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SHORT RESEARCH NOTES

A preservation method for *Peronospora viciae* conidia

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Abstract. Conidia of the obligate pathogen *Peronospora viciae* were stored for one year at −80°C on field pea (*Pisum sativum*) leaves. Stored conidia were used to inoculate susceptible pea seedlings resulting in infection on 61.7% of seedlings after 365 days of storage as compared to 92.5% when inoculated with fresh conidia.

Peronospora viciae, the causal agent of downy mildew of field peas, is an obligate parasite prevalent on pea crops in many countries, including Australia. The Australian Coordinated Pea Improvement Program (ACPIP) screens pea germplasm for resistance to this disease using controlled conditions (Davidson *et al*. 2004). The current method for maintenance of *P. viciae* conidia is tedious, as regular conidial transfers from source plants to new pea seedlings are required. The pathogen requires low temperatures (8°C/4°C day/night) combined with high humidity to initiate the infection process and to induce sporulation on the host plant. These specific conditions necessitate the use of a controlled environment room to ensure successful infection and sporulation. Storage of *P. viciae* conidia on infected pea leaves has been successful in European laboratories (Peter Spencer-Phillips and Viranyi Ferenc, personal communication). Other obligate pathogens have been stored using a variety of methods. Bromfield and Schmitt (1967) maintained viable and infective conidia of *P. tabacina* for a storage period of 25 months in liquid nitrogen at −180°C using dimethyl sulfoxide (DMSO) as a cryoprotectant. Conidia of the downy mildew pathogen (*Phytophthora phaseoli*) on lima bean have been stored for 1 year on lima bean epicotyl in a liquid-nitrogen vapour phase (−100 to −160°C) (San Antonio and Blount 1973). The zoosporangia of *Plasmopara halstedii*, causal agent of sunflower downy mildew, remained infective after storage in liquid nitrogen for over 4 years using a dry spore, fast-freeze technique (Gulya *et al*. 1993). This technique was also successful with *P. parasitica* and *Pseudoperonospora cubensis* sporangia, but not with *Peronosclerospora sorghi*.

Different preservation techniques were evaluated for long-term storage of *P. viciae* to be used in the ACPIP breeding programme. Pea plants in commercial crops that showed symptoms of systemic infection and sporulating conidia of *P. viciae* were collected from three locations (Port Vincent, Laura, Balaklava) within South Australia during 2002. Each population was maintained separately on pea plants (cv. Alma).

The method described by Davidson *et al*. (2004) was used to transfer the conidia from diseased plants to fresh seedlings. Seven-day-old seedlings of the susceptible cultivar Alma were placed in a controlled environment room at 12°C/4°C for 12 h day/night cycle for a period of 7 days. After this stage, *P. viciae* conidia were collected from source plants using a fine hair brush, dispersed into sterilised water with a drop of 0.1% Tween 20 (BDH Laboratory Supplies) added as a surfactant and mixed to produce a conidial suspension of 1.8×10^6 spores/mL. The seedlings were inoculated with the conidial mixture by placing four droplets from a Pasteur pipette into the apical bud. The inoculated seedlings were incubated for 4 days at 8°C/4°C day/night temperature in plastic tents ($160 \times 80 \times 80$ cm). Each tent contained an ultrasonic humidifier to maintain high humidity (RH > 95%). At the end of this period, the humidifiers were turned off and the plastic tents were opened to allow equilibrium with the ambient humidity. The temperature was maintained at 12°C/4°C day/night cycle for the next 18 days. At the end of this period, the plants were subjected to another 3-day period of constant high humidity and low temperature (8°C/4°C day/night) to induce sporulation.

Pea leaves with profuse sporulation of *P. viciae* were collected, packed loosely into Petri plates that had been lined top and bottom with dry sterile filter papers to avoid condensation or ice formation, and sealed with Parafilm. The plates were put into a closed plastic container and placed into a freezer at −80°C.

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The viability of conidia was assessed after storage periods of 99 days and 365 days by inoculating on to pea seedlings. At each time period, samples of each *P. viciae* population were removed from the −80°C freezer and kept at room temperature for 30 min. Conidia were collected from the leaves using a fine-hair brush, dispersed into 5 mL of sterilised RO water in McCartney bottles, surfactant (0.1% Tween 20) added and suspended using a vortex mixer. Conidia that had been maintained on living plants in the controlled environment room were collected at the same time from fresh sporulating pea leaves. Each population of fresh and stored conidia was inoculated separately onto prepared seedlings and plants were maintained as described before. After 21 days, the plants were assessed for downy mildew infection by rating sporulation on leaves and stems using the 0–4 rating scale described by Davidson *et al*. (2004) where 0 = no sporulation on plant, 1 = sporulation on less than 10% of the leaf and stem, 2 = sporulation on 10–30% of leaf and stem, 3 = sporulation on 30–70% of the leaf and stem, and 4 = sporulation on greater than 70% of leaf and stem. The mean disease scores on infected plants were calculated for fresh conidia and each storage period, for all three populations. Significance was tested based on homogeneity of variances using a two-sample *t*-test in Statistix 8.0 (Analytical Software, Tallahassee). The number of infected plants was recorded as a percentage of the total number of inoculated plants.

Successful infection was observed on plants inoculated with conidia that had been stored for 99 days and for 365 days (Table 1). The mean disease scores were significantly reduced ($P < 0.05$) for both storage periods when compared with fresh conidia but differences between means of 99 days and 365 days were non-significant for all three populations. When the data from the three populations were combined, the differences between disease scores were significant at all time periods ($P < 0.05$). Gulya *et al*. (1993) also reported a reduction in infection rate of zoosporangia of *Plasmopara halstedii* after storage in liquid nitrogen.

Table 1. Infectivity of three populations of *Peronospora viciae*, compared with fresh conidia, after storage in a freezer at −80°C

Collection site	Storage period	Mean disease score (0–4 scale) on diseased plants	Diseased plants (%)
Port Vincent	Fresh conidia	2.34 a[A]	95.0
	99 days	2.00 b	90.0
	365 days	1.81 bc	67.5
Balaklava	Fresh conidia	2.27 a	92.5
	99 days	1.82 b	85.0
	365 days	1.78 bc	57.5
Laura	Fresh conidia	2.31 a	90.0
	99 days	1.85 b	85.0
	365 days	1.79 bc	60.0
Mean of populations	Fresh	2.31 a	92.5
	99 days	1.89 b	86.7
	365 days	1.79 c	61.7

[A]Means followed by the same letter are not significantly different at $P < 0.05$.

Alternative storage methods were also tested using the following cryoprotectant solutions; 10% DMSO (V/V); 15% DMSO, skim milk 8.5% + glycerol 10% (stock solutions of 17% skim milk and 20% glycerol mixed 1 : 1 after autoclaving), glycerol 10% (V/V), and sterilised water. Freshly produced conidia were harvested from plants with a fine hair brush and dispersed into 2 mL cryogenic vials containing 1.5 mL of the cryoprotective solution.

Sixteen vials per treatment were prepared. Eight vials per treatment were placed directly into the −80°C freezer. The remaining eight vials per treatment were placed in liquid nitrogen for 5 min before storage at −80°C. After 45 days storage, four vials were removed from the freezer and placed in a 15°C water bath for 30 min. Pea seedlings were inoculated with these conidia and plants were maintained using the procedure described above. Conidia preserved using these methods were not viable.

Our results indicate that conidia of *P. viciae* can be stored directly on pea leaves for 365 days under ultra low temperatures and retain high levels of infectivity on susceptible pea seedlings after this time. Sufficient quantities need to be stored to compensate for the decrease in infection rate. The storage technique described here is simple, does not require cryoprotectants or liquid nitrogen and does not require a controlled rate of freezing. It should prove useful for the preservation of *P. viciae* conidia for periods of at least 12 months.

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