The effect of drying a water suspension of *Pasteuria penetrans* on spore viability

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Summary. *Pasteuria penetrans* (*Pp*) is a mycelial and endospore forming bacterium parasitising root-knot nematodes (*Meloidogyne* spp.), with potential as a biocontrol agent. It is stored either in the form of ground dried roots containing infected females, or water suspensions deriving from crushed infected females. For its transportation between laboratories the dried root material and the liquid formulation may be blocked by posting regulations. Water suspensions containing *Pp* spores (*Pp*3 and *Pp* blend) were left to dry and remained dried for 2 or 12 months. After hydration, juveniles of *M. incognita* and *M. javanica* were exposed in Petri dishes containing spores of *Pp* blend and *Pp*3 respectively, either hydrated after the drying period or kept continuously in water suspension (control) and the number of attached spores recorded after 24 h incubation. Spore encumbered juveniles of *M. incognita* were inoculated on pepper and of *M. javanica* on tomato. The plants grew for 50 days in a growth room and afterwards the number of egg masses per root was recorded. Females without egg masses that were suspected to be infected were checked for the presence of spores inside. Both spores dried for 2 or 12 months and then hydrated and spores kept continuously wet, attached on juveniles at similar rates, and had a similar efficacy in reducing the number of egg masses and infection rate of females. Therefore, a dried water suspension of *Pp* can overcome posting restrictions and can be safely used for further multiplication of the parasite.

Key words: biocontrol, *Meloidogyne javanica*, *Meloidogyne incognita*, nematode parasite, tomato, pepper.

Pasteuria penetrans (*Pp*) is a mycelial and endospore forming bacterium parasitising root-knot nematodes (*Meloidogyne* spp.), with biocontrol potential. Spores of *Pp* are immobile and attach on second-stage juveniles (J2) when they move. The J2 encumbered with spores invades the root and develops while the spores germinate and proliferate inside its body. The development of *Pp* does not impair feeding, moulting or growth of the nematode, but it selectively destroys the reproductive system of females. The infected female becomes filled with spores and does not lay eggs (Chen & Dickson, 1998). For use as a biocontrol agent, roots containing spore-infected females are thoroughly air-dried to destroy egg masses produced by non-infected females and afterwards the roots are ground. *Pp* is stored in the form of this dried root material at room temperature (Stirling & Wachtel, 1980). The spores contained in dried roots maintain their ability to attach to J2 and subsequently infect females after being hydrated. Alternatively, infected females can be extracted from fresh roots and crushed in water to release the spores. Therefore, a suspension of *Pp* spores, free of any surrounding root material is obtained and can be stored in a refrigerator.

Resistance of *Pp* spores to desiccation for a brief period, was tested by drying a spore suspension in an open glass Petri dish overnight at 28°C. Afterwards, spores sticking to the glass were re-suspended with a toothbrush in water and designated as ‘dried spores’ to differentiate them from the original ‘wet spores’. Attachment tests indicated, that ‘dried spores’ adhered to *M. incognita* much more rapidly and at higher rates than ‘wet spores’ but their ability to infect was not tested (Netscher & Duponnois, 1998).

The idea for the current work originated from a ‘casual’ observation, when a spore suspension of *Pp* kept in a fridge, was by a mistake left to dry for an unknown period, less than 1 year. It was found that after subsequent hydration, spores attached on J2 and infected females. Therefore, two experiments were conducted to investigate whether spores of *Pp*, derived from squashed females in a water suspension, retain their ability to attach to J2 and infect females of *M. javanica* and *M. incognita* after

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the suspension dries and remains dry for periods of 2 months and 1 year.

MATERIAL AND METHODS

Nematodes and *Pasteuria penetrans*. Eggs of *M. javanica* and of *M. incognita* were collected from roots of tomatoes and peppers grown in pots (Hussey & Barker, 1973) and incubated in extraction dishes for 4 days to obtain J2 for the experiments. Both nematode populations originated from Crete, Greece and had been maintained in pots for several years.

A spore suspension of *Pp* blend (a blend of six isolates of different continental origins, *e.g.*, Africa, Oceania, USA; Nasiou *et al.*, 2020), with a density of 1.7×10^4 spores ml^{-1}, had been produced by squashing spore-infected females of *M. javanica*. After using it for experiments, a small quantity (*ca* 6 ml) remaining in the bottom of a small glass beaker, was stored in a refrigerator and kept for reference. The glass beaker had been covered with parafilm which by a mistake was loosely tighten and the suspension progressively dried. The suspension was observed to be dry after storage for 1 year, but the period that the suspension had been dry could not be determined. For spore recovery, 6 ml of distilled water was added to the beaker, vortexed and remained for 2 weeks in a refrigerator, so as to ensure hydration of spores. Afterwards, the suspension was vortexed to dispersing the spores and the density was estimated with a haemocytometer and found to be 10^4 spores ml^{-1}. Hatched J2 of *M. javanica* were transferred to a 5.5 cm diam. Petri dish and 4 ml of the spore suspension was pipetted inside. After 48 h incubation at 25-28°C, spores were observed on J2 using an inverted microscope at ×200. The J2 encumbered with spores were used to inoculate a tomato plant (*Solanum lycopersicum* 'Ace') grown in 250 ml plastic pot filled with a commercial soil substrate. The plant was kept in a growth room at 24-26°C and 14 h photoperiod for 50 days. Afterwards it was uprooted, the roots washed thoroughly and females without egg masses were selected and extracted from the root under a dissecting microscope. Those females were put in drops of distilled water on glass slides, crushed with cover slips and examined at ×400 for the presence of mature spores of *Pp*. Since infected females were found, it demonstrated that the spores of *Pp* did not lose their ability to infect after the suspension dried.

The cover slips were removed and the pieces of the squashed females stuck on glass slides and coverslips were washed in a glass beaker and vortexed to disperse the spores. This fresh suspension of *Pp* blend was kept stored in a refrigerator for 2 weeks and afterwards was used to prepare 10 ml distilled water dilutions with *ca* 70,000 spores ml^{-1} in three glass vials. Similarly, three other vials were prepared with another *Pp* isolate, called *Pp*3 (from South Africa; Spaull, 1981), containing 3 ml dilutions with *ca* 700,000 spores ml^{-1}.

Drying and hydration process of *Pasteuria penetrans*. The one vial for *Pp* blend and *Pp*3 was capped tightly (wet spore treatment) while the other two remained opened (dried spore treatment) and all were placed in an incubator at *ca* 26°C, for 1 month, until the water in the opened vials had completely evaporated.

Afterwards, the opened vials of both *Pp* isolates were capped tightly and were put in the refrigerator with the vials containing the aqueous spore suspensions. After a period of 2 months, one empty vial was filled with 10 ml (for *Pp* blend) or 3 ml (for *Pp*3) distilled water, vortexed and was placed back to refrigerator, so as to ensure spore hydration. Ten days later, all vials were vortexed to disperse the spores and the spore density was determined. The spore densities were: *Pp* blend wet spore treatment 75,000 spores ml^{-1}, *Pp* blend dried spore treatment 57,000 spores ml^{-1}, *Pp*3 wet spore treatment 1,000,000 spores ml^{-1} and *Pp*3 dried spore treatment 765,000 spores ml^{-1}. Twelve months from the beginning of the experiment, water was added in the second set of vials with the dried spores and processed as previously described. The spore densities were: *Pp* blend wet spore treatment 76,000 spores ml^{-1}, *Pp* blend dried spore treatment 33,500 spores ml^{-1}, *Pp*3 wet spore treatment 1,500,000 spores ml^{-1} and *Pp*3 dried spore treatment 744,000 spores ml^{-1}.

Experiment 1. Hatched J2 of *M. incognita* were transferred to 3.5 cm diam. Petri dishes (*ca* 300 J2 per dish) containing *ca* 10,000 spores of *Pp* blend to a total volume of 2.5 ml, each with five replicates, for *Pp* blend wet spore and *Pp* blend dried spore (for 2 months) treatments. After 24 h of incubation at 25-28°C, spores were observed on 10 J2 per dish in an inverted microscope at ×200. The J2 encumbered with spores from each dish were used to inoculate one pepper plant (*Capsicum annuum* 'California Wonder') which grew in 250 ml plastic cup filled with a commercial soil substrate. Five pepper plants, inoculated with J2 free of spores incubated in distilled water to 3.5 cm diam. Petri dishes for 24 h, served as controls. Plants were kept in a growth room at 24-26°C and 14 h photoperiod for 50 days.

Table 1. Mean number of *Pasteuria penetrans* (*Pp*) spores per second-stage juvenile (J2) of *Meloidogyne* spp. exposed to spore suspensions that were kept continuously wet or dried for 2 and 12 months and number of egg masses per root and number of infected females (n = 10), when spore encumbered J2 were used to inoculate plants.

Treatments	Spores (J2)$^{-1}$		Egg masses (root)$^{-1}$		Infected females (n = 10)	
	Exp. 1 *Pp* blend (*M.i.*)	Exp. 2 *Pp*3 (*M.j.*)	Exp. 1 *Pp* blend (*M.i.*)	Exp. 2 *Pp*3 (*M.j.*)	Exp. 1 *Pp* blend (*M.i.*)	Exp. 2 *Pp*3 (*M.j.*)
2 months drying period						
Control	–	–	20.80 a	26.2 a	–	–
Wet spores	5.46 a	13.26 a	4.7 b	2.0 b	8.0 a	8.6 a
Dried spores	5.58 a	15.32 a	4.8 b	2.6 b	7.6 a	8.4 a
LSD 5%	1.11	3.13	8.6	10.57	1.38	1.08
12 months drying period						
Control	–	–	83.4 a	62.4 a	–	–
Wet spores	8.56 a	7.94 b	13.4 b	18.4 b	5.8 a	10.0 a
Dried spores	9.06 a	9.08 a	10.6 b	14.4 b	5.6 a	10.0 a
LSD 5%	2.14	1.04	23.75	16.77	1.26	0

Mean of five replicates per treatment; means within columns followed by the same letter are not significantly different according to LSD test ($P \leq 0.05$); 300 J2 (plant)$^{-1}$ for the 2 month drying period and 1,000 J2 (plant)$^{-1}$ for the 12 month drying period. Exp. 1: *Meloidogyne incognita* (*M.i.*) exposed to spores of *Pp* blend and inoculated on pepper; Exp. 2: *M. javanica* (*M.j.*) exposed to spores of *Pp*3 and inoculated on tomato.

Afterwards they were uprooted, the roots were thoroughly washed and the number of egg masses was recorded. The roots remained in water for 2 days for the tissue to be softened. Single females without egg masses in galls were observed under a stereoscope. The *Pp* infected females were distinguished by their dull creamy white colour (Mankau & Prasad, 1977), which sometimes had traces of gelatinous matrix without eggs inside. From each replicate plant, ten females suspected to be infected were removed and were put in drops of water on a glass slide, crushed with a cover slip and examined at ×400 for the presence of mature spores of *Pp*. The *Pp* blend spore suspension dried for 12 months and a non-treated control were used to encumber J2 (*ca* 1,000) in 3.5 cm diam. Petri dishes containing *ca* 30,000 spores to a total volume of 3 ml with five replicates for each treatment. The encumbered and spore free J2 were used to inoculate pepper plants, which were maintained in the growth room for 50 days and afterwards the number of egg masses per root was recorded and females suspected to be infected were extracted and examined, as described above.

Experiment 2. The same procedures as in Experiment 1 were repeated with *Pp*3 and *M. javanica*. The J2 were inoculated on tomato plants.

All results were analysed with single ANOVA and treatment means compared using LSD test at 5% level of significance. The analysis was conducted with the SAS University Edition.

RESULTS

The results are presented in Table 1. There are no significant differences between treatments in spore attachment on J2, number of egg masses per root and number of infected females after the 2 months drying period for both experiments. For the 12 months drying period, while there was no difference on spore attachment on J2 for *Pp* blend (Exp. 1), dried spores of *Pp*3 attached on J2 at higher rate than the wet spores (Exp. 2). Both wet spore and dried *Pp* for twelve-month treatments (Exp. 1 and Exp. 2) had a similar efficacy in reducing the number of egg masses and in infection of females compared with the controls.

DISCUSSION

It has been known that spores of *Pp* inside dried root material lose their ability to infect females over time. A period of 6 or 11 years of storage did not affect the ability of spores to attach but significantly decreased the infection rate of females (Espanol *et al.*, 1997; Giannakou *et al.*, 1997). Spores of *Pp* stored for 24 years in the form of dried root powder attached to J2 but did not infect females (Nasiou *et al.*, 2020). However, spores maintained in water suspensions in a domestic refrigerator for 24 years, retained their ability to infect females (Tzortzakakis, 2022).

Drying spore suspension of *Pp* offers the advantage of transporting the parasite for research

purposes between laboratories. *Pp* is a beneficial organism that occurs naturally and is harmful exclusively to plant-parasitic nematodes. However, its transportation may be blocked by posting regulations since dried root material may be considered 'suspicious' while a liquid form may be forbidden for post services. This could be overcome easily if it is in the form of a dried aqueous suspension contained in a small 'empty' vial.

The results of this work indicate that *Pp* spores in a water suspension, which remains dry for 2 and 12 months and hydrated afterwards, were able to attach to J2 and subsequently to infect the females. Therefore, a dried water suspension of *Pp* for up to 1 year can be safely used for further multiplication of the parasite.

The viability of *Pp* spores in dry conditions may cause problems in the laboratories with possible contamination of the infrastructure (benches, glassware *etc.*). It has been demonstrated that treatment of dried spores with alcohol did not prevent subsequent spore attachment, while a dilution of 0.5% commercial sodium hypochlorite, added to dried spores on a microscope slide, destroyed them (Netscher & Duponnois, 1998). Cleaning of glassware from attached spores can be achieved by autoclaving, while methods for spore elimination from non-autoclaved items (*e.g.*, bench surface, plastic ware) should be further investigated.

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E.A. Tzortzakakis. Влияние высушивания водной суспензии *Pasteuria penetrans* на жизнеспособность спор.

Резюме. Паразитирующая на галловых нематодах бактерия *Pasteuria penetrans* (*Pp*) – потенциальный агент биологической борьбы. Возможно сохранение споры бактерий в высушенных корнях, с зараженными самками мелойдогин, или в водных суспензиях измельченных зараженных самок. В связи с таможенными правилами интерес представляет высушивание самих спор, без корней и самок нематод. Высушенные водные суспензии со спорами *Pp* высушивали в течение двух или 12 месяцев. После гидратации высушенных спор оценивали их способность прикрепляться к личинкам *M. incognita* и *M. javanica* за 24 часа инкубации. Личинки *M. incognita* с прикреплёнными спорами были использованы для заражения перца, а личинки *M. javanica* для заражения томата. Количество яйцевых мешков и наличие спор в самках без них определяли после 50 дней выращивания в инкубационной комнате. Показана одинаковая скорость прикрепления спор и их эффективность в снижении количества яйцевых мешков и степени заражения самок, что доказывает возможность использования высушивания водных суспензий *Pp* для почтовой пересылки и применения этой бактерии.