Field trial of a genetically improved baculovirus insecticide

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IMPROVEMENT of biological pesticides through genetic modification has enormous potential and the insect baculoviruses are particularly amenable to this approach[1,2]. A key aim of genetic engineering is to increase their speed of kill, primarily by the incorporation of genes which encode arthropod or bacterially derived insect-selective toxins[3–11], insect hormones[12,13] or enzymes[14,15]. We report here the first, to our knowledge, field trial of a genetically improved nuclear polyhedrosis virus of the alfalfa looper, *Autographa californica* (AcNPV) that expresses an insect-selective toxin gene (AaHIT) derived from the venom of the scorpion *Androctonus australis*[16–18]. Previous laboratory assays with the cabbage looper, *Trichoplusia ni*, demonstrated a 25% reduction in time to death compared to the wild-type virus, but unaltered pathogenicity[6] and host range[19]. In the field, the modified baculovirus killed faster, resulting in reduced crop damage and it appeared to reduce the secondary cycle of infection compared to the wild-type virus.

The trial was designed to test the genetically modified virus under challenging conditions where insect density was high and the larvae were large and capable of causing serious crop damage. The genetically modified virus (AcST-3)[6] and parent clone (AcNPV C6) were compared at low, medium and high doses, which were applied as a spray against third instar *T. ni* on cabbage. Laboratory data suggested that peak AcST-3 mortality should occur earlier than that caused by the wild-type virus[6]. Four samples were taken: two before either virus caused insect death (and release of secondary inocula), one between the two peaks of virus mortality and a final sample after both virus epizootics had occurred, to measure crop damage and larval survival.

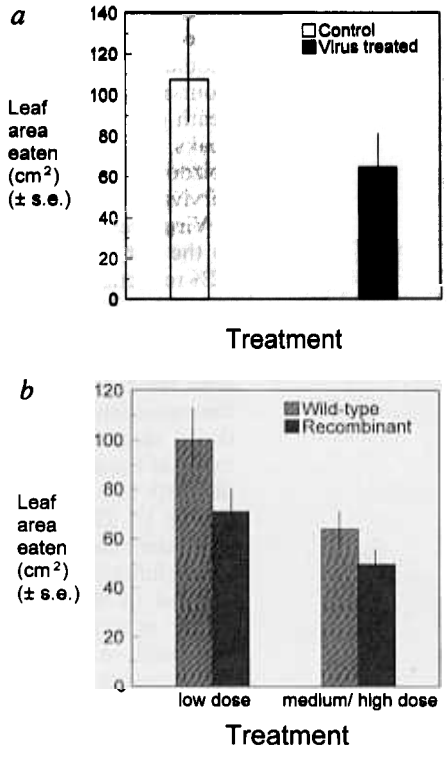
Virus treatment significantly reduced insect damage compared to the untreated controls (Fig. 1*a*). Untreated larvae caused a 22% reduction in leaf area, whereas the loss in virus-treated plots was only 12.5% (mean leaf area 183 $cm^2 \pm 5$ s.e.). Recombinant-treated plots had significantly less crop damage than those treated with the unmodified virus (Fig. 1*b*, *c*). Insects infected with the recombinant virus consumed 29% and 23% less than the equivalent wild-type treatment at low and medium plus high doses, respectively. This reduction in leaf damage was clearly a result of the earlier death of recombinant-infected insects: larvae died 10–15% earlier than insects infected with the wild-type (Table 1). This reduction was not as large as that reported in laboratory studies[6]. This could be the result of numerous factors which influence baculovirus pathogenicity, including insect age, method of dosing, food plant and environmental variables. Toxin expression by the recombinant virus induces larval paralysis[6]: in the field this resulted in many larvae falling off the plants before death (for example at the AcST-3 high dose (day 11), 62% of larvae were found paralysed/dead on the soil, compared to none in wild-type treatments). The earlier peak in recombinant-induced paralysis/mortality is clearly illustrated by comparison of the number of live larvae recovered at each sampling date in the high-dose treatment (Fig. 2). There was no difference between treatments at 2 and 7 days after spraying. However, by day 11, peak mortality had already occurred in the recombinant treatment but not in the wild-type treatment (Fig. 2). By day 16, the number of larvae collected from the wild-type treatment had also declined.

The patterns of virus-induced mortality obtained in the trial were more complex. Overall recovery of live larvae was high with 10,442 larvae recovered of the 14,000 released (74.6%).

TABLE 1 Mean time to death (days ± s.e.) for *T.ni* Larvae

Sample time and size	Wild-type virus dose (polyhedra per m^2)			Recombinant virus dose (polyhedra per m^2)		
	10^6	10^7	10^8	10^6	10^7	10^8
day 2	8.0 (±0.15)	7.5 (±0.15)	7.3 (±0.14)	7.0 (±0.14)	6.4 (±0.13)	6.2 (±0.12)
sample size =	122	369	468	131	358	444
day 7	12.6 (±0.25)	11.7 (±0.23)	10.9 (±0.21)	11.3 (±0.22)	10.1 (±0.20)	9.5 (±0.19)
sample size =	224	396	410	177	293	401

Mean time to death for larvae collected from the trial at different times after virus application. Treatments and sampling regime as described for Fig. 1. On each sampling occasion five cabbages were taken from each plot and the *T.ni* larvae removed and counted. They were then returned to the laboratory and were reared individually on artificial diet[23] at 24 °C ± 1 °C and checked daily for death or pupation. Viral deaths were usually identified by eye. Suspicious deaths were smeared, stained with Giemsa and diagnosed using oil-immersion light microscopy at ×1,000. Only those larvae collected from the first two sampling dates were used in the analysis because insects collected at the two later time points could have become infected from either the initial or secondary inoculum (see text). Time to death was analysed using the Weibull distribution which allows the hazard function to vary with time. Recombinant virus-infected larvae died faster than wild-type virus-infected larvae on all sampling occasions ($F = 20.48$; d.f. = 4, 71; $P < 0.001$). Speed of kill also increased with dose on all sampling occasions ($F = 12.23$; d.f. = 8,71; $P < 0.001$).



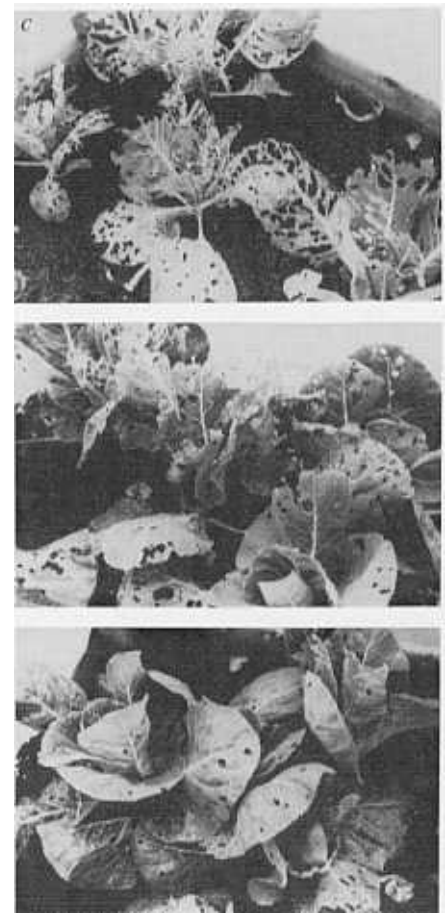


FIG. 1 Mean leaf areas eaten ($cm^2 \pm$ s.e.) per cabbage (total of three leaves) in infested control and virus-treated plots at 16 days after virus application. Twenty cabbage seedlings were planted within 1 m^2 plots on the University Farm, Wytham, UK, on August 2nd 1993. Plots were caged in fine mesh netting 1 m high and buried to a depth of 10 cm. Soil between the plots was treated fortnightly with glyphosate herbicide until virus application. Four weeks after planting, 500 third-instar *T.ni* larvae were introduced to each plot (25 per plant) from a laboratory culture. Wild-type and recombinant virus were applied at 10^6, 10^7 and 10^8 polyhedra per m^2 using a handheld, ULV spinning-disc sprayer (11,000 r.p.m., 50 ml min^{-1}). Plots were lined during spraying to eliminate drift. Tween 20 (1%) was added to the purified virus before application. Controls with and without insects were also set up and treated with 1% Tween 20 in water. Each treatment had four replicates. Plots were randomly sampled at 2, 7, 11 and 16 days after spraying. On the last sampling occasion the leaf areas of three leaves from each of the remaining five cabbages were measured using a leaf area analyser. *a*, Leaf areas eaten in control and virus-treated plots. Error bars represent the least significant difference between control and virus-treated plots. Virus-treated plots sustained significantly less damage ($F = 5.86$; d.f. = 1,26; $P < 0.05$; ANOVA on $\log_e$(area eaten) with contrasts and normal errors). *b*, Leaf areas eaten in wild-type and recombinant virus-treated plots. Model simplification resulted in medium and high doses being grouped together ($F = 0.26$; d.f. = 1,20; n.s.). The recombinant-treated plots sustained significantly less damage ($F = 4.34$; d.f. = 1,21; $P < 0.05$), and the low-dose sustained significantly more damage ($F = 8.01$; d.f. = 1,21; $P < 0.05$). *c*, Damage to cabbage plants in (top) infested control, (middle) high-dose wild-type and (bottom) high-dose recombinant-virus treatments at the final timepoint.

Recovery from control plots was even higher (92%), indicating low levels of natural mortality. Non-virus mortality in the larvae recovered was <5% in the controls and <1% in the treated plots. As expected, there was a significant increase in mortality with increasing virus dose, with over 95% mortality at the highest dose in both treatments. On the first two sampling occasions there was no difference in mortality between virus treatments, indicating that they did not differ in pathogenicity (Fig. 3*a*, *b*). However, on the last two sampling dates the recombinant virus caused significantly less mortality (Fig. 3*c*, *d*). These samples are likely to be biased, because of the loss of paralysed/dead larvae in the recombinant-treated plots, which would lead to an underestimate of mortality. However, the number of survivors (comparable between treatments because an equal number of larvae were released initially) followed a similar pattern: there were more survivors in the recombinant-treated plots at day 16 ($\chi^2 = 20.25$, $P < 0.01$, Poisson errors, scale parameter 1.87). As this difference in survivorship occurs only at the last timepoint, it suggests that it may arise from variation in secondary transmission (release of virus from larvae killed by the original

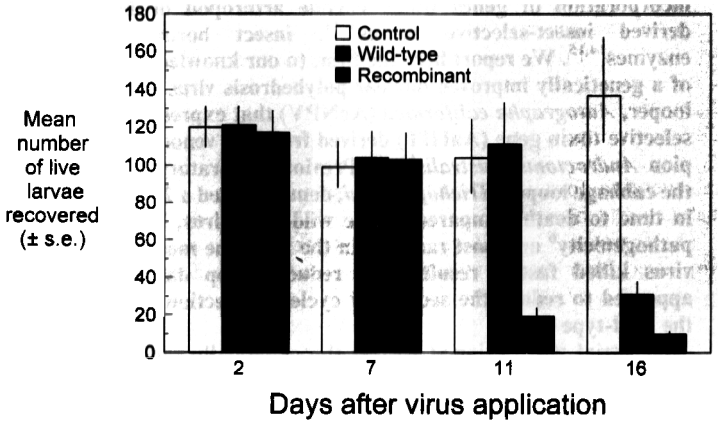


FIG. 2 Mean number (±s.e.) of live *T.ni* larvae recovered from controls and high-dose wild-type and recombinant-virus treatments on four separate sampling occasions. For 2 and 7 days after application, there was no significant difference in the numbers recovered from the three treatments ($F = 0.45$; d.f. = 2,25; n.s.; GLIM with Poisson errors and a scale parameter of 3.6; and $F = 0.75$; d.f. = 2,25; n.s., GLIM with Poisson errors and a scale parameter of 5.7, respectively). Eleven days after application, recovery differed between treatments ($F = 6.53$; d.f. = 2,25; $P < 0.01$, GLIM with Poisson errors and a scale parameter of 15.2): significantly fewer larvae were recovered from the recombinant-treated plots. For this timepoint, the model may be simplified to recombinant plots versus other plots (that is, control and wild-type plots combined) without significant loss of explanatory power ($F = 0.16$; d.f. = 1,25; n.s., GLIM with Poisson errors and a scale parameter of 15.2). On the final sampling occasion the three treatments are significantly different ($F = 10.3$; d.f. = 2,25; $P < 0.01$, GLIM with Poisson errors and a scale parameter 20.3). Retrieval is at its lowest in the recombinant-treated plots (recombinant versus wild type: $t = 2.52$; d.f. = 25; $P < 0.02$) and is at its highest in the control plots (control versus wild type: $t = 2.69$; d.f. = 25; $P < 0.02$). Model checking involved inspection of residuals and a normal order statistical plot.

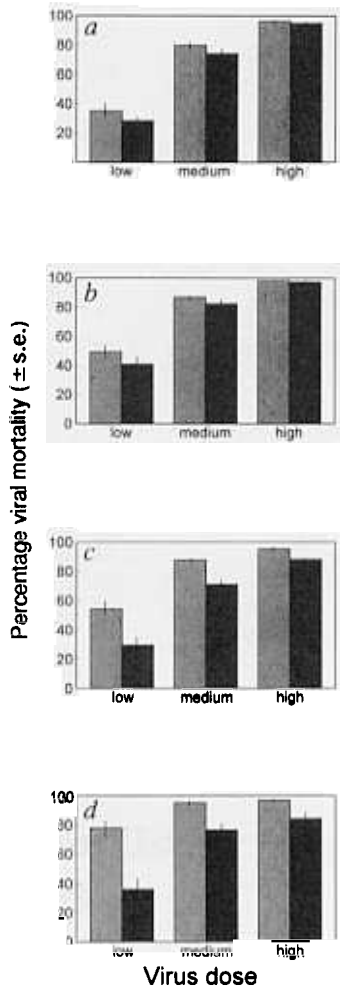


FIG. 3 Percentage virus-induced mortality in wild-type (shaded bars) and recombinant (black bars) treatments at three virus doses, sampled on four occasions: *a*, 2 days; *b*, 7 days; *c*, 11 days, and *d*, 16 days after release. Error bars are the least significant difference between treatments. Each timepoint was analysed separately. For the first two timepoints, mortality varied between doses (day 2; $F=155.2$; d.f. = 2,20; $P<0.001$; ANOVA with binomial errors and scale parameter 2.98; day 7; $F=154.8$; d.f. = 2,20; $P<0.001$, ANOVA with binomial errors and scale parameter 2.39), but mortality did not differ significantly between virus treatments (day 2; $F=3.04$; d.f. = 1,20; n.s.; ANOVA as above; day 7; $F=3.97$; d.f. = 1; n.s.; ANOVA as above). However, at days 11 and 16, both virus type and dose were significantly different, with the recombinant virus causing significantly lower mortality (day 11: virus type: $F=30.28$; d.f. = 1,20; $P<0.01$; dose: $F=73.8$; d.f. = 2,20; $P<0.01$; Day 16, virus type: $F=41.33$; d.f. 1,20; $p<0.01$; dose: $F=17.29$, d.f. 2,20; $p<0.01$). In each case the analysis is weighted according to the sample size, so allowing for the difference in recovery rates illustrated in Fig. 2.

sprayed inoculum). This variation may be due to differences in the pathology and behaviour of insects infected by the two viruses. Insects infected with wild-type NPV usually remained on the plant after death, where they liquified and thus aided release of large quantities of virus, whereas recombinant infected larvae fell onto the soil and did not lyse. The yield of virus was also reduced in larvae infected with the recombinant (such as 9.6×10^8 polyhedra/larva (C6) and 9.9×10^7 polyhedra/larva (AcST-3) for *T.ni* larva infected as fourth instars), further reducing the likelihood of transmission. Thus the recombinant virus may be less readily transmitted to other larvae, resulting in reduced secondary infection, which, if confirmed, could have important implications for risk assessment. The environmental safety of genetically modified baculoviruses has been discussed[20], particularly regarding host range[21,22]: these results indicate that evaluating the safety of such organisms requires the integration of several factors, including transmissability, environmental persistence and host range to arrive at a full risk assessment.

In summary, we have demonstrated that a genetically improved baculovirus insecticide expressing an insect-selective toxin kills *T.ni* larvae more rapidly in the field than the wild-type virus, resulting in improved crop protection. There was also evidence that secondary transmission was lower in insects treated with recombinant virus. These results highlight the importance of speed of action in achieving reduced crop damage and demonstrate that genetic modification can improve the efficacy of bio-insecticides in the field. □

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