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Effects of Cryopreservation at –80°C on the Formulation and Pathogenicity of the Obligate Aphid Pathogen *Pandora nouryi*

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Abstract

Cryopreservation at –80°C is an alternative to liquid nitrogen storage for Entomophthorales. However, detailed studies about its effects on fungal pathogenicity and formulation are very limited. In the present study, the obligate aphid pathogen *Pandora nouryi* was formulated as mycelia grown on millet-gel granules after preservation as primary spores at –80°C for 3–18 months, although its ability to produce infectious conidia gradually diminished. The sporulation capacity of this granular formulation was reduced to 18.5×10^4 conidia/mg after 18 months of storage, which was still higher than that of mycotized aphids. The half-decline time of sporulation capacity was computed as 13.6 months. The infectivity to the green peach aphid *Myzus persicae* had no significant decline in 12 months. The ability to yield resting spores within host carcasses remained unchanged, and the probability of resting spore formation increased with the conidial concentrations that infect aphids. Therefore, cryopreservation at –80°C exerted a marginal impact on formulation and pathogenicity of *P. nouryi* and can substitute for costly liquid nitrogen storage in routine laboratory studies. The potential of the formulation in aphid biocontrol can be maintained although there is a risk of losing fungal sporulation ability in long-term preservation.

Key words: Cryopreservation at –80°C, *Pandora nouryi*, *Myzus persicae*, formulation and pathogenicity, sporulation capacity, resting spore formation

Introduction

Entomopathogens belonging to the order Entomophthorales (Entomophthoromycota) are widespread in the natural environment. They impede the overgrowth of arthropod populations or even decimate host cohorts (Pell *et al.*, 2001). Conidia actively project from mycotized carcasses to infect adjacent hosts and propagate the pathogens to a great extent. The resting spores produced *in vivo* endure the host-lacking period for renewing infection cycles during favorable seasons (Hemmati *et al.*, 2001; Castrillo *et al.*, 2007). Thus far, 223 species of Entomophthorales have been documented. Their enormous isolates derived from the carcasses of diverse host species or geographical origins exhibit high variability in terms of genetic characteristics as well as sporulation and infection capacities (Rohel *et al.*, 1997; Sierotzki *et al.*, 2000; Jensen *et al.*, 2006; Keller 2007; Sosa-Gómez et al. 2010). Isolates of high conidial production and infectivity formulated or not have been applied for decades in classical and conservation biological control programs against agricultural and forest pests (Pell *et al.*, 2010; Hajek and Delalibera 2010). The preservation of these valuable isolates is an essential step for exploring their practical potential, but there obstacles to be overcome.

Entomophthorales are difficult to culture and preserve *in vitro*. These fungi grow slowly even in rich media, such as Grace's insect tissue culture medium plus fetal bovine serum and Sabouraud dextrose agar plus egg yolk and milk (SEMA) (Papierok 2007). Their routine maintenance at low temperatures above freezing requires periodical serial transferring, which inevitably decreases their viability and virulence (Hajek *et al.*, 1990; Feng and Xu 2001; Grundschober *et al.*, 2001). The fungal body in host carcasses can survive at –14°C with reduced sporulation, but their cultures hardly stay viable at –20°C (López-Lastra *et al.*, 2002; Vingaard *et al.*, 2003). Other simple storage methods with deionized water, mineral oil, or silica gel also prove undesirable for Entomophthorales (López-Lastra *et al.*, 2002). Given their high vacuolar volumes in cells and

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intolerability to rapid freezing, the best recommended preservation protocol is to immerse fungal cultures in liquid nitrogen (–196°C) with a controlled freezing rate and a cryoprotectant (Humber 1997). However, this method is too costly to be available for most laboratories and also has several flaws, *e.g.*, the sporulation and virulence of *Entomophaga maimaiga* isolates decline after seven years of storage (Hajek *et al.*, 1995). Many studies have focused on preserving the isolates of Entomophthorales at –80°C for routine studies. This method has a simpler procedure and similar efficacy to liquid nitrogen storage (López-Lastra *et al.*, 2001, 2002; Delalibera *et al.*, 2004). However, studies on the survival quality of Entomophthorales under such a condition, which are necessary for their further utilization, are very limited. The following are some of the few reports on the survival quality: *Neozygites parvispora* can sporulate and infect hosts after 7 months of cryopreservation at –80°C (Grundschober *et al.*, 2001), *Conidiobolus thromboides* and *Zoophthora radicans* can grow on fresh media after 18 months of preservation (López-Lastra *et al.*, 2002), *Entomophaga aulicae* survives with decreased cellular viability for 10 months and *E. maimaiga* can still kill gypsy moth larvae by injection with its protoplasts stored for months (López-Lastra *et al.*, 2001).

In the present study, the effects of cryopreservation at –80°C on the formulation and pathogenicity of the obligate aphid pathogen *Pandora nouryi* (Remaudière & Hennebert) Humber were evaluated. The fungus was formulated as mycelia grown on the granules comprising 90% millet powder and 10% acrylate-acrylamide copolymer after storage for 3, 6, 9, 12, 15, and 18 months. The sporulation capacity was then assessed. A multi-concentration aphid bioassay was also performed on the green peach aphid, *Myzus persicae* (Sulzer) after one year of preservation, to determine the effects on both the infectivity of *P. nouryi* and probability of its resting spore formation *in vivo*.

Experimental

Materials and Methods

Fungal isolate and culture. The isolate of *P. nouryi*, ZJU0604, which has a high sporulation and infectivity to aphids, was used in this study. The isolate was derived from the mustard aphid *Lipaphis erysimi* (Kaltenbach) in Yunnan, southwest China, and preserved in the USDA-ARS Collection of Entomopathogenic Fungal Cultures (RW Holley Center for Agriculture and Health, Ithaca, NY, USA; ARSEF accession number: 8931) (Huang *et al.*, 2008; Zhou and Feng 2009). Fungal liquid cultures were obtained by adding small pieces of colony cultures on SEMA (v/v: 80% Sabouraud dextrose agar, 11.5% egg yolk, and 8.5% milk) to 50 ml of Sabouraud dextrose broth (SDB; w/v: 4% glucose, 1% peptone, and 1% yeast extracts) plus 0.1% (v/v) emulsified sesame oil in a 150-ml flask. The mixture was incubated on a rotary shaker (150 rpm) for 6 days at 20°C.

Spores collection and storage. To collect primary conidia of *P. nouryi* for storage, plates tiled with sporulating mycelial mats were prepared by dehydrating liquid cultures in 90 mm petri dishes (50 ml of culture per dish; excess water removed by filter paper). After leaving the plates undisturbed at 20°C overnight, conidia ejected from the mycelial mats were to be collected. After inverting on a 90-mm petri dish containing 20 ml of SDB, each sporulating plate released conidia for 3 h at 20°C. Most suspended conidia were without germination as either second conidia or germ tubes in SDB. After 1-min of vortexing and counting using standard hemocytometers, the conidial suspensions were adjusted to 2×10^5 conidia/ml and then transferred into 2-ml cryovials (1 ml per vial). After leaving all vials undisturbed at 4°C for 2 h, they were placed in an isopropanol-filled polycarbonate container (Cryo 1°C Freezing Container, "Mr. Frosty" Nalgene Co.), which can produce a semi-controlled freezing rate of *ca*. –1°C/min at –80°C. After 3 h of freezing, the vials were transferred into a plastic box for long-term preservation at –80°C.

Formulation and assessment of sporulation capacity. The conidial suspensions were thawed at 3-month intervals by immersing the cryovials into 500-ml of warm water (*ca*. 37°C) until no visible remnant of unthawed contents was observed. The suspensions were then added to 10 ml of fresh SDB in a 100-ml flask (2 ml of suspension per flask), which incubated for 12 days on a rotary shaker (150 rpm) at 20°C. About 12-ml of the liquid culture was transferred into a 150-ml flask containing autoclaved millet-gel granules (4-g of dry granules per flask), and stirred evenly before static incubation at 20°C and 12:12 L:D cycle. Details about the millet-gel granular culture have been described elsewhere (Zhou and Feng, 2009). The sporulation capacity of the 8-day-incubated granular culture was computed as the number of primary conidia released from every milligram of granule. Conidial counting with special spore collectors (13 mm diameter × 22 mm high) was performed as previously described (Zhou and Feng 2009). The upper lids of the collectors, on which six granular samples from each flask were separately mounted, were inverted onto the concave bottoms containing 0.5% dodecyl sodium sulfate. This surfactant inactivates conidia ejected from granules without morphological change. After 6-day of monitoring at 20°C, the conidia collected from each of the lower parts were counted using a hemocytometer under a microscope at 100 × magnification. The obtained result was then transformed to number of conidia per milligram of dry granule.

Aphid bioassay. To evaluate infectivity of *P. nouryi* after cryopreservation at –80°C, a multi-concentration bioassay was carried out (Feng *et al.*, 1998). The 3 ml vials containing the conidial suspensions stored for 1 year were thawed as above and added to 50 ml of fresh SDB in a 150-ml flask. After 12 days of shaking incubation, the liquid culture was converted into sporulating mats on a 90-mm plate for inoculating cohorts (*ca.* 50 nymphs per leaf disc) of second-instar *M. persicae*. By adjusting the length of exposure under the sporulating plate, the cohorts on leaf discs were inoculated with mean (±SD) conidial concentrations of 3.9 (±0.4), 13.1 (4.4), 23.4 (2.6), and 34.0 (±7.3) conidia/mm^2. Four cohorts were separately exposed to each concentration, and an additional four cohorts of nymphs without inoculation were included as blank control. All the cohorts were maintained at 20°C and 12:12 L:D cycles for 7 days. At 24-h intervals, the carcasses were promptly removed and individually laid on glass slides to verify the mycosis of *P. nouryi* and resting spore formation under a microscope.

Data analysis. To estimate the 50% decline time of sporulation capacity, observations over the full period of storage (in months) were fitted to the logistic equation $y = K/[1+\exp(a+rt)]$, where K is the maximal potential of sporulation for the granular culture, a is the intercept of the fitted equation, and r is the decline rate of sporulation capacity over storage time. With the fitted parameters, the 50% decline time was computed as $\{\ln[(2K/y_0)-1]-a\}/r$, where y_0 is the observed initial sporulation capacity. Daily mortalities from the multi-concentration bioassay were corrected using the control mortality and then fitted to a time-concentration-mortality model (Feng *et al.*, 1998). The fitted parameters and associated variances for the effects of time and concentration and the interaction of both were used to assess the median lethal concentration (LC_{50}) and 95% confidence interval for determining variation in fungal infectivity. The probability of resting spore formation within aphid carcasses was also examined. The proportional observations over the conidial concentrations inoculating the host cohorts were fitted to the linear equation $y = a + bx$. All analyses were performed using the DPS software (Tang and Feng, 2007).

Results

Variations in sporulation capacity. The formulation of the stored *P. nouryi* never failed during the 18-months of storage, but its sporulation capacity decreased significantly. These observations well fitted to the logistic equation $y = 69.5/[1+\exp(-1.5+0.1x)]$ ($r^2 = 0.93$, $F_{2,4} = 25.1$, $P < 0.01$) (Fig. 1). The last count of 18.5×10^4 conidia/mg was only 30.8% of the initial value. Based on the fitted parameters, the half-decline period of sporulation capacity over the storage time was computed as 13.6 months. This result was similar to that obtained from a previous study where fewer cadavers injected with *E. maimaiga* protoplasts and stored at –80°C abundantly produce conidia after 10 months (López-Lastra *et al.*, 2001). This finding indicates detrimental effect of cryopreservation on the fungal ability to sporulate.

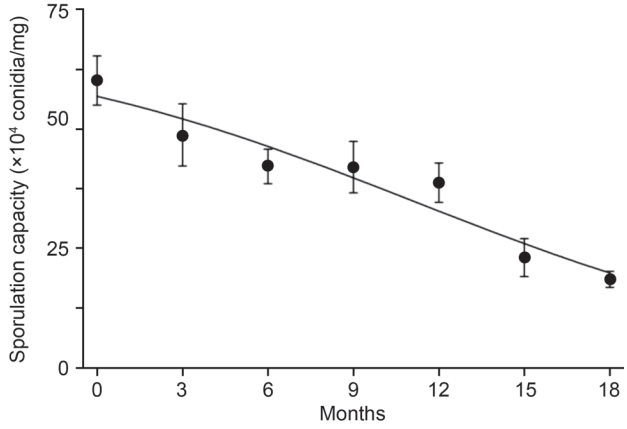

Fig. 1. Declining sporulation capacity (number of conidia per milligram) of the millet-gel granular formulation of *Pandora nouryi* after storage at –80°C for 0, 3, 6, 9, 12, 15, and 18 months. ●, observations (error bar: SD) with the curve fitted to the logistic equation $y = 69.5/[1+\exp(-1.5+0.1x)]$ ($r^2 = 0.93$, $F_{2,4} = 25.1$, $P < 0.01$).

Infectivity of *P. nouryi* to *M. persicae.* In the multi-concentration bioassay, the cumulative mortalities of *M. persicae* nymphs corrected with the control mortality (2.3%–7.7% on days 2–7) increased with both the aphid-inoculating conidial concentrations and days after inoculation (Fig. 2). The data well fitted the

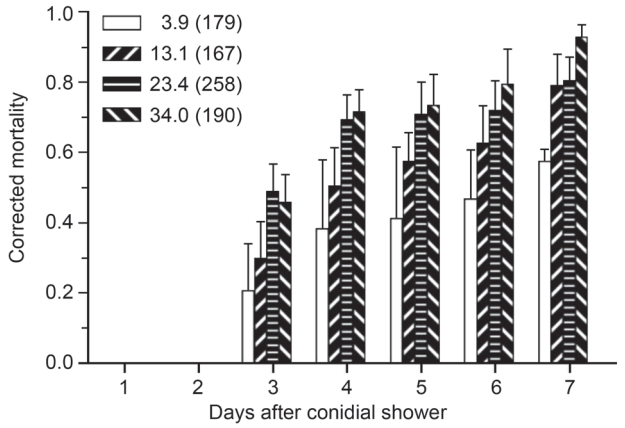

Fig. 2. Corrected mortalities of *Myzus persicae* nymphs after exposure to a shower of *Pandora nouryi* conidia discharged from dehydrated liquid cultures of conidial suspensions stored for 1 year. The cumulative mortalities at different conidial concentrations (number of conidia per square millimeter) were corrected with the control mortality (2.3–7.7% on days 2–7). Four cohorts of aphids (with the total number in parentheses) were separately exposed to these conidia.

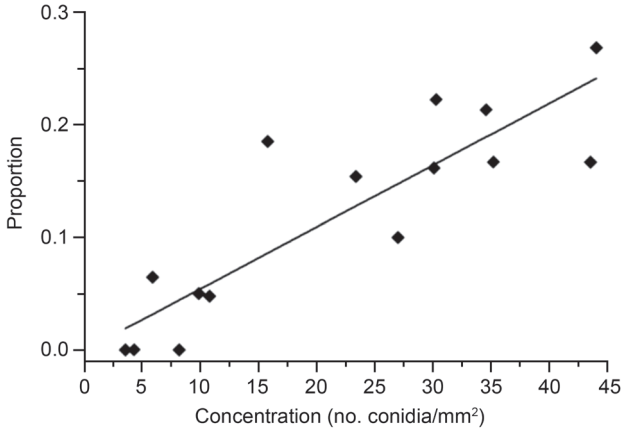

Fig. 3. Probability of resting spore formation *in vivo* increasing with the number of *Pandora nouryi* conidia inoculating aphid cohorts. ♦, observations (proportions of carcasses producing resting spores per cohort) with the fitted straight line $y = 0.0008 + 0.0055x$ ($r^2 = 0.78$, $F_{1, 14} = 48.4$, $P < 0.01$).

time-concentration-mortality model with no significant heterogeneity for the goodness of fit (Hosmer-Lemeshow $C = 1.23$, df = 8, $P = 0.996$). Based on the fitted parameters for the effects of the concentration, post-inoculation time and interaction of both, the LC_{50} values were computed as 8.6, 7.1, and 5.6 conidia/mm^2 on days 4, 5, and 6, respectively. The LC_{50} value of 2.5 (95% confidence interval: 1.6–3.8) conidia/mm^2 on day 7 were slightly larger than those of 1.4 (0.6–3.2) and 0.9 (0.3–2.4) from the repeated bioassays of the same host-pathogen interaction and regime in a previous study (Huang and Feng 2008). These estimates were not significantly different from one another because of the overlapped 95% confidence intervals.

Resting spore formation *in vivo*. In the bioassay, the proportions of carcasses bearing spherical resting spores increased with the concentrations of the showering conidia. The values fell within a range of 0.048–0.268 at 3.6–44.1 conidia/mm^2 (Fig. 3). The observations were linearly correlated to the conidial concentrations ($r^2 = 0.78$, $F_{1, 14} = 48.4$, $P < 0.01$), in agreement with the reported dependence of the resting spore formation *in vivo* of *P. nouryi* on the concentration of conidia that infect aphids (Huang and Feng 2008; Zhou and Feng 2010a, 2012). This result proved that the fungal ability to produce resting spores was unaffected by cryopreservation.

Discussion

Entomophthorales need a cryoprotectant to protect their protoplasts, conidia, or hypha from the lethal effects of large intracellular ice crystal formation and hyperosmotic injury under ultracold conditions. However, most cryoprotective solutions are toxic to cells at ambient temperatures, and should be eliminated by centrifugation or dilution after thawing (Humber, 1997; Hubálek 2003). Glycerol is a widely used cryoprotectant for Entomophthorales under cryopreservation at –80°C or –196°C, and dimethylsulfoxide (DMSO) preserves a few species, such as *N. parvispora* which cannot survive well in glycerol-inclusive cryoprotective medium (Humber, 1997; Grundschober *et al.*, 1998; Hubálek 2003). Recent studies indicate that assorted cryoprotectants in a mixture can generate synergic effects on protecting cells, especially those with diverse permeabilities functioning in different cell sites (Hubálek 2003). For example, 2% DMSO plus 1% trehalose can preserve *Neozygites tanajoae* at –80°C (Delalibera *et al.*, 2004). Our pre-study has proven that SDB is a better cryoprotective medium for primary conidia of *P. nouryi* than separate glycerol, trehalose or DMSO solution. There is also a potential cryoprotectant preference for the preservation of different species of entomophthoralean fungi.

To function in biocontrol programs, Entomophthorales must produce consistent high pathogenicity, which entails steady outcomes of fungal formulation and prolonged storage. None of the currently available preservation techniques for Entomophthorales have proven perfect (Hajek *et al.*, 1995; Humber, 1997; Feng and Xu, 2001; López-Lastra *et al.*, 2001, 2002; Vingaard *et al.*, 2003). Hence, the detailed effects of these techniques on fungal survival quality need to be determined before being adopted in long-term studies. Among the studies on cryopreservation at –80°C for Entomophthorales, only fungal survival is generally observed and not latent changes in fungal pathogenicity, formulation, or other biological attributes. The present study modified the method to collect infectious conidia and preserve them at –80°C, as opposed to culturing sensitive wall-less protoplasts as stored materials in previous studies (López-Lastra *et al.*, 2001, 2002; Delalibera *et al.*, 2004). The formulation, pathogenicity, and fungal ability to produce resting spores within aphid carcasses were also proven impervious to the effects of cryopreservation at –80°C. However, the diminished sporulation capacity of the formulated *P. nouryi* indirectly implied that the storage process damaged fungal viability such as growth rate *in vitro* or recovery rate after cryopreservation. Thus, further studies are required to explain this decline. The 50% decline time of 13.6 months was incomparable because no relevant study exists. Nevertheless, the last count of sporulation capacity was sufficiently large in terms of entomopthoralean formulation, i.e., four times higher than that (4.2×10^4 – 4.5×10^4 conidia per capita) of mycotized carcasses (Zhou and Feng, 2009; 2010b). Therefore, cryopreservation at –80°C meets the requirement of important isolate stocks, but potential declines in fungal viability and sporulation capacity should be closely monitored.

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