so inclusion of this type of treatment in future trials should be questioned.

Among the spray treatments, in terms of number of PTM present the WP sprays appear to have outperformed the crude aqueous (CA) and ULV sprays but the results are not conclusive enough to draw any sound recommendations on whether to drop either of the other two formulations. This is especially true when the level of damage is brought into consideration, results there having shown no significant difference between any treatments.

The ULV treatments caused the highest level of GV infection in the PTM larvae overall, consistently causing higher levels of infection than the other formulations when compared dose-to-dose with them. Other studies have shown that ULV application causes higher levels of infection with baculoviruses, and therefore, if a purely liquid-based formulation is to be considered, a ULV formulation may be the most favourable option to choose.

The results strongly indicated that the powder alone in both the wettable powder and the dry-powder formulations had a highly significant effect on the PTM. Although significant differences occurred between the dry and wet formulations, actually within the formulations there were no significant differences. That is to say that with regard to pest numbers and damage, the effect of Kaolin alone was equal to that shown by Matapol formulations. Again in the wettable powder treatments, WP alone was as effective as the WP formulated with low, medium and high doses of PoGV. This brings in the question of whether any active ingredient is required at all?

From the results of this bioassay there are a few points to draw for discussion with regard to future trials.

- Trials should include the crude-aqueous and ULV treatments as liquid-based treatments alone but the doses should be increased by 30 and 100 times the PoGV rate currently used.
- Due to the issue of the powder alone having a significant effect the WP formulation should be looked at only as inert powder compared to the local Kaolin inert powder in a separate study of different concentrations sprayed in a "slurry" format.
- Harvest data from the field trial should be collected to give a clear indication of overall effect or treatments.
- Dipping treatments appear ineffective and could be dropped form future trials.
- The effect of the Kaolin alone on the secondary pest should be considered.

APPENDIX I List of treatments

T1 Control
T2 Kaolin only
T3 Matapol (commercial)
T4 Matapol Plus (commercial)
T5 Crude Aqueous Spray low-dose
T6 Crude Aqueous Spray Medium-dose
T7 Crude Aqueous Spray High-dose
T8 WP Spray Control
T9 WP Spray low-dose
T10 WP Spray medium-dose
T11 WP Spray high-ose
T12 WP Dip Control
T13 Crude Aqueous Dip low-dose
T14 Crude Aqueous Dip medium-dose
T15 Crude Aqueous Dip high-dose
T16 WP Dip low-dose
T17 WP Dip medium-dose
T18 WP Dip high-dose
T19 ULV Control
T20 ULV low-dose
T21 ULV medium-dose
T22 ULV high-dose

APPENDIX II Sprayer Calibration

- For each sprayer type, 25 Kg of potato tubers were placed out on the ground in a single layer.
- Four potatoes were completely covered using small squares of water or oil sensitive paper and placed among the other tubers.
- The sprayer was then "loaded" with a measured quantity of spray liquid whether it was oil or water based.
- The potatoes were then sprayed and the spray liquid allowed to dry.
- All of the tubers were then turned over and the spraying repeated.
- The remaining spray liquid was then measured.
- Initial and final volumes were then compared to give the volume of spray liquid used and thus the volume required per Kg of tubers.
- This procedure was performed three times for each type of sprayer.

APPENDIX 3

Protocol for field trials of PoGV against *P.operculella* in Bolivia, October 2002.

Introduction

Bioassays of material used in the previous field trial of May-June 2002 have indicated that GV application rates in aqueous formulations as used in that trial are far to low to provide adequate control of PTM. Even at a 10 times higher dose than that currently used in Matapol formulations, there was not good control and infection levels in PTM larvae did not reach higher than 55%. It has therefore been proposed that much higher dose-rates be tested in a single formulation in order to gain an insight into the feasibility of using an aqueous formulation.

Materials and methods

Field inoculum

The material used for dose preparation originated from PROINPA and was supplied to NRI during the first field visit made by David G. The material consisted of 490 infected PTM larvae that had been freeze-died individually before being carried to NRI. For purposes of dose preparation the freeze-dried larvae had been amalgamated suspended in distilled H_2O and homogenised using a laboratory homogeniser. Before doses were prepared, eye-counts of GV concentration were performed on three separate sub-samples that were taken from the stock homogenate. The counts revealed an average concentration of $4.70x10^{11}$OB/ml and the total volume of inoculum produced was 65mls. From this and previous quantification of GV content in infected PTM larvae it appears that an average of $8.0X10^{10}$ OBs are produced in a single fully infected larva.

Application Rates (Treatments)

Treatments for the trial to be tested against standards will be freeze-dried Crude Aqueous PoGV applied at 30 and 100 times the original dose used in Matapol formulations. Aqueous treatments will be applied to 25Kg of tubers using a hand-held sprayer.

- The dose at 30 times the standard will be at a quantity of $2.40\ x10^{12}$ OBs per 25Kg bag of tubers, which is 30 larval equivalents (Low dose).
- The dose at 100 times the standard will be at a quantity of $8.0x10^{12}$ OBs per 25-Kg bag of tubers, which is 100 larval equivalents (High dose).

Both of these treatments will be in the form of freeze-dried PoGV in vials of pre-measured doses. One vial will be supplied for each replicate of the two doses and vials are labelled LD-R1 to LD-R5 and HD-R1, HD-R2. LD and HD refer to low dose and high dose respectively. The standard treatments will be supplied by PROINPA at the start of the trial.

Storage trial

The full list of treatments is:

Untreated control (5 replicates)
Matapol (5 replicates)
Kaolin only (5 replicates)
Crude Aqueous "30LE" (5 replicates)
Crude Aqueous "100LE" (5 replicates)

Two people can complete 7 treatments of 5 replicates in one working day.

Treatment application

The standard treatments of Matapol and Kaolin will be applied in the usual way for powder-formulation application. The crude aqueous formulations will be applied using a hand held sprayer supplied by PROINPA. From the previous trial it is known that an application volume of 145mls will be required to deliver the PoGV dose of each replicate when using the hand-held sprayer.

For each replicate of the two aqueous treatments the following protocol should be followed:

- Weigh out 6Kg of potato tubers (125 tubers) and spread them out in a single layer over 1 metre square).
- Measure out 145mls of water and add the appropriate amount of locally available spreading agent.
- Add the contents of one vial of freeze-dried crude PoGV preparation and wash out the vial several times to ensure that all the GV is included in the treatment.
- Spray the meter square evenly with half of the measured dose and allow the liquid to dry.
- Turn the tubers to expose the unsprayed surfaces and spray the remaining treatment.
- Leave tubers to dry (15-25 minutes)
- Take a sub-sample of 5 tubers for laboratory bioassay.
- Bag the remaining potatoes and store in an area free from *S. tangolias* species.
- Repeat for the remaining replicates and the 100LE rate.

Apply kaolin and Matapol plus treatments to 6-Kg samples as normal.

Persistence Trial

This will be to determine the persistence of PoGV on potatoes on store. To be acceptable to farmers the PoGV formulation needs to stay active for 6 months to repel invasions by the 3-5 generations of PTM that occur over the storage season (Oct-March). In this trial samples will be treated as above but there will be

Five LD replicates
One HD replicate
Two controls

Once treated 6 Kg batches will be stored separately in boxes excluding Po or St at Toralapa in the dark at 10 deg. Standard bioassays will then be conduced at 0, 1, 2, 3, 4, 5 and 6 months post spraying to determine any loss of activity due to GV plant interaction.

Laboratory bioassay protocol

1. Put 5 treated tubers in a 700ml ice cream tub.

2. Infest with 50 eggs of PTM on paper as laid

3. Cover with muslin cover held on with elastic bands

4. Place in 18 deg C CT room

5. After 6 weeks take out tubers, score damage, count PTM larvae and numbers PTM infected.

APPENDIX 4

SEARCH FOR AN ENTOMOPATHOGENIC VIRUS AGAINST *SYMMETRISCHEMA TANGOLIAS*

INTRODUCTION

The granulovirus of *P. operculella* (*Po*GV) is an effective biological agent to control this particular pest. Due to the high specificity of granuloviruses *Po*GV is not virulent against *S. tangolias*. In view of the spread of *S. tangolias* in the Andean region, the need identify specific biological agents for this particular pest is urgent. Baculoviruses are very advantageous because they could be produced and used alongside the existing *Po*GV product. The possibility that a specific virus for *S. tangolias* exists was considered to be very prospective. The present activity is to search for a specific baculovirus infecting *S. tangolias*.

MATERIALS AND METHODS

Collection

Samples of *S. tangolias*-infected potatoes were collected from different farmers' stores in Peru and Bolivia as indicated in Table 1 and Figure 2. The samples were collected in paper bags and sent to CIP, Lima, for further investigation (see Fig 6 and 7). Sampling in Bolivia was conducted by PROINPA. At CIP, potatoes were cut and *S. tangolias* larvae individually macroscopically analyzed and classified for presence of pathogens according to the presence of symptoms. Thereafter, larvae were separated in Eppendorf tubes and homogenized in 500 µl in grind buffer TEP 1x (10.8 g Tris-base, 4.0 ml 0.5M EDTA and 0.168 g phosphate acid 85%) using a mechanic tissue mixer. The homogenate was filtered through muslin and centrifuged at 4.000 rpm for 1 min. The supernatant was poured into eppendorf tubes and stored in an ultra-refrigerator at - 70°C.

Light microscopy

All samples were screened for virus incidence using the light microscope with dark field optics (400x).

Serological assays

Samples were submitted to DAS-ELISA using the method described by Clark & Adams (1977) modified by CIP 1979. The reaction of baculovirus antiserum was quantified by measuring the light absorbance in a micro plate reader (Biorar model 550). 100µl of semi-purified larval material was used for this test (see Figure 8).

Transmission electron microscopy (TEM)

DAS-ELISA positive samples were inspected by electron microscopy to confirm the presence of granulovirus infection. 20 µl of the homogenate were placed on a carbon-coated grid with 20 drops of uranyl acetate (2%) for 1 minute. Seven fields of each grid were observed at 80 Kv in a Jeol 100S electron microscope.

Virus multiplication tests

When presence of granulovirus was evident or expected in a sample, both *S. tangolias* and *P. operculella* were inoculated by pouring 250 µl of the homogenate over eggs placed on filter paper (egg-dip bioassay technique). Larvae were reared on potato tuber at 24°C and natural photoperiod. First after 14 and secondly after 20 days the number of infected larvae

was assessed. Due to the fact that multiplication in *S. tangolias* trails further efforts were made to multiply the virus in this host by stressing the larvae. Virus inoculated egg were incubated at different temperature (17, 20, 25°C) until pupae emergence. Furthermore, *P. operculella* eggs were inoculated with homogenates.

Virus purification

Only DNA from viruses multiplied in *P. operculella* could be extracted because multiplication failed in *S. tangolias*. Diseased larvae were harvested and macerated in distilled water with a mechanic tissue mixer (GlasCol, USA). The homogenate was filtered through muslin (four layers of cheesecloth) to remove the gross debris. The filtrate was transferred to centrifuge tubes (Beckmann) and lipids, soluble material, and small contaminants were removed by 10,000 g centrifugation for 20 min. The supernatant fluid was discarded and the pellet resuspended in small volumes of 0.1% sodium dodecyl sulfate (SDS) layered onto a 40/65/80% (w/w) sucrose gradient. After ultra centrifugation (50,000 g, 60 min, 4°C) the bands containing the virus were harvested with a Pasteur pipette and diluted in distilled water. Rests of sucrose were removed (washed) twice by centrifugation at 10,000 g for 15 min and resuspending the pellet in sterile distilled water. The virus was stored at -20°C.

DNA extraction and Restriction Enzyme analysis

Virus granules were dissolved in 0.05 M-Na_2CO_3 1% SDS, and incubated with proteinase K (10 µl/300 µl final concentration) at 37°C for 1 h. The solution was double extracted with phenol and dialyzed against 10 mM-Tris-HCl, 1 mM-EDTA pH 8.

The viral DNA was digested with a list of restriction enzymes (*Eco*RI, *Xho*I, *Bgl*II, *Hind*III, *Pst*I) and submitted to electrophoresis in 0.5% to 1.5% agarose gels using standard methods (SAMBROOK *et al.* 1989).

RESULTS AND DISCUSSION

A total of 10,435 larvae collected within Peru and Bolivia (Table 1, Figure 2). From this number, 383 larvae showed abnormalities or symptoms of diseases during the macroscopic analysis, which could be attributed to bacteria, fungus, or protozoa (see Figure 3). In none of the 10,435 samples incidence of baculoviruses could be noted through observations using light microscopy.

However, DAS-ELISA detected baculovirus in 29 larvae of the sample. Using TEM, the incidence of baculovirus could be confirmed in 23 of this sample and the virus could be identified as a granulovirus (in all cases, see Figure 4). Furthermore, incidence of granulovirus could be detected in additional 23 larvae of the DAS-ELISA negative sample (Table 3). However, the presence of granulovirus could be classified as low.

The attempts to multiply the new viruses using original *S. tangolias* homogenates failed in *S. tangolias.* However, *P. operculella* became infected producing infection levels between 2 to 20% while no infection occurred in the control. The virus titer in homogenates could not be determined before because of their very low virus concentrations. Therefore, the infection level does not clear the question if differences in virulence between this new isolates exists.

The virus progenies obtained from *P. operculella* larvae were purified (ultra-centrifugation) and quantified stock solutions prepared with relative high virus titers (1.6×10^8 granules/ml). These stock solutions also failed in further attempts to infect *S. tangolias*. Only few larvae (<1% of larvae showed symptoms of diseases (see Figure 1)

Consequently, the virus found in *S. tangolias* larvae may be one and the same as *Po*GV, causing only chronic infections in *S. tangolias* larvae in certain circumstances.

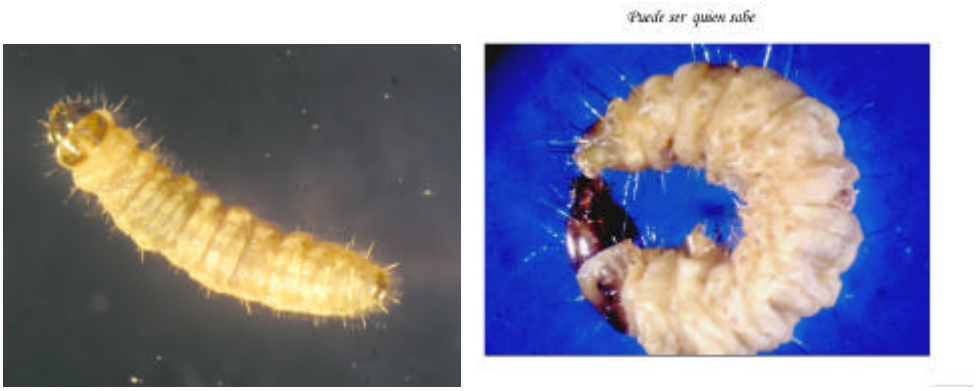


Figure 1: *S. tangolias* larvae with symptoms of infection.

The restriction endonuclease analysis (REN) demonstrated no differences between new virus isolates and *Po*GV according to the fragment length pattern (see Figure 2a-e: electrophoresis profiles). This result strength the theory that the new viruses identified in *S. tangolias* is *Po*GV. Most virus-positive samples were from Huancayo in Peru, where generally both PTM species prevail and occur together in potato stores. Because *Po*GV is naturally abundant in that zone and the two species occur frequently together in potato stores *Po*GV particles could be consumed by *S. tangolias* larvae. These virus particles could be detected with the technique used, but the possibility exists that these particles had not pass from the midgut through the peritrophic membrane causing infection of midgut cells.

CONCLUSIONS

No highly efficient baculovirus could be identified from the samples. It seems that new virus isolates are the same as *Po*GV, which is effective agains *P. operculella* and causes only chronic infections in the close related PTM species *S. tangolias* under certain circumstances. These isolates are not favorable for in pest control programs against *S. tangolias*.

Figure 2: Sampled sites in 6 departments of Peru

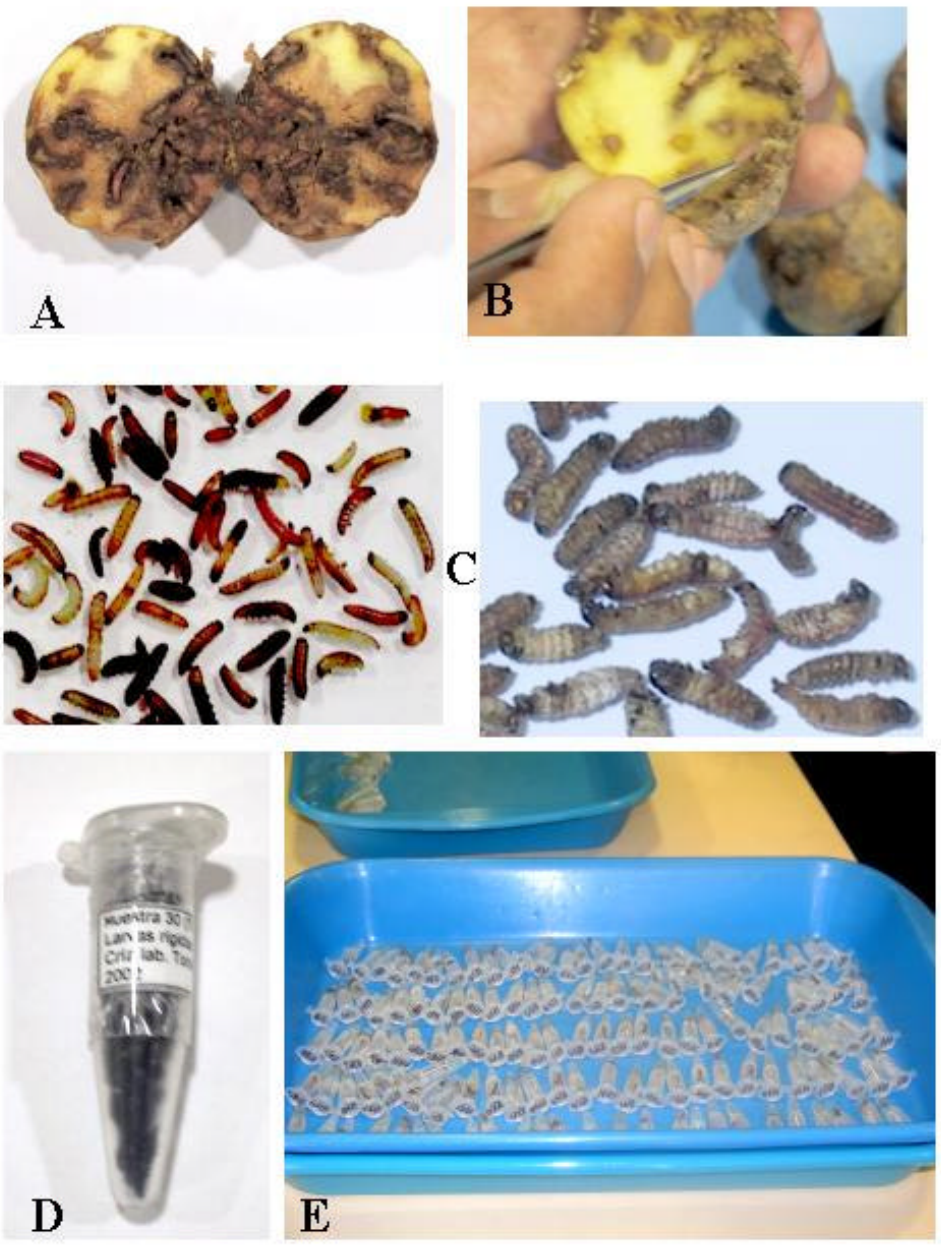


Fig # 3. - Evaluación Macroscópica de las larvas de *S. tangolias*, hasta su Individualización

Figure 3: Collection of *S. tangolias* larvae from potato tuber samples sent to CIP (A, B), larvae showing deformations or symptoms of diseases (C), and separation of individual larva in eppendorf tubes (D, E).

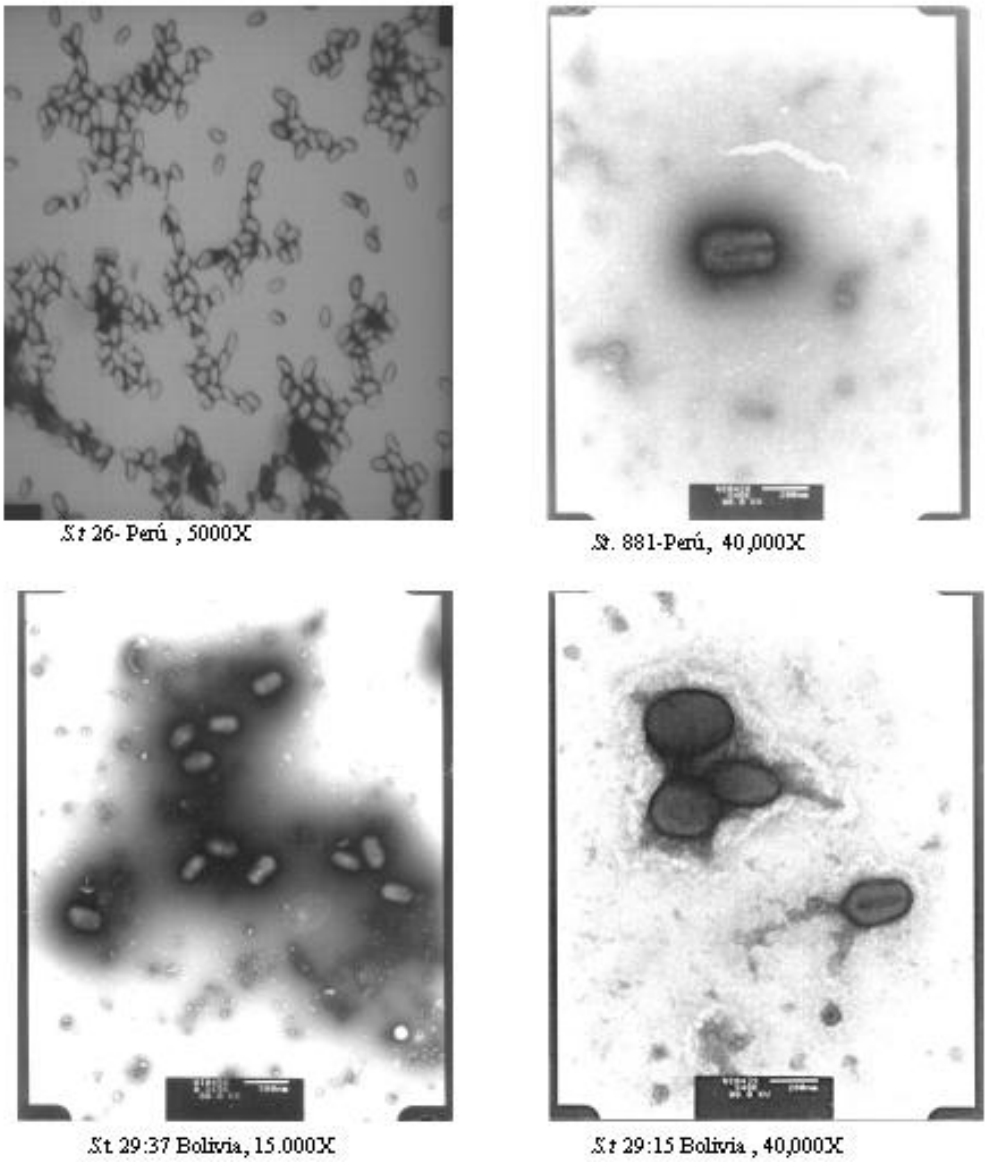


Figure 4: Transmission electron microscopy of granuloviruses detected in *S. tangolias* larvae.

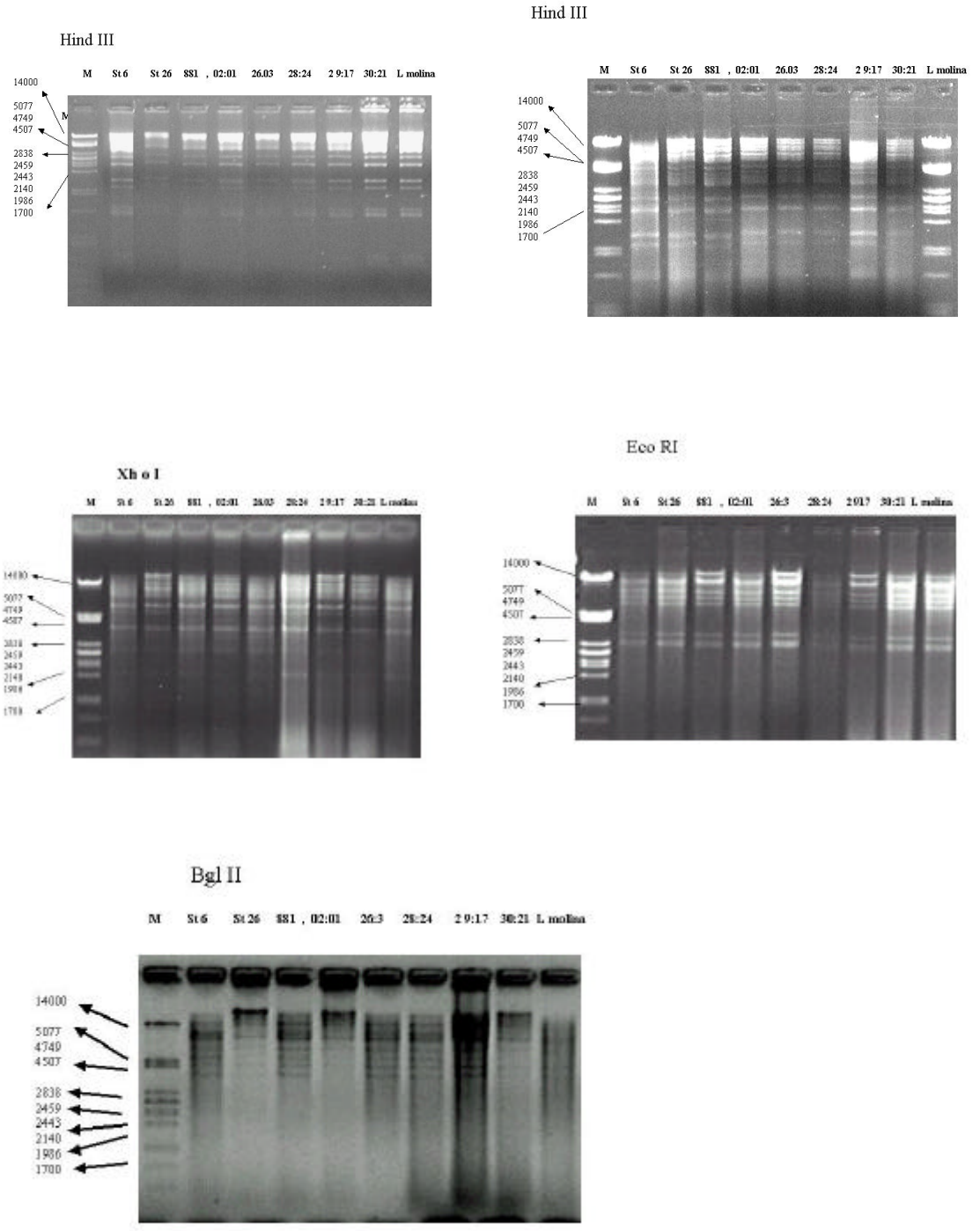


Figure 5: Comparison restriction endonuclease (REN) of DNA extracted from new virus isolates multiplied in *P. operculella*. Lane 1 shows Lambda PstI digest as molecular size marker; lane 2 = St-6-Peru (Huancayo), 3 = St-26- Peru (Huancayo), 4 = St 881 Peru (Huancayo), 5 = St-2:1-Bolivia (Cochabamba), 6 = St-26:3-Bolivia (Tarija), 7 = St-28:24-Bolivia (rearing unit Toralapa, Cochabamba), 8 = St-29:17-Bolivia (rearing unit Toralapa, Cochabamba), 9 = St-30:21-Bolivia (rearing unit Toralapa, Cochabamba), 10 = PoGV standard (isolate “La Molina”).

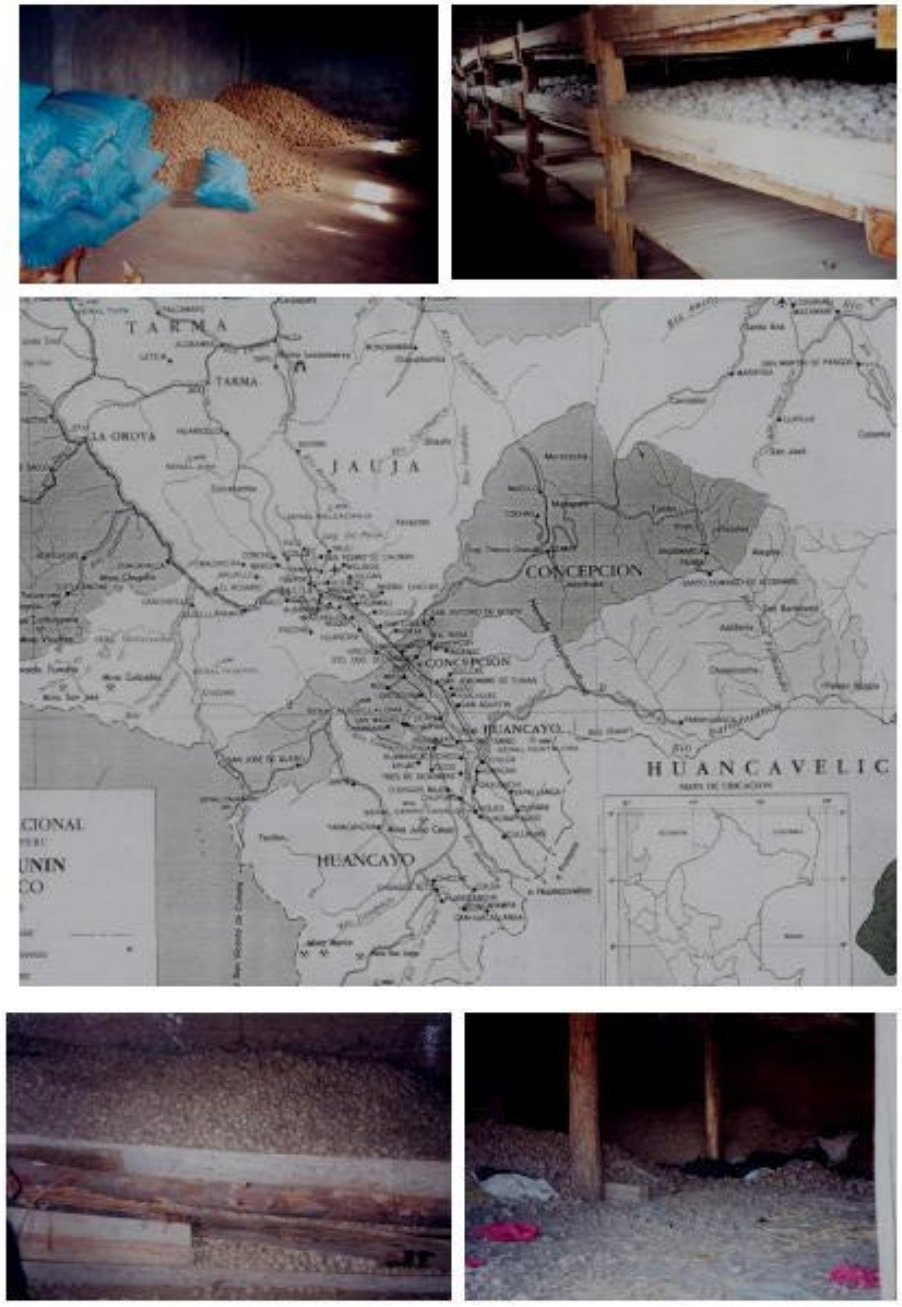


Fig # 1 .- Diferentes almacenes muestreados en el valle de Huancayo durante los años 2002-2003

Fi

Figure 6: A stores evaluated in Peru, assessment of *S. tangolias* presence, and samples sent to CIP, Lima.

Fig # 2 .- Busqueda del nuevo agente en los tuberculos alamacenados que muestran diferente grado de intencidad de daño causado por *Symmetrischema tangolias*

Figure 7: Assessment of *S. tangolias* presence during evaluation of potato stores, and sampled material sent to CIP, Lima.

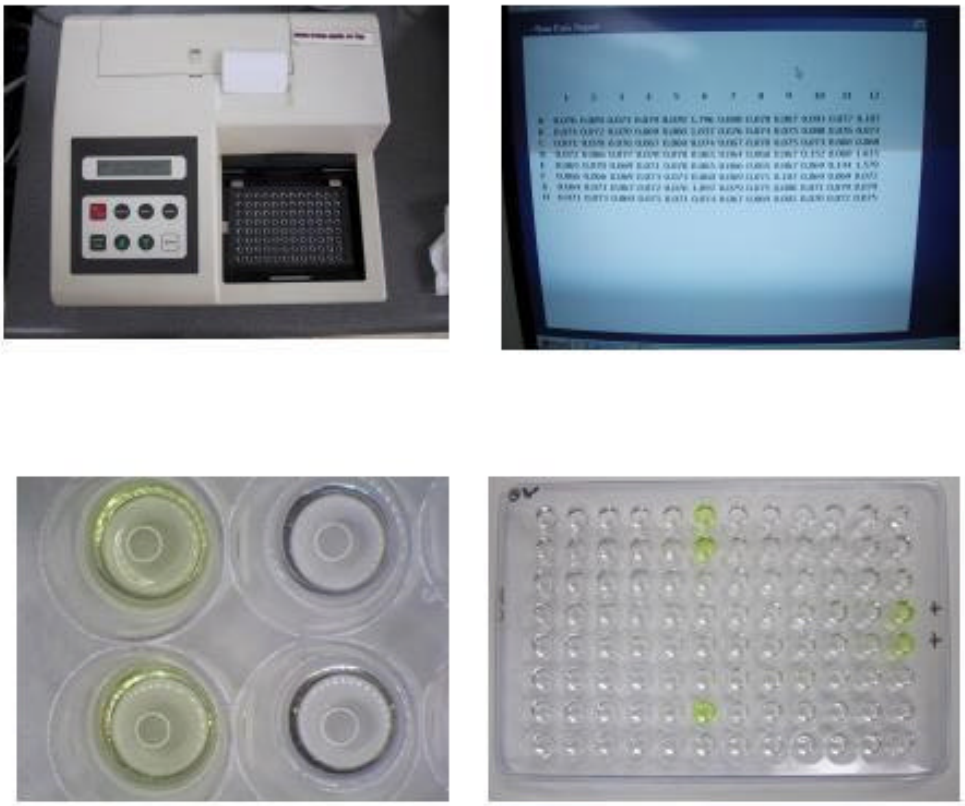


Fig # 5. Placa DAS-ELISA con muestras de reacción positiva (Color amarillo) al Antisuero PoGV, en larvas de *S. tangolia* de Bolivia y Perú.

Figure 8: Measuring light absorbance of DAS-ELISA preparations in a micro plate reader

Table 1. Sites, year, and numbers of *Symmetrischema tangolias* larvae collected in Bolivia and Peru during the project period September 2001- August 2004.

Year	Department	Province	District	Place	No. larvae	Total
		Jauja		Apata		
		Concepción		Concepción		
		Concepción		Orcotuna		
		Concepción		Mathawasi		
		Concepción		Santa Rosa		
		Concepción		Allao		
	Junin	Huancayo		Chacon	1904	
		Huancayo		Culluhas		**2389**
2001		Huancayo		Huamali		
2002		Huancayo		Huanchoc		
		Huancayo		Huancan		
		Huancayo		Huancani		
				Quilca		
	Ayacucho				485	
	Cochabamba			Candelaria	210	
				Crianza Toralapa		
	Chuquisaca				126	
				Campanario		**377**
	Tarija			El molino	34	
				San Lorencito		
	La paz				3	
2002	**Huancayo**				3852	**3852**
	Huancayo				2458	**2458**
2003	**Cochabamba**			Sapanani	4	
	San Andrés			Tarija	1	**6**
				Tscagachi	1	
	Huanuco	Huanuco	Umari	La punta	107	**169**
			Chinchao	Nueva Independencia	62	
		Huaraz	independencia	Unchus	44	
		Yungay	Musho	Musho	26	
		Yungay	Tumpo	Tumpo	12	
		Yungay	Shupiuy		3	
		Huaraz	Huaraz	San nicolas	44	
		Huaraz	Atipayan		19	
		Huaraz	Tarica		11	**330**
2003	**Huaraz**	Huaraz	Olleros	Huaripampa	25	
2004		lares	lares	Rosapata	41	
		Lares	Ayllu		12	
		Carhuaz	Vices		10	
		Carhuaz	Macora	Chancos	10	
		Huanta	Huaylas	Huanta	9	
		Recuay	Cotaparaco		30	
	Cajamarca	Cajamarca			34	
		La convención	Vilcabamba	Yupanco	89	
		Urubamba	Maras	Cruzpata	44	
		Calca	Calca	Llipllec	84	
		Urubamba	Huayllabamba	Urquillos	78	
	Cuzco	La Convención	Vilcabamba	Huancacalle	78	**854**
		La Convención	Vilcabamba		189	
		La Convención	Vilcabamba	Lucuma	76	
		Anta	Izcuchaca	Anta alta	61	
		Anta	Izcuchaca	Compone	155	
						10,435

Table 2. Total numbers of individual *S. tangolias* larvae tested for country during the period 2001 to 2004.

Country	Larvae
Bolivia	383
Peru	10052
Total	**10435**

Table 3. Number of granulovirus positive S tangolias samples detected by electron microscopy (TEM) and serological tests (DAS-ELISA).

Isolate	Origin	DAS-ELISA	TEM	Isolate	Origin	DAS-Elisa	TEM
St-6-Perú	Huancayo	-	+	St 29:22-Bolivia	Cochabamba	+(2002)	+(2002)
St-26-Perú	Huancayo	+(2002)	+(2002)	St 29:24-Bolivia	Cochabamba	+(2002)	+(2002)
St- 881-Perú	Huancayo	+(2002)	+(2002)	St 29:28-Bolivia	Cochabamba	+(2002)	+(2002)
St. 2:1-Bolivia	Cochabamba	-(2002)	+(2003)	St 29:34-Bolivia	Cochabamba	+(2002)	+(2002)
St 2:3- Bolivia	Cochabamba	-(2002)	+(2003)	St 29:36-Bolivia	Cochabamba	+(2002)	+(2002)
St 2:5 -Bolivia	Cochabamba	-(2002)	+(2003)	St 29:37-Bolivia	Cochabamba	+(2002)	+(2002)
St 2:21- Bolivia	Cochabamba	-(2002)	+(2003)	St 29:41-*Bolivia*	Cochabamba	+(2002)	+(2002)
St 3:26-Bolivia	Cochabamba	-(2002)	+(2003)	St 29:42-Bolivia	Cochabamba	+(2002)	+(2002)
St 26:3-Bolivia	Tarija	-(2002)	+(2003)	St 29:43-Bolivia	Cochabamba	+(2002)	+(2002)
St 28:5-Bolivia	Cochabamba	-(2002)	+(2003)	St 30:21-Bolivia	Cochabamba	+(2002)	+(2002)
St 28:9-Bolivia	Cochabamba	-(2002)	+(2003)	St1- Peru	Huancayo	+ (2004)	+(2004)
St 28:11-Bolivia	Cochabamba	-(2002)	+(2003)	St2-Peru	Huancayo	+(2004)	_(2004)
St 28:12-Bolivia	Cochabamba	-(2002)	+(2003)	St3-Peru	Huancayo	+(2004)	+(2004)
St 28:13-Bolivia	Cochabamba	-(2002)	+(2003)	St4- Peru	Huancayo	+(2004)	+(2004)
St 28:15-Bolivia	Cochabamba	-(2002)	+(2003)	St5- Peru	Huancayo	+(2004)	+(2004)
St 28:16-Bolivia	Cochabamba	-(2002)	+(2003)	St6A- Peru	Huancayo	+(2004)	+(2004)
St 28:17-Bolivia	Cochabamba	-(2002)	+(2003)	St7- Peru	Huancayo	+(2004)	-(2004)
St 28:20-Bolivia	Cochabamba	-(2002)	+(2003)	St8- Peru	Huancayo	+(2004)	_(2004)
St 28:24-Bolivia	Cochabamba	-(2002)	+(2003)	St9- Peru	Huancayo	+(2004)	_(2004)
St 28:27-Bolivia	Cochabamba	-(2002)	+(2003)	St10- Peru	Huancayo	+(2004)	+(2004)
St 28:29-Bolivia	Cochabamba	-(2002)	+(2003)	St11- Peru	Huancayo	+(2004)	_(2004)
St 29:01-Bolivia	Cochabamba	-(2002)	+(2003)	St12- Peru	Huancayo	+(2004)	+(2004)
St 29:07-Bolivia	Cochabamba	-(2002)	+(2003)	St13- Peru	Huancayo	+(2004)	+(2004)
St 29:15-Bolivia	Cochabamba	-(2002)	+(2003)	St14- Peru	Huancayo	+(2004)	+(2004)
St 29:17-Bolivia	Cochabamba	-(2002)	+(2003)	St15- Peru	Huancayo	+(2004)	+(2004)
St 29:19-Bolivia	Cochabamba	+(2002)	+(2002)	St16- Peru	Huancayo	+(2004)	(2004)

Table 4. Positive Results from Examination of *S. tangolias* Cadavers by Double-Antibody Sandwich Enzyme-Linked Immunoadsorbent Assay (DAS-ELISA) and Transmission Electron microscopy (TEM)

ASSAY	No. POSITIVE
DAS-ELISA only	6
TEM only	23
DAS-ELISA and TEM	23
TOTAL	52

APPENDIX 5

LABORATORY BIOASSAYS

MATERIALS AND METHODS

Bioassays were carried out at Toralapa during the three cropping seasons of 2001 – 02, 2002 – 03 and 2003 – 04.

Insect material. For bioassays in 2001 – 02, adults of *Rhigopsidius piercei* were collected from inside stored potato tubers and were assumed to be virgin. There are no obviously sexually dimorphic characteristics, and sexing was done arbitrarily on size taking males as smaller than females. Adults of *Premnotrypes latithorax* were obtained from the field either on plants or in traps and were assumed to be mated. This species can be reliably sexed as adults. In the subsequent two seasons adults of both species were obtained from cultures maintained on potato tubers in the laboratory under ambient temperature and humidity conditions. Adults were sexed soon after emergence and kept in single sex groups. Thus they were virgins when tested.

Bioassay procedure. The basic experimental testing unit was a dual-choice pitfall bioassay chamber. This was formed by a petri-dish through which two holes were punched. Eppendorf tubes whose ends had been cut off were fitted into these holes and the tubes were then inserted into glass sample tubes (Fig. 1). This arrangement allowed weevils to fall into the glass tubes but prevented them returning. Each petri-dish rested on the two glass tubes. An array of up to 18 bioassay chambers was formed by fixing the glass tubes of each within a sheet of polystyrene (Fig. 2). In each petri-dish a piece of filter-paper was placed covering the entire surface to make it easier for the insects to move around. Several smaller pieces of paper were put into each petri-dish to permit the weevils to hide from the light.

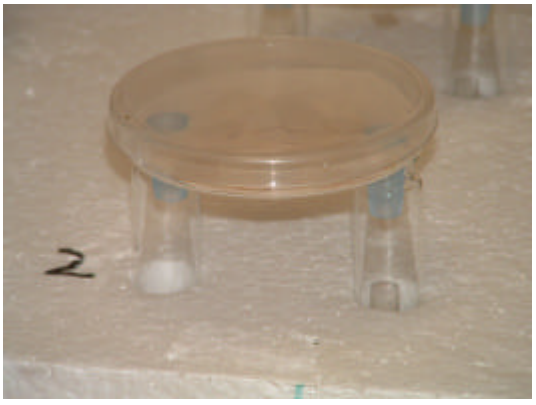


Fig. 1. Dual-choice pitfall bioassay chamber formed from petri-dish, Eppendorf and glass tubes.

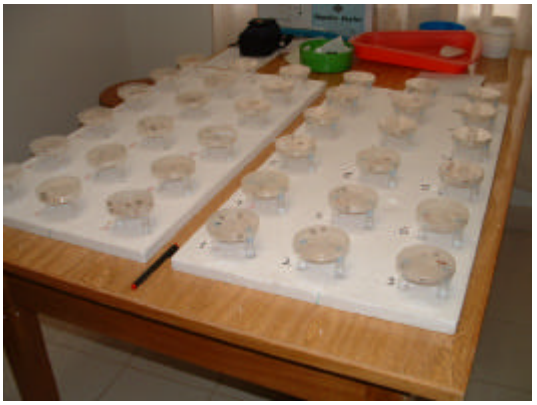


Fig. 2. Two arrays of 18 bioassay chambers set into polystyrene sheets.

The experiment consisted of placing one or more test weevils in the centre of the petri-dish in between the holes above each of the two glass tubes. For each test and within each array of 18 petri-dishes, nine were used to test males and nine with females for each test. Treatment details are summarised for each bioassay group in Table 1.

Data were collected up to three times a day (bioassays were run during the approximate periods 8.00 – 13.00, 13.00 – 17.00 and 17.00 – 8.00), although in later bioassays only the overnight period was used as this was when weevils were generally more responsive. Where more than one test period per day was employed, the same individual weevils were normally used. Numbers in each treatment tube, together with any non-responders, were counted and recorded at the end of each bioassay period.

Statistical Analysis. Contingency tables and χ^2 tests were used to check for significant differences between treatments with equal distribution as the null hypothesis. With some of the bioassays in 2003 – 04 the level of replication was such as to allow analysis of variance to be carried out (using arc-sine transformed proportions as the data to be analysed). Non-responders were not included in either of these analyses. Results are summarised as the Response Index (RI) = (number in Treatment 1 - number in Treatment 2)/Total number tested (Prokopy *et al.* 1995).

Table 1. Summary of treatments used for each bioassay set (each set was carried out with both weevil species although number replicates varied slightly).

Bioassay set	Number of test insects used	Treatment pairs (choices) from among
Feb – March 2002*	1 per petri-dish	weevil of same or opposite sex, fresh potato leaves, weevil+leaf, blank
Oct – Nov. 2002	1 per petri-dish	weevil of same or opposite sex, fresh potato leaves, weevil+leaf, blank
Feb – March 2003	1 per petri-dish	fresh potato leaves, damp filter-paper, blank
Nov – Dec 2003	5 per petri-dish	100 µl Z3-hexenol, 200 µl E2-hexenal in polyethylene vials, blank
Dec 2003	5 per petri-dish	0.2 mg Z3-hexenol, 0.2 mg E2-hexenal on filter-paper pieces (initially in 96% ethanol solvent), blank
Jan 2004	5 per petri-dish	0.04 mg Z3-hexenol, 0.04 mg E2-hexenal (initially in 96% ethanol solvent) on filter-paper pieces, blank
Feb – March 2004	5 per petri-dish	0.04 mg Z3-hexenol + 0.04 mg E2-hexenal (initially in 96% ethanol solvent) on filter-paper pieces, fresh potato leaves, blank

* Bioassays from this group were carried out by Ms M. Kruidhof, a visiting Dutch student at Toralapa, as part of her Ph.D studies.

RESULTS

Bioassays in 2001 – 02. For the first set of bioassays with *P. latithorax*, in Feb – March 2002, the percentage of responding weevils varied from 72 – 99%, but was greatest for the overnight period. Generally, the majority of treatment pairs produced positive values of RI, indicating attraction to conspecifics over leaves or blank tubes. However only a few of these results were statistically significant and there was little consistency with respect to attraction

to the same or opposite sex (Table 2). One consistent result, across test periods, was of attraction to potato leaves relative to blank tubes. For treatment pairs in which the choice was between weevils and leaves, leaves were favoured but not significantly in most cases. None of the corresponding results with *R. piercei* were statistically significant and there was no strong indication of attraction to other weevils or to leaves (Table 3). This was in part due to the much lower proportion of responding insects – around 50% – compared to *P. latithorax*.

Bioassays in 2002 – 03. Essentially, the bioassays of Oct – Nov 2002 repeated the earlier set in terms of the treatments evaluated. In respect of *P. latithorax*, the only significant results were of attraction to potato leaves, compared to blank tubes (Table 4). For *R. piercei* there were a few significant results (Table 5). In two cases these suggested attraction to the opposite sex, but this was only seen during the day and not the night and was negated by the apparent attraction of males to other males in one treatment pair.

The bioassays of Feb – March 2003 were intended to check the finding (of the earlier bioassays) of apparent attraction of weevils – at least for *P. latithorax* - to fresh potato leaves. It was thought this might be due to the humidity produced by the leaves in a confined space so to check for this damp filter paper was used in comparisons with leaves and blank tubes. For *P. latithorax*, although not all results were statistically significant, leaves were confirmed as more attractive than blank tubes (Table 6). Leaves were also clearly more attractive than damp paper for males. The same was true for females, although this result was not significant. Paper was not significantly more attractive than blank tubes for either sex, suggesting that little, if any of the attraction to leaves was due to humidity. For the bioassays with *R. piercei* (Table 7), response rates were again lower than for *P. latithorax*. Males showed significant attraction to leaves, relative to blanks and paper, but only during the morning test period. Female *R. piercei* evinced no significant results.

Bioassays in 2003 – 04. Following indications of the attraction to leaves, the bioassays of the 2003 – 04 season aimed to examine responses to two volatile components of potato leaves, Z-3-hexenol and E-2-hexenal, presented individually and, in one case, together. When presented in the form of polyethylene vials both compounds were significantly repellent to both sexes of *R. piercei* in overnight tests (Table 8) when compared against blank tubes. There was also evidence that given a choice between the two compounds, most weevils preferred Z-3-hexenal. The same results were found for the respective bioassays with *P. latithorax*, although results in respect of Z-3-hexenol were not significant (Table 9).

When the same compounds were presented as 0.2 mg doses on small filter-paper pieces, no significant repellency or attraction was noted for either species or any treatment pair except for a repellent effect seen in respect of E-2-hexenal with *P. latithorax* females (Tables 10 and 11).

Identical bioassays with serial five-fold dilutions of the compounds (i.e. 0.04 mg) also produced mostly insignificant results, although at this lower dose there was significant attraction to E-2-hexenal for *R. piercei* females. Strangely, for *P. latithorax* both sexes showed repellency to both compounds presented against blanks, but a preference for E-2-hexenal against Z-3-hexenol, however only the Z-3-hexenol *vs.* E-2-hexenal and E-2-hexenal *vs.* blank results for males were statistically significant (Tables 12 and 13).

When the two synthetic compounds were presented together in the same 0.04 mg doses, individually, and compared with blank tubes the combination proved significantly repellent to

R. piercei males and females (Table 14). Male *R. piercei* preferred potato leaves to the binary blend, while although both sexes preferred leaves to blank tubes neither result was significant. None of the corresponding results for *P. latithorax* were statistically significant (Table 15).

Responsiveness vs. bioassay observation period. During the course of the bioassays *P. latithorax* weevils generally showed higher levels of responsiveness (*i.e.* made a clear choice between the treatment pairs) during overnight test periods. On the other hand, *R. piercei* weevils showed no preferred activity period. Accordingly, overnight was the only period used to test both species during bioassays conducted after November 2003.

Table 2. *P. latithorax* bioassays, Toralapa, February – March 2002 (11 - 12 reps)

				am		pm		overnight	
#	TEST	TRT1	TRT2	RI*	P**	RI*	P**	RI*	P**
1	F	M+L	L	0.33	NS	0.25	NS	0.45	NS
2	M	M+L	L	0.42	NS	0.50	NS	-0.09	NS
3	M	F+L	L	0.45	NS	0.67	< 0.05	-0.09	NS
4	F	F+L	L	0.50	< 0.05	0.00	NS	-0.27	NS
5	F	M	blank	0.33	NS	0.17	NS	0.36	NS
6	M	M	blank	0.67	< 0.05	0.17	NS	0.45	NS
7	M	F	blank	0.50	NS	0.17	NS	0.82	< 0.01
8	F	F	blank	0.75	< 0.01	0.58	< 0.01	0.09	NS
9	F/M†	L	blank	0.47	< 0.01	0.47	< 0.01	0.88	< 0.01
10	F	M	L	-0.30	NS	-0.10	NS	-0.50	NS
11	M	M	L	-0.70	< 0.05	-0.14	NS	-0.60	NS
12	M	F	L	-0.40	NS	-0.25	NS	-0.80	< 0.05
13	F	F	L	-0.50	NS	-0.30	NS	-0.80	< 0.05
responsiveness				79%		72%		99%	

F=female, M=male, L=potato leaf

* Positive value = treatment 1 preferred; negative = treatment 2 preferred. ** Distribution of responses differed from equality ($?^2$, 2 × 2 goodness of fit, 1 d.f.). † Separate data for male and female test insects pooled, following determination that respective data-sets were homogenous.

Table 3. *R. piercei* bioassays, Toralapa, February – March 2002 (12 - 14 reps)

				RI*		
#	TEST	TRT1	TRT2	am	pm	overnight
1	F	M	L	0.13	-0.20	-0.23
2	M	M+L	L	0.04	-0.47	-0.03
3	M	F+L	L	-0.05	-0.23	-0.23
4	F	F+L	L	0.19	-0.01	-0.13
5	F	M	blank	-0.01	0.03	0.45
6	M	M	blank	-0.10	-0.29	-0.13
7	M	F	blank	-0.14	-0.25	0.04
8	F	F	blank	-0.23	-0.46	-0.07
9	F/M†	L	blank	0.04	-0.25	-0.05
responsiveness				40%	55%	52%

F=female, M=male, L=potato leaf

* Positive value = treatment 1 preferred; negative = treatment 2 preferred.

† Separate data for male and female test insects pooled, following determination that respective data-sets were homogenous.

Table 4. *P. latithorax* bioassays, Toralapa, October 2002 (11 reps)

				daytime		overnight	
#	TEST	TRT1	TRT2	RI*	P**	RI*	P**
1	F	male/leaf	leaf	-0.09	NS	-0.45	NS
2	M	male/leaf	leaf	0.27	NS	0.00	NS
3	M	female/leaf	leaf	-0.18	NS	0.00	NS
4	F	female/leaf	leaf	0.36	NS	0.36	NS
5	F	male	blank	0.18	NS	0.09	NS
6	M	male	blank	0.18	NS	0.27	NS
7	M	female	blank	0.09	NS	0.27	NS
8	F	female	blank	0.09	NS	0.00	NS
9	F	leaf	blank	-0.18	NS	0.64	<0.05
10	M	leaf	blank	0.18	NS	0.73	<0.01
		responsiveness		51%		77%	

F=female, M=male

* Positive value = treatment 1 preferred; negative = treatment 2 preferred. ** Distribution of responses differed from equality ($?^2$, 2 × 2 goodness of fit, 1 d.f.)

Table 5. *R. piercei* bioassays, Toralapa, November 2002 (10 reps)

				daytime		overnight	
#	TEST	TRT1	TRT2	RI*	P**	RI*	P**
1	F	male/leaf	leaf	0.60	< 0.05	0.20	NS
2	M	male/leaf	leaf	-0.30	NS	0.60	< 0.05
3	M	female/leaf	leaf	0.30	NS	-0.10	NS
4	F	female/leaf	leaf	0.10	NS	0.20	NS
5	F	male	blank	0.00	NS	-0.20	NS
6	M	male	blank	0.40	NS	0.20	NS
7	M	female	blank	0.60	< 0.05	0.60	NS
8	F	female	blank	0.40	NS	-0.70	< 0.05
9	F	leaf	blank	-0.50	NS	0.00	NS
10	M	leaf	blank	0.30	NS	-0.20	NS
		responsiveness		65%		68%	

F=female, M=male

* Positive value = treatment 1 preferred; negative = treatment 2 preferred. ** Distribution of responses differed from equality ($?^2$, 2 × 2 goodness of fit, 1 d.f.)

Table 6. *P. latithorax* bioassays with potato leaves and damp filter-paper, Toralapa, February 2003 (15-18 reps)

			am		pm		overnight	
Test	TRT1	TRT2	RI*	P**	RI*	P**	RI*	P**
F	leaf	blank	0.47	NS	0.60	<0.01	0.50	<0.05
F	paper	blank	0.13	NS	0.20	NS	0.28	NS
F	leaf	paper	0.13	NS	0.20	NS	0.44	NS
	responsiveness		91%		64%		96%	
M	leaf	blank	0.53	<0.05	0.33	NS	0.50	<0.05
M	paper	blank	0.00	NS	0.33	NS	0.33	NS
M	leaf	paper	0.53	<0.05	0.00	NS	0.50	<0.05
	responsiveness		84%		67%		96%	

* Positive value = treatment 1 preferred; negative = treatment 2 preferred. ** Distribution of responses differed from equality ($?^2$, 2 × 2 goodness of fit, 1 d.f.)

Table 7. *R. piercei* bioassays with potato leaves and damp filter-paper, Toralapa, February – March 2003 (18-24 reps)

			am		pm		overnight	
Test	TRT1	TRT2	RI*	P**	RI*	P**	RI*	P**
F	leaf	blank	0.17	NS	-0.04	NS	0.14	NS
F	paper	blank	-0.17	NS	-0.17	NS	0.00	NS
F	leaf	paper	0.28	NS	0.13	NS	0.10	NS
	responsiveness		57%		58%		68%	
M	leaf	blank	0.44	< 0.05	0.21	NS	-0.14	NS
M	paper	blank	-0.17	NS	-0.13	NS	0.05	NS
M	leaf	paper	0.39	< 0.05	0.00	NS	0.10	NS
	responsiveness		44%		69%		57%	

* Positive value = treatment 1 preferred; negative = treatment 2 preferred. ** Distribution of responses differed from equality ($?^2$, 2 × 2 goodness of fit, 1 d.f.)

Table 8. *R. piercei* bioassays with synthetic green leaf volatiles in polyethylene vials, Toralapa, November 2003 (overnight, 6 reps)

			overnight		am	
TEST	TRT 1	TRT 2	RI*	P**	RI*	P**
5 F	Z3	E2	0.17	NS	0.13	NS
5 F	E2	blank	-0.57	< 0.01	-0.10	NS
5 F	Z3	blank	-0.37	< 0.05	-0.20	NS
	responsiveness		63%		57%	
5 M	Z3	E2	0.37	< 0.05	0.13	NS
5 M	E2	blank	-0.53	< 0.01	-0.13	NS
5 M	Z3	blank	-0.37	< 0.01	0.07	NS
	responsiveness		58%		56%	

* Positive value = treatment 1 preferred; negative = treatment 2 preferred. ** Distribution of responses differed from equality ($?^2$, 2 × 2 goodness of fit, 1 d.f.)

Table 9. *P. latithorax* bioassays with synthetic green leaf volatiles in polyethylene vials, Toralapa, December 2003 (overnight, 6 reps)

TEST	TRT 1	TRT 2	RI*	P**
5 F	Z3	E2	0.37	< 0.05
5 F	E2	blank	-0.47	< 0.01
5 F	Z3	blank	-0.13	NS
	responsiveness		86%	
5 M	Z3	E2	-0.10	NS
5 M	E2	blank	-0.43	< 0.01
5 M	Z3	blank	-0.30	NS
	responsiveness		83%	

* Positive value = treatment 1 preferred; negative = treatment 2 preferred. ** Distribution of responses differed from equality ($?^2$, 2 × 2 goodness of fit, 1 d.f.)

Table 10. *R. piercei* bioassays with synthetic green leaf volatiles as 0.2 mg doses on filter-papers, Toralapa, December 2003 (overnight, 18 reps)

TEST	TRT 1	TRT 2	RI*	P**
5 F	Z3	E2	0.06	NS
5 F	E2	blank	-0.03	NS
5 F	Z3	blank	-0.04	NS
	responsiveness		78%	
5 M	Z3	E2	0.09	NS
5 M	E2	blank	0.09	NS
5 M	Z3	blank	0.10	NS
	responsiveness		73%	

* Positive value = treatment 1 preferred; negative = treatment 2 preferred. ** Response rates to the two treatments differed significantly (1-way ANOVA on arc-sine transformed data)

Table 11. *P. latithorax* bioassays with synthetic green leaf volatiles as 0.2 mg doses on filter-papers, Toralapa, December 2003 (overnight, 18 reps)

TEST	TRT 1	TRT 2	RI*	P**
5 F	Z3	E2	0.10	NS
5 F	E2	blank	-0.19	< 0.01
5 F	Z3	blank	-0.11	NS
	responsiveness		87%	
5 M	Z3	E2	0.12	NS
5 M	E2	blank	-0.08	NS
5 M	Z3	blank	-0.03	NS
	responsiveness		80%	

* Positive value = treatment 1 preferred; negative = treatment 2 preferred. ** Response rates to the two treatments differed significantly (1-way ANOVA on arc-sine transformed data)

Table 12. *R. piercei* bioassays with synthetic green leaf volatiles as 0.04 mg doses on filter-papers, Toralapa, January 2004 (overnight, 18 reps)

TEST	TRT 1	TRT 2	RI*	P**
5 F	Z3	E2	0.13	NS
5 F	E2	blank	0.21	< 0.05
5 F	Z3	blank	-0.13	NS
	responsiveness		75%	
5 M	Z3	E2	-0.10	NS
5 M	E2	blank	0.08	NS
5 M	Z3	blank	-0.11	NS
	responsiveness		66%	

* Positive value = treatment 1 preferred; negative = treatment 2 preferred. ** Response rates to the two treatments differed significantly (1-way ANOVA on arc-sine transformed data)

Table 13. *P. latithorax* bioassays with synthetic green leaf volatiles as 0.04 mg doses on filter-papers, Toralapa, January 2004 (overnight, 12 reps)

TEST	TRT 1	TRT 2	RI*	P**
5 F	Z3	E2	-0.18	NS
5 F	E2	blank	-0.23	NS
5 F	Z3	blank	-0.17	NS
	responsiveness		95%	
5 M	Z3	E2	-0.32	< 0.01
5 M	E2	blank	-0.17	< 0.01
5 M	Z3	blank	-0.17	NS
	responsiveness		94%	

* Positive value = treatment 1 preferred; negative = treatment 2 preferred. ** Response rates to the two treatments differed significantly (1-way ANOVA on arc-sine transformed data)

Table 14. *R. piercei* bioassays with synthetic green leaf volatiles as 0.04 mg doses of both compounds combined on filter-papers, Toralapa, February - March 2004 (overnight, 18 reps)

TEST	TRT 1	TRT 2	RI*	P**
5 F	Z3+E2	leaves	-0.09	NS
5 F	Z3+E2	blank	-0.21	~ 0.05
5 F	leaves	blank	0.09	NS
	responsiveness		63%	
5 M	Z3+E2	leaves	-0.20	< 0.05
5 M	Z3+E2	blank	-0.16	~ 0.05
5 M	leaves	blank	0.04	NS
	responsiveness		60%	

* Positive value = treatment 1 preferred; negative = treatment 2 preferred. ** Response rates to the two treatments differed significantly (1-way ANOVA on arc-sine transformed data)

Table 15. *P. latithorax* bioassays with synthetic green leaf volatiles as 0.04 mg doses of both compounds combined on filter-papers, Toralapa, March 2004 (overnight, 6 reps)

TEST	TRT 1	TRT 2	RI*	P**
5 F	Z3+E2	leaves	-0.23	NS
5 F	Z3+E2	blank	-0.07	NS
5 F	leaves	blank	0.13	NS
	responsiveness		66%	
5 M	Z3+E2	leaves	0.00	NS
5 M	Z3+E2	blank	0.03	NS
5 M	leaves	blank	0.13	NS
	responsiveness		28%	

* Positive value = treatment 1 preferred; negative = treatment 2 preferred. ** Distribution of responses differed from equality ($?^2$, 2 × 2 goodness of fit, 1 d.f.)

DISCUSSION

Taking all the bioassays conducted in the period up to March 2003 (i.e. all those involving live weevils and potato leaves as attractant sources) together a number of consistent trends emerged. Firstly, *P. latithorax* was the more responsive, or active, of the two species, particularly overnight while *R. piercei* showed no preferred activity period. Typically, rates of response, overnight, to one of the presented attractant choices were of the order 80 – 100% for *P. latithorax* and 50 – 70% for *R. piercei*. On this basis the later bioassays were carried out over the overnight period only.

For neither species was there any consistent evidence of weevil-weevil attraction and this strongly indicated the absence of any pheromonal communication, in contrast to the field trapping results reported by Calvache (1985) for *Premnotrypes vorax* in Colombia. There was quite good evidence for attraction of both sexes of *P. latithorax* to fresh potato leaves, but there was no corresponding result for *R. piercei.* The attraction of *P. latithorax* to leaves was shown not simply to be the result of a hydrophilic response by comparing the relative degree of attraction to leaves and damp filter-paper.

The first bioassays involving Z-3-hexenol and E-2-hexenal, compounds previously shown to be present in potato leaves and to elicit EAG responses in the weevils, demonstrated quite strong repulsive effects to both compounds, particularly in respect of *R. piercei*. For *P. latithorax* the repellent effects were only statistically significant in respect of E-2-hexenal. The compounds were dispensed from polyethylene vials at what were probably super-natural doses, particularly within the closed environment of the bioassay chambers, and it was considered likely that the repellent effects were due to this.

The subsequent bioassays were intended to test physiologically lower doses in the expectation that these might produce attractive effects. This was borne out with *R. piercei.* For the species, repellent effects disappeared at the higher dose on filter-papers, whilst at the lower dose there was significant attraction of females to E-2-hexenal, but not Z-3-hexenol. For *P. latithorax*, while the repellent effects of E-2-hexenal weakened slightly, they were still significant with females at the higher dose on filter-papers and with males at the lower dose. There was no evidence of attraction at any dose.

APPENDIX 6

The attracting capacity of adult APW by potato leaves (feeding attractant) and by adult weevils (pheromone).

Background/Justification

Food attractants and sex pheromones are useful tools for integrated pest management. However, for the Andean potato weevil no extensive research is available at present. A few reports have been published on the capture of *P. vorax* by using other adults of the same species as attractants (Calvache, 1985 and Valencia, 1989) where the probable existence of an aggregating pheromone is mentioned. On the other hand, host studies in *P. suturicallus* and *P.*
latithorax allude to potatoes as the only host where the weevil completes its life cycle; adults are powerfully attracted to potato leaves as is demonstrated when using refuge traps prepared with potato leaves and potato trap plants to control *P. vorax* in Ecuador (Gallegos,1999). For this reason it's a priority to investigate the existence of pheromones or food attractants and to identify insects' and plants' volatile chemicals for further synthesis. This study is being developed as part of a collaborative research project between CIP, PROINPA and NRI.
Identification and synthesis will be developed by NRI (UK), while product evaluation will be completed by CIP in Peru and PROINPA in Bolivia

Methodology

A. The attracting capacity of APW adult by potato leaves (feeding attractant) and by adult weevils (pheromone).

Laboratory bioassay at CIP

a) To determine sex pheromone production by weevils:

Experiment 1 (20 virgin males released). Treatments: 1. Five virgin males with cotton wick of water, 2. Five virgin female with cotton wick of water, 3. Check, cotton wick of water.

Experiment 2 (20 virgin females released). Treatments: 1. Five virgin male with cotton wick of water, 2. Five virgin female with cotton wick of water, 3. Check, cotton wick of water.

b) To determine the attracting capacity of APW adult by potato leaves, cv. Yungay.

Experiment 3 (20 virgin males released). Treatments: 1. Potato leaves with cotton wick of water, 2. Corn leaves (Amarillo) with cotton wick of water, 3. Check, cotton wick of water.

Experiment 4 (20 virgin females released). Treatments: 1. Potato leaves with cotton wick of water, 2. Corn leaves with cotton wick of water, 3. Check, cotton wick of water.

The methodology used in each experiment was chosen of three pit-fall bioassays. The pit-fall traps were distributed by using a complete randomized design consisting of three treatments, three replicates and each experiment was replicated three times. In all experiments we used *Premnotrypes suturicallus*. Evaluation was carried out after 24 hours by recording the number of weevils captured in the pit-fall trap. Results were analyzed with SAS system, analysis of variance, and Waller – Duncan test.

Results and discussion

A. The attracting capacity of APW adult by potato leaves (feeding attractant) and by adult weevils (pheromone).

- Laboratory bioassay between virgin weevils of *Premnotrypes suturicallus* showed no evidence of pheromones. Records were 3.33, 4.77, and 6.22 males/pit-fall trap, and 4.0, 3.55 and 3.77 females/pit-fall trap, in pit-fall traps baited with males, females and check respectively. No differences were found when virgin males or females were released (Table 1).
- Laboratory bioassay to test the attracting capacity of the adult weevil of *Premnotrypes suturicallus* by potato leaves showed high evidence of attraction to potato leaf volatiles. Records were 14.83, 3.25 and 0,50 males/pit-fall trap, and 14.16, 3.75 and 1.16 females/pit-fall, in pit-fall baited with potato leaves, corn leaves and check, respectively. Differences among potato leaf, corn leaf and the check were found. Similar reactions were shown by male and female weevils (Table 2).

Major conclusions

- Laboratory bioassay and field trapping showed no evidence of pheromones.
- Laboratory bioassay showed some evidence for attraction to volatiles of the potato leaf .

. **Table 1. Weevils, *Premnotrypes suturicallus* captured in pit-fall traps baited with male and female virgins. La Molina, 2003.**

Treatments	Males liberated		Females liberated	
1. Males	3.33	n.s.	4.00	n.s.
2. Females	4.77	n.s.	3.55	n.s.
3. Check	6.22	n.s.	3.77	n.s.

Table 2. Weevils, *Premnotrypes suturicallus* captured in pit-fall traps baited with potato and corn leaves. La Molina, 2003.

Treatments	Males liberated		Females liberated	
1. Potato leaves	14.83	a	14.16	a
2. Corn leaves	3.25	b	3.75	b
3. Check	0.50	c	1.16	c

Lima, 22 de Enero, 2004

Realize el experimento con dos especies de gorgojo: *Premnotrypes latithorax* (Procedente de Cusco) y *Premnotrypes suturicallus* (de Huancayo). En cada caso utilize 20 gorgojos (10 Machos y 10 hembras).

Los resultados de este primer ensayo son los siguientes:

Experimento 1.

1. (E)-2-hexenal
2. Hojas de papa, Vr. Yungay
3. Testigo

Experimento 2.

1. (Z)-3-hexenol
2. Hojas de papa, Vr. Yungay
3. Testigo

Experimento 3.

1. (E)-2-hexenal
2. (Z)-3-hexenol
3. Testigo

Experimento realizado con la especie *Premnotrypes latithorax*

Exp. 1	**(E)-2-hexenal**	**Hoja**	**Testigo**	**Afuera**
Rep. I	0	17	0	3
Rep. 2	0	17	0	3
Rep. 3	0	19	0	1
Exp. 2	**(Z)-3-hexenol**	**Hoja**	**Testigo**	**Afuera**
Rep. I	0	17	1	2
Rep. II	0	20	0	0
Rep. III	0	17	0	3
Exp. 3	**(E)-2-hexenal**	**(Z)-3-hexenol**	**Testigo**	**Afuera**
Rep. I	0	0	0	20
Rep. II	0	0	2	18
Rep. III	0	0	3	17

Experimento realizado con la especie *Premnotrypes suturicallus*

Exp. 1	**(E)-2-hexenal**	**Hoja**	**Testigo**	**Afuera**
Rep. I	0	19	0	1
Rep. 2	0	16	0	4
Rep. 3	1	12	0	7
Exp. 2	**(Z)-3-hexenol**	**Hoja**	**Testigo**	**Afuera**
Rep. I	0	14	4	2
Rep. II	1	12	1	6
Rep. III	2	14	0	4
Exp. 3	**(E)-2-hexenal**	**(Z)-3-hexenol**	**Testigo**	**Afuera**
Rep. I	4	1	4	11
Rep. II	0	0	3	17
Rep. III	3	0	8	8

Comentarios:

- Los productos tienen un olor muy fuerte y parece que han actuado como repelentes
- Esta reaccion se ha visto mas claro en el experimento 3.
- Estas son las primeras prueba que realize la semana pasada en Huancayo.
- Voy a repitir estos experimentos unas 5 veces mas con poblaciones nuevas de gorgojos adultos para confirmar estos resultados
- Quizas la concentracion es muy alta y se podria probar a bajas concentraciones.
- Si usted tiene alguna sugerencia para seguir realizando pruebas, este tiempo es ideal porque hay muchos gorgojos adultos en campo.
- Tambien he iniciado los ensayos comparando hojas de papa de variedades comerciales, nativas y silvestres. Mas adelante le estare enviando estos resultados

PREFERENCIA RELATIVA DE GORGOJOS ADULTOS POR HOJAS DE PRINCIPALES VARIEDADES COMERCIALES , NATIVAS y SILVESTRES

Los bioensayos se realizaron con adultos de la especie *Premnotrypes suturicallus* en el laboratorio de Entomologia del CIP en Huancayo, Junin. Durante los meses de Enero y Febrero del 2004.
En cada caso se utilizaron 20 gorgojos (10machos y 10 hembras) y las evaluaciones se realizaron a las 24 horas.

Preferencia de gorgojos adultos, *Premnotrypes suturicallus* por hojas de variedades comerciales

Fecha	Repeticion.	Yungay	Perricholi	Amarilis	Afuera
29/01	I	5	4	9	2
	II	5	1	8	6
	III	4	4	6	6
30/01	I	6	3	8	3
	II	4	4	5	7
	III	6	3	8	3
03/02	I	8	3	7	2
	II	4	4	9	3
	III	10	2	6	2
04/02	I	7	4	8	1
	II	6	5	6	3
	III	6	3	10	1
05/02	I	6	6	8	0
	II	2	6	9	3
	III	3	4	13	0
06/02	I	7	0	11	2
	II	10	4	4	2
	III	4	4	10	2

Preferencia de gorgojos adultos, *Premnotrypes suturicallus* por hojas de variedades nativas

Fecha	Repeticion.	Peruanita	Huayro	Amarilla	Afuera
29/01	I	6	8	5	1
	II	8	7	4	1
	III	5	10	3	2
30/01	I	8	6	3	3
	II	8	10	2	0
	III	11	3	6	0
03/02	I	13	6	0	1
	II	10	4	4	2
	III	8	7	3	2
04/02	I	2	10	5	3
	II	8	6	1	5
	III	7	6	4	3
05/02	I	5	8	6	1
	II	11	3	4	2
	III	9	6	2	3
06/02	I	8	5	5	2
	II	9	7	4	0
	III	10	3	4	3

Preferencia de gorgojos adultos, *Premnotrypes suturicallus* por hojas de especies silvestres de papa

Fecha	Repeticion.	OCH-11322	OCH-14267	OCH-14397	Afuera
29/01	I	8	7	2	3
	II	7	6	6	1
	III	10	5	4	1
30/01	I	4	9	6	1
	II	9	5	6	0
	III	8	3	7	2
03/02	I	6	6	7	1
	II	8	4	8	0
	III	7	0	10	3
04/02	I	8	7	3	2
	II	7	9	4	0
	III	7	7	6	0
05/02	I	5	2	11	2
	II	12	2	6	0
	III	8	2	10	0
06/02	I	4	6	9	1
	II	9	7	4	0
	III	6	3	7	4

APPENDIX 7

FIELD TRAPPING EXPERIMENTS

MATERIALS AND METHODS

Trapping experiments were conducted during each of the 2001 – 02, 2002 – 03 and 2003 – 04 cropping seasons.

Experiments using adult weevils and leaves as bait, 2001 – 02. Two field trapping experiments to catch *P. latithorax* were conducted from 22 January – 27 February 2002 in farmers' fields a few hundred metres from the research station at Toralapa. One experiment employed pitfall traps while the other employed sticky stake traps.

The pitfall traps were made from white, plastic flowerpots (12 cm diameter, 9 cm deep, 6 openings 3.2 × 1.5 cm) to which a green, corex lid was attached by elastic string (Fig. 1). The trap was set into a small hole in the ground so that the bottom edges of the openings were level with the soil, allowing insects to crawl in. Traps were baited with fresh potato leaves, two adult male or female *P. latithorax* (collected from old potato stores) or were left un-baited. The leaves and weevils acting as baits were retained inside small plastic containers (old photographic film holders, 3 cm diameter × 4.5 cm) which had been adapted with fine plastic mesh, to allow airflow through. These bait holders were attached under the centre of the trap-lid.

Fig. 1. White "flowerpot" pitfall trap used throughout the trapping experiments.

Fig. 2. Sticky stake trap used only from Jan. – Feb 2002.

The sticky stake traps consisted of wooden stakes, 1 × 2 cm in cross-section, driven into the soil so that they stood 30 cm high. Beginning 5 cm above soil a 20 cm length of the stake

was coated with sticky insect glue; at the top of the stake a small plate was used to hold a small plastic container (details as for the pitfall traps) containing the adult weevils or potato leaves that acted as bait (Fig. 2).
In each of the two experiments, traps were positioned 10 m apart along the edge of potato field facing a potato store. This location was used in the expectation that weevils moving into the field from the store would encounter the traps. Each of the experiments was set out to a randomized complete-block (RCB) design with three replicates. Traps were checked at least once a week when the numbers of male and female *P. latithorax*, and of other invertebrates were recorded separately. At the same time the bait leaves and weevils were replaced.

Experiments using adult weevils and leaves as bait, 2002 – 03 season. In 2002 – 03 a further experiment at Toralapa also compared numbers of male and female *P. latithorax*, and of other invertebrates, caught in pitfall traps. Traps were baited with fresh potato leaves alone, two virgin adult male or female *P. latithorax* (each with 1 – 2 fresh leaves for food) or left un-baited. The design of the pitfall trap was the same as for the previous season except that the number of trap openings was reduced from six to five. The experiment ran from 4 December 2002 – 27 March 2003. Six replicate blocks were set out to a RCB design, three each in two fields (one belonging to a farmer) a short distance from the research station. Individual traps were positioned 5 m apart along the edges of fields, and the spacing between blocks was 10 m. Traps from one field had to be redeployed to another in early January following vandalism. Traps were checked approximately two times per week.

A very similar experiment was also carried out with "white flowerpot" pitfall traps in respect of *Rhigopsidius piercei* catches in farmers' fields at Yampara, near Sucre from 12 January – 29 March. The experiment differed in that the virgin adult *R. piercei* were not supplied with leaves as food. This was because traps were only checked weekly, so leaves would not have been fresh, and because this species is better able to survive periods with feeding. Initially, traps containing the same treatments were grouped together, rather than being arranged in a randomised block design, but this was corrected on 4 February.

Trap design experiments, 2002 – 03 season. From 11 February to 19 March a trap design comparison was carried out in experimental fields within the Toralapa station. This was between with the hitherto standard "white flowerpot" trap and three other designs of pitfall trap made from various plastic containers (Table 1, Fig. 3). All traps were baited with fresh leaves.

Table 1. Respective dimensions of the four trap designs compared February – March 2003 at Toralapa (all dimensions in cm).

	Black flowerpot (*Maceta negra*)	White flowerpot* (*Maceta blanca*)	Plastic bucket (*Balde 1 litro con tapa*)	Plastic bottle (*Recipiente 1-litro, tapa roja*)
Height (incl. lid)	12.3	9.3	12.6	16.0
Height (excl. lid)	12.0	9.1	12.5	15.8
Diameter (top)	13.8	13.5	12.9	
Diameter (bottom)	10.1	11.0	9.9	9.6
Openings	Five, each 2.0 × 3.0 cm	Five, each 1.5 × 3.0 cm	Five, each 2.0 × 3.0 cm	Four, each 2.0 × 2.0 cm

* standard trap

In parallel with this experiment some laboratory observations on the ability of weevils to escape from each of the four trap designs were carried out, in order to provide additional information on their relative efficiency. Ten weevils were placed in one trap of each of the different designs overnight, in the laboratory, and the respective numbers retained and escaped were recorded. This procedure was done once for *R. piercei* and three times with *P. latithorax*.

Fig. 3. Four trap designs tested February – March 2003 at Toralapa; clockwise from left, plastic bucket, 1-l plastic bottle, black flowerpot, white flowerpot.

Experiment with synthetic host plant volatiles, 2003 – 04 season. Following the findings of statistically significant attraction of both weevil species to potato leaves in laboratory bioassays in Feb – March 2003, and results from linked GC-EAG studies, indicating responses to the potato leaf volatiles, (Z)-3-hexenol and (E)-2-hexenal, trapping studies during 2003 – 04 aimed to confirm the attractiveness of these compounds in the field.

The experiment in respect of *P. latithorax* ran from 3 December 2003 – 10 March 2004 in farmers' fields close to the Toralapa station. Eight treatments were deployed in a RCB design with five replications: the Z3 and E2 compounds (as used in the bioassays) dispensed individually and together, in polyethylene vials and 2.5 × 2.5 cm white sachets, plus traps baited with potato leaves and control (blank) traps. Vials and sachets contained 100 µl of one or both compounds (*i.e.* lures dispensing both compounds initially contained 200 µl of material).

It was originally intended that a similar experiment should be conducted near to Sucre to trap *R. piercei*. Unfortunately, scarce and very late rains prevented almost all potato planting in the region and hence no suitable site for the experiment could be found.

Statistical Analysis. The data analysed were total catches of each sex in each trap over the respective trapping period. One-way analysis of variance was used to examine the data for treatment effects. Blocking effects were accounted for in the models except in the case of the experiment with *R. piercei* at Sucre in 2002 – 03 (when traps were not initially set up with a blocked design). Data were not transformed.

RESULTS

Experiments using adult weevils and leaves as bait. All of the experiments of 2001 – 02 and 2002 – 03 produced low catches, and none at all were observed using the sticky stake traps at Toralapa in 2001 – 02. Average catches in the other three experiments mostly did not exceed 10 individuals of each sex per trap (Figs. 4 - 6). No significant treatment differences were noted in any of the individual experiments ($P > 0.05$ for treatment effects, F-ratio, ANOVA). As might be expected with pitfall traps large numbers of "other" invertebrates, mainly spiders and a variety of non-target beetle species, were trapped in all experiments. For clarity these are omitted from some of the figures.

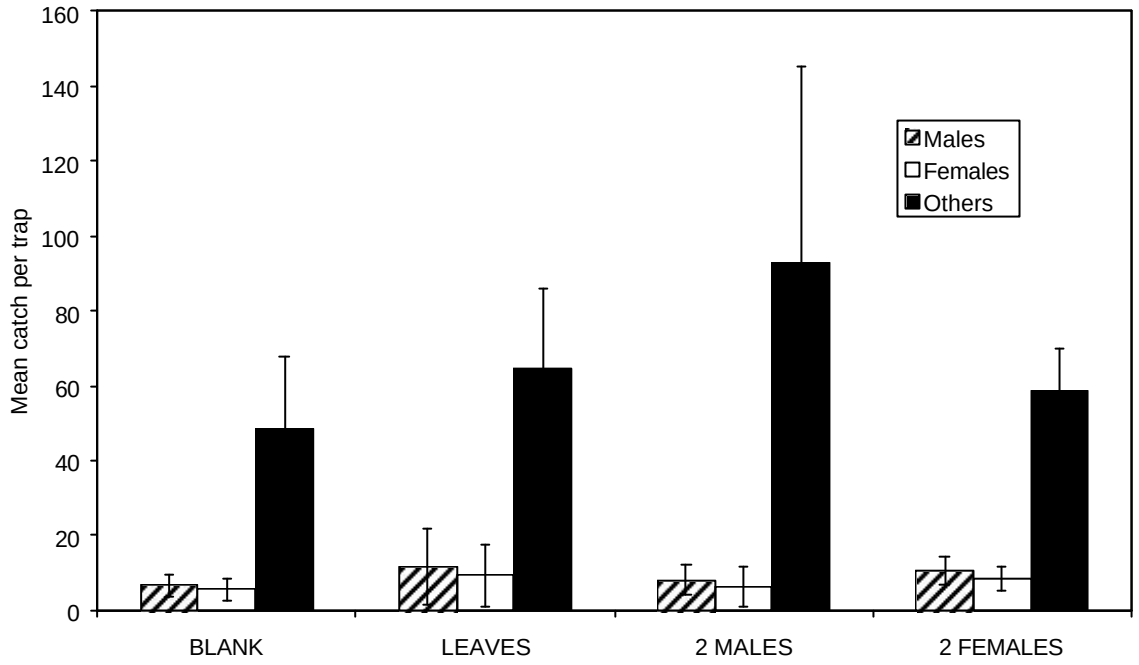


Figure 4. Mean total catch per trap of *P. latithorax* and "other" invertebrates in "white flowerpot" pitfall traps, un-baited or baited with fresh potato leaves, 2 male or 2 female *P. latithorax* weevils, 23 January – 27 February 2002 (3 reps).

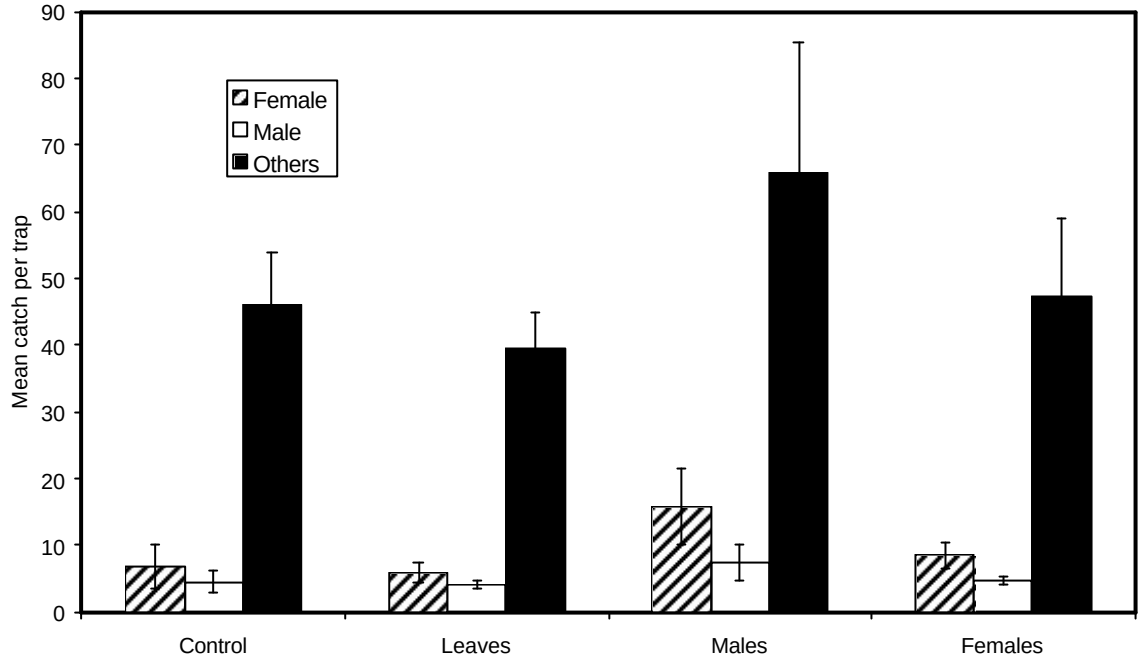


Figure 5. Mean total catch (± S.E.) per trap of *P. latithorax* and "other" invertebrates in "white flowerpot" pitfall traps, un-baited or baited with fresh potato leaves, 2 male or 2 female *P. latithorax* weevils, at Toralapa, 4 December 2002 – 27 March 2003 (6 reps).

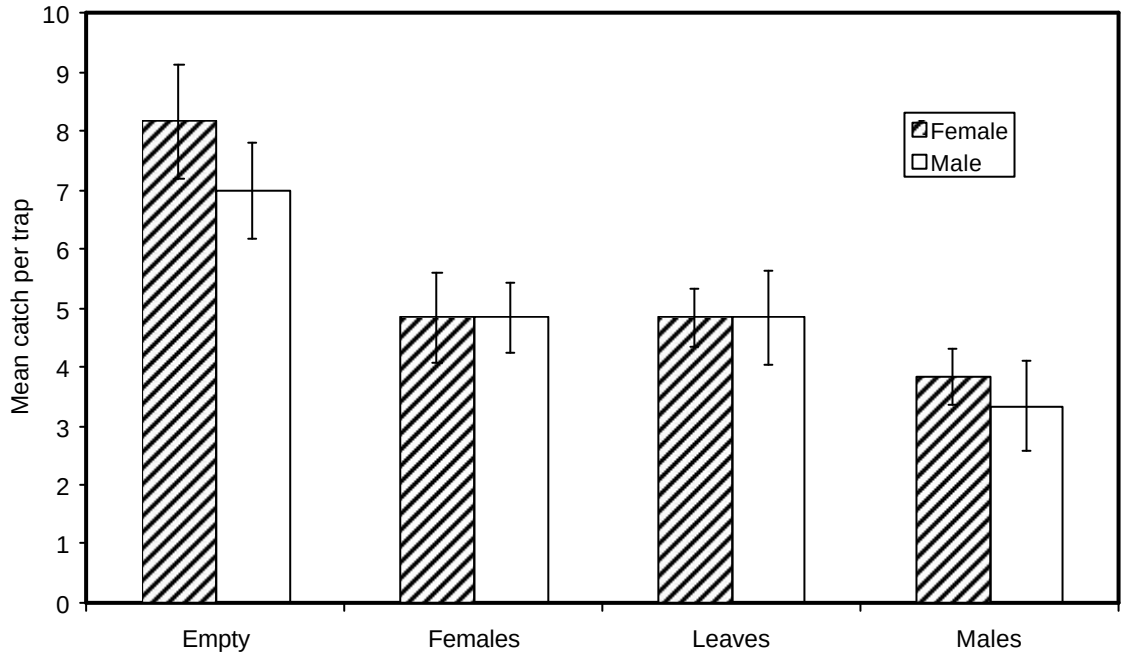


Figure 6. Mean total catch per trap (± S.E.) of *R. piercei* in "white flowerpot" pitfall traps, un-baited or baited with fresh potato leaves, 2 male or 2 female *R. piercei* weevils, at Yampara (Sucre), 12 January - 29 March 2003 (6 reps).

Trap design experiments. The trapping experiment to compare different locally-made designs commenced late in the season, hence overall catches mostly did not exceed 1 male or female

P. latithorax per trap (Fig. 7). Catches were highest in the plastic bottle traps (1 litre) but no significant treatment differences were noted (P > 0.05 for treatment effects, F-ratio, ANOVA).

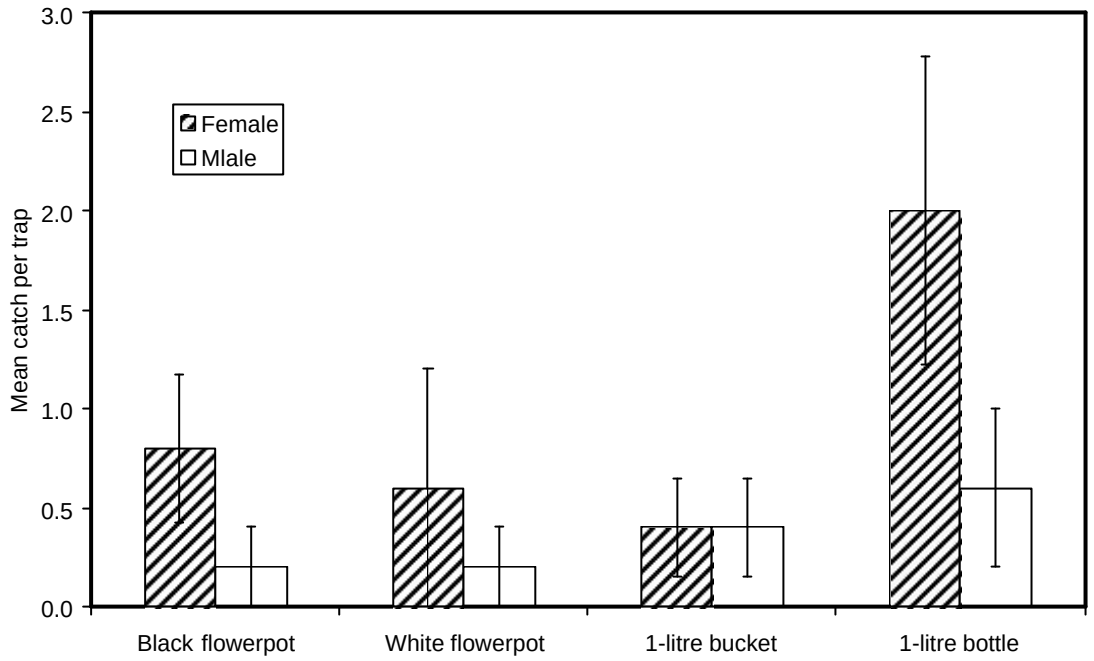


Figure 7. Mean total catch (± S.E.) per trap of *P. latithorax* in different designs of pitfall traps baited with 1 – 2 fresh potato leaves at Toralapa, 11 February - 19 March 2003 (5 reps). The plastic bottle traps were the deepest or highest of the four designs. This hampered the retrieval of trapped weevils, and was considered by PROINPA staff to be a practical disadvantage. Against this the screw-top nature of the bottle obviated the need for an elastic cord to retain the lid, as was the case with the two flowerpot designs. In addition, there was some evidence from the laboratory test of the retention efficiency that the bottle trap was most effective at preventing escape of *P. latithorax* weevils (Table 2). No *Rhigosidius* escaped from any of the trap four designs in the laboratory test.

Table 2. Results for laboratory test of the retention efficiency of the four pitfall trap designs used in the trapping comparison of 2002 – 03.

	P. latithorax (mean of 3 reps)		*R. piercei* (single rep)	
	retained	escaped	retained	escaped
White flowerpot	5.7	4.3	10	0
Black flowerpot	6.0	4.0	10	0
Plastic bucket	6.0	4.0	10	0
Plastic bottle 1-l	7.7	2.3	10	0

Experiment with synthetic host plant volatiles, 2003 – 04 season. Mean catches in this experiment was somewhat higher than previous seasons, averaging around 20 males and 20 females per trap for most of the treatments, including fresh leaves. The blank control treatment produced less than half this figure but, due to some large variability in catches between replicate blocks, this difference was not statistically significant. Results are summarised in Fig. 8.

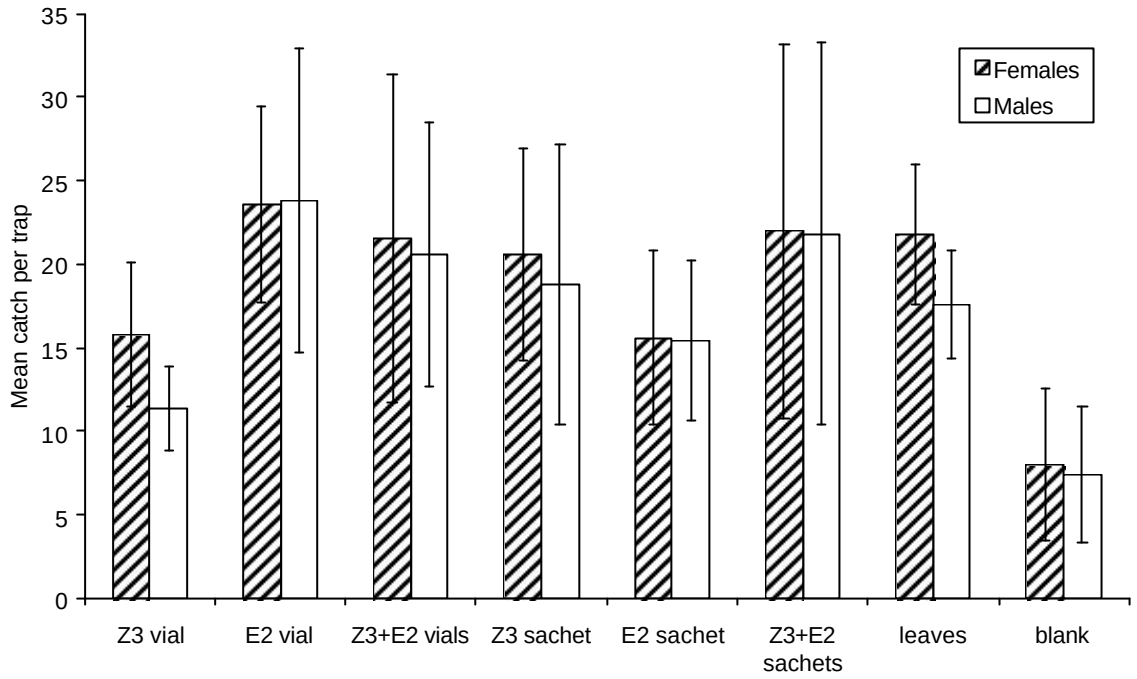


Figure 8. Mean total catch (± S.E.) per trap of *P. latithorax* in "white flowerpot" pitfall traps baited with synthetic host volatile lures at Toralapa, 3 December 2003 – 10 March 2004 (5 reps).

DISCUSSION

It was clear from trapping results of the first two seasons that when placed in traps as baits, neither adult weevils plus leaves, nor leaves alone, caught significantly more weevils of either sex than un-baited traps. This contrasts with the findings by Calvache (1985) that simple pitfall traps baited with live *P. vorax* adults attracted large numbers of both male and female weevils of the same species in Colombia and further work by Valencia (unpublished CIP report, 1986) showing that potato plants were attractive to adult *P. vorax* and this attractiveness could be enhanced with a hexane extract of mixed sex weevils.

With respect to the adult weevils, this can be interpreted as supporting the similar conclusions of the laboratory bioassays that there is no weevil-weevil attraction, and hence no pheromonal communication, in either species of APW. However, the absence of clear attraction to leaves as baits, in any of the trap experiments, runs contrary to the corresponding data from the bioassays. This may be explained by noting that the leaves were not always fresh (up to a week old, between trap checks). In addition volatiles from the leaves may not have diffused out of the trap at adequate rates for attraction. Finally, as the season progressed, host plant volatiles originating from the fields may have obscured, or competed with, those emanating from the traps.

Results from the 2003 – 04 trapping experiment at Toralapa suggested, but did not clearly prove, that *P. latithorax* weevils were attracted to traps containing either or both E-2-hexenal and Z-3-hexenol. This, taken with the results of the bioassays indicating dose-dependent effects and some attraction for *R. piercei*, but not *P. latithorax*, indicates the need for further experimentation.

In general terms dose-dependent effects are likely to be important and these could be investigated further, although under open field conditions where large aerial concentrations of the chemicals cannot build up (as in the bioassays), the effects may be less important. The two compounds are known to be fairly ubiquitous among green plants and it would be logical if the weevils' attraction to potato were mediated by more specific volatile compounds. If some of these can be identified they should be tested along with E-2-hexenal and Z-3-hexenol. Certainly, no field testing at all with *R. piercei* has yet taken place and this should be rectified in any further work. Thought should also be given to the manner and timing of trap deployment in order to address the issue of "competition" with synthetic host volatile lures from the potato crop itself.

On the issue of trap design, the experiment in 2001 – 02 with sticky stake traps indicated clearly that these were not effective. For a predominantly ground dwelling, crawling rather than flying, insect it is not surprising that pitfall traps are more effective. The limited experimentation to date with different forms of pitfall traps has been inconclusive, due to low catches. Ideally this would be repeated during periods of high weevil populations. Results in terms of weevil catches may need to be balanced against conflicting factors such as cost and the ease of fabrication and use of each design.

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