

Susceptibility of the Taro Beetle, *Papuana uninodis* (Coleoptera, Scarabaeidae) to Two New *Bacillus popilliae* Isolates from *Papuana* spp.

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Two morphological types of *Bacillus popilliae*, causal agent of the milky disease, have been isolated from taro beetles (*Papuana* spp, Coleoptera: Scarabaeidae). *B. popilliae* from *P. woodlarkiana woodlarkiana* (Papua New Guinea) was a type A1 with a small sporangium ($4.1 \times 1.6 \mu\text{m}$) and a large spore ($2.1 \times 1.4 \mu\text{m}$) and parasporal body ($1.8 \times 1.2 \mu\text{m}$) that sometimes overlap. *B. popilliae* from *P. uninodis* and *P. woodlarkiana laevipennis* (Solomon Islands) was a type B2 with a small sporangium ($2.8 \times 1.3 \mu\text{m}$), a small eccentric spore ($1.1 \times 0.7 \mu\text{m}$), and no parasporal body. The infectivity of these *B. popilliae* to *Papuana uninodis* larvae was compared with two *B. popilliae* samples from *Popillia japonica* in injection tests. The hemolymph of *P. uninodis* supported the germination and growth of isolates from *Papuana* and *P. japonica*. Results were similar in third instars and adults. Highest infection (spores present) and mortality was caused by the isolates from *Papuana*: mortality reached almost 100% 4 weeks after injection of the B2 type *B. popilliae* with 40% of larvae and 52% of adults infected. Injection of type A1 caused lower mortality but a similar percentage infected. Of two A1 *B. popilliae* from *P. japonica*, one caused a mortality comparable to type A1 from *Papuana* but lower infection; an older isolate resulted in low mortality and only one infected larva. *B. popilliae* type A1 from *P. woodlarkiana* was produced in the Solomon Islands by injection of spores in *P. uninodis*. Thirty four percent of the injected larvae and 31% of the adults produced spores with an average yield of 3.2 and 0.8×10^9 spores/insect, respectively. Oral application of a single dose of 10^7 spores of the *B. popilliae* isolates from *P. uninodis* or *P. japonica* did not cause infection and similarly inoculation of the food with spores of *B. popilliae* type B2 did not result in infections. However, when different rates were applied to the food of second- and third-instar *P. uninodis*, the *B. popilliae* type A1 from *P. woodlarkiana* caused up to 15% infection and concentration-related mortality. © 1999 Academic Press

Key Words: *Bacillus popilliae*; *Papuana uninodis*; Scarabaeidae; pathogenicity; biological control; milky disease.

INTRODUCTION

In Fiji, Kiribati, Papua New Guinea (PNG), the Solomon Islands (SI), and Vanuatu 8 of 19 described *Papuana* spp. (Coleoptera; Scarabaeidae) have been reported as major agricultural pests. The adult beetles tunnel into root crops and cause extensive damage. They prefer taro (*Colocasia esculenta*) but also damage other aroids and banana; yams, potatoes, and some other crops are minor hosts. The larvae are soil feeders; they cause no damage and are found in a range of soil habitats usually outside of the gardens (Waterhouse and Norris, 1987; Thistleton *et al.*, 1995; Waterhouse, 1997).

Only persistent organochlorine chemicals were found effective in trials by the Ministries of Agriculture of PNG, SI, and Fiji (unpublished internal reports). Lindane was temporarily recommended (Thistleton, 1984; Macfarlane, 1987) but is no longer considered environmentally acceptable. Until recently, *Metarhizium anisopliae* was the only entomopathogenic microbe reported to be naturally associated with *Papuana* spp. (Prior, 1986; Shaw, 1984).

The regional EU/SPC Taro Beetle Project was therefore set up to find environmentally acceptable measures for taro beetle control. The emphasis is on biological control, particularly the search for pathogens. Several pathogens naturally associated with taro beetles have been found; these and other potential biocontrol agents are being tested against *Papuana* spp. (Theunis *et al.*, 1993, 1996; Theunis, 1998; Theunis and Aloali'i, 1998).

Bacillus popilliae has played a major role in biological control of scarabs, particularly for the suppression of the Japanese beetle in the U.S. (Klein, 1992). *B. popilliae* has been reported from at least 29 scarabs, mostly Melolonthinae and Rutelinae. Only Scarabaeidae are susceptible to natural or artificial *B. popilliae* infections and species are generally not susceptible to *B. popilliae* isolated from other species (Klein and Jackson, 1992). The discovery of two *B. popilliae* causing milky disease in the dynastid *Papuana* spp. de-

scribed in this paper was thus of major potential importance for our study. We further report here on laboratory tests on *Papuana uninodis* conducted in the Solomon Islands to examine the potential of *B. popilliae* as a biological control agent.

MATERIAL AND METHODS

Papuana uninodis Mass Culture

P. uninodis was mass reared for pathogen tests. Adults were fed taro and kept in boxes with soil for oviposition; eggs were transferred to boxes with a heat-sterilized 1:1 mixture of sawdust and cow dung (Perry, 1977). Larvae feed on this medium. Third instars and adults used in experiments were laboratory bred. For production of *B. popilliae* type A1, a mixture of field-collected and laboratory-bred insects (larvae or adults) was used.

B. popilliae Isolates

Two *B. popilliae* isolates were found in *Papuana* spp. *B. popilliae* type A1 (for description of different types, see Milner, 1981) was isolated in PNG from *P. woodlarkiana woodlarkiana* collected in sugar cane fields at Ramu Sugar, PNG, and the isolate will be referred to as Bp papuana A1. *B. popilliae* type B2 was isolated from *P. uninodis* and *P. woodlarkiana laevipennis* in the Solomon Islands and will be further referred to as Bp papuana B2. Heavily infected larvae were surface sterilized and bled onto a slide for storage and transportation (Klein, 1992).

The two isolates were measured at a 1000-fold magnification using a phase-contrast microscope. Mean and standard error were calculated from 10 measurements.

Two type A1 *B. popilliae* isolates from *P. japonica* were received from Dr. Michael Klein, USDA, Wooster, OH; one had been stored on slides since 1994 and the other since 1957. They are referred to as Bp japonica 1 and 2, respectively.

Bioassays

Injection experiments. Spores stored on glass slides were resuspended and diluted with 0.9% NaCl and counted in a Helber bacterial counting chamber (depth 0.02 mm).

P. uninodis third instars and adults were injected with 20 μ l of sterile 0.9% NaCl containing 10^6 spores (5×10^7 /ml) using a 1-ml syringe with 10- μ l divisions and a thin needle (30G1/2). Control treatments received NaCl only.

Larvae were washed in 70% alcohol before injection dorso-lateral in the thorax; adults were rinsed free of soil and injected dorsally through the membrane between thorax and abdomen. Three replicates of 10 insects each were injected with Bp papuana A1 and the

B. popilliae from *P. japonica*. Bp papuana B2, which was isolated later was tested separately in six replicates of 10 insects. Cadavers and insects surviving after 6 weeks were examined microscopically for the presence of *B. popilliae* spores in the hemolymph. The early stages of infection characterized by the presence of vegetative rods may have escaped detection or if suspected could not be confirmed to be Bp and were not included in the number infected.

Spore production. Bp papuana A1 originates from *P. w. woodlarkiana*, which is indigenous to PNG. It was logistically not feasible to produce Bp in PNG its original host and for quarantine reasons these beetles or big amounts of bacterial spores could not be imported into the Solomon Islands. Fortunately the spores could be produced in the Solomon Islands for laboratory tests by injection of *P. uninodis* third instars and adults (as described above). A total of 1740 third instars in batches of 100–500 larvae was injected and observed every 2–3 days for 4, 5, or 6 weeks. A batch of 2000 adults was injected and observed for 10 weeks. Infection was confirmed microscopically and cadavers were stored in the freezer.

Sufficient Bp papuana B2-infected larvae (infections found on average in 1% of the population were confirmed by microscopical examination) were found in regular field collections to prepare spore suspensions and spore-talc for tests.

Insects were mashed up in a small volume of water. The suspension was filtered subsequently through a thin sieve and through a muslin cloth. The number of spores in the suspension was determined (bacterial counting chamber) and 1 g of CaCO₃ was added for every 10^9 spores. The mixture was dried and ground. The concentration was checked by dissolving 1 g with 100 ml of a 10% HCl solution (Warren and Potter, 1983). Talc was added to reach a final concentration of 10^8 spores/g which was added to the medium in the required dose.

Oral application. Spores resuspended from glass slides were diluted to 10^9 spores/ml of which 10 μ l or 10^7 spores were fed to a third-instar larva. The drop was applied to the mouth parts of the larva with a 10- μ l capillary and the larvae were observed under a dissection microscope until the drop was ingested. Larvae that regurgitated were replaced. Three replicates of 10 third instars were tested; control larvae were fed water only. Tests were examined three times weekly and cadavers were examined microscopically for the presence of *B. popilliae* spores in the hemolymph. As before, early stages of infection may have gone unnoticed. Cases in which only vegetative rods were present for which identity could not be confirmed by sporulated cells were not included in the count.

Food-application. Five concentrations, 10^5 , 3.2×10^5 , 10^6 , 3.2×10^6 , 10^7 spores/g dry medium, were applied to larval food as a talc-spore formulation (10^8 spores/g talc). A control treatment of 0 spores/g was included and used to correct the mortality data. Corrected mortality was calculated with 'Abbott's Formula' (Finney, 1952).

For Bp papuana A1, four replicates of 10 larvae each were tested. The possible influence of talc was examined by including three control treatments of 10 insects each, which were exposed to a low, medium, and high amount of talc, corresponding to the amounts in the 10^5 , 10^6 , and 10^7 spores/g treatments. For Bp papuana B2, two replicates were tested; Bp papuana A1 at a concentration of 10^6 spores/g was tested simultaneously.

Tests were examined three times weekly for 6 weeks. Cadavers and insects surviving after 6 weeks were examined microscopically for the presence of *B. popilliae* spores in the hemolymph. As before, early stages of infection with vegetative rods only may have gone unnoticed. Also, cases in which only the vegetative stages of Bp were assumed to be present, without spores to confirm the identity, were not included in the number infected.

RESULTS

B. popilliae Isolates

B. popilliae type A1 (with parasporal body) was isolated from *P. woodlarkiana woodlarkiana* collected from sugarcane plantations in Ramu Sugar, Papua New Guinea (Theunis *et al.*, 1997). The sporulated bacterium has a sporangium of $4.1 \times 0.28 \times 1.6 \pm 0.19$ μm , a large spore of $2.1 \pm 0.10 \times 1.4 \pm 0.17$ μm , and an almost equally large parasporal body of $1.8 \pm 0.25 \times 1.2 \pm 0.15$ μm . In comparison with the 10 varieties of *B. popilliae* examined by Milner (1981), the spore is average size but the parasporal body is very large; so there is little extra space in the small sporangium and the spore and parasporal crystal are never separated and sometimes overlap.

B. popilliae type B2 (no parasporal body) was isolated from *P. uninodis* and *P. woodlarkiana laevipennis* collected from village gardens in South Guadalcanal (Theunis *et al.*, 1997). The sporangium was small, $2.8 \pm 0.39 \times 1.3 \pm 0.18$ μm , and eye-shaped, and the small spore, $1.1 \pm 0.08 \times 0.7 \pm 0.16$ μm , was eccentric and sometimes set at an angle across the sporangium. Compared to the 10 varieties listed by Milner (1981), the sporangium is shorter and quite narrow; the spore is of regular B2 size.

Injection Experiments

Injection of *B. popilliae* isolates in *P. uninodis* caused similar results in third instars and adults (see Table 1).

TABLE 1

Effect of Injection of Different *B. popilliae* Isolates on *P. uninodis* Third Instars and Adults

Injected isolate	Third instars		Adults	
	% Mortality	% Infected	% Mortality	% Infected
Bp papuana A1	64 \pm 15	38	53 \pm 7	53
Bp papuana B2	97 \pm 2	40	95 \pm 2	52
Bp japonica 1	48 \pm 20	28	47 \pm 14	18
Bp japonica 2	22 \pm 12	3	8 \pm 8	0

Note. $N = 60$ for Bp papuana B2; $N = 30$ for other treatments. Percentage mortality (control mortality 20–30%) \pm standard error 6 weeks after injection and percentage infection including infected surviving insects.

Control mortality was 20–30%. Highest infection (with Bp spores present) and mortality was caused by the isolates from *Papuana* spp. Bp papuana B2 caused almost 100% mortality when injected in larvae and adults of its original host; this level was reached after 4 weeks. Bp papuana A1 caused less mortality, but a similar percentage infected. Bp japonica 1, the 1994 *B. popilliae* isolate from *P. japonica*, caused a comparable percentage mortality but lower infection. Injection of the 1957 isolate, Bp japonica 2, resulted in low mortality and only one larva ($n = 30$) was infected.

Bp Papuana A1 Production by Injection of P. uninodis

Mortality of injected larval and adult *P. uninodis* in the first week was usually high: 20% in adults and an average of 30% in larvae (see Fig. 1). This mortality was probably unrelated to *B. popilliae* infections and the percentage mortality and infection was corrected accordingly. Thirty-one percent of the adults and 34% of the larvae were found infected with *B. popilliae*. The average yield was 0.8×10^9 spores and 3.2×10^9 spores per infected adult and larva, respectively.

Oral Application

Mortality of third instars fed *B. popilliae* spores was highest with Bp papuana B2 but spores were not present in any of the dead or surviving larvae.

Food Application

Application of Bp papuana A1 caused concentration-related mortality in both second and third instars (see Table 2). Mortality was high in talc controls but not related to the amount of talc added (13–47% and 23–47%, respectively in 3rd and 2nd instars from weeks 2–6). Control mortality after 6 weeks in third-instar larvae was on average 20% and in second-instar larvae was 40%.

Highest mortality was found in second instars treated with 10^6 – 10^7 spores/g. Bp spores were found in only one

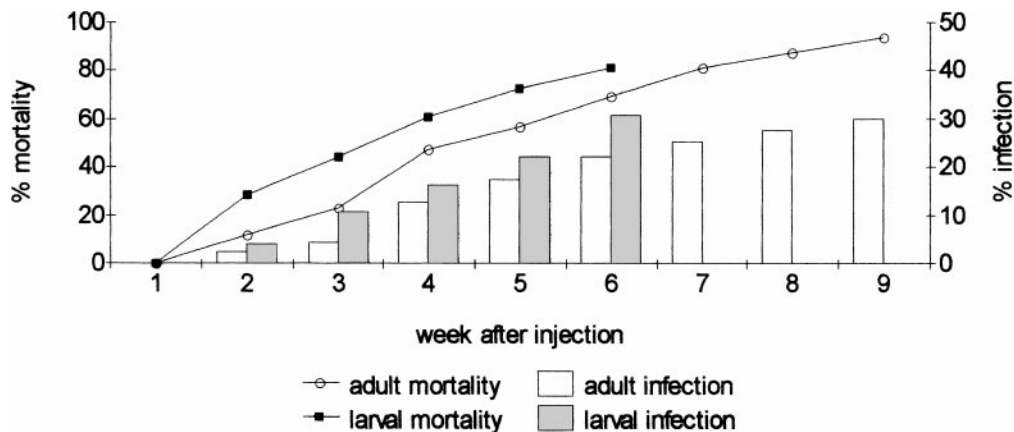


FIG. 1. Effect on third instar and adult *P. uninodis* of injection of 10^6 spores of *Bacillus popilliae* type A1 (from *P. woodlarkiana*). Percentage mortality and infection are based on the number of surviving larvae ($n = 1215$) and adults ($n = 1606$) 1 week after injection.

larva in each of the two treatments of 10^6 and 3.2×10^5 spores/g.

The highest number of infected larvae was found at 10^7 spores/g in third instars. No infection was found in the lowest concentration used.

Application of Bp papuana B2 to larval food in two replicates of 10 insects each did not cause any treatment-related infection. In a Bp papuana A1 treatment (10^6 spores/g) tested simultaneously, 3 infected larvae (of 20) were found, 2 of which were alive after the 6-week test period. In the second replicate, a Bp papuana B2-infected larvae was found in this treatment and in each of two treatments of Bp papuana B2 with a fully developed infection 2 weeks after treatment. It was assumed that these were carried over from the field,

although none of the larvae in the control treatments showed infections.

DISCUSSION

B. popilliae was reported for the first time from *Papuana* spp.: a *B. popilliae* type A1 from *P. woodlarkiana woodlarkiana* in PNG with a small sporangium and a large spore and parasporal body that sometimes overlap; and a type B2 from two species of *Papuana*, *P. uninodis* and *P. woodlarkiana laevipennis*, in South Guadalcanal (Solomon Islands) with a small eye-shaped sporangium, small eccentric spore, and no parasporal body.

Injection experiments show that *P. uninodis* larvae and adults can support the germination and growth of the *B. popilliae* isolates from *P. uninodis*, *P. woodlarkiana*, and two isolates from *P. japonica*. Infection (as indicated by the presence of a high number of spores in the hemolymph) was highest after injection of the *B. popilliae* isolates from *Papuana*. Unexplained high mortality was noted with the type B2 and, possibly, early stages of infection went unnoticed.

Since in a natural infection germination in and penetration of the gut is necessary, injection tests can obviously only be an indication of the susceptibility of the insect and do not necessarily relate to susceptibility to 'per os' infection. Indeed, oral application of a single dose of 10^7 spores of the *B. popilliae* isolates did not cause infection. Similarly, inoculation of the food with spores of *B. popilliae* type B2 (without parasporal body, isolated from the target species itself) did not result in infections, despite the high mortality and infection found in injection experiments. The *B. popilliae* type A1 from *P. woodlarkiana*, however, caused concentration-related mortality and infections when applied to the food of second- and third-instar *P. uninodis*. The lack of infection in direct oral application and subsequent

TABLE 2

Effect of Five Inoculum Rates of *B. popilliae* Type A1 ($n = 40$) and B2 ($n = 20$) from *Papuana* spp. to Third and Second Instars of *P. uninodis*

Concentration $\times 1000$ spores/g	B. popilliae A1				B. popilliae B2	
	3 rd instar		2 nd instar		3 rd instar	
	% mortality	% inf.	% mortality	% inf.	% mortality	% inf.
10000	77 \pm 9	15	100	0	29	
3200	53 \pm 11	10	100	0	29	
1000	16 \pm 11	7.5	91 \pm 19	2.5	29	<i>b</i>
320	6 \pm 13	7.5	42 \pm 43	2.5	0	<i>b</i>
100	0	0	13 \pm 14	0	7	
1000 ^a					29 ^a	15 ^b

Note. Percentage corrected mortality (Abbott's formula) after 6 weeks \pm standard error and percentage infected larvae; control mortality after 6 weeks averaged 20% in third instar and 40% in second instar.

^a *B. popilliae* A1 treatment (10^6 spores/g) tested simultaneously with type B2 treatment.

^b One larva infected with *B. popilliae* type B2 died 2 weeks after treatment.

positive result with food-applied spores of this *B. popilliae* may be due to the constant exposure of the larvae to a high dose (10^5 – 10^7 spores/g food) as opposed to a one-time exposure to 10^7 spores in the oral application. Possibly mixing the spores with food or repeated ingestion enhances germination in the gut. The mortality data (high mortality with often no causative infection detected) indicate larvae died before the bacteria could complete sporulation. This may have been due to high concentration and high ambient laboratory temperature (26–27°C) (Milner *et al.*, 1980; Klein, 1981).

The results of the tests with food-applied spores on *P. uninodis* correspond with field collection data in SI and PNG. Type B2, which does not cause infection or concentration-related mortality, was found in only 1% of 23,425 *P. uninodis* collected in SI (Theunis *et al.*, 1997). Type A1, which causes concentration-related mortality and up to 15% infections, was found in 16 and 24% of *P. woodlarkiana* larvae of two collections in 1994 in sugar cane plantations of Ramu Sugar and in an average of 11% third instars, 4% second instars, and 1% first instars in collections of 1996–1997. Correlation between insect density and percentage *B. popilliae* infection suggested that *B. popilliae* A1 was a major factor in population control of *P. woodlarkiana* in sugar cane fields of Ramu Sugar, PNG (Theunis *et al.*, 1997). Possibly the presence (type A) or absence (type B) of a parasporal body in these *B. popilliae* isolates is associated with the mortality in the feeding trials.

The effect of the *B. popilliae* type A1 and B2 is being investigated in controlled field trials in PNG and SI against the original host species. Application of pathogens to larval breeding sites may, however, prove to be a problem; different species of taro beetles seem to have different preferred breeding sites (under logs, grasses; Thistleton *et al.*, 1995) and the areas to be considered are vast. Preference for breeding sites and the possibility of using *B. popilliae* in artificial breeding sites are being investigated; the long persistence and, consequently, the possibility of building up high concentrations of spores could make *B. popilliae* an important control agent for taro beetles.

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