Techniques for Formation, Maturation, and Germination of *Plasmopara viticola* Oospores Under Controlled Conditions

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ABSTRACT

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Sexual reproduction of *Plasmopara viticola* was induced in detached leaves inoculated with sporangia under controlled conditions. Oogenesis was stimulated by thermal shock from 20 to 10 C. Oospore maturation in controlled conditions required incubation at low temperatures of 10 and −5 C, alternating weekly. The transfer of oospores separated from leaf tissues to water agar in petri dishes at 20 C enabled the dynamics of oospore germination to be determined. Oospores germinate within 6–10 days, but a period of at least 5 mo after inoculation was necessary for optimum germination. The technique for assessing germination of mature oospores can also be used on oospores maturing naturally, and thus allows an evaluation of primary infection risks in the spring.

The biological cycle of grapevine downy mildew caused by the fungus *Plasmopara viticola* (Berk. & Curt.) Berl. & de Toni comprises an asexual multiplication phase occurring during the vegetative period of the vine, and a sexual phase that ensures survival of the pathogen over winter (2,11,17). Unlike the asexual phase, whose parameters of development are relatively well known, there is relatively little knowledge of the conditions of formation, maturation, and germination of oospores. This stage is particularly important since zoospores, liberated from macrosporocysts released by oospores, are responsible for initiation of the disease in the spring. However, it is still unknown whether the quantity and quality of available primary inoculum in spring plays a decisive role in the later epidemic development. The principal risk factor recognized by plant protection services is the speed of germination of the oospores under conditions of optimal temperature (20 C). These services consider that there is the risk of contamination in spring when the oospores are able to germinate in less than 24 hr. Indeed, at such a stage in maturity, any rainy period of 24 hr may provide the conditions for primary infection. However, it is not possible to determine whether this probability affects a high or low proportion of oospores. The role of rainfall for survival of the oospores has been largely emphasized (2), but the role of temperature seems more complex. Temperature should be above 11 C for the germination of mature oospores to take place (1), but the most favorable conditions for their maturation remain unclear.

Using the meteorological data of CAPUS between 1907 and 1915, coupled with observations on mildew evolution, Strizyk (14–16) has recently developed a biomathematical model establishing the fundamental role of winter conditions on epidemic risk. This model has been under test since 1981 (5–7).

In order to have accurate biological bases for improving the model, one must determine the sexual cycle of *P. viticola* under controlled conditions, and measure the principal parameters (oospore formation, length of maturation, germination dynamics) under the effects of the natural environment. For this, we developed methods for manipulating and assessing oospore maturation and germination under artificial or natural conditions. These methods are described in the paper.

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MATERIALS AND METHODS

Oospore acquisition on detached leaves. The oospores of *P. viticola* can be formed in the vineyard at any time during the season. In nature, we have observed them in large numbers in autumn, but also on young vine plants in May and on older vines at the beginning of August. Our hypothesis was that a drop in temperature, after a sufficiently warm and damp period for the intercellular mycelium to form, would favor oospore formation.

The presence of oospores in leaf tissue is not necessarily associated with the characteristic "patchwork" symptoms. This is particularly true when the leaves are scorched by drought; they can have brown necrotic areas, which are not characteristic, but contain abundant oospores.

From these observations, we developed a technique for obtaining oospores on sporocyst-inoculated leaves in the laboratory, under the following specific controlled conditions.

The leaves were taken from Muscadelle vine cuttings and cultivated in a greenhouse at 25 C in pots filled with coarse sand watered with nutrient solution every 3 days. The Muscadelle cultivar was chosen for its susceptibility to downy mildew.

The leaves were inoculated by spraying with a fresh aqueous suspension containing 2.5×10^4 sporocysts per milliliter prepared from a natural population of sporangia of *P. viticola* picked up with a paint brush. They were kept alive in mini greenhouses in conditions of saturated humidity, with the petiole immersed in water in a hemolysis tube. The first week after inoculation, the leaves were placed in a room at 18–20 C and received 12 hr light ($65\ \mu E \cdot s^{-1} \cdot m^{-2}$) per day. Under such favorable conditions for downy mildew, the parasite developed abundant inter-

cellular mycelium and sporulations appeared. At the end of the first week, the temperature was lowered to 10–12 C, thereby stopping fungal sporulation. About a month after inoculation, the oospores thus obtained were placed under favorable conditions for maturation.

Maturation and germination of oospores formed in natural conditions was studied on mildew-affected vine leaves (cv. Malbec) that were picked on 31 October at the INRA Research Centre in Bordeaux. The Malbec cultivar was chosen for the thinness of its leaves, which facilitates the location of oospores under a stereomicroscope (magnification ×75).

Once the foliar zones that were rich in oospores were located in naturally or artificially contaminated leaves, 6-mm-diameter disks were taken with a punch and stored inside plaster modelling tubes (L = 40 mm; O int. = 12 mm; O ext. = 30 mm), as is the common practice in the agricultural forecasting stations of the French Plant Protection Services (13) (Fig. 1).

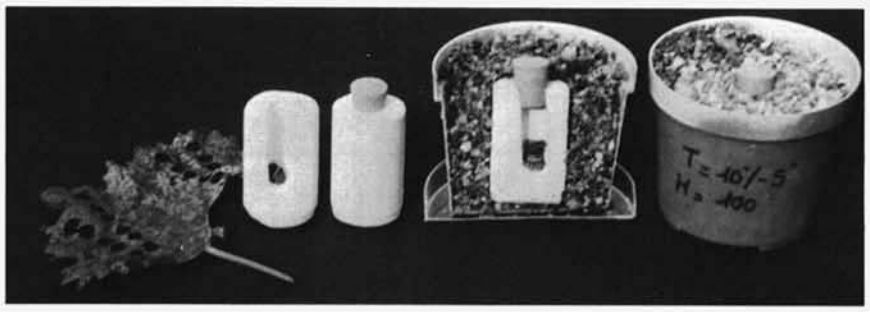

Fig. 1. Procedures in the treatment of leaf disks containing oospores of *Plasmopara viticola*.

Table 1. Climatic conditions at Bordeaux during experiments on oospore maturation

Temperature (C)	1984		1985					
	Nov.	Dec.	Jan.	Feb.	March	April	May	June
Average	11.6	6.8	1.4	8.8	7.8	12.9	14.2	18.3
Minimum	8.4	3.8	−1.9	4.7	3.4	7.9	9.6	13.4
Maximum	14.8	9.9	4.8	12.9	12.3	17.8	18.9	23.1
Rainfall (mm)	139.6	65.4	51	82.1	107.5	68.4	114.3	44.9

Oospore maturation. *Artificial and controlled conditions.* After a comparative study of several thermal and water parameters, we decided on the following method: the tubes were placed in 8-cm-diameter plastic pots filled with coarse sand, and then placed in controlled environment chambers, alternating 1 wk at 10 C and 1 wk at −5 C. The tubes at 10 C were watered every 2 days; the porosity of the plaster made it possible to maintain the disks through the entire phase of oospore maturation in a totally saturated atmosphere (Fig. 1). Three months after inoculation, oospore germination was measured every 15 days.

Natural conditions. A tube containing 60 disks of leaves with oospores was buried at a depth of 5 cm in soil at the INRA Centre of Bordeaux, and thus exposed to natural climatic conditions. The conditions were as follows (Table 1).

Oospore germination. To estimate the percentage of oospores likely to germinate, we used an isolation method of oospores on water agar, which was adapted from work by Morgan on *Bremia lactucae* (10). The disks were removed from the plastic tubes, placed on a slide holder, and chopped finely with a blade to free the oospores from the foliar parenchyma. The blend, which was suspended in water, was filtered on a 45-μm gauze. The new suspension, which contained mainly oospores, was concentrated by filtration on a 12-μm micropore filter. The oospores were regularly moistened to avoid desiccation.

Under a stereomicroscope (enlargement ×75), and with a very fine Pasteur pipette, the oospores were aspirated by capillarity and placed on 1% water agar in petri dishes at a rate of 10 lots of 10 oospores each per dish. For this purpose, a 5 × 2 cm rectangle of graph paper (10 squares of 1 cm^2) was stuck to the base of each dish on the outside. Each lot of 10 oospores was placed over a square in order to facilitate future counting of the macrosporocysts. Each dish was checked under the stereomicroscope to ensure that it contained 100 oospores from each leaf sample.

The dishes were then placed in a controlled environment chamber at 20 C for optimal speed of oospore germination. Every day for 2 wk, the number of macrosporocysts formed was counted for each dish under a stereomicroscope (Fig. 2). After counting, the macrosporocysts were removed with a fine needle so that they were not included in later counts.

With these measurements, it was possible, for each date of sampling, to determine the percentage of oospores capable of germination, and their speed of germination. The latter parameter was measured by the minimum speed (minimum number of days necessary for the appearance of the first sporocyst) and by the average speed (aS) according to the following formula:

$$aS = \sum_{i=o}^{15} (\text{number of germinated oospores on day } i \times Ji)/\text{total number of germinated spores, where } Ji = \text{number of days in climatic chamber.}$$

Pathogenicity of macrosporocysts. From the macrosporocysts formed in similar conditions, we tried to obtain primary infection in the laboratory. For this, the macrosporocysts were taken from the pedicel with a fine needle and placed in a drop of water on the lower side of a vine leaf disk that was placed in a petri dish on filter paper soaked in deionized water and incubated in a culture chamber at 20 C.

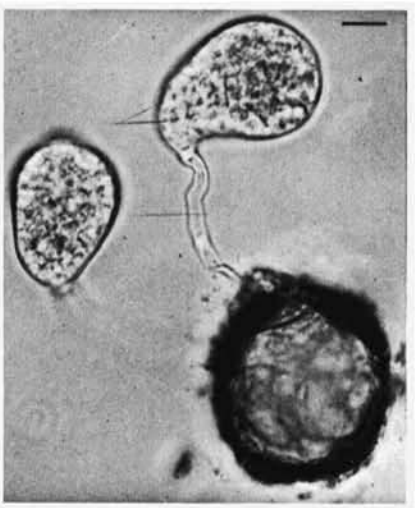

Fig. 2. Oospore germination on water agar to form a macrosporocyst (bar = 10 μm).

RESULTS

Artificial induction and maturation of oospores. From the 18th day following inoculation in the described conditions, the first oospores were visible around the slightly necrotic areas. Afterwards, symptoms comparable to those observed in nature appeared. The oospores became more and more abundant but they varied in quantity from leaf to leaf.

Under controlled maturation conditions, the first mature oospores were obtained after 3.5 mo, and the greatest number of mature oospores was obtained after 4 mo (Fig. 3). After 5 mo, the percentage of germination decreased. The minimum germination time varied between 6 and 10 days, but on the average it was about 7 days. The average

germination period, calculated for 498 oospores, was 8.48 days. This result shows that there is no relation between the maturation level and the germination speed.

From the macrosporocysts formed, it was possible to obtain primary infection in the laboratory. Liberation of zoospores (of which there may be as many as 60 in the large macrosporocysts) occurred in less than 30 min at 20 C. Sporulation occurred also from the lesions initiated from single sporocysts.

Natural maturation. Maturation in natural conditions occurred earlier than in artificial conditions (about 2 mo). Indeed, certain oospores were already mature in January and the optimal rate of germination occurred at the beginning of March. However, in natural conditions, only a quarter of the oospores germinated. Moreover, from 10 April onwards, the rate of germination decreased considerably and was nil at the end of June (Fig. 4).

As a parallel to the evolution of the germinative power of the oospores, one may note a progressive decrease in the time necessary for germination at 20 C, i.e., the time of incubation in the climate chamber (Fig. 4). Therefore, an average time of 7.1–7.8 days during the optimal germination phase (4 March–10 April) dropped to only 5.2 days by 6 May. As for the minimum time, it decreased regularly to 24 hr from 22 May onwards.

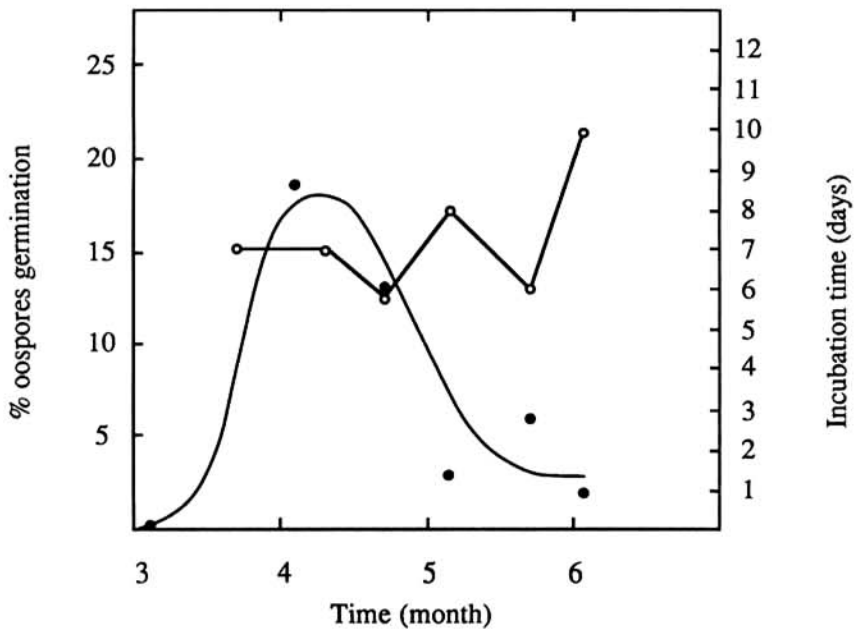

Fig. 3. Germination period (days) at 20 C of oospores of *Plasmopara viticola* under controlled maturation conditions of alternating 7 days at 10 C, 7 days at −5 C. Open circles = minimum days to germination, solid line = percentage of germination.

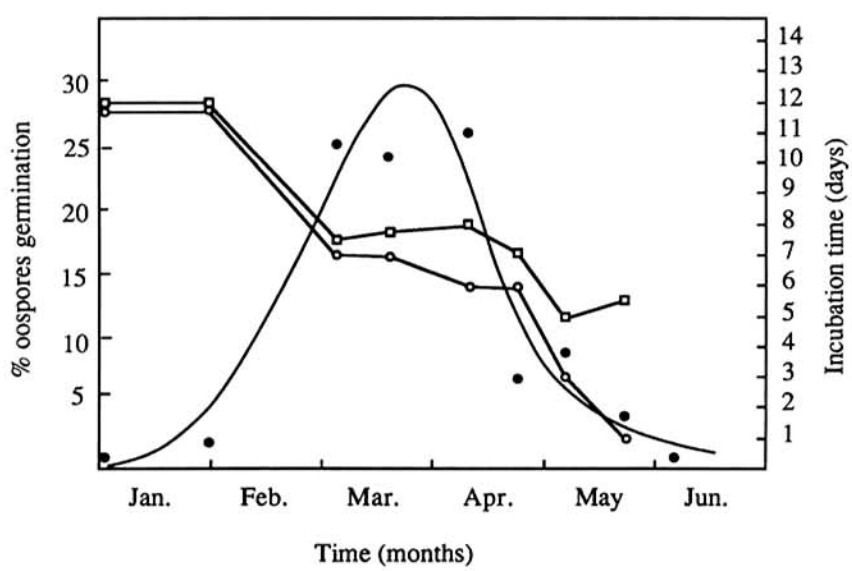

Fig. 4. Percent and time to germination at 20 C of naturally matured oospores. Open circles = minimum days to germination, open squares = average days to germination, solid line = percentage of germination.

DISCUSSION

The simple techniques described above make it possible to control each phase of the sexual reproduction of *P. viticola*, but they have certain limitations.

The first concerns the number of oospores formed in the infected leaf tissues, which is generally variable and often insufficient for experimental purposes. However, the percentage of oospores able to germinate did not appear significantly different in natural or artificial conditions of maturation. Similar difficulties occurred in *B. lactucae* infecting lettuce (3,9). But in that case, those difficulties were minimized by selecting the tissues to be infected, as well as the strain of the heterothallic fungus (8). In *P. viticola*, in which heterothallism has never been demonstrated, it might also be possible to improve the induction of oospore formation with better knowledge of the compatibility between strain and the ontogenetic stage of leaf tissue that is the most favorable to oogenesis in the host.

The second factor determining the supply of mature oospores of *P. viticola* lies in the duration of the maturation phase (4 mo minimum). This is comparable to that observed in nature and may be connected to the need for the fungus, which is an obligate parasite, to produce macrosporocysts after the appearance of susceptible leaves on the vine. In the other genera of Peronosporales, the period of maturation seems to be shorter. Maturation is a minimum of 23 days for *B. lactucae* infecting lettuce (10) and about 1 mo in a *Phytophthora* sp. cultivated on an artificial medium (12). Maturation time may be related to the intervegetative stages of the host crop.

The rate of oospore germination obtained with *P. viticola* may reach 50% and is normally satisfactory for experimental purposes. Germination of oospores of *P. viticola* appeared to be similar in natural oospores and in those formed and matured in artificial conditions. In contrast, germination of oospores is rarely greater than 5% in *Phytophthora* sp., despite the stimulating effect of certain factors such as light quality or the addition of enzymes in the medium (12). In lettuce, the proportion of viable *B. lactucae* oospores is often lower than 10% (9). By eliminating oospores that are apparently defective, and in the presence of lettuce seeds that play a stimulating role, this proportion may be as high as

23–33%, according to the age of the oospores (10).

To compare and explain the differences in the germination kinetics of oospores placed in artificial or natural conditions for maturation, it must be noted that in the former case, the germination of mature oospores inside plaster tubes was not possible because the temperatures were too low (less than 11 C). In natural conditions, however, from April onwards, the temperatures around the oospores progressively moved higher above 11 C, and the process of germination was, therefore, able to take place.

The decrease in incubation time at 20 C, which is necessary for the formation of the sporocysts, may be explained by the fact that the germination process is already under way in a certain number of oospores. Those that are capable of germinating in the space of 24 hr during May have, evidently, reached the end of this process. So it may be that the incubation time necessary for oospore germination at 20 C does not decrease progressively to a few hours only for mature oospores according to the state of maturation of the oospores, as the general opinion has been until now. On the contrary, the incubation time of mature oospores may be constant at a given temperature, e.g., an average of 7–8 days at 20 C. In effect, these values correspond to those obtained in both artificial or natural conditions of maturation when a low temperature precludes germination. It is possible that the maturation time is longer (11–12 days) in oospores that mature earlier.

The methodology described above, in which all stages of the sexual cycle of *P. viticola* may be controlled, makes it possible to study the influence of climatic factors on oospore maturation, and thereby to establish bases on which to construct epidemiological risk models. The study of the dynamics of oospore germination helps us to evaluate the risk of primary infection in spring. Such criterion is probably better adapted than measurements of the speed of oospore germination that are currently in use for assessing risk, because the speed and the optimal rate of germination may not be correlated.

Control of the sexual cycle of *P. viticola* in the laboratory is also opening up new horizons concerning the genetic variability of the fungus with regard to its pathogenic ability (4), resistance to fungicides (13), and to the susceptibility of oospores to fungicides.

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