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1 **INACTIVATION OF BACULOVIRUS BY ISOFLAVONOIDS ON CHICKPEA**
2 **(*Cicer arietinum*) LEAF SURFACES REDUCES THE EFFICACY OF**
3 **NUCLEOPOLYHEDROVIRUS AGAINST *Helicoverpa armigera*.**

4
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10 Chickpea isoflavonoids inhibit *Helicoverpa armigera* NPV

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13
14 **ABSTRACT**-Biological pesticides based on nucleopolyhedroviruses (NPVs) can provide
15 an effective and environmentally benign alternative to synthetic chemicals. On some
16 crops, however, the efficacy and persistence of NPVs is known to be reduced by plant
17 specific factors. The present study investigated the efficacy of *Helicoverpa armigera*
18 NPV (HearNPV) for control of *H. armigera* larvae and showed that chickpea reduced the
19 infectivity of virus occlusion bodies (OBs) exposed to the leaf surface of chickpea for at

20 least one hr. The degree of inactivation was greater on chickpea than on previously
21 reported on cotton and the mode of action is different to that of cotton. The effect was
22 observed for larvae that consumed OBs on chickpea leaves but also occurred when OBs
23 were removed after exposure to plants and inoculated on to artificial diet, indicating that
24 inhibition was leaf surface related and permanent. Despite their profuse exudation from
25 trichomes on chickpea leaves and low pH, organic acids – primarily oxalic and malic acid
26 – caused no inhibition. When HearNPV was incubated with biochanin A and sissotrin,
27 however, two minor constituents of chickpea leaf extracts, the OB activity was reduced
28 significantly. These two isoflavonoids increased in concentration by up to 3 times within
29 one hr of spraying the virus suspension onto the plants and also when spraying only
30 carrier, indicating induction was in response to spraying and not a specific response to the
31 HearNPV. Although inactivation by the isoflavonoids did not account completely for the
32 level of effect recorded on whole plants this work constitutes evidence for a novel
33 mechanism of NPV inactivation in legumes. Expanding the use of biological pesticides
34 on legume crops will be dependent upon the development of suitable formulations for
35 OBs to overcome plant secondary chemical effects.

36

37 **Key Words-** Baculovirus, Biopesticide, Nucleopolyhedrovirus, *Helicoverpa armigera*,
38 Chickpea, Induced resistance, Plant leaf chemistry, Isoflavonoid.

39

40 INTRODUCTION

41

42 *Helicoverpa armigera* (Hubn.) is a major crop pest in Asia, Africa and Australasia
43 attacking a wide range of important crops including cotton, maize, tomato, peppers,
44 chilies, and legumes such as chickpea and pigeonpea (Gowda, 2005; King, 1994). Its
45 status as arguably the world's most important agricultural pest can be attributed to its
46 wide geographical and host range coupled with its ability to develop high levels of
47 resistance to chemical insecticides (Armes et al., 1992b; Kranthi et al., 2002). The
48 baculovirus biopesticide *Helicoverpa armigera* nucleopolyhedrovirus (HearNPV) is an
49 ecologically benign alternative to chemical insecticides that is effective and can
50 overcome problems of chemical insecticide resistance (Moscardi, 1999; Grzywacz et al.,
51 2005). HearNPV is now commercially produced in Australia, Thailand, India and China
52 for control of *H. armigera* (Buerger et al., 2007, Sun and Peng, 2007, Singhal, 2004).
53 However, the utility of baculoviruses for insect pest management is compromised by the
54 fact that some host plants adversely influence the severity of viral disease in insects and
55 so reduce pest control efficacy (Felton and Duffey, 1990; Duffey et al., 1995; Hoover et
56 al., 1998a; Cory and Hoover, 2006). It has for some time been recognized that *Heliothis*
57 *zea* NPV, a closely related baculovirus, performed poorly on some crops such as cotton
58 (Young and Yearian, 1974; Forschler et al., 1992), a phenomena linked to the direct
59 action of glandular secretions in reducing the persistence of occlusion bodies (OBs) the
60 infective stage of the virus (Young and Yearian, 1977; Ellerman and Entwistle, 1985).
61 OBs are a protective crystalline protein matrix in which virions are embedded during
62 transmission and in hostile environments (Hunter-Fujita et al., 1998). The maintenance
63 of OB integrity is crucial to viral persistence outside the host and for initiating infections
64 in new host insects. Host plant effects on biological pesticides are not restricted to

65 baculoviruses, as plants such as cotton have been shown to reduce the efficacy of other
66 biopesticides, especially *Bacillus thuringiensis* (Kushner and Harvey, 1962; Johnson,
67 1982; Ali et al., 2004). Inhibition of NPV infections on cotton has also been attributed to
68 high peroxidase activity and subsequent free radical generation which was associated
69 with an increase in the sloughing off of midgut cells that are the point of entry for the
70 NPV virions, thereby reducing virus-induced mortality (Hoover et al., 1998a; Hoover et
71 al., 1998b; Hoover et al., 2000). While the use of HearNPV has been shown to be
72 effective on chickpea (Jayaraj et al., 1987; Rabindra et al., 1992; Cherry et al., 2000) field
73 trials have indicated OB persistence and activity to be much lower on chickpea leaf
74 surfaces than on other crops such as tomato (Rabindra et al., 1994), suggestive of some
75 degree of adverse interaction on chickpea. Chickpea produces copious glandular
76 secretions rich in organic acids and the leaf surface can subsequently have a very low pH
77 (<3) (Rembold and Weigner, 1990; Stevenson and Aslam, 2006). This could make it a
78 challenging host plant for biopesticide use because earlier work on *Lymantria dispar*
79 NPV has shown that larvae can be less susceptible to OBs when inoculated on highly
80 acidic (pH 3.8-4.6) oak foliage rather than other less acidic aspen foliage (Keating and
81 Yendol, 1987) an effect associated with low pH and high levels of organic acids (Keating
82 et al., 1989).

83 The present study was undertaken to investigate the efficacy of HearNPV on
84 chickpea in comparison with tomato, a known favorable host (Forschler et al., 1992;
85 Farrar et al., 2000), and cotton, a host plant known to impair OB infectivity, to better
86 understand what plant factors affect virus efficacy with a view to developing better
87 recommendations for the efficacy of NPV-based insecticides on legume crops and to

88 assist in the development of a suitable formulation for OBs for use on crops such as
89 chickpea.

90

91

MATERIALS AND METHODS

92

93 Virus. The virus strain (NRI#0210) was provided by Professor R.J. Rabindra of
94 Tamil Nadu Agricultural University, India, and stored at -80°C. This strain is typical in
95 activity of strains of HearNPV used in biopesticides products in India having a mean
96 LC_{50} of 2.78×10^3 OB ml⁻¹ for neonate larvae similar to that reported by others including
97 Somasekar et al. (1993) and had been used previously in field trials on chickpea in India
98 (Cherry et al., 2000). It was multiplied up in third instars of *H. armigera* then harvested
99 and purified using a standard NPV purification protocol (Hunter-Fujita et al., 1998). The
100 virus was enumerated using a standard Neubauer haemocytometer and phase contrast
101 microscope at X400 magnification (Wigley, 1980). The identity of the source and
102 progeny of the virus was checked using a standard DNA restriction analysis protocol for
103 NPVs with EcoR1 (Hunter-Fujita et al., 1998).

104 Insects. The insects for the bioassays were derived from a culture of *H. armigera*
105 provided by the NERC Centre for Ecology and Hydrology at Oxford which had been
106 maintained there for a number of years. The insects were reared at 26 ± 2 °C with a
107 relative humidity of $50 \pm 5\%$ and a 14:10 hr light:dark regime. Larvae were reared in
108 groups in 250 ml plastic pots on an artificial wheatgerm casein diet until the second instar
109 and then individually in 30 ml plastic pots on wheatgerm diet using a method previously
110 described (Armes et al., 1992a).

111 Plants. The plants used in the study were cotton (*Gossypium hirsutum*,) variety
112 Ankur 651 (Ankur Seeds Ltd. Nagpur, India), chickpea (*Cicer arietinum*) variety ICC
113 11322 provided by ICRISAT, Hyderabad, India and tomato (*Lycopersicon esculentum*)
114 ‘Moneymaker’ variety. All were grown in plastic pots on John Innes no. 2 potting
115 compost at $28 \pm 2^\circ\text{C}$ in a glasshouse with a 14:10 hr light:dark cycle and a relative
116 humidity of 60%. Plants were used at 5 weeks old. The surface area of leaves was
117 measured using a Quantimet 520-image analyser (Leica Microsystems Cambridge Ltd.,
118 UK). Thus, the concentration of different compounds in a sample could be equated to an
119 area of leaf surface to ensure that insects were presented with naturally occurring
120 concentrations during feeding bioassays. These data together with the chemical analysis
121 were used to calculate chemical concentration of leaf extracts in terms of unit area so that
122 surface contamination bioassays could be calibrated to match concentrations found on
123 leaf surfaces.

124 Viral Bioassays. To assess OB activity both leaf dip and surface contamination
125 neonate larval bioassays were used under standard larval rearing conditions, 26°C with a
126 14/10 hour light dark cycle. In the leaf dip assays a standard methodology was used
127 (Evans and Shapiro, 1997). The HearNPV stock suspensions were prepared as fivefold
128 dilution series in 50 ml of 0.02 % Triton X-100 immediately prior to use in bioassays.
129 The leaves were cut from the plant at the stem and dipped in the HearNPV dilutions.
130 Control leaves were dipped in 0.02 % Triton X-100 only. After dipping, the stem of the
131 treated leaves was mounted in molten agar in 250 ml round plastic containers, either one
132 cotton leaf, two tomato leaves and six compound chickpea leaves were used per container
133 ; fifty neonate larvae less than 18 hours old were used for each treatment with 25 being

134 placed in each container. Larvae were allowed to feed on the leaves for 24 h, after which
135 they were transferred to 25 ml individual pots and reared individually on clean artificial
136 diet, the mortality was recorded after 5 and 7 days. To ascertain OB activity separately
137 from leaf surfaces OB treatments the mass surface contamination bioassay was employed
138 (McKinley, 1985; Jones, 2000). Again fivefold series dilutions of OBs in distilled water
139 were prepared and then dispensed as 75 μ l aliquots onto the surface of artificial diet in
140 30ml plastic pots, spread evenly by tilting and left to dry. Two larvae were added to each
141 pot, reared for 7 days under standard conditions and mortality counted on days 5 & 7.
142 Fifty larvae were used for each treatment replicate. All assays were replicated 5-7 times
143 with each assay including a control and a stock solution positive control and the results
144 were subjected to probit analysis (Finney, 1971) in SPSS. Comparisons of LC₅₀ were
145 performed on log transformed data, to equalize variances, using ANOVA procedure in
146 SIGMASTAT software and treatment means were compared using LSD test. In some
147 bioassays where means differed by several orders of magnitude transforming the data did
148 not normalize variances so the non-parametric Kruskal-Wallis test with Tukey multiple
149 comparison procedure was adopted.

150 Effect of exposure of HearNPV to cotton, tomato and chickpea leaf surfaces. To
151 study plant surface chemistry and its effect on HearNPV, OBs suspended in distilled
152 water were applied to the leaf surfaces on whole plants at a concentration of 3×10^7 OB
153 ml^{-1} in 0.02% triton using a hydraulic hand sprayer and applied at a rate sufficient to
154 evenly wet the leaves. The plants used in experiments were after application of OB
155 maintained in the laboratory at 26°C under the 14/10 hour light dark cycle and the virus
156 was then left on the leaves for 1 or 24 hr after which OBs were recovered using a

157 standard washing technique in water containing 0.1% sodium dodecyl sulphate for one
158 hr (Jones 1988). The samples and the OBs concentrated by centrifugation at 2500g at
159 5°C for 30 min (Hunter Fujita et al., 1998a). The supernatant was discarded, and the OBs
160 were re-suspended in distilled water then stored at -20°C prior to counting and bioassay.
161 This procedure was found to have no significant effect on the LC₅₀ of virus and recovery
162 of OBs from leaf surfaces was ascertained to be >95%; similar to that reported by other
163 workers using this technique (McKinley, 1985; Jones, 1988).

164 Analysis of organic acids in methanol extract of chickpea leaf surface by GC-MS.
165 The surfaces of 50 leaves were extracted in methanol 300ml and analyzed by GC-MS.
166 Purification of organic acid fraction was carried out according to Stumpf and Burris
167 (1979). The residue was resuspended in pyridine (50µl) (Sigma-Aldrich) with a glutaric
168 acid internal standard (1mg ml⁻¹) (Sigma-Aldrich). Ten min before injection 25 µl of N,
169 O-bis (tri-methylsilyl)-acetamide (Supelco) was added; the vial shaken and left to stand at
170 room temperature for 5 min before injection. GC-MS was carried out on a Hewlett
171 Packard HP6890 GC linked to an Ion detector (HP 5973 Mass Selective Detector)
172 operated in Electron Ionisation (EI) mode. A fused silica capillary column (30 m x 0.25
173 mm i.d., coating 0.25µm) coated with non-polar HP-5MS (5% Phenyl Methyl Siloxane,
174 Agilent 1909 IS-433) was used with a split/splitless injector and helium as a carrier gas
175 (0.5kg cm⁻²). The oven temperature was held at 60°C for 2 min and then raised to 250°C
176 at 6°C per min. Compounds were identified by comparing EI-MS and GC retention
177 indices with synthetic standards under the same operating conditions. A set of organic
178 acid standards as reported to occur on chickpea leaf surfaces (Rembold and Weigner,
179 1990) was prepared in sterile distilled water, derivatised and analysed as described above.

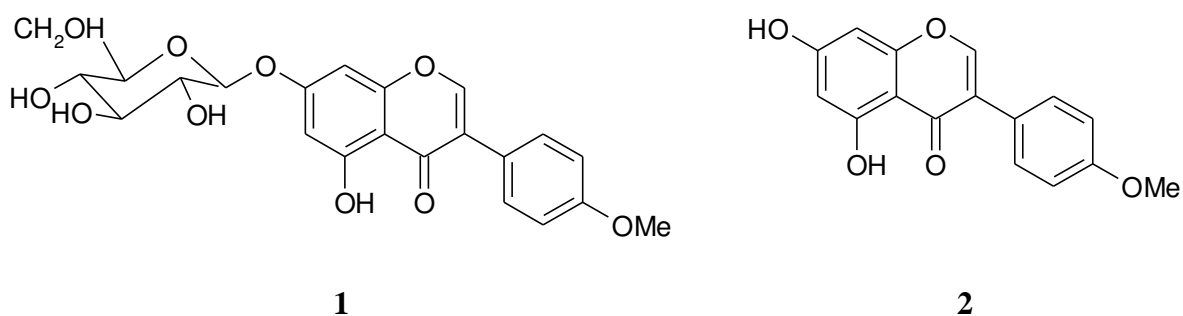
180 Effect of organic acids present on the chickpea leaf surface on the infectivity of
181 OBs against *H. armigera* neonates. Organic acids (Sigma Aldrich, USA) were mixed
182 together, at the concentration present on leaf surface as determined above, in 10 ml of
183 sterile distilled water. A sample of HearNPV (1×10^{10} OB) was added to the organic acid
184 solution and then left in a rotator at 30 rpm for one hr. OBs were then recovered by
185 centrifuging at 2500g for 30 minutes then re-suspended in 5 ml of distilled water and
186 counted. Serially diluted suspensions of OBs in distilled water were bioassayed alongside
187 a control OB suspension not exposed to the organic acids.

188 HPLC analysis of chickpea leaf extracts after spraying with OB suspension. To
189 determine the effect of HearNPV OBs on the chickpea leaf chemistry, a suspension of 3
190 $\times 10^7$ OB ml⁻¹ in 0.02% Triton was sprayed onto to the leaf surfaces of whole plants using
191 a hydraulic hand sprayer sufficient to evenly wet the leaves. Control plants were sprayed
192 with 0.02% Triton. The leaves were excised within 5 min or after 1, 4 or 24 h after
193 spraying and surface extracted in methanol for 40 sec, and the extracts filtered (Whatman
194 No. 1), and evaporated to dryness under reduced pressure. The dried extracts were
195 redissolved in 1 ml of 100% HPLC grade methanol for analysis. Aliquots (10 ul) were
196 injected onto a reverse-phase column (Spherisorb 5ODS analytical column, 4.6 mm i.d. x
197 250 mm) and eluted at 1 ml/min using the gradient 90% A: 10% B at t = 0 min to 50% A:
198 50% B at t = 20 mins to 20% A: 80% B at t = 25 mins to 100% B at t = 30 mins and 90%
199 A: 10% B at t = 37 mins (A is 2% acetic acid and B is 2% acetic acid in acetonitrile).

200 Isolation of leaf surface compounds and their effect on the activity of HearNPV
201 OBs against *H. armigera* larvae. Compounds **1** and **2** were isolated by repetitive HPLC as
202 described above and fractions were collected manually at approximately 22 and 29 min.

203 The combined fractions were evaporated under reduced pressure and weighed. LC-MS
204 was carried out on a Thermo-Finnigan LC/MS/MS system consisting of a ‘Surveyor’
205 autosampling LC system interfaced to a LCQ Classic quadrupole ion trap mass
206 spectrometer. Chromatographic separation was performed on a 150 mm × 4.6 mm i.d. (5
207 µm particle size) Phenomenex Luna C18 column using a linear mobile phase gradient of
208 1 ml min⁻¹ flow rate with water (A): MeOH (B): 5% Acetic Acid in MeOH (C). Initial
209 conditions were 80% A, 0% B and 20% C changing to 0% A, 80% B and 20% C at t = 20
210 min and maintained at these conditions to t = 25 min. Injection volume was 10 µl and
211 data analysis was performed using Xcalibur 1.2 software. The ion trap MS was fitted with
212 an Atmospheric Pressure Chemical Ionisation (APCI) source operated under standard
213 conditions; i.e. vaporiser temperature 450 °C, needle current 5 mA, heated capillary
214 temperature 150 °C, sheath and auxiliary nitrogen gas pressure 80 and 20 psi, and the
215 source voltages tuned for the optimal transmission of protonated rutin. The ion trap was
216 set to monitor ions from m/z 125-1200 with collision energy of 45 %. Authentic samples
217 of genistein, daidzein, pratensein, biochanin A and formononetin (Aldrich-Sigma) were
218 co-chromatographed with methanol leaf extracts of chickpea leaf surface that had been
219 sprayed with HearNPV (suspended in 0.02% Triton X-100) and indicated that **2** was
220 biochanin A. Compound **1** had a similar UV spectrum to **2** but eluted earlier (22 min)
221 indicating a more polar nature and suggesting a glycoside. An aliquot of **1** that had been
222 isolated from the leaf extracts as described above was analysed by LC-MS and recorded a
223 molecular ion signal in positive mode [M + H]⁺ at m/e = 447 indicating the molecular
224 weight of 446 and a molecular formula C₂₂H₂₂O₁₀. Comparison of the mass spectrum
225 with the library confirmed the structure to be biochanin A 7-O-glucoside (sissotrin) with

226 good match in the lower range ($m/e = 100-300$) of the spectrum. For example, the signal
227 observed at $[M + H]^+$ $m/e = 285$ indicated loss of a glucose moiety $[M - 162 + H]^+$ and
228 corresponded to biochanin A with a base peak at $m/e = 270$ correlating to the loss of
229 glucose and a methyl from the methoxy at C-4' and a further fragment at $m/e = 253$
230 correlating to $[M - 162 - OCH_3]^+$ with the loss of the methoxy group. Subsequent co-
231 chromatography using an authentic standard of sissotrin from natural products collection
232 at Royal Botanic Gardens, Kew, confirmed this identification.



233 Compounds **1** and **2** were used subsequently in bioassays to evaluate their effects on
234 HearNPV.

235 The surface area of the leaves was measured as described above. A 200 μl aliquot
236 of sissotrin ($25 \mu\text{g ml}^{-1}$) in methanol containing the equivalent sissotrin from 1250 mm^2 of
237 chickpea leaf surface and equal to the surface area of artificial diet in a 30ml container
238 was placed onto the diet surface and allowed to evaporate. The control diets were treated
239 with 200 μl methanol. HearNPV concentrations on a five-fold dilution scale were
240 prepared in distilled water. A control dose containing only distilled water was also
241 prepared. An aliquot of each virus concentration was dispensed in a volume of 75 μl onto
242 the surface of the diet and allowed to dry after which 10 neonate larvae were released into
243 each of the 5 pots. Larvae were allowed to feed for 24 hr and then were transferred to

244 clean artificial diet pots at a rate of two per pot and reared under standard conditions and
245 mortality recorded after 7 days. The experiment was replicated three times

246 Effect of biochanin A on the efficacy of HearNPV against *H. armigera* larvae.
247 Biochanin A (Sigma Aldrich, USA) was diluted to 500, 250, 100 and 10 ppm in distilled
248 water and was also tested against HearNPV. A 200 μ l aliquot of biochanin A at 500, 250,
249 100 or 10 ppm was spread over the surface of artificial diet. Control pots were treated
250 with same amount of biochanin A. Bioassays were carried out as described above for
251 sissotrin with 50 larvae treatment⁻¹ and the experiment was again replicated three times.

252

253 RESULTS

254 Effect of cotton, tomato and chickpea plants on HearNPV against *H. armigera*
255 larvae using a leaf dip bioassay method. The leaf dip bioassay showed that exposure of
256 HearNPV on chickpea leaf could impair HearNPV activity. The LC₅₀ values (Fig 1) for
257 the different plants were significantly different ($F = 14.6$, $df = 2,20$, $P = <0.001$) and the
258 LC₅₀ for HearNPV on chickpea was of 3.96×10^4 OB ml⁻¹ was significantly higher than
259 that on tomato (2.65×10^3 OB ml⁻¹) and cotton (9.36×10^3 OB ml⁻¹). The result on
260 tomato was not different from the mean LC₅₀ of this virus strain obtained on artificial diet
261 which was 2.78×10^3 OB ml⁻¹. The bioassays of HearNPV OBs exposed to tomato,
262 cotton and chickpea leaf surfaces also showed highly significant differences after 1 hr (H
263 = 10.851, $df = 3$, $P = 0.017$) and 24 hr ($H=11.033$, $df = 3$, $P = 0.012$) (Fig 2); OBs on
264 chickpea were markedly less infectious than OBs on tomato or cotton which did not
265 differ significantly from the LC₅₀ of unexposed control OBs. Thus, exposure of OBs to
266 the surface of chickpea for 1 and 24 hr resulted in inactivation even after OBs were

267 removed from the leaf surface. The LC₅₀ values of HearNPV OBs exposed to chickpea
268 for 1 and 24 hr did not differ significantly, indicating that the observed inactivation
269 reaches its maximum effect within one hr and exposure beyond that does not further
270 affect OB infectivity.

271

272 Analysis of organic acids in methanol extract of chickpea leaf surface by GC-MS.
273 The leaf surfaces of chickpea extracted with 100% methanol contained oxalic, malonic,
274 malic, citramalic and citric acid (Fig 3). The compounds with retention times 13.47-13.48
275 and 16.01 min were silane impurities while those at 24.80-24.81 min were sugars.
276 Glucose-6-phosphate, oxalacetate, succinic and fumaric acids were not found in any of
277 the solvent extracts despite having been identified earlier by Rembold et al. (1980).

278 Effect of organic acids present on the chickpea leaf surface on the efficacy of
279 HearNPV against *H. armigera* neonates. The mean LC₅₀ values of HearNPV exposed to
280 organic acids and for untreated HearNPV using a surface contamination bioassay system
281 to neonates of *H. armigera* were 8.05×10^2 OB ml⁻¹ and 6.16×10^2 OB ml⁻¹ respectively
282 and were not significantly different ($t = 0.484$, $P = 0.762$).

283 HPLC analysis of chickpea leaf surfaces after spraying with NPV. Chickpea
284 plants were sprayed with HearNPV in a 0.02% Triton X-100 suspension (to optimize
285 spreading) and surface extracted in methanol within 5 min and after 1, 4 and 24 hr. After
286 1 hr there was a more than four-fold increase in the concentration of **1** to $22 \mu\text{g cm}^{-2}$
287 compared with unsprayed leaf surfaces ($5 \mu\text{g cm}^{-2}$) in which the presence of **1** is
288 constitutive. After 2, 4 and 24 hr the concentration of **1** was similar to pre spray quantities
289 and remained there up to 24 hr. Analysis of control plants that were sprayed with 0.02%

290 Triton only also showed higher levels of **1** after 1hr indicating that the process of
291 spraying in the absence of virus was itself sufficient to induce the production of this
292 compound and was not induced by the presence of the HearNPV.

293 Effect of sissotrin on the efficacy of HearNPV against *H. armigera* larvae. The
294 mean LC_{50} after exposure of HearNPV to sissotrin for 1 hr at a concentration equivalent
295 to that found on the leaf surface after spraying was 1.23×10^4 OB ml^{-1} and was
296 significantly higher than untreated HearNPV at 2.30×10^3 OB ml^{-1} ($F = 44.24$, $df = 1,4$, p
297 $= 0.003$). However, this increase in LC_{50} for sissotrin treated HearNPV are small
298 compared to the LC_{50} values when HearNPV OBs were exposed to chickpea plant
299 surface for 1 hr suggesting that sissotrin does reduce the efficacy of HearNPV but does
300 not account for all the inhibition observed when HearNPV was applied to the leaf.

301 The mean LC_{50} s of HearNPV after exposure to different concentrations of
302 biochanin A are shown in Fig.4. There was a significant difference ($F = 4.16$, $df = 4, 10$,
303 $p = 0.031$) between the treatments and it was shown using least significant difference
304 tests that mean LC_{50} values for HearNPV exposed to biochanin A were not significantly
305 different from each other but were significantly greater than the untreated sample,
306 indicating that biochanin A even at concentrations as low as 10 ppm. As with sissotrin,
307 however, the effect of biochanin A does not explain fully the 5-fold increase in LC_{50} seen
308 in HearNPV after exposure on chickpea plants suggesting that other factors must be
309 involved.

310 DISCUSSION

311 This study showed that the efficacy of HearNPV OBs was inhibited considerably
312 more on chickpea than on cotton and that the effect was caused, at least in part, by

313 surface isoflavonoids and not by organic acids. This was surprising since chickpea leaf
314 surfaces have pH of <3 due the presence of organic acids (Rembold and Weigner, 1990),
315 and there is a well known association between low pH with NPV inactivation (Ignoffo
316 and Garcia, 1966). This study has also demonstrated that the inactivation of OBs on
317 leaves is caused by their direct interaction with surface chemicals since OBs that had
318 been exposed to the leaf surface were still inactive once removed and thus differs from
319 the mechanism of peroxidase inactivation reported previously for cotton (Hoover et al.,
320 1998a; 1998b.). The present work does not support an earlier proposition that the
321 reduced efficacy of HearNPV on chickpea could be related to a slower feeding rate of *H.*
322 *armigera* on chickpea, thus reducing the rate of OB ingestion (Rabindra et al., 1992).
323 Sissotrin accumulated on the leaf surface at least for a short period of time after plants
324 that were sprayed with the OB suspension in 0.02% Triton or even with the 0.02% Triton
325 control. This indicates that the process of spraying was sufficient to induce the
326 production of these compounds and was not induced by the presence of the HearNPV.
327 Thus the induction of these compounds is not a specific response to the application of
328 HearNPV but a response to either wetting or the presence of surfactant. The increased
329 secretion of biologically active antimicrobial compounds by chickpea in response to
330 wetting would be biologically explicable as chickpea is subject to the damaging fungal
331 diseases such as *Botrytis* grey mould during periods of heavy dew or precipitation (Pande
332 et al., 2005).

333 Plant chemicals have previously been shown to inhibit OB dissolution by binding
334 irreversibly to OB structural proteins (Schultz and Keating, 1991), a mechanism that is
335 enhanced at least for orthodihydroxy moieties in the presence of peroxidases and

336 polyphenoloxidases, particularly in damaged plant tissues (Felton and Duffey, 1990). The
337 present data do not shed light on the mechanism by which isoflavonoids impair NPV
338 infectivity. Further work to understand this would be useful since the inactivation
339 mechanism reported here may impact on other biological pesticides such as Bt or
340 entomopathogenic fungi, given that chickpea isoflavonoids are toxic to numerous
341 organisms including viruses, bacteria, fungi and insects (Aslam et al., 2009; Getti et al.,
342 2007; Ito et al., 2003; Simmonds and Stevenson, 2001; Stevenson and Haware, 1999;
343 Stevenson et al., 1997). The identification of a new group of compounds affecting OBs,
344 however, adds to the existing literature on this topic and the importance of the finding is
345 highlighted by the LC₅₀s of OBs exposed on leaf surfaces being 3-5 orders of magnitude
346 greater than that reported in cotton in both the present and earlier studies (Young and
347 Yearian, 1974; Forschler et al., 1992). It is not known if this mechanism is present or as
348 profound in all chickpea varieties. However, selective breeding for disease resistance
349 (Pande et al., 2005) may have resulted in varieties with more biologically active
350 compounds and may explain the high OB inactivation reported here.

351 This study showed that HearNPV OBs were inactivated when consumed on
352 cotton leaf material, but showed no sign of inactivation when bioassayed on diets after
353 exposure on and then removal from cotton; a result that concurs with those of Hoover et
354 al. (1998a; 1998b). However, there was no evidence of the OB inactivation by ionic
355 cotton gland secretions reported previously (Ellerman and Entwistle, 1985) on Ankur
356 651, the cotton variety tested here. This may again be explained by varietal differences in
357 the chemistry of Ankur 651 and the Deltapine varieties studied earlier. Some Indian

358 cotton are reportedly more detrimental to OB infectivity than chickpea (Rabindra et al.,
359 1994).

360 While sissotrin and biochanin A have a significant inactivating action, the
361 magnitude of inactivation by these compounds did not fully account for the effects
362 observed on leaf surface assays. Therefore, other chemicals are likely to contribute to this
363 inactivation and further work will be required to identify these.

364 In considering the results reported here it may be surprising that HearNPV is
365 effective as a biopesticide on chickpea (Jayaraj et al., 1987; Rabindra et al., 1989; Cherry
366 et al., 2000; Ahmed and Chandel, 2004). However, on some crops 90% of *H. armigera*
367 larvae killed by HearNPV sprayed onto plants acquire the infection within one hr of
368 application (D Murray, pers. comm.). The interaction of HearNPV with chickpea may
369 also be influenced by the variety of chickpea. Cowgill and Bhagwat (1996) for example
370 reported a field trial in which HearNPV was more effective at killing *H. armigera* when
371 applied to the *H. armigera* susceptible genotype (ICCC 37) of chickpea than on a *H.*
372 *armigera* resistant genotype (ICC 506). This may have been due to differences in their
373 chemistry since the production of isoflavonoids in chickpeas is known to vary between
374 cultivars at least in association with resistance to plant pathogens such as *Botrytis* and
375 *Fusarium* (Stevenson et al., 1997).

376 Additives, including milk powder, casein, molasses and Robin blue dye are
377 reported to improve HearNPV performance on chickpea (Rabindra et al., 1989) and
378 although it has been assumed that they improved UV stability (Rabindra and Jayaraj,
379 1988) given the present findings, it is possible that some additives may also contribute to

380 improving OB efficacy by inhibiting chemical inactivation of OBs or by encouraging
381 feeding and rapid viral acquisition before the OB inactivation processes have taken effect.

382

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386

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