Zeitschrift: Botanica Helvetica
Herausgeber: Schweizerische Botanische Gesellschaft
Band: 96 (1986)
Heft: 1

Artikel: Factors affecting spore germination in Pilobolos crystallinus as evidence for an ancient association between fungi, plants and animals

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DOI: https://doi.org/10.5169/seals-67193

Botanica Helvetica 96/1, 1986

0253-1453/86/010073-04 $ 1.50+0.20/0

Factors affecting spore germination in *Pilobolos crystallinus* as evidence for an ancient association between fungi, plants and animals

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Manuscript accepted March 7, 1986

Abstract

Fisher, P. J. and Anson, A. E. 1986. Factors affecting spore germination in *Pilobolus crystallinus* as evidence for an ancient association between fungi, plants and animals. Bot. Helv. 96: 73–76.

Maximum percentage germination in *Pilobolus crystallinus* was induced by applying a heat stimulus of 40–45 °C for 1 h. Alkaline pancreatin treatment had no effect on germination but incubation in alkaline conditions (pH 8.9) resulted in higher percentage germination than did neutral conditions (pH 7.1). A rich nutrient medium supported higher percentage germination than did a very low nutrient medium.

Introduction

Freshly voided rabbit dung frequently contains the spores of *Pilobolus crystallinus* (Wiggers) Tode. These spores readily germinate in the dung and within 1–2 days sporangiophores of the fungus are formed. The sporangia are violently discharged onto adjoining vegetation where they adhere on leaves to be subsequently eaten by a rabbit or other herbivores. This cyclic relationship between herbage-animal gut-dung-herbage is typical of enterocoprophilus fungi where the fungal spores not only survive the passage through the animal gut but also derive some benefit from the gut environment (Webster 1970). The coprophilous habit is widespread amongst fungi and includes numerous genera (Richardson & Watling 1982).

Most of the taxa in the genus *Pilobolus* are well adapted to a coprophilous way of life and it is known that spores of *P. kleinii* van Teigh require a short heat stimulus to induce germination (Webster 1970).

In the present study spores of *P. crystallinus* were subjected to different regimes of temperature, pH, digestive enzyme treatment and nutrient supply to ascertain optimum conditions for germination

Material and Methods

P. crystallinus isolated from rabbit pellets was maintained at 20 °C on nutrient agar (NA) (Oxoid nutrient broth CM 1 plus 1.5% agar). Spore suspensions were obtained as follows. Mycelial plugs (1 cm diam) were transferred to the centre of a ring of five sterile pellets embedded to half their depth in NA in 9 cm diam plastic Petri dishes. These were illuminated from above by natural daylight and sporulation occurred after 5–7 days at 20–27 °C. Sporangia were far more numerous using daylight rather than the fluorescent lights described in an earlier paper (Anson, Fisher & Kuthubutheen, 1985). Sporangiophores of *P. crystallinus* are positively phototropic, so that illumination from above caused sporangia to be discharged vertically enabling them to be collected over a period of three days on sterile, 7 cm diam, clear cellophane discs held on the inside of the Petri dish lids. The discs were then removed, the sporangia dislodged and their spores released in 20 cm^3 sterile distilled water (SDW) using a stomacher (Colworth Lab-Blender 80). The suspension was diluted in SDW to the required density and stored at 6 °C until required. Preliminary experiments indicated no significant effect of ageing on spore germination (with or without heat stimulus at neutral or alkaline pH's) over a 15-day period.

The effect of nutrient concentration on germination was tested as follows. A suspension containing 1.0×10^6 spores cm^{-3} was placed in a pre-warmed universal vial in a waterbath at 45 °C for 1 h. Aliquots (0.1 cm^3) were then surface-spread in triplicate 5 cm diam Petri dishes containing either 5 cm^3 of the surface dried yeast peptone agar (YPA) (bacterial peptone, 5 g; yeast extract, 3 g; agar, 15 g; DW, 1 litre; pH 7) or the same volume of 0.1% YPA. Plates were incubated at 25 °C for 11 h and spores examined using a phase microscope (magnification × 400). Spores were counted as germinated as soon as they showed initial stages of germ tube formation, spores which were only swollen being counted as ungerminated. A minimum of 400 spores were counted per plate. An average of 77% (range 75–80%) spores germinated on YPA compared with an average of 46% (range 42–50%) on the low nutrient medium, 0.1% YPA. Thereafter, the high nutrient medium, YPA, was used in all experiments.

The effect of heat treatment on germination was investigated as follows. Aliquots of suspension containing 1.0×10^6 spores cm^{-3} were placed in pre-warmed universal vials in water baths at 30, 35, 40, 45, and 50 °C for 1 h. Aliquots (0.1 cm^{-3}) were then surface-spread on triplicate YPA plates and incubated as before. The combined effect of alkaline pH and heat were studied as follows. A spore suspension in SDW was prepared (2×10^6 spores cm^{-3}) and diluted 1 : 1 in universal vials in either 0.1 M-Tris/HC 1 buffer pH 8.9 or 0.1 M-Tris/HC 1 buffer pH 7.1 or SDW and incubated for 24 h at 25 °C at which time suspensions were checked to confirm that germination had not taken place. Each suspension was then subdivided between three pre-warmed universal vials, one vial per treatment then being incubated for 1 h at 25, 40, and 45 °C. Aliquots (0.1 cm^3) were then surface-spread on triplicate YPA plates, incubated for 11 h and scored for percentage germination.

The effect of pancreatin was studied as follows. Porcine pancreatin grade III (Sigma London Chemical Company, Poole, Dorset) was made up as a 1 $mg.cm^{-3}$ suspension in Tris/HC 1 buffer pH 8.9 and then passed through a 0.45 μm membrane filter. A spore suspension (2×10^6 spores cm^{-3}) was diluted 1 : 1 with either the freshly-prepared enzyme in buffer or with buffer alone in pre-warmed universal vials and held at 35 or 40 °C for 1 h. Aliquots (0.1 cm^3) were then surface-spread on triplicate YPA plates and scored for germination as described previously.

Results

Results in Table 1 indicate clearly that a heat stimulus above 35 °C and below 50 °C for 1 h has a significant effect on subsequent germination at 25 °C.

Results shown in Table 2 were analysed using the G-statistic and the unplanned test of homogeneity of replicates (Sokal & Rohlf, 1981) and showed that at whichever temperature was selected there was a significantly higher ($P < 0.001$) percentage germi-

Tab. 1. Effect of heat treatment on subsequent germination of *P. crystallinus* at 25 °C

Temperature (°C) for 1 h	Germination %			
	Replicates			Average
30	1.5	1.1	1.3	1.3
35	1.6	1.8	1.3	1.6
40	43	47	54	48
45	65	74	73	71
50	0	0.6	0.7	0.4

Tab. 2. Effect of alkalinity and heat treatments on subsequent germination of *P. crystallinus*. Figures are percentage spores germinated (average of triplicate plates) with the range in brackets

Pre-treatment for 24 h at 25 °C in:	Temperature for 1 hour		
	25 °C	40 °C	45 °C
Tris/HCl buffer pH 8.9	9 (7–11)	70 (69–70)	62 (58–66)
Tris/HCl buffer pH 7.1	2 (1.9–2.1)	22 (21–24)	37 (35–42)
Sterile distilled water	1 (0.3–2.0)	19 (17–21)	33 (30–38)

Tab. 3. Effect of alkaline pancreatin treatment on germination. Figures are percentages with the range in brackets

Treatment	Temperature	
	35 °C	40 °C
Pancreatin (buffer pH 8.9 for 1 h)	4 (3–5)	47 (45–49)
Control (buffer pH 8.9 for 1 h)	6 (4–8)	48 (46–52)

nation at pH 8.9 than at pH 7.1 or in SDW, whereas the differences between pH 7.1 and SDW were not significant ($P = 0.05$). The percentage germination at 25 °C was significantly lower than at 40 and 45 °C in all cases ($P < 0.001$). The difference between spores treated at 40 and 45 °C was significant only at pH 7.1 ($P < 0.01$).

Presence of the enzyme had no significant effect ($P = 0.05$) on percentage germination (Table 3) which is in accordance with the findings of Webster (1970) for *P. kleinii*. This supports a result described earlier (Tables 1, 2) that the optimum temperature to stimulate spore germination in *P. crystallinus* lies in the region of 40–45 °C when the spores are heated at this temperature for 1 h.

Discussion

These results confirm that the spores of *P. crystallinus* require the heat stimulus they receive in the gut of herbivores for efficient subsequent germination. In the rabbit for example gut temperatures are 38.7–39.7 °C (Fenner & Ratcliffe, 1965) which lie close to the temperature that gave maximum germination in our experiments (40–45 °C). Indeed under alkaline conditions which are typical in the guts of herbivores (Prosser 1973) maximum germination was achieved at 40 °C rather than 45 °C. The dependence of the entero-coprophilous fungi on the food plants of herbivores which act as transportation for their spores to the animal gut raises the question of their evolutionary origins.

Bakker (1975) suggested that therapids which lived during the late Permian and early Triassic 230–200 million years ago were the first warm blooded animals. He bases these ideas on evidence that these mammal-like reptiles invaded southern Africa, South America and other parts of the southern cold temperature realm during the late Permian when severe latitudinal temperature gradients existed and some glaciation continued in Tasmania and the southern end of Gonduanaland (present day South America) retained its cold-adapted *Glossopteris* flora.

Some Dinosaurs which date back to the Jurrasic 200–70 million years ago are also thought to have been warm blooded (Charig 1979). These early endothermic herbivorous reptiles must have had an important influence on the evolution of the coprophilous fungal habit long before the advent of mammals.

It is of interest that although a large number of species of fungi have been reported from dung of birds and mammals, relatively few have been reported from the dung of reptiles and amphibia. One notable exception is *Basidiobolus ranarum* which is unusual in that its life cycle involves entering into the guts of beetles which are in turn devoured by frogs (Levisohn 1927). It is, therefore, likely that the cyclic relationship herbage-animal gut-dung-herbage proved a successful combination early on in the evolution of this group of fungi and was later continued by the substitution of mammals once the warm blooded reptiles had become extinct.

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