Reduced efficacy of Helicoverpa armigera nucleopolyhedrovirus (HearNPV) on chickpea (*Cicer arietinum*) and other legume crops, and the role of organic acid exudates on occlusion body inactivation.

Authors: Aliyu Aminu[1], Philip C. Stevenson[2&3] & David Grzywacz*[3]

Addresses:

[1] Department of Agricultural Education, Adamu Augie College of Education, Argungu, P.M.B 1012, Kebbi State, Nigeria.

[2] Royal Botanic Gardens, Kew, Kew Green, Richmond, Surrey TW9 3AE, UK.

[3]Agriculture Health and Environment Department, Natural Resources Institute, University of Greenwich, Central Avenue, Chatham Maritime, Chatham, Kent, ME4 4TB, UK.

d.grzywacz@gre.ac.uk Tel. +44-1634-883360

*Correspondence: David Grzywacz, Agriculture Health and Environment Department, Natural Resources Institute, University of Greenwich, Central Avenue, Chatham Maritime, Chatham, Kent, ME4 4TB, UK.

d.grzywacz@gre.ac.uk Tel. +44-01634-883360

Abstract.

Baculoviruses, such as the nucleopolyhedroviruses (NPV), are used widely as commercial biopesticides, however, their efficacy is reduced on some crops including chickpea (*Cicer arietinum*) due to the inactivation of the baculoviruses on the leaf surface. Here, we report for the first time, that a reduction in infectivity of the baculovirus, Helicoverpa armigera nucleopolyhedrovirus (HearNPV), also occurs on cowpea (*Vigna unguiculata*) and pigeonpea (*Cajanus cajan*), though at much lower levels than that recorded in chickpea. The isoflavonoids formononetin and biochanin A, known to be present in chickpea, pigeonpea and cowpea, were associated with reduced infectivity of HearNPV occlusion bodies (OB) *in vitro* comparable to that seen on cowpea and pigeon pea, but did not explain the greatly higher level of reduced infectivity recorded on chickpea *in vivo.* Exposure of OB to malic and oxalic acids, two organic compounds produced naturally at high concentrations from chickpea leaf trichome exudates, dramatically reduced the infectivity. The activity of baculovirus OB is known to be reduced by low pH equal to that recorded on chickpea leaf surfaces by malic and oxalic acid. Thus, we hypothesise that chickpea leaf surface acids, and in particular oxalic acid, are the primary leaf surface factors responsible for the reduced infectivity of OB on chickpea reported earlier in field and laboratory studies. The mechanism of this inactivation is not yet completely resolved, although electron microscopy suggests that the acids do not affect the OB structural integrity. The implications for the use of baculovirus insecticides on cowpea, pigeonpea and chickpea in the light of these findings are discussed.

keywords

Biological control, Nucleopolyhedrovirus, Chickpea, *Cicer arietinum,* Pigeonpea, *Cajanus cajan,* Cowpea, *Vigna unguiculata, Helicoverpa armigera*, plant leaf chemistry, Host plant resistance, Isoflavonoids, organic acids.

Key message

- The efficacy of virus-based pesticides on legume crops reduced by the plant leaf chemistry.
- Reduced infectivity of a baculovirus was observed on three legume crops, although this effect was much greater on chickpea.
- On pigeonpea and cowpea reductions in infectivity could be related to the presence of host defence isoflavonoids formononetin and biochanin A.
- On chickpea baculovirus inactivation was associated with the low leaf surface pH due to the presence of oxalic acid in plant leaf exudates

Introduction

Biopesticides that use an insect virus as the active ingredient have an established, though currently limited role in plant protection (Glare et al., 2012; Lacey et al., 2015). Existing commercial virus biopesticides, and most of those under development are based upon baculoviruses whose fast kill, specificity and robustness make them especially attractive as products for controlling pest species of global importance (Harrison and Hoover, 2012; Gwynn, 2014). While baculoviruses are an established part of the crop protection arsenal, they form only a small part of the $3 billion dollar a year market in biopesticides let alone the much larger market for insect control products that is still dominated by chemical insecticides (Trimmer, 2017). Constraints to expanding their use include regulation/registration costs (Chandler et al., 2011) and their higher production cost which limits their uptake particularly in field crops (Reid 2014).

A third limitation to wider uptake of baculoviruses has been their uncertain or limited field performance on some key crops where limited efficacy and short persistence make their use problematic (Lacey et al., 2015). Short post-application persistence of baculoviruses on plants is frequently a consequence of them being inactivated by ultraviolet radiation in sunlight (Shapiro, 1995; Lacey et al., 2008; Grzywacz and Moore, 2017). With specific crops such as cotton and chickpea, the reduced efficacy or short persistence was reported to be because of phytochemicals present in or on crop leaf surfaces (Elleman and Entwistle, 1985; Rabindra et al., 1994; Cory and Hoover, 2006; Williams, 2017).

Short post-application persistence of baculoviruses has been reported as a particular problem in chickpea (*Cicer arietinum*). A major pest of this crop in South Asia is the caterpillar of the cotton bollworm (*Helicoverpa armigera)*. Insecticide resistance is frequently reported in this insect and therefore it has been difficult for farmers to control and a target for novel pest management approaches (Kranthi et al., 2002; Srinivas et al., 2004; Patil et al., 2017). In response to this challenge the use of Helicoverpa armigera nucleopolyhedrovirus (HearNPV) as an IPM component has been developed and evaluated in India and Nepal (Rabindra and Jayaraj, 1988; Pande et al., 2005). Field studies have, however, highlighted the problem that while initial control is good, field persistence of HearNPV rarely extends beyond one day even in the absence of sunlight, resulting in the need for frequent reapplication (Rabindra et al., 1994; Cherry et al., 2000; D'Cunha, 2007) which is demanding and costly.

Previous studies on chickpea have identified that the poor persistence of HearNPV occlusion bodies (OB) is in part due to the presence on the leaf surface of isoflavonoid plant defence chemicals, specifically sissotrin and biochanin A (Stevenson et al. 2010). These compounds are secreted onto the leaf surface, possibly as a natural plant defence response (Wang 1998, Stevenson and Haware, 1999; Simmonds and Stevenson, 2001; Stevenson and Aslam, 2006). The reduced efficacy of HearNPV OB, and this was ascribed to the direct effect of these chemicals in inactivating OB on the leaf surfaces (Stevenson et al., 2010), though other possible mechanisms were not ruled out. These include increased sloughing of infected midgut cells reducing infectivity to hosts (Hoover et al., 2000), increased thickness of the peritrophic matrix reducing access of virions to the midgut cells (Plymale et al., 2008), upregulation of insect immune responses (Shikano et al., 2010) or the antifeedant activity of

phytochemicals (Simmonds and Stevenson, 2001). Experiments *in vitro* showed that the presence of chickpea isoflavonoids accounted for only about 20% of the inactivation observed on the chickpea plants *in vivo* (Stevenson et al., 2010). It is likely therefore, that other, as yet unidentified factors, contribute to the inactivation of baculovirus OB on chickpea. Here we report on the marked OB inactivating effect of organic acids exuded on to leaf surfaces which could explain the full extent of virus inactivation observed on this crop.

The use of baculoviruses OB has also been advocated for the control of pests on other important legume crops such as legume pod borer (*Maruca vitrata*) on cowpea (*Vigna unguiculata*) in Africa and pigeonpea (*Cajanus cajan*) in Asia (Srinivasan et al., 2009; Srinivasan et al., 2013) but there is little evidence on the efficacy or persistence of these NPVs on these other legume crops to determine if they have similar persistence challenges to those reported on chickpea. There has been a report of limited efficacy of NPV's on alfalfa (Santiago-Alvarez and Ortiz-Garcia, 1992) so such detrimental NPV- host plant interactions may be more widespread in legume crops than just chickpea. This paper therefore also reports for the first time on studies of the efficacy and inactivation of baculovirus OB in cowpea and pigeonpea.

Materials & methods

Insects

The *H. armigera* larvae were obtained from a laboratory culture kindly supplied by AgBioTech Australia having been established by them for many years. Insects were reared and maintained using procedures developed at Natural Resources Institute, Chatham (NRI)

for this species (Armes et al., 1992) and as previously described (Grzywacz et al., 2004; Stevenson et al., 2010). These involved rearing at 26 ± 2ºC with a relative humidity 50 ± 5% and a 14:10 light: dark regime. Larvae in culture were fed on a standard wheat germ diet communally in 200 ml pots until the second instar and were then reared individually to pupation in 30 ml plastic pots (Armes et al., 1992). All bioassays were conducted on neonate larvae as described below.

Viruses

The HearNPV virus strain used in these studies was obtained from the commercial Helicovex® HearNPV formulation produced by Andermatt Biocontrol of Switzerland. The occlusion bodies (OB) from this formulation were purified using a standard NPV purification protocol before being re-suspended in sterile distilled water and stored at -20º C for use in experiments (Hunter-Fuijita et al., 1998). This strain of HearNPV had a LC_{50} of 2.260 x 10^3 OB ml^{-1} for neonate *H. armigera*, similar to that reported for Indian HearNPV isolates (2.78 x 10^3 OB ml^{-1}) used earlier in the field (Cherry et al., 2000) and laboratory work on HearNPV and chickpea baculovirus inactivation studies (Stevenson et al., 2010). The virus was counted under phase contrast microscopy at X400 using a Leica DMR microscope using a standard Neubauer haemocytometer following the accepted protocol for counting OB (Wigley 1980).

Plants

The plants grown for use in the experiments were chickpea (*C. arietinum*) cultivar WR-315 and pigeonpea (*Cajanus cajan*) cultivar ICPL-87 kindly provided by the International Crop Research Institute for the Semi-Arid Tropics (ICRISAT), Hyderabad India. The cowpea (*Vigna unguiculata* (L.) Walp) variety IT84E-124 was provided by IITA, Cotonou, Benin, and the

tomato (*Lycopersicum esculentum*) used was the widely used commercial variety `Money maker'. Tomato was included as a plant control as this was reported widely as showing no baculovirus inactivation (Forschler et al., 1992; Farrar and Ridgway, 2000; Stevenson et al., 2010; Simón et al., 2015). All these plants were grown in the plant culture facilities at NRI (UK) using methods previously described (Stevenson et al., 2010). The seeds of the three plants were sown directly in John Innes No.2 potting compost at two seedlings per pot and maintained in a glass house at 28±2^{o}C under a 14:10 hour light dark cycle and 60% relative humidity. For pigeonpea, seeds were first sown between moist folded tissue paper and covered with two sand box lids. This process helped to enhance germination (to about 100%) that was difficult to achieve using the direct seeding method. After germination, pigeonpea seedlings were transplanted into pots at 1 seedling per pot, all other procedures were as mentioned above. Plants were cultured for 4-6 weeks before being used in the experiments.

Exposure of plants to HearNPV OB and recovery

Chickpea, cowpea, pigeonpea and tomato leaf surfaces were sprayed with HearNPV OB suspended in sterile distilled water at a concentration of 3×10^{8} OB ml^{-1} in 0.02% Triton using a 10 ml chromatographic reagent sprayer a technique previously used with chickpea in earlier studies (Stevenson et al., 2010). The plants were sprayed at the rate of 2 ml plant^{-1} for chickpea, 2 ml per branch of plant for tomato and 2 ml per trifoliate leaf for pigeon pea (only the largest trifoliate leaves were selected), which coincided with equal spray on each plant. Spraying was carried out such that the plants or selected parts were evenly wet. All treated plants were maintained in the laboratory at 25°C and 14 h photoperiod, and the HearNPV remained on the leaves of the treated plants for periods of 2, 8, 24 or 48 h. The HearNPV OB

were recovered from the plants after the specific exposure period (48 h for cowpea, pigeonpea and tomato or 2-48 h for chickpea) by cutting the leaves of each treated plant and placing them in a 50 ml conical centrifuge tube prior to washing in 30 ml of 0.1% sodium dodecycl sulphate (SDS) in sterile distilled water (SDW) on a sonicator for 3 minutes then placed on a rotator for 60 minutes at 30 rpm, a technique that was previously shown to recover >95% of OB applied (D'Cunha 2007). After one hour of washing, the leaves were removed and the OBs concentrated by centrifugation at 2500 g at 5°C for 25 min (Hunter-Fujita et al., 1998). After centrifugation the supernatant was discarded, and the virus pellet re-suspended in SDW in a 1.5 ml Eppendorf tube and pelleted again in a micro centrifuge at 2550 g for 25 min at room temperature. The process was repeated at least 3 times, OB were then re-suspended in 1 ml of SDW in a 1.5 ml micro centrifuge tube and stored at -20°C until needed for bioassay or examination by scanning electron microscopy (SEM). This method of exposing OB to leaf surfaces and washing off and cleaning prior to bioassaying was adopted to separate OB from other chemicals present on leaf surfaces so that the potency of OB could be determined in the absence of other plant surface chemicals that might interfere either through antifeedant effects (Stevenson and Aslam, 2006) or because their presence affected post-ingestion infection dynamics (Hoover et al., 2000).

Accumulation of isoflavonoid compounds in chickpea leaves

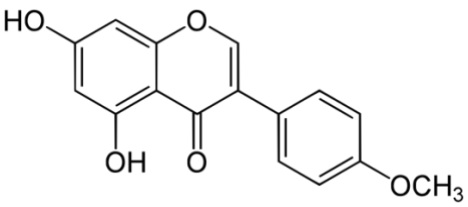
Some isoflavonoids can function as phytoalexins meaning their production is induced as a defence against invading disease-causing microorganisms (e.g. Stevenson and Haware, 2006), but they can also be induced by UV light and some chemical treatments. To determine whether the treatments in our assays induced the production or secretion of

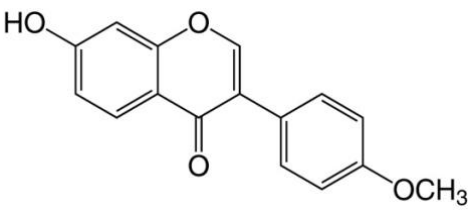
isoflavonoids onto chickpea leaves influencing OB infectivity, plants were sprayed with (a) distilled water (b) 0.2% Triton surfactant in distilled water and (c) HearNPV in distilled water at 3 x10^8 OB ml^{-1} in 0.2% Triton in distilled water. These treatments were applied with a 10 ml chromatographic sprayer to the leaves of five-week old chickpea plants at a rate of 2 ml per plant, two plants were used for each treatment replicate and the experiment repeated 5 times. Controls were left unsprayed. In each replicate 10 compound leaves were selected randomly from similar positions on two different plants giving a total of 20 leaves per replicate. Each treatment was replicated 5 times, therefore 10 different plants were used in total per treatment. The compound leaves were excised at 2, 8, 24 and 48 h after spraying. The concentrations of isoflavonoids present on the leaf surface were determined as previously described (Stevenson et al. 2010). Leaf samples were extracted in 5 ml HPLC grade methanol by rinsing the leaves individually for one minute each. Extracts were dried at 40°C using a sample concentrator (Techne Dri-Block) under a stream of nitrogen. The dried extracts were re-suspended in HPLC grade methanol and stored at 4°C prior to chemical analysis.

Chemical analysis of chickpea leaf extracts

Chickpea leaf surface extracts were analysed using an LC-MS (Agilent Technologies, 1200 series) interfaced with a photodiode array ultraviolet (PDA-UV) and single quadrupole mass-spectrometer using an electrospray ionization (ESI) source operating in positive mode under standard conditions. Extracts were filtered (Acrodisc 0.45 µm) and co-analysed with commercial standards (Sigma-Aldrich, Dorset, UK) on a Zorbax Eclipse Plus C18 analytical column (4.6mm i.d. x 150 mm; 5 µl particle size) with 90% A; 10% B gradient at t = 0 min to 90% C: 10%; B at t = 30 min (A = 100% water, B = 1% formic acid in acetonitrile and C = 100%

acetonitrile) solvent system and flow rate 0.5 mL min^{-1}. Column temperature was maintained at 30 °C and injection volume was 10 µL. Compounds were identified by comparison of their retention time and UV and MS spectra, where fragmentation in ESI-MS (+) was characterized by the molecular ion of each compound. Biochanin A recorded a molecular ion in positive mode $[M+H]^+$ at m/z =285 indicating the molecular weight of 284 corresponding to a molecular formula of $C_{16}H_{12}O_5$ and a UV spectrum similar to that recorded previously for biochanin A (Stevenson et al., 2010), while formononetin recorded a molecular ion in positive mode $[M+H]^+$ at m/z =269 indicating the molecular weight of 268 corresponding to a molecular formula of $C_{16}H_{12}O_4$ with a UV spectrum similar to that previously recorded for formononetin (Stevenson and Veitch, 1998). Data from LC-MS were analysed using analysis of variance (ANOVA), having first tested the data was suitable with equal variance and normal distribution. Where significant differences were detected among the treatment means, differences were separated using Holm-Sidak multiple comparison tests. Where data was not normal/equal variance appropriate non-parametric tests i.e., Kruskal-Wallis were used. All analysis was carried out using Sigmaplot software (11.0).





Biochanin A Formononetin

The leaf area of chickpea leaves was measured with a Quantimet 520 image analyser (Leica Microsystems) using 150 leaf samples. These results were then combined with the chemical

analyses to quantify surface compounds in terms of leaf area of the plants as previously described (Stevenson et al., 2010).

Virus bioassays

To assess the activity of OB, surface contamination larval bioassays were employed in all cases using neonate larvae as described previously (McKinley, 1985; Jones, 2000; Stevenson et al., 2010). A five-fold dilution series of the OB samples were prepared and dispensed in 100 µl aliquots onto the surface of artificial diet in 30 ml plastic pots. The aliquots were then spread evenly by tilting and left to dry. Ten larvae were then added to each pot and left to feed for 24 hours, after which they were transferred to pots containing clean artificial diet. They were then reared under standard conditions before survival was counted at five days post-dosing. In each bioassay 50 larvae were used per treatment and the assays were replicated 5-7 times on different days with each replicate bioassay including a treatment control. The bioassay results were analysed using probit analysis (Finney, 1971) with Poloplus® probit software to determine the 50% lethal concentration of OBs (LC_{50}) (Robertson et al., 2007). For comparison of experimental treatments LC_{50} values were obtained from a minimum of five separate replicate assays carried out on different days. To obtain estimates of HearNPV LC_{25} and LC_{75} needed for chemical assays, data from seven different LC_{50} bioassays carried out within short intervals (<10 days) using *H. armigera* neonates were used. The data were analysed using the Poloplus software package. The determination of LC_{50} values allowed for easier comparison of the results reported here with earlier studies of phytochemical inactivation on chickpea and other species (D'Cunha, 2007; Stevenson et al., 2010) and allowed us to quantify the differences in OB potency on different plant species. To compare treatments, comparisons of LC_{50} were performed, using

log transformed data to normalise variances using ANOVA procedures in the SIGMASTAT® 11.0 software package. using Holm-Sidak multiple comparison test for parametric data and Kruskal-Wallis tests where data were non-parametric.

Scanning electron microscopy of Hear*NPV* OB

To investigate any physical changes to HearNPV OB resulting from exposure to chickpea plant surfaces samples were studied using scanning electron microscopy (SEM). OB exposed to tomato leaf surface, known not to reduce OB efficacy, were also examined as were unexposed samples of OB as controls. The OB samples were washed and concentrated by centrifugation as above. Samples of OB (1µl) were pipetted onto 5 mm squares of silicon wafer that were attached to aluminium stubs with a carbon adhesive (Leit-C Agar Scientific). Samples were then allowed to dry at room temperature then examined using a Hitachi model SU8030 cold-cathode field emission gun scanning electron microscope, using an accelerating voltage of 1.5kV, decelerating voltage of 1.0 kV and landing voltage of 0.5 kV. The samples were examined at x10000 -x15000 magnification at 3 mm working distance.

Determination of the effect on OB infectivity after exposure to mixtures of chickpea isoflavonoids and organic acids

To determine the effects of specific legume leaf surface chemicals on HearNPV, a series of experiments was conducted exposing HearNPV OB to various combinations of isoflavonoids and chickpea organic acids. These OB were then bioassayed against neonate *H. armigera* to determine the effects of exposure on OBLC_{50} values. The exposure/bioassay used protocols previously described (Stevenson, 2010) with some modifications detailed below.

306

307 The chemical components present on chickpea leaf surfaces were known from earlier studies
308 (Rembold and Weigner, 1990; Stevenson and Aslam, 2006). Solutions of the most abundant
309 components, malic and oxalic acid (Sigma Aldrich, USA) were prepared in sterile distilled
310 water to match the levels reported to be present on the leaf surfaces, namely oxalic acid
311 119.60 mg ml^{-1}, malic acid 3.80 mg ml^{-1} (Rembold and Weigner, 1990).

312

313 The concentrations of formononetin and biochanin A used to treat OB were 25 µg g^{-1}, 50 µg
314 g^{-1}, 100 µg g^{-1} and 500 µg g^{-1}, as these extended over as well as above and below the natural
315 concentrations of 100-200 µg g^{-1} determined in this study (see results below). In the first
316 series of assays a low concentration of 100 µL (1.1×10^3 OB /pot) of HearNPV equivalent to
317 LC_{25} was employed with low concentrations of 25 and 50 µg g^{-1} isoflavonoids. The treatments
318 were as follows (1) control with chickpea organic acids mixture (oxalic acid plus malic acid
319 119.6 and 3.80 mg ml^{-1} respectively) (2) HearNPV OB alone (3) organic acid mixture plus
320 *Hear*NPV OB LC_{25} (4) Formononetin 25 µg g^{-1} plus biochanin A 25 µg g^{-1} plus organic acids
321 mixture plus HearNPV LC_{25}. (5) Formononetin 50 µg g^{-1} plus the organic acids mixture plus
322 HearNPV OB LC_{25} (6) Biochanin A 50 µg g^{-1} plus organic acids plus HearNPV OB LC_{25}. In this
323 series of assays the chemical solutions were applied to the surface of 29 ml bioassay diet pots
324 in aliquots of 100 µl and were spread evenly over the surface. The different chemicals were
325 applied to the surface of bioassay diet pots sequentially, finally 100 µl of HearNPV OB LC_{25}
326 was then applied (equivalent to 1.1 x 10^3 OB/pot). Treated pots were tilted and rotated
327 gently until both aliquots spread evenly over the surface to allow the chemical treatments to
328 mix. Treated pots were then allowed to dry at room temperature. All other procedures were
329 same as mentioned above.

A second series of assays was conducted with similar treatments as mentioned in the first series of bioassays but using a higher OB concentration equal to the estimated LC_{75} (5.8×10^3 OB ml^{-1}) and a higher range of concentrations of isoflavonoids (100 µg g^{-1} and 500 µg g^{-1}). In this assay series, the acids, isoflavonoids and OB were mixed in an Eppendorf tube (1.5 ml) in aqueous solution, prior to application to the bioassay pots. This protocol was adopted to reduce any potential effect of the diet on the chemical interactions, and to increase the interaction of the three components. The treatments for the second series of assays are given in Table 1. These two series of assays ANOVA was used to compare percent mortality for each treatment after checking that the data was appropriate, i.e. normally distributed and of equal variance.

A third series of assays was carried out to explore the role of chickpea acids alone on HearNPV lethal concentration values, and to help determine which of the acids was more active against the virus. The different concentrations of the organic acids used were as follows; (1) half the reported leaf surface concentration i.e., 2.0 and 60 mg ml^{-1} for oxalic and malic acid respectively (2) the reported leaf surface concentration, 4.0 and 120 mg ml-1 for oxalic and malic acids (3) three times the reported leaf surface concentration, 12 and 360 mg ml^{-1} for oxalic and malic acids (4) the reported concentrations of oxalic and malic were transposed in value, 120 and 4.0 mg ml^{-1} oxalic and malic acid respectively. The pH of the organic acid combinations was measured using a microprocessor pH meter (Hanna Instrument, Bedfordshire, UK) in aqueous suspension and with pH paper immediately after applying to the diet surface. The details of the treatments for the third series of assays are

given in Table 2. All other procedures were the same as mentioned in the second series of assays.

Results

Chemical analysis of isoflavonoids

Formononetin was recorded on chickpea leaf surfaces at between 69-102 µg g^{-1} leaf with a mean of 84.7 µg g^{-1} (SE = 9.563), while biochanin A was recorded between 157 and 225 µg g^{-1} with a mean of 177 µg g^{-1} (SE = 24.111). These data were used to inform the concentrations chosen for later tests on the influence of these two isoflavonoids on the infectivity of HearNPV OB. The formononetin concentrations found in this study on chickpea leaves were similar to those of other isoflavonoids, such as sissotrin previously reported on chickpea leaf surfaces (Stevenson et al., 2010).

Effect of exposing OB to different crop surfaces on infectivity of HearNPV

Figure 1 shows the infectivity of HearNPV OB when exposed to different plant surfaces. The LC_{50} of OB exposed to chickpea leaf surface for one hour was 6.9 x 10^8 OB ml^{-1}, which was significantly higher than the LC_{50} of OB exposed to tomato, cowpea and pigeonpea leaf surfaces or that of the control OB ($p < 0.001$, $H = 21.619$, df = 4,). The LC_{50} of *Hear*NPV exposed to tomato (2.382 x10^3 OB ml^{-1}) was not significantly different to the control OB at 2.261 x10^3 OB ml^{-1}, ($p = 0.91$, $t = 0.101$ df 3). The LC_{50} of OB exposed to pigeonpea was 8.817 x 10^3 OB ml^{-1} which was significantly higher (diff. of means = 4360, $t = 4.996$ df 3, p =

376 0.001) than that of the control OB, as was the LC_{50} of OB exposed to cowpea at 6.626×10^3
377 OB ml^{-1} (diff. of means 6651, t = 3.226, df 3, p = 0.007)

378

379 *Effects of exposing OB to combinations of isoflavonoids and chickpea acids on infectivity of*
380 *HearNPV*

381

382 All treatments including exposure of OB to (1) chickpea acids alone, (2) acids plus a mixture
383 of 25µg g^{-1} formononetin plus 25 µg g^{-1} biochanin, (3) acids plus 50µg g^{-1} biochanin A, and
384 (4) acids plus formononetin 50µg g^{-1}, produced significantly lower mortality than with the
385 control of unexposed HearNPV OB and were also not significantly different to the mortality
386 of control insects that did not consume OB (p <0.001, F = 5.326, df = 5, 36) (Figure 2a).

387

388 When tested at a concentration equivalent to LC_{75} and a higher range of concentrations of
389 isoflavonoids (100 and 500 µg g^{-1}) the mortalities of larvae given OB from all OB treatments
390 exposed to organic acids, and organic acids plus isoflavonoids were not significantly
391 different to that of the no virus control and significantly lower than larvae bioassayed with
392 untreated OB (p = <0.001, F = 42.079, df 6,26) (Figure 2b). Exposure to chickpea acids alone
393 resulted in inactivation that did not differ significantly from OB treated with acids in
394 mixtures with either formononetin or biochanin A at 25-500 µg g^{-1} (Fig 2a and 2b).

395

396 *Effects of exposing OB to chickpea organic acids alone*
397

Larvae exposed to samples of HearNPV OB that had been treated with any of the chickpea organic acids at concentrations between 62 and 372 µg ml^{-1} showed significantly lower mortality (p <0.001, F = 16.873, df = 6,15) than larvae treated with HearNPV OB that had not been exposed to organic acids (Fig 3). At the highest total acid concentration of 372 µg ml^{-1} the mortality of larvae was not significantly different to that of the no virus control (p = 0.105, F =4.495, df = 1,4). In the two treatments with a total acid concentration of 124 µg ml^{-1} the treatment that corresponded to the acid proportions present on leaves (120 µg ml^{-1} malic and 4 µg ml^{-1} oxalic) 124N in Fig 3 resulted in a lower (25.5%)mortality than that of the treatment 124R (9.0%) in which the proportions of the acids were reversed (4 malic µg ml^{-1} and 120 oxalic µg ml^{-1}) although this difference was not significant (p> 0.05).

Exposure of HearNPV to concentrations of oxalic acid between 2-120 µg ml^{-1} (Fig 4) resulted in significantly lower mortality than the untreated HearNPV OB (p <0.001, F = 19.698, df = 5,13). The exposure of OB to concentrations of oxalic acid of 2 µg ml^{-1} and above was associated with the progressive loss of OB activity and at 4 -120 µg ml^{-1}l larval mortality was not significantly different to that of the control insects that had not consumed OB (p > 0.05).

The mortality of larvae treated with the LC_{75} that had been exposed to acid mixtures was plotted against the measured pH of the oxalic acid/malic acid mixtures (Fig 5) and was shown to be pH dependent with lower pH reducing infectivity of the OB. Larval mortality differed significantly among treatments (p <0.001, F = 24.479, df = 5, 12) with the mortality in the HearNPV LC_{75} exposed to pH 1.4 - 0.8 not significantly different from the no virus control indicating that the OB in these samples showed negligible or no activity. However, at

421 the LC_{75} OB that had not been exposed to acids and with pH 7.4 showed significantly higher
422 mortality than any of the other treatments.

423

424 *Electron micrograph studies of OB exposed to leaf surfaces.*

425

426 Examples of SEM images of HearNPV OB are shown in fig 6, with OB exposed to chickpea
427 surfaces for 2 hours (Fig 6 A), OB exposed to chickpea leaf surface for 48 hours (Fig 6 B)
428 alongside OB exposed to tomato leaf surface for 48 hours (Fig 6 C) and unexposed OB (Fig 6
429 D). No physical differences were observed between OB exposed to chickpea leaf surfaces or
430 exudates and unexposed OB.

431

432 *Discussion*

433

434 The main finding from this study was that exposure of OB to chickpea leaf surfaces resulted
435 in a very high level of reduction in infectivity (> x100,000) compared with controls and
436 exposure to the leaf surfaces of other crop legumes. This finding is in line with earlier
437 studies on the reduced activity of OB in both field (Rabindra et al., 1992; Cherry et al., 2000)
438 and in laboratory studies (Rabindra et al., 1994; Stevenson et al., 2010). Our data further
439 indicate for the first time that oxalic and malic acid on the surface of chickpea likely account
440 for this reduced OB infectivity. The presence of organic acids on chickpea leaves causes the
441 surface to have a very low leaf pH, in the range 0.4-1.3, (Launter and Munns, 1986; Rembold
442 and Weigner, 1990), similar to the pH of the solutions tested here. Concentrations
443 equivalent to those found in chickpea leaf extracts had a pH of 1.4; this is well within the

444 ranges reported as inactivating NPV OB (Ignoffo and Garcia, 1966; Gudaukas and Canerday,
445 1968) thus the acidity of the leaf surfaces may explain the potent inactivation.
446
447 Oxalic and malic acid confer *H. armigera* resistance in chickpea cultivars (Rembold, 1981;
448 Rembold, 1989) and contribute to fungal resistance in chickpea (Nene and Reddy, 1987;
449 Armstrong-Cho and Gossen, 2005). Furthermore, oxalic and malic acid concentrations are
450 higher in *H. armigera* resistant chickpea cultivars (Yoshida, 1995; Yoshida et al., 1997)
451 suggesting that resistance is in part conferred by an antifeedant effects of oxalic and malic
452 acid (Rembold, 1981; Rembold and Winter, 1982; Lateef, 1985; Rabindra et al., 1992). By
453 reducing feeding on leaves, this could cause reduced consumption of OB and lead to lower
454 efficacy of NPV on chickpea cultivars, a known effect (Rabindra et al., 1992; Cowgill and
455 Bhagwat, 1996). However, the resistance mechanisms in chickpea to *H. armigera* was
456 associated with antibiosis of oxalic acid leading to growth and survival reduction (Yoshida et
457 al., 1995) and chickpea organic acids can even act as feeding stimulants at lower
458 concentrations (Yoshida et al., 1995) so reduced consumption of OB is unlikely to explain
459 reduced OB infectivity.
460
461 HearNPV had just a two- to four-fold reduction in infectivity after exposure to the leaves of
462 two other legume crops, pigeonpea and cowpea while tomato had no adverse effect on OB
463 infectivity as expected from earlier reports (Forschler et al. 1992; Farrar and Ridgway 2000;
464 Moore et al. 2004). Previously, isoflavonoids in chickpea were reported to be partly
465 responsible for the inactivation of OB owing to their biological activity and thus may
466 similarly explain here the modest reduction in infectivity in pigeonpea and cowpea since
467 they also produce related isoflavonoids (Ingham, 1976; Williams and Harborne, 1989;

Dakora and Phillip 1996; Duker-Eshun et al., 2004; Nix et al., 2015). In contrast, isoflavonoids have not been reported from tomato.

Combining formononetin and biochanin A with organic acids had no greater reduction in infectivity than that seen with the acids alone suggesting that the organic acids were the primary influence on OB infectivity in chickpea. Increasing isoflavonoid concentrations from 50 to 500 μg ml^{-1} had no additional effect. The treatments in which the concentrations of malic and oxalic acid were not changed such that malic acid has the higher concentration, but the mixture has the same total acid concentration of 124 μg ml^{-1}, did not produce significantly higher levels of inactivation than the naturally occurring ratio of oxalic and malic acids. However, the lower mean value in this treatment in the reversed sample where the oxalic acid was predominant suggested that oxalic acid might be more potent at OB inactivation. If the data is replotted against only the oxalic acid concentration (Fig 4) there was a pronounced association of reduced mortality with increasing oxalic acid concentration with concentrations of oxalic acid of ≥ 4 μg ml^{-1} and a reduction in OB induced mortality that was not significantly different to the non-virus control.

In the plot of virus-induced HearNPV mortality against the pH of solution used to treat OB (Fig 5) when OB were exposed to pH ≤ 1.4 (i.e. p H of between 1.4 – 0.4)larval mortality was not significantly higher than that seen in the non-virus control, indicating that complete inactivation of the OB can occur with mixtures of chickpea acids at concentrations equal to those found on chickpea leaves.

In the leaf surface exposure experiments reported here OB removed from chickpea, cowpea and pigeonpea all showed reduced activity, even after recovery and washing, which would be consistent with the observed reduced infectivity being caused by a permanent reduction in OB infectivity. A finding in line with earlier studies of OB inactivation in chickpea (Stevenson et al., 2010). This is more consistent with the hypothesis that involves a physical change to the OB that persists even when the phytochemicals have been removed after exposure. Felton and Duffey (1990) proposed that the binding of plant phenolics to OB could lead to impaired solublisation and release of virions. Other mechanisms of inactivation have included chemical binding leading to aggregation of OB impairing virion release, masking through phenolic binding or direct damage (Cory and Hoover, 2008).

SEM alone is unable to visualise the range of biochemical changes to OB structure that might impair infectivity, however, the SEM micrographs did not show any evidence of physical degradation of OB after exposure to chickpea leaf surface, implying no gross physical damage of OB. This was a similar finding to that observed in another recent study that investigated inactivation of a baculovirus in amaranth (*Amaranthus hypocondriacus*) in which post exposure electron microscopy of inactivated OB showed no overt damage to the surface structure of the OB (Lasa et al., 2018). Thus, a mechanism that involved isoflavonoids binding to OB surface, leading to the impairment of OB functionality such as impaired virion release seems more consistent with this result.

The reduced efficacy of HearNPV OB on cowpea and pigeonpea was comparatively low compared to that seen with chickpea. These levels of inactivation do not necessarily preclude the use of baculovirus based insecticides on these crops entirely. The results for

515 cowpea and pigeonpea are of interest because the sustainable management of the key
516 lepidopteran pest *Maruca vitrata* in Africa can be provided by a biological pesticide that is
517 similar to HearNPV, based upon the baculovirus *M. vitrata* NPV (MaviNPV) (Lee et al., 2007)
518 and may be similarly susceptible to inactivating factors on the surfaces of legume crops.
519 MaviNPV has undergone development and field trials in which it has shown promise as an
520 IPM intervention for use on cowpea and pigeonpea (Srinivasan et al 2009; Traore et al.,
521 2019; Sokame et al., 2015). Mild impairment of baculovirus infectivity may not represent a
522 major barrier to the successful use of biopesticides, though overcoming this inactivation
523 through formulation might make virus -based pesticides even more effective.
524
525 Researchers in India and Australia have reported that HearNPV can be effective in
526 controlling *H. armigera* on chickpea (Rabindra et al., 1992; Cherry et al., 2000; Beurger et al.,
527 2007) even though the efficacy and persistence of OB activity on these crops is reportedly a
528 matter of hours (Cherry et al., 20000; Stevenson et al., 2010). The impairment of baculovirus
529 infectivity on chickpea through the high acidity of chickpea exudates may not be relevant to
530 the use of baculoviruses alone. Armstrong-Cho and Gossen (2005) demonstrated that
531 germination of fungal conidia (*Ascochyta rabiei*) was completely inhibited at high
532 concentrations of chickpea leaf exudate (1.5 µg ml^{-1}, pH 2.8) and partially inhibited at lower
533 concentration (0.3 µg ml^{-1}, pH 3.1). This ability of chickpea exudates to inactivate plant
534 pathogenic fungi might be a key defence function of these acids in the plants. This could
535 account for the incompatibility between HearNPV and fungi resistant chickpea cultivars
536 reported previously (Rabindra et al. 1992; Cowgill and Bhagwat 1996). These cultivars were
537 bred specifically to maximise their antifungal activity (Pande et al., 2005). It is possible that

538 variations in efficacy reported in the use of HearNPV on chickpea may in part be related to
539 the differing antimicrobial properties of the specific chickpea cultivars.
540
541 Since the phytochemistry of cultivars differs with respect to their organic acids (Rembold
542 1990; Yoshida et al., 1995; Toker et al., 2006) this may explain field trials reporting that in
543 chickpea NPV efficacy against *H. armigera* varies with cultivar (Rabindra et al., 1992; Cowgill
544 and Bhagwat, 1996). The organic acid levels are also reported to vary with location,
545 temperature, and growth stage (Kaudal and Sinha, 1981; Rembold, 1990), thus further
546 assessment of different cultivars is required to fully understand how widespread this
547 phenomenon is and how it impacts the use of NPV insecticides on these three crops.
548
549 Felton et al. (1987) proposed that biological control might not be compatible with host-plant
550 resistance (HPR) when conferred by antibiosis owing to conflicting bioactivities against
551 beneficial organisms. However, while plant induced reduction in infectivity of a baculovirus
552 on a particular crop may occur, it does not necessarily invalidate the use of baculovirus
553 where field trials show a useful degree of pest control can still be provided. It may be that
554 formulations with additives that help overcome these plant surface effects could
555 significantly improve their efficacy on that crop (Grzywacz and Moore, 2017). The results
556 reported here indicate that such a technology could markedly improve infectivity on
557 chickpea where efficacy and persistence is lower.
558
559 In conclusion, the results reported here identify that the presence of organic acids in
560 chickpea leaf exudates, especially oxalic acid, contribute to the impairment of infectivity and
561 short persistence of a baculovirus insecticide on chickpea cultivars. On two other legume

crops some loss of infectivity and persistence was also observed though at a much lower level which may be attributable to the presence of isoflavonoid defence chemicals known to be produced by these species.

References

Armes, N.J., Bond, G.S., Cooter, R.J., 1992. The laboratory culture and development of *Helicoverpa armigera*. Natural Resources Institute Bulletin 57, Chatham, United Kingdom: Natural Resources Institute, 21pp.

Armstrong-Cho, C., Gossen, B.D., 2005. Impact of glandular hair exudates on infection of chickpea by *Ascochyta rabiei*. Can J Botany, 83, 22-27. https://doi.org/10.1139/b04-147

Ashraf, N., Basu, S., Narula, K., Ghosh, S., Tayal, R., Gangisetty, N., Biswas, S., Aggarwal, P.R., Chakraborty, N., Chakraborty, S., 2018. Integrative network analyses of wilt transcriptome in chickpea reveal genotype dependent regulatory hubs in immunity and susceptibility. Sci Rep 8, 1-16. https://doi.org/10.1038/s41598-018-19919-5

Bailey, J.A., 1973. Production of antifungal compounds in cowpea (*Vigna sinensis*) and pea (*Pisum sativum*) after virus infection. J Gen Microbiol 75, 119-123. https://doi.org/10.1099/00221287-75-1-119

Buerger, P., Hauxwell, C., Murray, D., 2007. Nucleopolheydrovirus introduction in Australia. Virol. Sinica. 22,173–179. https://doi.org/10.1007/s12250-007-0019-y

583 Chandler, D., Bailey, A.S., Tatchell, G.M., Davidson, G., Greaves, J., Grant, W.P., 2011. The
584 development, regulation and use of biopesticides for integrated pest management. Philos.
585 T. Roy. Soc. B: Biol. Sci. 366, 1987–1998. https://doi.org/10.1098%2Frstb.2010.0390

586 Cherry, A.J., Rabindra, R.J., Parnell, M.A., Geetha, N., Kennedy, J.S., Grzywacz, D., 2000. Field
587 evaluation of *Helicoverpa armigera* nucleopolyhedrovirus formulations for control of the
588 chickpea pod-borer, *H.armigera* (Hubn.), on chickpea (*Cicer arietinum* var. Shoba) in
589 southern India. Crop Prot 19, 51–60. https://doi.org/10.1016/S0261-2194(99)00089-7

590 Chun-Hat. S., Yuanling, C., Mingfu, W., Ivan, K.C., Clive, L., 2008. Accumulation of isoflavone
591 genistin in transgenic tomato plants overexpressing a soybean isoflavone synthase gene. J.
592 Agric. Food Chem. 56, 5655-5661. https://doi.org/10.1021/jf800423u

593 Cory, J.S., Hoover, K., 2006. Plant mediated effects in insect-pathogen interactions. Trends
594 Ecol Evol 21, 278–286. https://doi.org/10.1016/j.tree.2006.02.005

595 Cowgill, S.E., Bhagwat, V.R., 1996. Comparison of the efficacy of chemical control and
596 *Helicoverpa* NPV for the management of *Helicoverpa armigera* (Hübner) on resistant and
597 susceptible chickpea. Crop Prot 15, 241–246. https://doi.org/10.1016/0261-2194(95)00100-
598 X

599 Cowgill, S.E., Lateef, S.S., 1996. Identification of antibiotic and antixcnotic resistance to
600 *Helicoverpa armigera* (Lepidoptera: Noctuidae) in chickpea. J Econ Entomol 89, 224-229.
601 https://doi.org/10.1093/jee/89.1.224

602 Dakora, F.D., Phillips, D.A., 1996**.** Diverse functions of isoflavonoids in legumes transcend
603 anti-microbial definitions of phytoalexins. Physiol Mol Plant Pathol 49, 1–20.
604 https://doi.org/10.1006/pmpp.1996.0035

D'Cunha, R.F., 2007. Impact of host plants on the efficacy of nucleopolyhedrovirus as a biopesticide against insect pest *Helicoverpa armigera* Hubner (Lepidoptera: Nuctudae). Dissertation, Natural Resources Institute, University of Greenwich. http://gala.gre.ac.uk/id/eprint/6146

Duker-Eshun, G., Jaroszewski, J.W., Asomaning, W.A., Oppong-Boachie, F., Brøgger Christensen, S., 2004. Antiplasmodial constituents of *Cajanus cajan*. Phytother Res 18, 128-130. https://doi.org/10.1002/ptr.1375

Elleman, C.J., Entwistle, P.F., 1985. Inactivation of a nuclear polyhedrosis-virus on cotton by the substances produced by the cotton leaf surface glands. Ann Appl Biol 106, 83–92. https://doi.org/10.1111/j.1744-7348.1985.tb03097.x

Farrar, R.R., Ridgway, R.L., 2000. Host plant effects on the activity of selected nuclear polyhedrosis viruses against corn earworm and beet armyworm (Noctuidae: Lepidoptera). Environ Entomol 29, 108–115. https://doi.org/10.1603/0046-225X-29.1.108

Felton, G.W., Duffey, S.S., Vail, P.V., Kaya, H.K., Manning, J., 1987. Interaction of nuclear polyhedrosis virus with catechols: potential incompatibility for host-plant resistance against noctuid larvae. J Chem Ecol 13, 947–957. https://doi.org/10.1007/BF01020174

Finney, D.J., 1971. Probit Analysis. Cambridge University Press, London.

Forschler, B.T., Young, S.Y., Felton, G., 1992. Diet and the susceptibility of *Helicoverpa zea* noctuidae: Lepidoptera to nuclear polyhedrosis virus. Environ Entomol 21, 1220–1223. https://doi.org/10.1093/ee/21.5.1220

Glare, T.R., Caradus, J., Gelernter, W., Jackson, T., Keyhani, N., Kohl, J., Marrone, P., Morin, L., Stewart, A., 2012. Have Biopesticides come of age? Trends Biotechnol. 30, 250-258. https://doi.org/10.1016/j.tibtech.2012.01.003

Gudauskas, R.T., Canerday, D., 1968. The effect of heat, buffer salt and H-ion concentration, and ultraviolet light on the infectivity of *Heliothis* and *Trichoplusia* nuclear polyhedrosis viruses. J. Invert. Pathol. 12, 405-411. https://doi.org/10.1016/0022-2011(68)90346-7

Grzywacz, D., Moore, S., 2017. Production, formulation, and bioassay of baculoviruses for pest control. In: Lacey LA (ed) Microbial control of insect and mite pests. Academic, New York, pp 109–124. https://doi.org/10.1016/B978-0-12-803527-6.00007-X

Gwynn, R., 2014. Manual of biocontrol agents, 5th edition, British Crop Protection Council, Alton. ISBN 978 1 901396 87 4.

Harrison, R., Hoover, K., 2012. Baculoviruses and other occluded insect viruses. In: Vega F, Kaya H (eds) Insect pathology. Elsevier, Amsterdam, pp. 73–131. ISBN 978-0-12-384984-7

Hoover, K., Stout, M.J., Alaniz, S.A., Hammock, B.D., Duffey, S.S., 1998. Influence of induced plant defenses in cotton and tomato on the efficacy of baculoviruses on noctuid larvae. J. Chem. Ecol. 24, 253–271. https://doi.org/10.1023/A:1022528324344

Hoover, K., Washburn, O.J., Volkman, E.L., 2000. Midgut-based resistance of *Heliothis* presence to baculovirus infection mediated by phytochemicals in cotton. J. Insect. Physiol. 46, 999–1007. https://doi.org/10.1016/S0022-1910(99)00211-5

Hunter-Fujita, F.R., Entwistle, P.F., Evans, H.F., Crook, N.E., 1998 Insect viruses and pest management. John Wiley & Sons Ltd, Chichester. ISBN: 978-0-471-96878-8

646 Ingham, J.L, 1976. Induced and constitutive isoflavonoids from stem of chickpeas (*Cicer*
647 *arietinum* L.) inoculated with spores of *Helminthosporium carbonum* Ullstrup. J.
648 Phytopathol. 87, 353–367. https://doi.org/10.1111/j.1439-0434.1976.tb01741.x

649 Jones, K.A., 2000. Bioassays of entomopathogenic viruses. In A. Navon, and K. R. S. Ascher
650 (eds) Bioassays of entomopathogenic microbes and nematodes. CAB International,
651 Wallingford, pp 95–140. http://dx.doi.org/10.1079/9780851994222.0000

652 Ignoffo, C.M., Garcia, C., 1966 The relation of pH to the activity of inclusion bodies of a
653 *Heliothis* nuclear polyhedrosis. J. Invert. Pathol. 21, 426-427.

654 Kaudal, K.K., Sinha, S.K., 1981. Malic acid exudation and photosynthesis characteristics in
655 *Cicer arietinum*. Phyotochemistry 20, 1251-1252. https://doi.org/10.1016/0031-
656 9422(81)80015-5

657 Keating, S.T., Hunter, M.D., Schultz, J.C., 1990. Leaf phenolic inhibition of gypsy moth
658 nuclear polyhedrosis virus: Role of polyhedral inclusion body aggregation. J. Chem. Ecol. 16,
659 1445-1457. https://doi.org/10.1007/BF01014080

660 Kranthi, R.K., Jadhav, D.R., Kranthi, S., Wanjari, R.R., Ali, S.S., Russsell, D.A., 2002.
661 Insecticide's resistance in five major insect pests of cotton in India. Crop. Prot. 21, 449-460.
662 https://doi.org/10.1016/S0261-2194(01)00131-4

663 Lacey, L.A., Thomson, D., Vincent, C., Arthurs, S.P., 2008. Codling moth granulovirus: a
664 comprehensive review. Biocontrol Sci. Technol. 18, 639-663.
665 https://doi.org/10.1080/09583150802267046

Lacey, L.A., Grzywacz, D., Shapiro-Ilan, D.I., Frutos, R., Brownbridge, M., Goettel, M.S., 2015. Insect pathogens as biological control agents: back to the future. J. Invert. Pathol. 132, 1-41. https://doi.org/10.1016/j.jip.2015.07.009

Lasa, R., Guerrero-Analco, J.A., Monribot-Villanueva, J.L., Mercado, G., Williams, T., 2018. Why do *Spodoptera exigua* multiple nucleopolyhedrovirus occlusion bodies lose insecticidal activity on amaranth (*Amaranthus hypocondriacus* L.). Biol. Control 126, 74–82. https://doi.org/10.1016/j.biocontrol.2018.07.014

Lateef, S.S., 1985. Gram pod borer (*Heliothis armigera* Hub.) resistance in chickpeas. Agric. Ecosys. Environ. 14, 95-102. https://doi.org/10.1016/0167-8809(85)90087-8

Lee, S.T., Srinivasan, R., Wu, Y.J., Talekar, N.S., 2007. Occurrence and characterization of a nucleopolyhedrovirus from *Maruca vitrata* (Lepidoptera, Pyralidae) isolated in Taiwan. BioControl 52, 801-819. https://doi.org/10.1007/s10526-006-9049-x

Nene, Y.L., Reddy, M.V., 1987. Chickpea diseases and their control. In: Saxena, M.C. & Singh, K.B. (eds.), The Chickpea. CAB International, Oxon, pp. 233-270. ISBN 0851985718.

Nix., A., Paull, C.A., Colgrave, M., 2015. The flavonoid profile of pigeonpea, *Cajanus cajan*: a review. *SpringerPlus*, 4, 1-6. https://doi.org/10.1186/s40064-015-0906-x

Patil, S.B., Goyal, A., Chitgupekar, S.S., Kumar, S., El-Bouhssini, M., 2017. Sustainable management of chickpea pod borer. A review. *Agron. Sustain. Dev.* 37, 1-17. https://doi.org/10.1007/s13593-017-0428-8

Pande, S., Stevenson, P.C., Narayana-Rao, J., Neupane, R.K., Chaudhary, R.N., Grzywacz, D., Bourai, V.A., Krishna-Kishore, G., 2005 Rehabilitation of chickpea in Nepal through

integrated management of *Botrytis* gray mold. Plant Disease 89, 1252–1262. https://doi.org/10.1094/PD-89-1252

Rabindra, R.J., Jayaraj, S., 1988. Efficacy of nuclear polyhedrosis virus with adjuvants as high volume and ultra-low volume applications against *Heliothis armigera* Hbn. on chickpea. Int J Pest Manag. 34, 441-444. https://doi.org/10.1080/09670878809371298

Rabindra, R.J., Sathiah, N., Jayaraj, S., 1992. Efficacy of nuclear polyhedrosis virus against *Helicoverpa armigera* (Hbn.) on *Helicoverpa*-resistant and susceptible varieties of chickpea. J Crop Prot. 11, 320–322. https://doi.org/10.1016/0261-2194(92)90056-B

Rabindra, R.J., Muthuswami, M., Jayaraj, S., 1994. Influence of host plant surface environment on the virulence of nuclear polyhedrosis virus against *Helicoverpa armigera* (Hbn.) (Lep., Noctuidae) larvae. J. Appl. Entomol. 118, 453–460. https://doi.org/10.1111/j.1439-0418.1994.tb00824.x

Reid S, Chan L, Van Oers M (2014) Production of entomopathogenic viruses. In: Morales-Ramos JA, Guadalupe Rojas M, Shapiro-Ilan DI (eds.) Mass production of beneficial organisms, Elsevier, Amsterdam. pp 437-482. https://doi.org/10.1016/B978-0-12-391453-8.00013-3

Rembold, H., Weigner C 1990. Chemical composition of chickpea, *Cicer arietinum*, exudate. Z. Naturforsch. 45, 922–923. https://doi.org/10.1515/znc-1990-7-827

Robertson, J.L., Russell, R.M., Preisler, H.K., Savin, N.E., 2007. Bioassays with arthropods. 2nd Edition, CRC Press, Boca Raton. ISBN 9780367573485.

Santiago-Alvarez, C., Ortiz-Garcia, R., 1992. The influence of host plant on the susceptibility of *Spodoptera littoralis* (Boisd.)(Lep., Noctuidae) larvae to *Spodoptera littoralis* NPV

709 (Baculoviridae, Baculovirus). J. Appl. Entomol. 114, 24-130. https://doi.org/10.1111/j.1439-
710 0418.1992.tb01106.x

711 Seneviratne, G.I., Harborne, J.B., 1992. Constitutive flavonoids and induced isoflavonoids as
712 taxonomic markers in the genus *Vigna*. Biochem. Syst. Ecol. 20, 459-467.
713 https://doi.org/10.1016/0305-1978(92)90087-T

714 Shapiro, M., 1995. Radiation protection and activity enhancement of viruses. In: Biorational
715 Pest Control Agents: Formulation and Delivery. Am. Chem. Soc. Symp. 595, 153–164. ISBN
716 08-412-32261.

717 Sharma, H.C., 1998. Bionomics, host plant resistance, and management of the legume pod
718 borer, *Maruca vitrata*—a review. Crop Prot. 17, 373-386. https://doi.org/10.1016/S0261-
719 2194(98)00045-3

720 Shikano, I., Ericsson, J.D., Cory, J.S., Myers, J.H., 2010. Indirect plant-mediated effects on
721 insect immunity and disease resistance in a tritrophic system. Basic Appl. Ecol. 11, 15-22.
722 https://doi.org/10.1016/j.baae.2009.06.008

723 Shikano, I., 2018. Evolutionary ecology of multitrophic interactions between plants, insect
724 herbivores and entomopathogens. J. Chem. Ecol. 43, 586–598.
725 https://doi.org/10.1007/s10886-017-0850-z

726 Simmonds, M.S., Stevenson, P.C, 2001. Effects of isoflavonoids from *Cicer* on larvae of
727 *Helicoverpa armigera*. J. Chem. Ecol. 27, 965–976.
728 https://doi.org/10.1023/A:1010339104206

729 Simón, O., Bernal, A., Williams, T., Carnero, A., Hernández-Suárez, E., Muñoz, D., Caballero,
730 P., 2015. Efficacy of an alphabaculovirus-based biological insecticide for control of

Chrysodeixis chalcites (Lepidoptera: Noctuidae) on tomato and banana crops. Pest Manage. Sci. 71, 1623-1630. https://doi.org/10.1002/ps.3969

Sree Latha, E., 2003. Stability, inheritance and mechanisms of resistance to *Helicoverpa armigera* (Hub.) in chickpea *(Cicer arietinum Linn.)* Doctoral dissertation, Acharya NG Ranga Agricultural University.

Srinivas, R., Udikeri, S.S., Jayalakshmi, S.K., Sreeramulu, K., 2004. Identification of factors responsible for insecticide resistance in *Helicoverpa armigera*. Comp. Biochem. Physiol. Part C 137, 261-269. https://doi.org/10.1016/j.cca.2004.02.002

Srinivasan, R., Tamo, M., Lee, S.T., Lin, M.Y., Huang, C.C., Hsu, Y.C., 2009. Towards developing a biological control program for legume pod borer, *Maruca vitrata*. Proceedings of the International Conference on Grain Legumes, Kanpur, 2009 pp.183-196.

Srinivasan, R., Yule, S., Chang, J.C., Malini, P., Lin, M.Y., Hsu, Y.C., Schafleitner, R., 2013. Towards developing a sustainable management strategy for legume pod borer, *Maruca vitrata* on yard-long bean in Southeast Asia. In Proceedings of the Regional Symposium on High Value Vegetables in Southeast Asia: Production, Supply and Demand. AVRDC pp. 76-82.

Srivastava, C.P. Srivastava, R.P., 1990. Antibiosis in chickpea (*Cicer arietinum* L.) to gram pod borer, *Heliothis armigera* (Hubner)(Noctuidae: Lepidoptera) in India. Entomon. 15, 89-93. ISSN 0377-9335.

Stevenson, P.C., Aslam, S.N. 2006. The Chemistry of the Genus *Cicer* L., In Atta-Ur-Rahman (ed.). Studies in natural products chemistry vol. 33. Bioactive natural products (Part M). Elsevier, Amsterdam. pp 905–956. https://doi.org/10.1016/S1572-5995(06)80043-8

752 Stevenson, P.C., Haware, M.P. 1999. Maackiain accumulation in species of *Cicer* L.
753 associated with resistance to Botrytis Grey Mould (*Botrytis cinerea*). Biochem. Syst. Ecol. 27,
754 761–767.

755 Stevenson, P.C., D'Cunha, R.F., Grzywacz, D., 2010. Inactivation of Baculovirus by
756 isoflavonoids on chickpea (*Cicer arietinum*) leaf surfaces reduces the efficiency of NPV
757 against *H. armigera*. J. Chem. Ecol. 36, 227–235. https://doi.org/10.1007/s10886-010-9748-
758 8

759 Traore. F., Waongo, A., Ba, M.N., Dabiré, C., Sanon, A., Tamò, M., Pittendrigh, B.R, 2019.
760 Effects of *Maruca vitrata* multi-nucleopolyhedrovirus and neem oil, *Azadirachta indica* Juss
761 on the eggs of the cowpea pod borer, *Maruca vitrata* Fabricius (Lepidoptera:
762 Crambidae). Int. J. Trop. Insect Sci. 39, 333-339. https://doi.org/10.1007/s42690-019-00061-
763 2

764 Toker, C., Karhan, M., Ulger, S., 2004. Endogenous organic acid variations in different
765 chickpea (*Cicer arietinum* L.) genotypes. Acta Agric. Scand. B. Soil Plant Sci. 54, 42-44.
766 https://doi.org/10.1080/09064710310019720

767 Trimmer, M., 2017. Biological Control Global Market Overview, Western Region State
768 Liaison Representatives /Commodity Liaison Committee Meeting and Biopesticides
769 Workshop. 25-26 April 2017, Ft. Collins, Colorado.

770 Tripati, S. R, Shanna, S. K., 1985. Effect of some food plants on development and growth of
771 gram pod borer *Heliolhis armigera* (Hubn.) (Lepidoptera: Noctuidae). Niger J Entomol 6, 33-
772 38.

773 Wan, N.F., Li, X., Guo, L., Ji, X.Y., Zhang, H., Chen, Y.J., Jiang, J.X., 2018. Phytochemical
774 variation mediates the susceptibility of insect herbivores to entomoviruses. J. Appl.
775 Entomol. 142, 705–715. https://doi.org/10.1111/jen.12516

776 Wang, S.F., Ridsdill-Smith, T.J., Ghisalberthi, E.L., 1998. Role of isoflavonoids in resistance of
777 subterranean clover trifoliates to the redlegged earth mite *Halotydeus destructor*. J. Chem.
778 Ecol. 24**,** 2089–2100. https://doi.org/10.1023/A:1020789710706

779 Wigley, P.J., 1980. Counting micro-organisms. In: Kalmakoff J Longworth JF (eds). Microbial
780 control of insect pests, New Zealand Department of Science and Industrial Research, Bulletin
781 228, Wellington, New Zealand, pp 9–35. ISBN 0-477-06672-0.

782 Williams, T., 2017. Viruses. In: Hajek AE, Shapiro-Illan DI, (eds) Ecology of Invertebrate
783 Diseases, Wiley & Sons, Hoboken NY, pp.213-285. ISBN: 978-1-119-25607-6

784 Williams, C.A., Harborne, J.B., 1989. Isoflavonoids. In: Dey PM, Harborne JB (eds.). Plant
785 Biochemistry: Plant Phenolics Vol. 1. Academic Press Ltd. New York, pp 421-447. ISBN 978-
786 0124121089.

787 Yoshida, M., Cowgill, S.E. and Wightman, J.A., 1995. Mechanism of resistance to *Helicoverpa*
788 *armigera* (Lepidoptera: Noctuidae) in chickpea: role of oxalic acid m leaf exudate as an
789 antibiotic factor. J. Econ. Entomol. 88, 1783-1786. https://doi.org/10.1093/jee/88.6.1783

790 Yoshida, M., Cowgill, S.E. and Wightman, J.A., 1997. Roles of oxalic and malic acids in
791 chickpea trichome exudate in host-plant resistance to *Helicoverpa armigera*. J. Chem. Ecol.
792 23, 1195-1210. https://doi.org/10.1023/B:JOEC.0000006395.45516.e8

793

794

795

796 Declarations

797

798 Author contributions

799

800 PS & DG conceived the research. AA PS and DG developed the experimental design and
801 protocols, AA conducted the experiments and chemical analysis. All authors analysed data,
802 wrote and edited the manuscript

803

804 Acknowledgements

805

806 The authors wish to thank Dr Patrick Buerger (Agbiotech Austrialia) for providing the
807 *Helicoverpa armigera* insect culture. Dr H.C. Sharma of ICRISAT India for providing chickpea
808 and pigeonpea seeds and Dr M Tamo of IITA Benin for providing cowpea seeds. Dr Ian
809 Slipper of University of Greenwich for assistance with scanning electron microscopy. and D
810 Farman from Natural Resources Institute of University of Greenwich, for assistance with
811 chemical analysis and Stephen Young for advice on statistical analysis.

812

813 Funding

814

815 This work was funded by a grant from the Kebbi state government of Nigeria.

816

817 Compliance with ethical standards

818

819 Conflict of interest

820

821 The authors declare no competing financial interest.

822

Titles for Tables and Figs

Table 1 Details of treatments to explore the effects of mixtures of the formononetin and biochanin A in combination with organic acid mixtures of oxalic and malic acid on mortality of neonate larvae treated with the 75% lethal concentration of *Hear*NPV OB (LC_{75}).

Table 2 Details of treatments to explore effects of mixtures of the major organic acids of chickpea leaf exudate in combination with *Hear*NPV LC_{75} showing treatment concentrations of oxalic and malic acids, *Hear*NPV OB, total acids and pH of treatments.

Fig 1 Average median lethal concentrations (LC_{50}±SEM) of *H. armigera* neonates on artificial diets inoculated with *Hear*NPV OB that had been exposed to leaf surfaces of chickpea, cowpea, pigeonpea and tomato for 2 hours. Bars headed by identical letters did not differ significantly ($P > 0.05$).

Fig 2a Mean percentage mortality (± SEM) of *Helicoverpa armigera* neonates on artificial diets inoculated with *Hear*NPV OB at LC_{25} (1.1×10^3 OB ml^{-1}) after previous exposure to solutions of chickpea isoflavonoids in combination with chickpea acids. Bars headed by identical letters did not differ significantly ($P > 0.05$). Treatments were Control = no HearNPV, NPV = HearNPV LC_{25}, NPV+A = HearNPV LC_{25} plus oxalic acid 119.60 mg ml^{-1} and malic acid 3.80 mg ml^{-1} , NPV+A+FB = NPV LC_{25} plus oxalic and malic acid acids plus a mixture of 25µg g^{-1} formononetin plus 25 µg g^{-1} biochanin, NPV+A+F = NPV LC_{25} plus oxalic and malic acid acids plus formononetin 50 µg g^{-1}, NPV+A+B = NPV LC_{25} plus oxalic and malic acid plus biochanin A 50 µg g^{-1}.

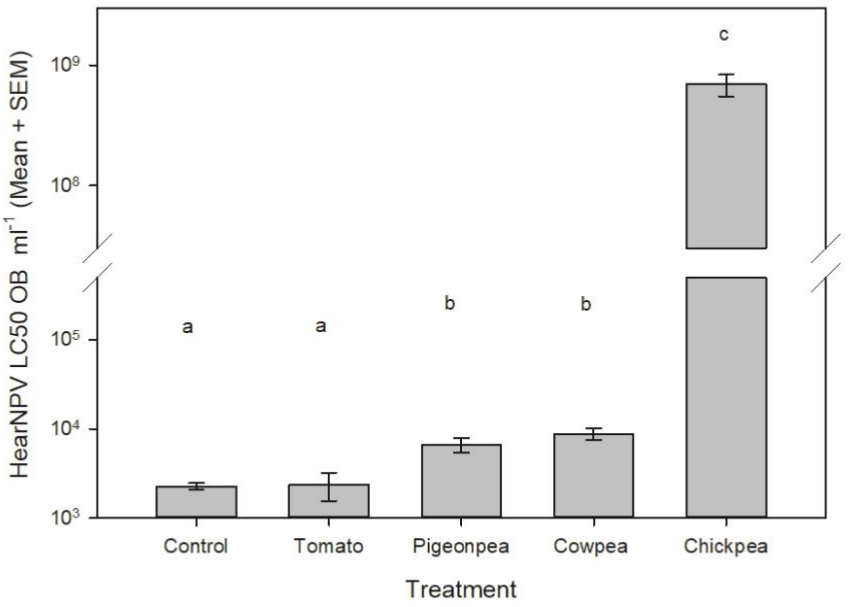
Fig 2b Mean percentage mortality (± SEM) of *Helicoverpa armigera* neonates on artificial diets inoculated with *Hear*NPV OB at LC_{75} (5.8×10^3 OB ml^{-1}) after previous exposure to solutions of chickpea isoflavonoids in combination with chickpea acids. Bars headed by identical letters did not differ significantly ($P > 0.05$). Treatments Control = no HearNPV, NPV = HearNPV LC_{75}, NPV + A + F + B 100 = HearNPV LC_{75} plus chickpea acids plus formononetin 100 µg g^{-1} plus biochanin 100 µg g^{-1}, NPV + A + F 100 = HearNPV LC_{75} plus chickpea acids plus formononetin 100 µg g^{-1}, NPV + A + B 100 = HearNPV LC_{75} plus chickpea acids plus biochanin 100 µg g^{-1}, NPV + A + F 500 = HearNPV LC_{75} plus chickpea acids plus formononetin 500 µg g^{-1},NPV + A + B 500 = HearNPV LC_{75} plus chickpea acids plus biochanin 500 µg g^{-1}.

Fig 3 Mean percentage mortality (± SEM) of *Helicoverpa armigera* neonates treated with samples of *Hear*NPV at LC_{75} (5.8 x10^3 OB ml^{-1}) previously exposed to chickpea acids, oxalic and malic at differing concentrations, or a non-virus control. Bars headed by identical letters did not differ significantly (P > 0.05). Treatments, 0 = HearNPV OB, 62 = HearNPV LC_{75} + half natural chickpea acid leaf surface concentration (2 µg g^{-1}oxalic acid plus 60 µg g^{-1} malic acid), 124 N = HearNPV LC_{75} + natural chickpea acids concentration (4 µg g^{-1}oxalic acid plus 120 µg g^{-1} malic acid), 124 R = HearNPV LC_{75} + natural chickpea acids concentration with proportions reversed (120 µg g^{-1} oxalic acid plus 4 µg g^{-1} malic acid), 372 = HearNPV OB + three times natural chickpea acids concentration (360 µg g^{-1} oxalic acid plus 12 µg g^{-1} malic acid), control = no OB and no acids.

Fig 4 Mean percentage mortality (± SEM) of *Helicoverpa armigera* neonates treated with samples of *Hear*NPV at LC_{75} (5.8 x10^3 OB ml^{-1}) following exposure to oxalic acid at differing concentrations, or a non-virus negative control. Bars headed by identical letters did not differ significantly (P > 0.05).

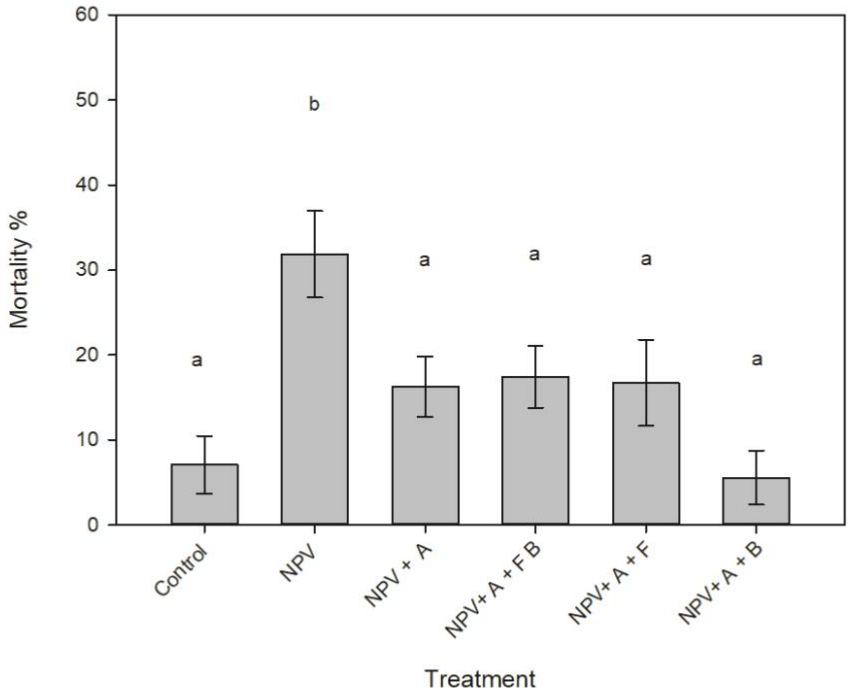
Fig 5 Mean percentage mortality (± SEM) of *Helicoverpa armigera* neonates treated with samples of *Hear*NPV OB at LC_{75} (5.8 x10^3 OB ml^{-1}) following exposure to chickpea acids at different pH's, or a non-virus negative control. Bars headed by identical letters did not differ significantly (P > 0.05).

Fig 6 Scanning electron micrograph images of *Hear*NPV occlusion bodies (OB) exposed to different leaf surfaces as compared to unexposed controls: (A) OB exposed to chickpea leaf surfaces for 2 hours (bar= 3 µm); (B) OB exposed to chickpea leaf surfaces for 48 hours (bar= 5 µm); (C) OB exposed to tomato leaf surfaces for 48 hour (Bar= 5µm); (D) Unexposed OB (bar = 3 µm).


HearNPV LC50 OB ml-1 (Mean + SEM)
10⁹
10⁸
10⁵
10⁴
10³
a
a
b
b
c
Control
Tomato
Pigeonpea
Cowpea
Chickpea
Treatment

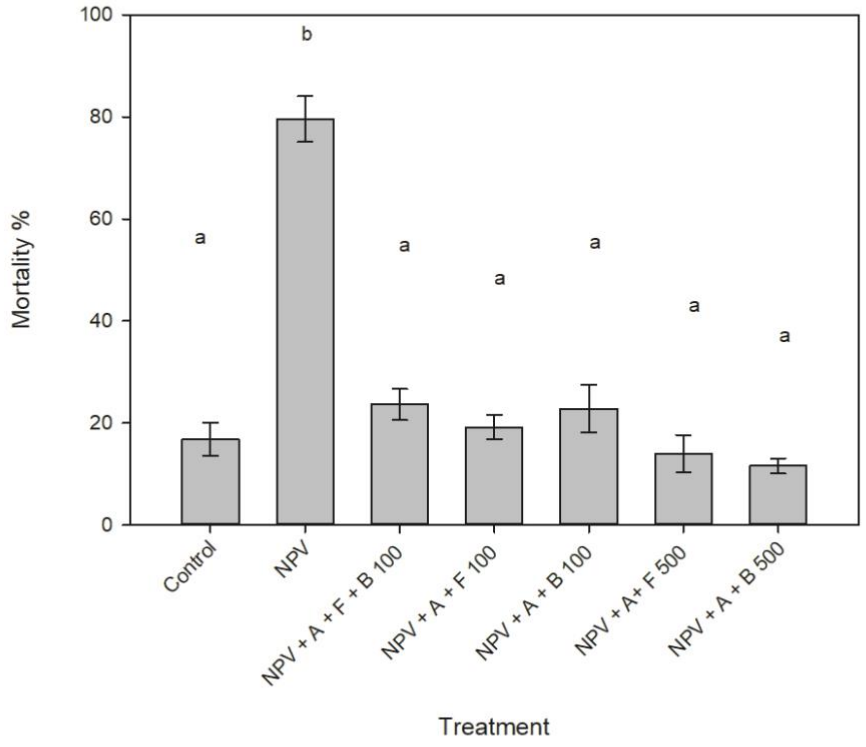
887

888 Fig 1



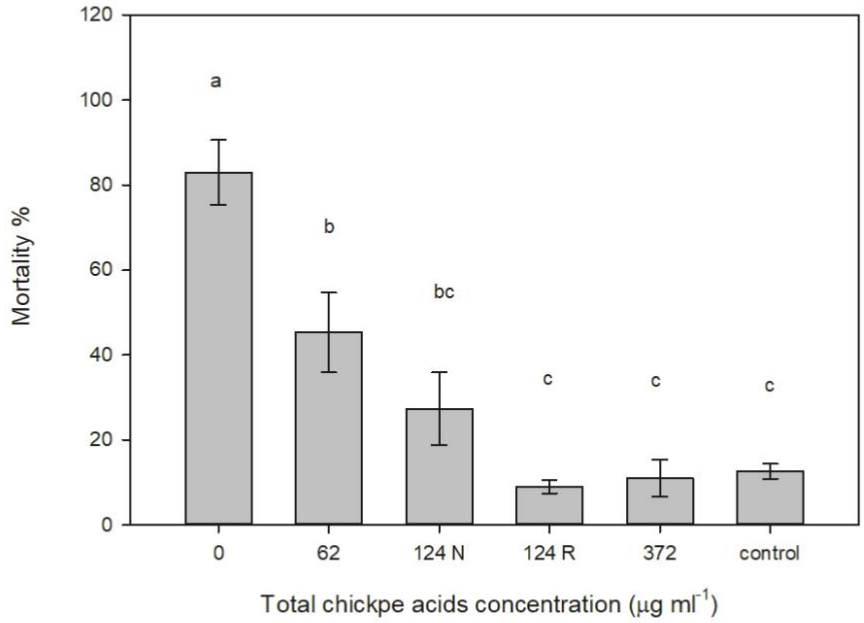
889

890 Fig 2a



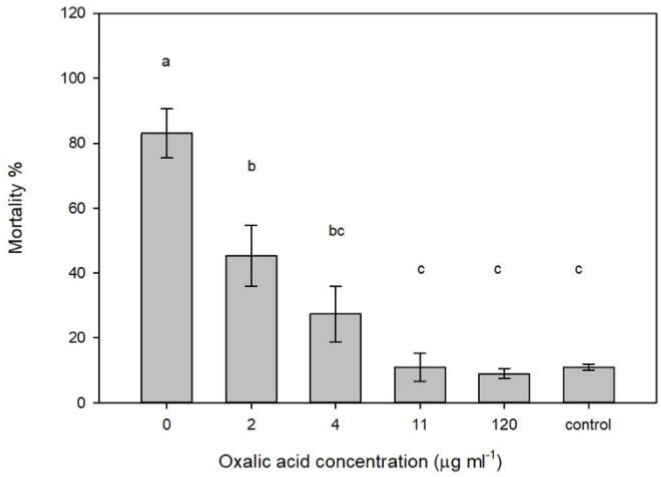
891

892 Fig 2b


120
100
80
60
40
20
0
Mortality %
a
b
bc
c
c
c
0
62
124 N
124 R
372
control
Total chickpe acids concentration (μg ml-1)

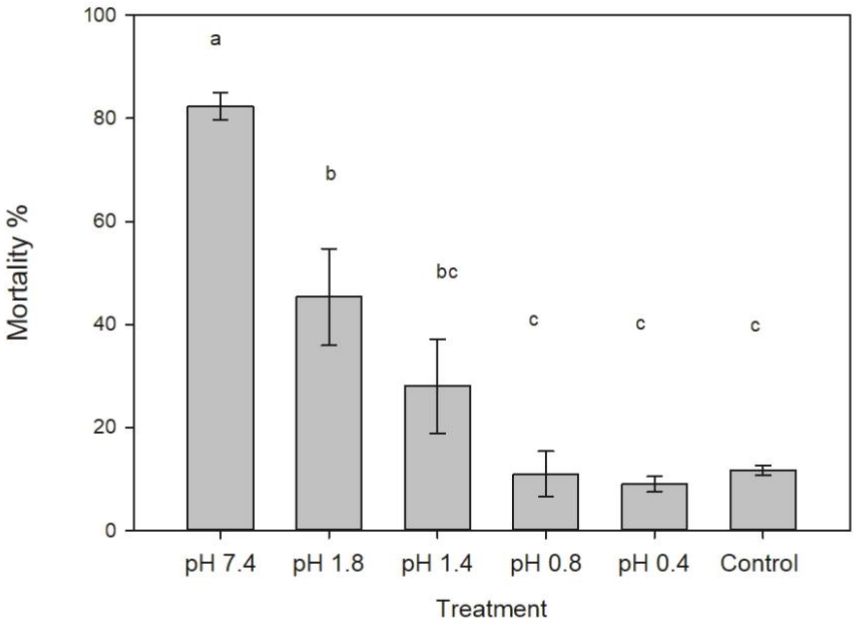
893

894 Fig 3


120
100
80
60
40
20
0
Mortality %
a
b
bc
c
c
c
0
2
4
11
120
control
Oxalic acid concentration (µg ml-1)

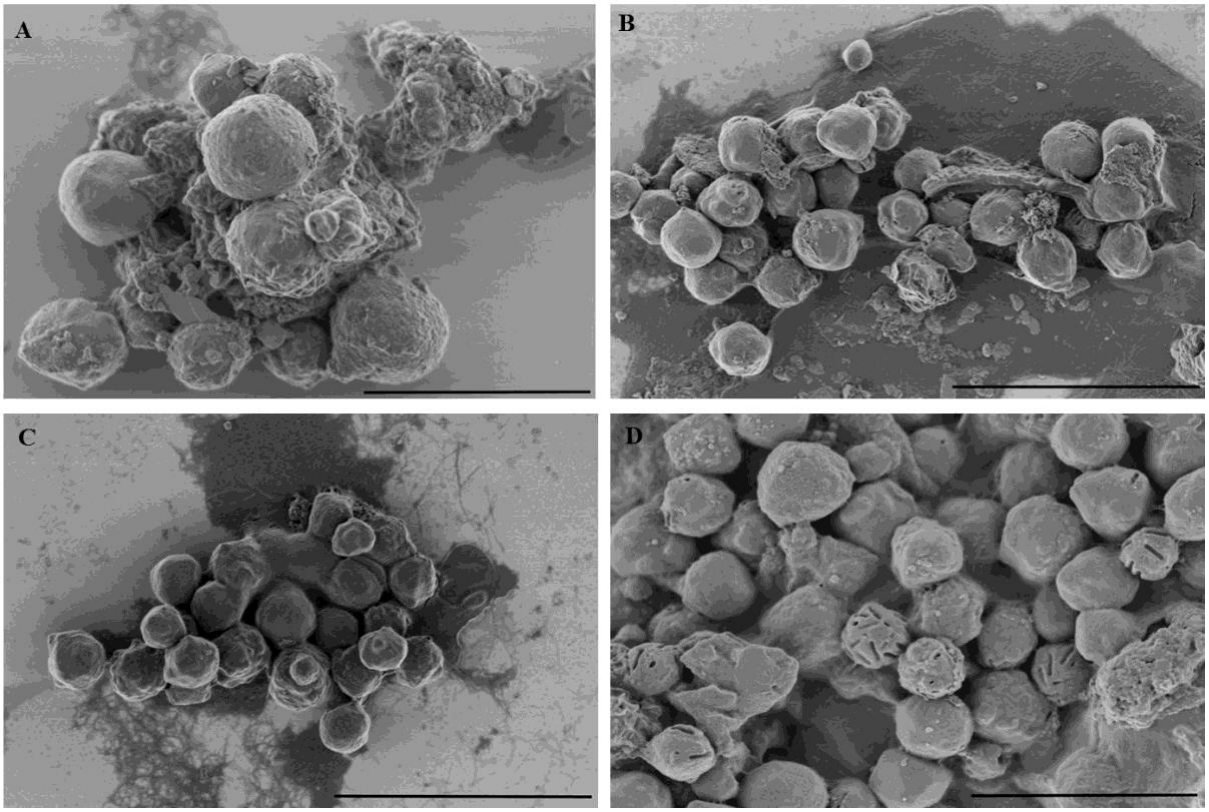
895

896 Fig 4


100
80
60
40
20
0
Mortality %
a
b
bc
c
c
c
pH 7.4
pH 1.8
pH 1.4
pH 0.8
pH 0.4
Control
Treatment

897

898 Fig 5



899

900

901

Table 1

Click here to access/download;Table;Table 1 Aliyu Stevenson and Grzywacz.docx

Table 1 Details of treatments to explore effects of mixtures of the Formononetin and Biochanin A in combination with organic acids mixture of Oxalic and Malic acid on mortality of neonate larvae dosed with *Hear*NPV LC_{75}

Treatment	*Hear*NPV LC_{75}*	Formononetin µg ml^{-1}	Biochanin A µg ml^{-1}	Organic acids mix**
1	0	0	0	0
2	Yes	0	0	0
3	Yes	100	100	Yes
4	Yes	100	0	Yes
5	Yes	0	100	Yes
6	Yes	500	0	Yes
7	Yes	0	500	Yes

**Hear*NPV concentration 5.8 × 10^3OB ml^{-1} **Mix of organic acids to give final concentration oxalic acid 119.60 mg ml^{-1} and malic acid 3.80 mg ml^{-1}

902

Table 2

Click here to access/download;Table;Table 2 Aliyu Stevenson & grzywacz.docx

Table 2 Details of treatments to explore effects of mixtures of the major organic acids of chickpea leaf exudate in combination with *Hear*NPV LC_{75} showing treatment concentrations of oxalic and malic acids, *Hear*NPV, total acids and pH of treatments

Treatment	*Hear*NPV LC_{75} (OB ml^{-1})	Oxalic acid (µg ml^{-1})	Malic acid (µg ml^{-1})	Total acids (µg ml^{-1})	pH
1 Water control	0	0	0	0	7.4
*Hear*NPV control	5.8×10^3	0	0	0	7.4
Organic acids at naturally occurring (NO) leaf concentration x1	5.8×10^3	4.0	120	124	1.4
Organic acids at NO leaf concentration x3	5.8×10^3	12	360	372	0.8
Organic acids at NO leaf concentration x 0.5	5.8×10^3	2.0	60	62	1.6
Organic acids NO leaf concentrations reversed	5.8×10^3	120	4.0	124	0.4

OAs = Organic acids (Malic + Oxalic), NPV = *Hear*NPV, * measured by pH meter

903

904