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Persistence of *Invertebrate iridescent virus 6* in soil

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Abstract. Soil represents an important reservoir for most entomopathogenic viruses. Invertebrate iridescent viruses (IIVs) (Iridoviridae) are non-occluded DNA viruses that infect agriculturally and medically important insect species, especially in damp or aquatic habitats. We used virus extraction and insect bioassay techniques to determine the effect of soil moisture and soil sterility on the persistence of *Invertebrate iridescent virus 6* (IIV-6) in a soil over a 90 day period in the laboratory. Loss of activity of IIV-6 in dry soil (6.4% moisture, −1000 kPa matric potential) was very rapid and was not studied beyond 24 h. Soil moisture did not affect the rate of inactivation of virus in damp (17% moisture, −114 kPa matric potential) or wet soil (37% moisture, −9.0 kPa matric potential). In contrast, soil sterilization significantly improved the persistence of IIV-6 activity, both in damp and wet soil. Control virus suspensions retained 0.72–0.87% of original activity after 90 days, which was significantly more than the activity retained in soil. These figures represent half lives of 4.9 days for IIV-6 in non-sterile soil, 6.3 days in sterilized soil (data pooled for moisture treatments), and 12.9 days for the control virus suspension. We conclude that extra-host persistence in soil habitats may be an important aspect of the ecology of IIVs.

Key words: half-live, Iridoviridae, moisture, soil, sterilization, virus persistence

Introduction

Soil represents an important reservoir for most pathogenic viruses of terrestrial insects (Fuxa and Richter, 1996). The persistence of viruses in soil has been recognized as particularly important for baculoviruses, entomopoxviruses and cypoviruses that form occlusion bodies specifically to protect them from degradation in the environment (Jaques, 1985). Much less is known about the abilities of non-occluded viruses to persist outside of the host.

Invertebrate iridescent viruses (IIVs) (Iridoviridae) are non-occluded, icosahedral particles of 120–130 nm diameter containing a dsDNA genome (Williams et al., 2000). These viruses infect many agriculturally and medi-

cally important insect species, particularly the aquatic stages of Diptera and soil-dwelling Lepidoptera and Coleoptera (Williams, 1998). IIVs have attracted little attention as potential biopesticides, principally because of a very low prevalence of lethal infections, although IIV epizootics have been reported occasionally (Ricou, 1975; Hernández et al., 2000). However, non-lethal inapparent (covert) infections may be common in certain insect populations (Williams, 1995; Tonka and Weiser, 2000) and such infections may seriously reduce the reproductive capacity, body size and longevity of infected individuals (Marina et al., 1999, 2003a, b).

In the present study, we evaluated the effect of soil moisture and the presence of microorganisms on the persistence of *Invertebrate iridescent virus 6* (IIV-6), originally isolated from the stem borer *Chilo suppressalis* (Lepidoptera: Pyralidae) (Fukaya and Nasu, 1966). IIV-6 is the type species of the genus *Iridovirus* and has been used as the standard model for studies on this group of viruses (Williams et al., 2000).

Materials and methods

IIV-6 was grown by injection into *Galleria mellonella* (Lepidoptera: Pyralidae). When larvae developed the characteristic iridescent coloration they were individually homogenized in sterile distilled water and the homogenate subjected to two cycles of centrifugation at 600 *g* for 10 min. The supernatant was respun at 9,500 *g* for 10 min. The resulting pellet was resuspended in 300 μl sterile water and layered onto 30% (wt/vol) sucrose and spun at 13,000 *g* for 30 min. The virus was washed twice and resuspended in a volume of 1 ml sterile water. The purified virus from 4–10 *G. mellonella* larvae was pooled and mixed thoroughly prior to virus quantification.

The concentration of each stock suspension of virus was quantified by the direct counting method adapted from Day and Mercer (1964) using latex spheres of 460 nm diameter (Sigma Chemical Co.) observed by SEM. Counts were made from 5 fields of vision for each of 5 replicate samples of the virus-latex mixture (Constantino et al., 2001).

Approximately 1 kg of soil was collected from a grassed area behind the ECOSUR laboratories in Tapachula, Chiapas, Mexico and sieved through a 250 μm mesh sieve. Half the soil was autoclaved at 1.04 kg/cm^2 for 45 min. The moisture content of sterilized and non-sterilized soils was determined by heating sub-samples to 80 °C followed by weighing at 12 h intervals. Samples calculated to contain 87.5 g dry wt soil were adjusted to a moisture content of 37, 17 or 6.4% by adding sterile distilled water. Measurements with a calibrated soil equitensiometer (EQ2 Probe, Delta-T Devices, Cambridge, UK) were noted when tensiometer readings reached a steady state; 24–48 h

after placing the probe in soil. Matric water potentials of −9.0 and −114 kPa were recorded for soil containing 37 and 17% moisture, respectively, whereas the matric potential of dry soil (6.4% moisture) was approximately −1000 kPa, at the limits of the measurement range for the equipment.

Soil was mixed with a quantified virus suspension to give a mean (±S.E.) of $4.0 \times 10^{11} \pm 9.3 \times 10^{10}$ particles per gram dry wt. soil. The soil samples were thoroughly mixed for 5 min each and then the equivalent of 10 g (dry wt.) of each sample was placed in each of seven sterile plastic Petri dishes. One Petri dish sample from each treatment was immediately used for the quantification of virus activity; the remaining samples were placed in sealed plastic containers and held in the laboratory at 25 ± 1 °C. Containers were weighed on a weekly basis and any loss of moisture was replaced by placing an equivalent weight of water in the bottom of the container. A 1 ml sample of the virus suspension was placed in a microcentrifuge tube and placed inside one of the containers as a virus control. The activity of this suspension was bioassayed at intervals of 0, 28 and 90 days. Sterilized and non-sterile control soil samples were treated identically but were not inoculated with virus.

In preliminary tests, virus extraction from soil was attempted using water, 2% KCl (wt/vol) (Evans et al., 1980), 1% Tween-80 or 1% Triton X-100, but these all proved to be extremely inefficient. Subsequently, a 0.5% solution of egg albumen was used which gave consistent levels of virus recovery. At intervals of 0, 7, 14, 21, 28 and 90 days a sample of soil from each treatment was placed in a polythene cup with 25 ml albumen solution and shaken with an orbital shaker for 30 min. Each sample was then centrifuged at 108 *g* for 10 min to sediment the soil. The supernatant was placed in a new 50 ml centrifuge tube and spun at 3,900 *g* in a swing-bucket rotor for 15 min. The resulting pellet was resuspended in 1 ml sterile water and serial dilutions were made. Volumes of 8.6 μl of each dilution between 10^{-1} and 10^{-10} (depending on the age of the sample) were injected into 30 *G. mellonella* larvae. These larvae were held in cups with semisynthetic diet at 25 °C and checked for signs of patent iridescent virus infection 12–14 days later. The experiment was performed three times.

Soil analysis was performed by the Analytical Chemistry Unit of CSIRO, Austraila. The soil had a pH of 6.7, a conductivity of 0.68 dS/m (1:5 soil:water) and comprised 3.1% total C, 0.28% total N and 1107 mg/kg total P. Oven dried soil was 17.2% clay, 25.7% silt and 52.7% sand (total 97.6%). The median abundance of microorganisms in non-sterilized soil was 5.5×10^6 colony forming units (cfu)/g on nutrient agar, 8.8×10^5 cfu/g on potato dextrose agar and 1.1×10^7 cfu/g on Czapek agar (Ogram and Feng, 1997). The majority of microorganisms detected appeared to be spore-

forming *Bacillus* type and non-spore-forming, rod-shaped bacteria (possibly *Pseudomonas* spp.) and actinomycete species.

The activity of the virus in each sample was calculated using the end-point dilution method (Reed and Muench, 1938) with the proportion of patently infected *G. mellonella* larvae as the response variable. The rate of loss of activity in each treatment was then compared by a linear regression of the logarithm of the proportion of virus activity remaining in each sample against time. Regression line gradients and intercepts were compared by *F*-tests for which a Bonferroni correction was applied giving a critical value of α = 0.0083 (rather than the conventional 0.05) to account for the simultaneous comparison of regression lines (Sen and Srivastava, 1990).

Results and discussion

Mean recovery of virus from soil using albumen solution was 0.09% (range: 0.35–0.03%) of the amount inoculated. Soil sterilization did not significantly affect the efficiency of virus recovery ($F_{1,10} = 0.51$, $P = 0.49$). Recovery using water, or solutions of KCl, Tween-80 or Triton X-100 was very low (>4 orders of magnitude lower) and varied greatly between samples and between sampling occasions.

Loss of activity of IIV-6 in dry soil (6.4% moisture) was very rapid (approximately 2 logarithms in 24 h) such that virus persistence at this water content was not studied further or included in any of the analyses described below. Other authors have reported a similar rapid loss of IIV activity in dry habitats (Linley and Nielsen, 1968; Mullens et al., 1999).

There was a significant reduction of virus activity over time in all treatments ($F_{1,65} = 534$, $P > 0.001$) (Figure 1). Soil moisture did not affect the rate of inactivation of virus in sterilized soil ($F_{2,33} = 0.392$, $P = 0.67$) or in non-sterile soil ($F_{2,33} = 1.189$, $P = 0.32$). Soil sterilization, however, significantly improved the persistence of IIV-6 activity both in damp soil (17% moisture) ($F_{2,33} = 6.813$, $P = 0.003$) and in wet soil (37% moisture) ($F_{2,33} = 7.329$, $P = 0.002$). Control virus suspension retained 0.72–0.87% of its original activity after 90 days, a decrease of approximately 2 logarithms, which was significantly less than the loss of activity that occurred in soil ($F_{4,10} = 779$, $P < 0.001$). These figures represent a half life of 4.9 days for IIV-6 in non-sterile soil, 6.3 days in sterilized soil (moisture data were pooled for both sterilized and non-sterilized soils), and 12.9 days for the control virus suspension (all held at 25 °C).

We assume that microbial activity or heat-sensitive agents such as microbially-produced enzymes were responsible for the greater rate of loss of IIV-6 activity in non-sterilized soil compared to sterilized soil. Virus inactiva-

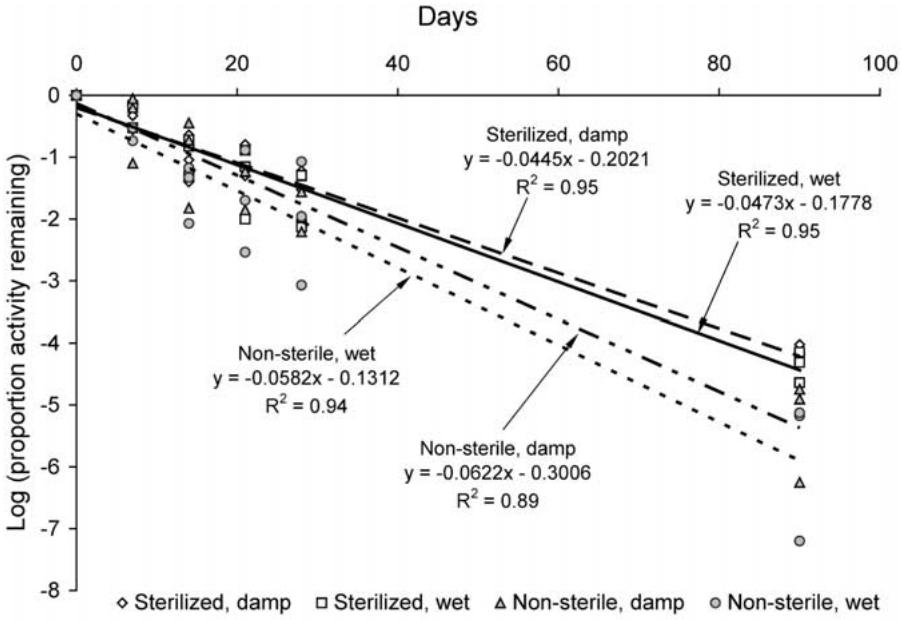

Figure 1. Linear regression of logarithm of proportion of activity of *Invertebrate iridescent virus 6* present in damp (17% moisture, −114 kPa matric potential) and wet (37% moisture, −9.0 kPa matric potential) sterilized and non-sterile soil over a period of 90 days at 25 °C. Activity was determined by insect bioassay in *Galleria mellonella* larvae.

tion in sterilized soil was presumably due to physico-chemical interactions between soil components and virus particles, leading to the breakdown of the particle, or the denaturing of viral proteins essential to the infection process or replication of the virus. The type of soil used in this study was a sandy loam with an almost neutral pH (6.8) that may have favoured virus persistence, although IIVs are not as sensitive to pH as baculoviruses, for example (Marina et al., 2000). The extremely rapid loss of activity in dry soil indicates that particle hydration is crucial to infectivity and probably explains why these viruses almost invariably infect invertebrates that inhabit damp or aquatic habitats during some stage of their lifecycle (Williams, 1998).

The persistence of baculoviruses in the soil has been studied more than other families of insect viruses, especially occluded viruses (Jaques, 1985). Indeed, the persistence of nucleopolyhedrovirus in marsh water was markedly improved by sterilization, suggesting that microbial degradation or substances associated with microbial activity are also important for baculovirus persistence (Peng et al., 1999).

An alternative interpretation for these results is that the strength of virus binding to soil increases over time leading to reduced efficiency of virus

recovery in later samples. This seems unlikely given that the rate of non-occluded virus binding to the clay component of soil reaches a plateau very quickly (<24 h) (P. Christian, unpublished data). Moreover, virus that becomes irreversibly bound to soil would be unavailable for transmission to susceptible hosts resulting in effectively the same biological outcome; a reduction in the density of active or available virus over time. Furthermore, soil sterilization did not effect the initial efficiency of recovery and the same concentration of virus was used in all treatments. Possible fluctuations in the efficiency of virus recovery observed over time were, therefore, the same for all treatments.

Clearly, temperature will affect the rate of deactivation as iridescent viruses are thermolabile and are inactivated within minutes at temperatures over 55 °C (Day and Mercer, 1964; Marina et al., 2003b). Aqueous suspensions of Invertebrate iridescent virus 2 (IIV-2) and IIV-6 had a half life of 32 days and 17 days respectively when stored in a laboratory refrigerator at 4 °C (calculated from data given in Day and Mercer, 1964 and Marina et al., 2000). In contrast, Linley and Nielsen (1968) reported that *Invertebrate iridescent virus 3* (genus *Chloriridovirus*), failed to cause patent infection of mosquito larvae 24 h after inoculation onto damp soil, although the technique employed for assay of viral activity was not highly sensitive.

Clearly, the persistence of IIV-6 in soil was far lower than that of occluded insect viruses. However, given the very high infectivity of iridescent viruses by injection and the potential of nematodes and parasitoids to act as vectors during the process of host penetration or oviposition (Hess and Poinar, 1985; Mullens et al., 1999; López et al., 2002), extra-host persistence in damp soil habitats may be an important aspect of the ecology of invertebrate iridescent viruses.

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