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Institut für Botanik, Universität Hohenheim, Stuttgart, Germany

Isolation of Oospores of Sunflower Downy Mildew, *Plasmopara halstedii*, and Microscopical Studies on Oospore Germination

O. SPRING and R. ZIPPER

Authors' address: Institut für Botanik, Universität Hohenheim, D-70593 Stuttgart, Germany (correspondence to O. Spring; e-mail: spring@uni-hohenheim.de)

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Abstract

A method was elaborated to isolate oospores of *Plasmopara halstedii* from tissue of its host, *Helianthus annuus*. Isolated oospores were studied microscopically and germination was documented with respect to the time course and the mode of germination. Formation of primary sporangia was similarly observed in oospores, harvested from 4- to 6-week-old systemically infected sunflower plants, grown under constant conditions at 16°C, as well as from field plants, harvested late in the season. Pretreatment of oospores with cold temperatures, previously assumed to stimulate the rate and to accelerate the speed of oospore germination, did not result in such effects. Germination usually occurred within 10–30 days of incubation at a highly variable rate of about 1 to 17% (average 6.7%) in deionized water.

Zusammenfassung

Gewinnung von Oosporen des Falschen Mehltau, *Plasmopara halstedii*, und mikroskopische Studien zur Bildung von Primärsporangien

Es wurde eine Methode zur Gewinnung von Oosporen von *Plasmopara halstedii* aus dem Gewebe seines Wirtes *Helianthus annuus* entwickelt. Isolierte Oosporen wurden mikroskopisch untersucht und ihr Keimverhalten wurde im Hinblick auf den cytologischen und zeitlichen Verlauf dokumentiert. Die Bildung von Primärsporangien konnte sowohl bei Oosporen aus 4 bi 6 Wochen alten, unter konstanten Bedingungen bei 16°C gehaltenen Sonnenblumen beobachtet werden, als auch bei solchen aus Freilandpflanzen am Ende der Vegetationsperiode. Eine Kältevorbehandlung der Oosporen, die bisher als notwendig für die Steigerung der Keimrate und für die Beschleunigung des Vorganges angenommen worden war, erwies sich als nicht erforderlich und zeigte keine stimulierenden Effekte. Die Keimung erfolgte üblicherweise in einem Zeitraum von 10 bis 30 Tagen. Die Keimrate erwies sich als stark schwankend und bewegte sich zwischen 1 und 17% (Mittelwert 6.7%) bei Versuchen in entionisiertem Wasser.

Introduction

Primary infection of sunflower with downy mildew, *Plasmopara halstedii* (Farl.) Berl. & de Toni is caused by zoospores which are released from a primary sporangium produced by an oospore (Nishimura, 1922). Within the *Peronosporales* this is one of three possible ways for oospores to germinate. Alternatively, zoospore production in sessile vesicles was found to be typical for Albuginaceae, and direct infection with the formation of a germ tube was reported from some *Pythium* and *Phytophthora* spp. (Shaw, 1981).

Reports on the ultrastructural, chemical and physiological changes that dormant oospores of downy mildews have to pass on their way to germination are generally rare (Tommerup, 1981). In most taxa of the obligate biotrophic Peronosporaceae even the methodology for gaining oospores and for maintaining them in a viable stage for subsequent investigations of the germination process is poorly elaborated. Some progress was recently reported for oospores of *Peronospora viciae* with respect to the method of isolation from plant tissue and from soil as well as to the evaluation of their viability by means of a vital staining (Van der Gaag, 1994; Van der Gaag and Frinking, 1996, 1997a, b).

For *Plasmopara halstedii*, it took 50 years from the formerly undocumented statement on oospore germination by Nishimura (1922) to the first published illustration of germinating oospores by Delanoe (1972). Unfortunately, the methodological aspects were not described in detail in Delanoe's publication. This may be the reason that no other successful experiments, either on methods of their isolation, or on factors affecting the germination process itself, have been published since.

Considering the fact that oospores of *Plasmopara halstedii* are the primary and therefore most important source of infection for sunflower seedlings in early springtime, this is in clear contrast to the wealth of publications on *P. halstedi*, which report on the use of asexually produced zoosporangia for studies on infection, on pathotype characterization or on host pathogen interactions (for ref. see Viranyi, 1992). This indicates that handling of oospores from sunflower downy mildew appears to be extremely difficult. Nevertheless, knowledge on the development and germination physiology of the oospores could be an important aspect in the search for a better control of downy mildew infection in sunflower cultivation. The current study therefore was aimed at elaborating a simple technique for the isolation of vital oospores from sunflower tissue and to investigate their readiness to germinate under laboratory conditions.

Materials and Methods

Extraction of oospores

The presence of oospores of *Plasmopara halstedii* in tissue of infected sunflower was checked microscopically after surface treatment of the plant material with hypochloride (1% solution, 5 min). Tissue with clusters of oospores was separated under a dissection microscope and transferred into 2 ml plastic vials (Eppendorf tubes) with 1 ml deionized water. The subsequent steps followed a method described by Van der Gaag and Frinking (1996) for *Peronospora viciae*. The tissue was homogenized in a micro blender (Ultra Turrax, T8; IKA Labortecknik, Staufen, Germany). The cell suspension was treated in an ultrasonic bath (Branson 1200; Branson Ultrasonic Corp., Danbury, CT, USA) for 1–5 min and then stirred on a magnetic stirrer for 1 h at 18–20°C in order to remove oospores from adhering plant cells. The suspension was sieved through nylon filter (mesh 150 μm). If microscopic control showed the occurrence of significant amounts of unreleased oospores the residue on the filter was resuspended and homogenized again as described above. Filtrates of the 150 μm mesh size were sieved again through mesh sizes of 70 and 30 μm, respectively. The residue on the 30 μm sieve was resuspended in deionized water and concentrated through centrifugation at $1000 \times g$ for 3 min. Purity of the oospore suspension was checked microscopically and the final steps were repeated if significant amounts of fragments of plant cell walls were detected.

If necessary, further purification of oospores was achieved in two ways: (i) plant residues were removed by digestion with cellulase as described elsewhere (Van der Gaag and Frinking, 1996). Such enzyme treatment can also be helpful at earlier steps of the preparation in order to release oospores from plant tissue. (ii) The suspension was spread out on the surface of a 1% water agar and oospores were selected individually with a 80 μm glass capillary (Eppendorf transfer tips; Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany).

Germination of oospores

Purified oospores were suspended in deionized water containing rifampicin (10 p.p.m.) to obtain a concentration of approximately 3000 oospores per ml. Sixty to 80 μl of this suspension was transferred on a common microscopic slide for germination. To avoid mechanical stress through pressure on the oospores, small spots of neutral grease (Vaseline) were placed at each corner of the cover slip. The slides were then placed in a plastic box (Cell Path, Hemel Hempstead, UK) and kept in a saturated atmosphere (wet filter paper at bottom and top of the box). To test the influence of temperature on germination, the boxes were stored under the desired conditions. Germination was controlled microscopically each day and water plus rifampicin was added if necessary.

Vital stain for oospores

3 - (4,5 - dimethylthiazol - 2 - yl) - 2,5 - diphenyl - 2H - tetrazolium bromide (MTT) (Aldrich GmbH, Steinheim, Germany) was used to stain oospores for the estimation of viability according to Jiang and Erwin (1990). Oospores were incubated for 24–36 h in a 1% MTT solution in 1 mM potassium phosphate buffer (pH 6.3) at 36°C. Colours of oospores were determined microscopically.

Results and Discussion

Selection of plant material and isolation of oospores

Systemically infected sunflower plants grown in a climate chamber for about 3–6 weeks after their inoculation with zoosporangia of *P. halstedii* were used for the extraction of oospores. Although sexual reproduction in downy mildew-infected plants has been reported to occur in almost all plant parts, in the present study it was very rarely found to be taking place in leaves. In contrast to Delanoe (1972), the extraction was started from the hypocotyl instead of the roots. This was advantageous with respect to a lower contamination with biotic and abiotic material from the soil. Oospores were found in the pith as well as in the cortical parenchyma and appropriate parts of the stem were selected from longitudinal sections (Fig. 1a). Homogenization of the plant tissue is a crucial point with respect to the ratio of released to destroyed oospores. Length and intensity of the employment of the micro-blender depended on the constitution of the source material and required microscopic control. Oospores were often surrounded by remnants of the oogonium and antheridium which impeded their release from host tissue. Short treatment in an ultrasonic bath followed by intensive stirring on a magnetic stirrer facilitated this process. After filtration through 150 and 70 μm nylon sieves, oospores were mostly cleared from plant tissue. Subsequent filtration through 30 μm mesh removed cellular debris and the oospores were retained on the sieve and could be retrieved for further investigation (Fig. 1b).

MTT staining of oospores

Homogenization of plant tissue obviously destroyed part of the oospores so that empty hulls were visible under the microscope. Other damage from the rough extraction might be invisible, but could still inhibit physiological functionality. A test for the estimation of oospore viability could be a useful means for setting up ger-

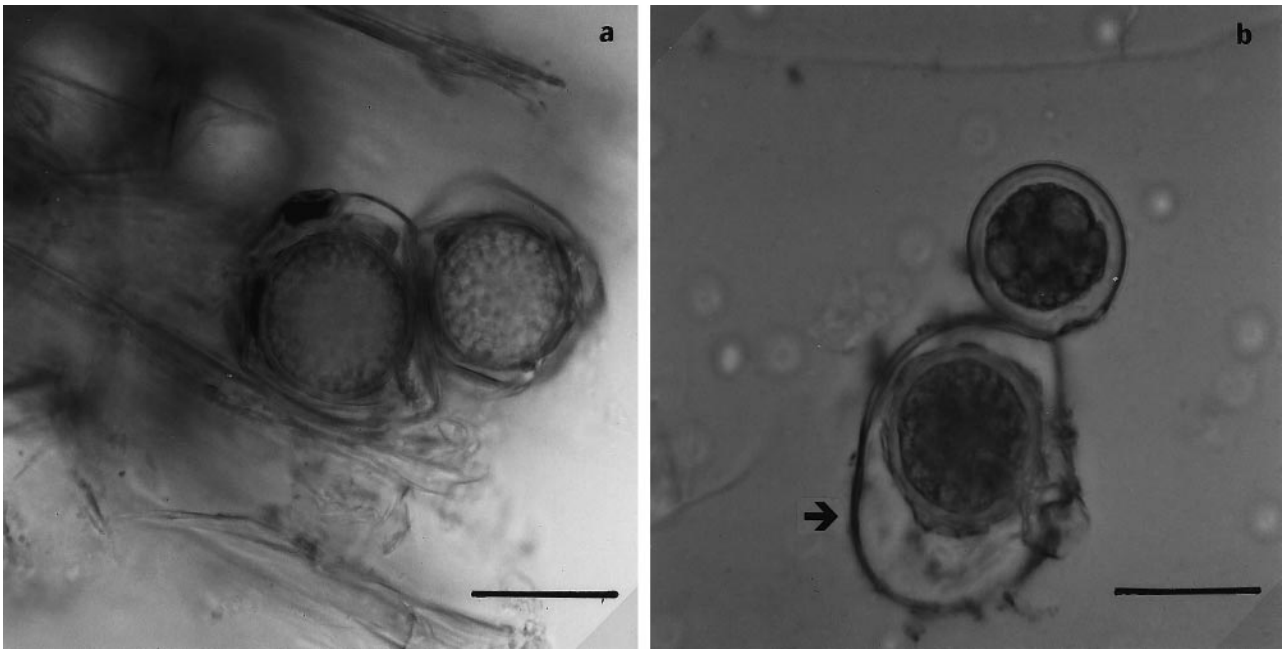

Fig. 1 Oospores of *Plasmopara halstedii*: (a) clusters of oospores in a longitudinal section of infected sunflower hypoctyl; (b) oospores with (arrow) and without adhering residues of the oogonium as separated from plant material after homogenization and sieving. Bar equals 30 μm

mination tests. The enzymatic reduction of tetrazolium bromide in the cytoplasm of oospores has been proposed as a measure for viability in several Oomycetes (Sutherland and Cohen, 1983; Cohen, 1984; Jiang and Erwin, 1990; Van der Gaag, 1994).

Oospores from *Plasmopara halstedii* stained either pale rose to bluish violet (as given in the literature for supposedly vital cells), or black or remained unstained (both types considered nonviable), although, colour intensity and shade from rose to violet varied considerably. The rate of rose plus violet-stained oospores in plant tissue sections ranged from about one-third to one-half. Homogenization in the micro-blender and short ultrasonification (up to 10 min) did not significantly reduce the rate of rose plus violet stained spores (Table 1). With respect to the reliability of this test it should be mentioned, however, that pale violet coloration also occurred in some oospores which were boiled for 5 min prior to staining. This coincides with the observation of Sutherland and Cohen (1983) who found up to 5% of rose-stained oospores of *Phytophthora megasperma* after autoclaving and therefore suggested that MTT cannot be used solely for determining oospore viability.

According to Jiang and Erwin (1990), rose-coloured spores are regarded as viable, but dormant, whereas violet staining should be regarded as indicative of activated cells. Since staining prohibited subsequent germination of oospores of *P. halstedii*, this correlation could not be investigated. Nevertheless, with some experience the MTT stain appeared to be a suitable test for a rough estimation of viable to nonviable cells in oospore preparations.

The process of germination

Germination of isolated oospores was assayed on microscopic slides in a volume of 60–80 μl liquid. Incubation over a period of up to 6 weeks in a small plastic container in a saturated atmosphere necessitated the addition of liquid every 3 to 4 days to keep the oospores continuously humid. Growth of bacteria was limited through the addition of rifampicin, an antibiotic which was previously shown not to affect the growth of sunflower downy mildew (Sackston and Anas, 1991; Spring et al., 1998). The rate and time course of germination in isolated oospores varied considerably. In successful experiments in deionized water at 16°C and in darkness the germination rate ranged from 1 to 17%, with an average of 6.7% (SEM 1.9; $n = 10$). It is worth mentioning that in about one-third of the preparations, germination failed completely, without obvious reasons. Germination in a sample

Table 1
Colour reaction after MTT staining of oospores of *Plasmopara halstedii* [number (percentage)] at different steps of preparation (results taken from a representative experiment)

Preparation step	Black	Unstained	Rose	Violet	Total no.
Oospores:					
in tissue sections	2 (1)	126 (64)	45 (23)	23 (12)	196
after homogenization	12 (7)	107 (60)	32 (18)	26 (15)	177
after 1 min ultrasonification	14 (7)	123 (63)	36 (18)	23 (12)	196
after 10 min ultrasonification	9 (4)	152 (69)	43 (19)	17 (8)	221

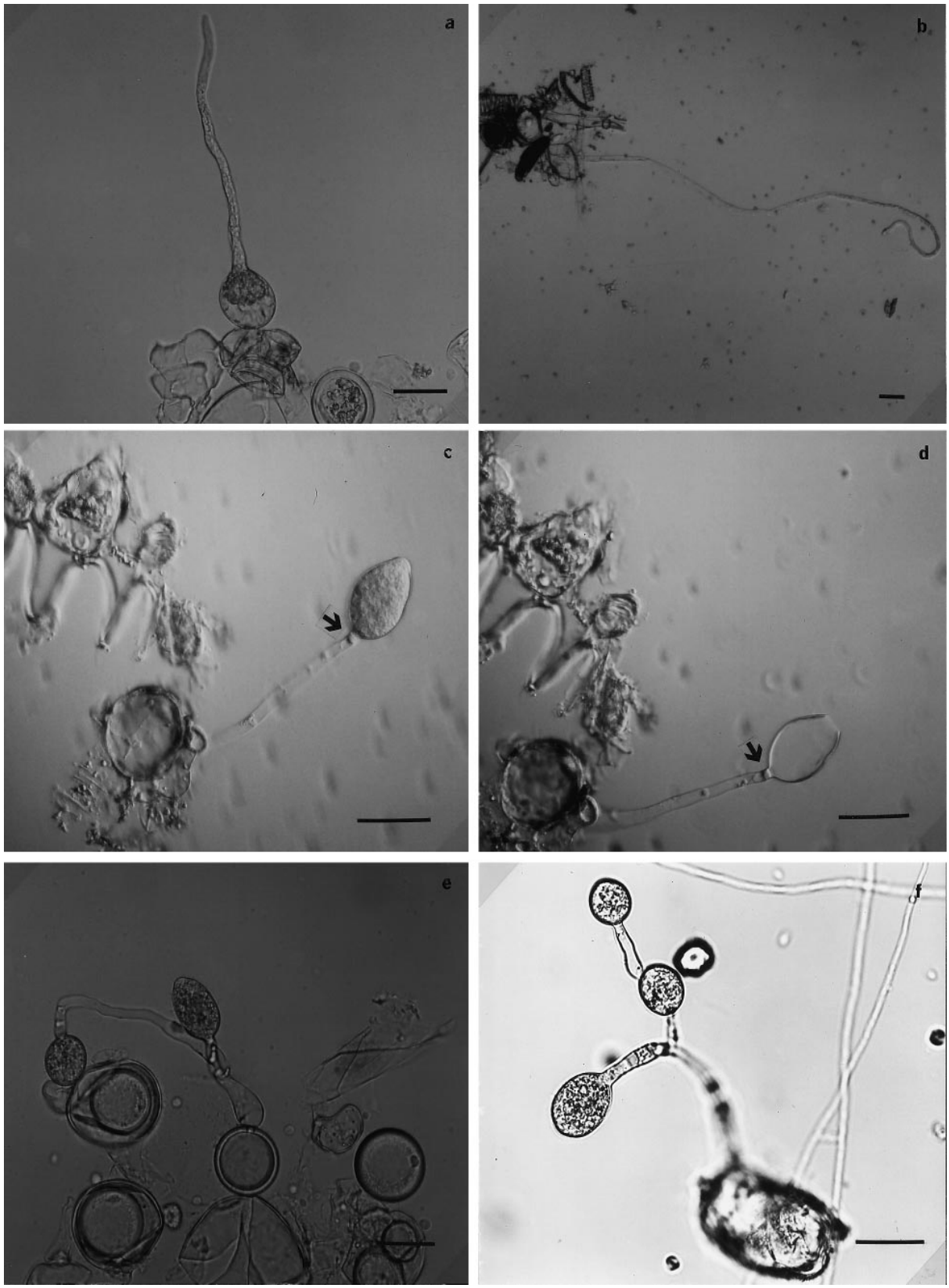

Fig. 2 Formation of primary sporangia from isolated oospores: (a) germ tube development and movement of the plasma towards the developing structure; (b) unusually long germ tube reaching a final length of 750 μm; (c) typical primary sporangium filled with plasma and separated from the germ tube by a septum of callose (arrow); (d) empty sporangium after release of zoospores through a circular opening at the tip; (e) and (f) formation of two or three sporangia after branching of the germ tube. Bar equals 30 μm

Table 2
Influence of temperature on the germination of *Plasmopara halstedii* oospores

Incubation temperature	5°C	11°C	16°C	22°C
Germination rate	0	1.9% (SD 2.2)	6.1% (SD 4.3)	2.5% (SD 3.5)

required a minimum of 6 days of incubation and occurred over a period of up to 4 weeks.

Once started, the formation of the primary sporangium usually required about 6–10 h. It started with the development of a germ tube and the plasma of the oospore moved towards its distal end (Fig. 2a). The length of the germ tube averaged about 100 μm, but extremes of up to 750 μm could be observed (Fig. 2b). At the end of the germ tube a sporangium was being formed that resembled in form and size the sporangia of the asexual part of the life cycle (Fig. 2c). Once the plasma had totally concentrated in the primary sporangium, a septum of callose (stainable with resorcin blue) prohibited backstreaming of the plasma. A few hours later zoospores (about 15–25 per sporangium) were released through a circular opening at the apical tip of the sporangium (Fig. 2d). They encysted and formed a germ tube with apressorium similar to the asexually formed zoospores.

In some cases two ore more sporangia were observed to develop from a single oospore (Figs 2e, f). This could be due to branching of the germ tube or by the formation of independent tubes. This coincided with the observations of Delanoe (1972) who documented several types of such variations.

Factors affecting germination

According to Delanoe (1972) oospores require a minimum amount of cold treatment before germination. This would simulate the natural stratification in most natural habitats of *Plasmopara halstedii*. Untreated oospores were reported to show very low germination rates (0–1%) and retarded development (germination time 20 days) when compared with those kept at 4°C to −11°C (up to 25% germination within 4–16 days).

No such influence from temperatures was observed in the present experiments. Pretreatment of plant material and oospores with low temperatures (5°C for 1 to 9 weeks or −18°C for 1 to 4 weeks, respectively) prior to the germination test showed no stimulatory effect in comparison to material kept at 16°C in a climate chamber or stored at room temperature after harvest of the host plant. Stratification was obviously not required for maturation of the oospores. Material gained from field plants in late September and in October did not show higher germination rates than oospores from 4-week-old plants grown under constant conditions at 16°C in a climate chamber. As for the latter, the highest germination rates were found at 16°C and germination did not occur, when oospores were kept below 11°C (Table 2).

So far, most germination tests were performed in deionized water. A diluted salt solution according to Machlis (1958), which was shown to give good results for the zoosporogenesis of asexually formed zoosporangia of *Plasmopara halstedii* (Spring et al., 1998) did not improve germination rates of oospores. Attempts to improve germination by means of other ingredients and to test the effect of host-derived compounds are the aim of future investigations.

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