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SHORT NOTES

Influence of environmental factors on germination of *Plasmopara viticola* sporangia sourced from mediterranean Western Australia

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Summary. Direct germination via the production of a germ tube was demonstrated in the absence of the host for *Plasmopara viticola* (causal agent of grape downy mildew) sporangia sourced from a major grape production area in mediterranean Western Australia (WA). In general, direct germination was favoured by environmental conditions considered less than optimal for infection by *P. viticola*. Most notable however was that sporangia had the capacity to germinate in the absence of free water, a factor known to be essential for the production and release of zoospores. The frequency of direct germination was low with only seven among 108 000 sporangia observed producing a germ tube. This is the first study to i) examine the influence of environmental factors *viz*: temperature, relative humidity (RH) and light, on the direct germination of *P. viticola* sporangia and ii) establish the frequency of this event. The infectivity of directly germinated *P. viticola* sporangia however remains unknown. Although rare, the capacity of sporangia to germinate directly and potentially infect the host, most likely when conditions are not conducive for zoospore production or survival, may provide an explanation for the source of the disease during the predominantly hot dry summer months in WA and other climatically similar viticultural areas.

Key words: epidemic, oomycete, survival.

Introduction

Grape downy mildew caused by the biotrophic oomycete *Plasmopara viticola* is one of the most important diseases of grapevines worldwide (McLean *et al.*, 1984). Grape downy mildew is not normally considered a problem in mediterranean climates (Mullins *et al.*, 1992) because of the usual dry periods between the wet conditions that favour the disease. Grape downy mildew is however a major disease in Australian viticulture (Magarey *et al.*, 1991).

Two mechanisms of sporangial germination are known for oomycete pathogens: direct, by the production of a germ tube, and indirect, via the production and release of zoospores. The particular germination mechanism is often species-specific; however, some species e.g. *Phytophthora infestans* and *Ph. cryptogea* can germinate by either mechanism depending on the prevailing environmental

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conditions (Colhoun, 1973, Erwin and Ribeiro, 1996). Usually, *P. viticola* sporangia liberate zoospores in free water (Lafon and Clerjeau, 1988). Zoospores then germinate to form a germ tube which penetrates through the stoma. Direct germination of *P. viticola* sporangia was however proposed in several historical studies conducted in Europe (Viala, 1893; Gregory, 1915). This phenomenon was reliably demonstrated much later in Germany by Kortekamp and Zyprian (1999).

To our knowledge, no attempts have been made i) to experimentally determine the direct germination of *P. viticola* sporangia in Australia; ii) to investigate the influence of the environmental factors *viz*: temperature, light and relative humidity (RH) on the process and iii) to establish the frequency of direct germination for *P. viticola*. The mechanisms by which *P. viticola* sporangia germinate directly or indirectly are still far from clear (Kortekamp and Zyprian, 1999).

In the mediterranean climate in Australia, for example in Western Australia (WA), the capacity of *P. viticola* sporangia to germinate directly and subsequently penetrate the host would provide the pathogen with an alternative way to initiate the infection process, most likely when environmental conditions are not conducive for zoospore survival and germination.

The aim of this study was to quantitatively assess the direct germination of *P. viticola* sporangia from WA under a variety of controlled environmental conditions. Germination was examined in a host-free system to avoid host-mediated effects and to facilitate assessment. The potential implications of the findings with regard to the development of grape downy mildew in environments such as those in WA are discussed.

Methods

Cultures and inoculum production

A collection of *P. viticola* isolates was obtained from infected grapevine leaf material sampled from naturally affected vines in the Swan Valley (-31.83 S, 116.02 E), a major grape production area in WA. To isolate the pathogen, the infected material was surface-sterilised (Emmett *et al.*, 1992) and incubated overnight at 22°C in a dark humid environment to induce sporulation.

The pathogen was maintained in the laboratory on detached glasshouse grown *Vitis vinifera* cv. Chardonnay laminae. Rootlings were planted into a mixture of steam sterilised composted pine, cocoa peat and river sand (2:2:1, v:v:v) in 200-mm free-draining black plastic pots (one rootling per pot). Pots were kept in a glasshouse where the temperature was regulated by air-conditioning and maintained so that the minimum temperature was 11°C and maximum 27°C. Vines were watered as needed and fertilised every 3–4 weeks using Phostrogen (Phostrogen Ltd., Bayer Crop Science Ltd., Cambridge, UK) at a rate of approximately 2 g l^{-1}.

Inoculum was produced by spraying the entire abaxial surface of fresh leaves with an aqueous suspension of *P. viticola* sporangia using a simple hand held atomiser. The inoculated leaves were incubated overnight at 22°C in a dark humid environment and then exposed to a natural diurnal light cycle in the laboratory under the same conditions of temperature and RH. Usually 5 to 6 d after inoculation, sporulation could be observed.

Experimental procedure and treatment application

Sporangia were deposited, without water, on the surface of microscope glass slides. Glass slides have been utilised in several previous studies to germinate spores of several different types of biotrophic fungi including *Erysiphe (Uncinula) necator* (grape powdery mildew) (Rea and Gubler, 2002) and *Puccinia graminis* f. sp. *tritici* (wheat stem rust fungus) (Maclean, 1982). To harvest and deposit sporangia, a heavily sporulating leaf was held approximately 100 mm above the glass slides and a small dry camel hair paint brush was used to gently dislodge the sporangia. The slides were placed in RH chambers (one slide per chamber) which consisted of a Petri dish (90 mm diameter and 20 mm deep) containing 15 ml of a saturated salt solution and a V-shaped glass rod to elevate the slide.

Saturated salt solutions of K_2SO_4, NaCl, NaBr and $MgCl_2$ were used to produce RH levels of 96–97, 75–75.8, 57–58 and 32.5–33.5% respectively (Sun, 2002). The RH chambers were sealed using Parafilm® (Pechiney Plastic Packaging, Chicago, IL, USA) and a weight was applied to the lid to ensure that the seal remained in place. The RH chambers were then placed inside lit temperature-controlled rooms set at 10, 22 or 37°C. To administer a dark treatment, half the chambers were kept inside plastic containers surrounded with aluminium foil during the incuba-

tion period. Slides were removed from the chambers after 1, 3 or 5 h incubation. To fix the sporangia, the slides were i) gently heated over a low flame and ii) sprayed with a water soluble fixative Spray-Cyte® (Clay Adams, Parsipanny, NJ, USA). Three slides were allocated to each combination of light, temperature, RH and sampling time (216 slides in total). As a control, the surface of three glass slides was sprayed with a fine mist of water before sporangia were deposited. These slides were maintained in darkness at 22°C and high RH for the duration of the investigation.

Light microscopy

Following fixation of sporangia, a drop of cotton blue and a cover slip was placed on each slide, after which they were observed under a light microscope. The numbers of directly germinated sporangia, out of the first 500 sporangia counted on each slide, were recorded.

Statistical analysis

The main effects of temperature, time, light and RH as well as the interaction of these treatment factors on direct germination was determined by ANOVA using a general analysis of variance design in Genstat 7th edition (VSN Internationl Ltd., Hemel Hempstead, UK).

Results and discussion

Sporangia that had germinated directly were observed on six of the 216 slides examined in this study. The germinated propagules had an obvious protrusion, resembling a typical conidial germ tube, extending from the apex (Fig. 1a). In earlier studies, the production of a germ tube directly was described as a potential mode of germination for *P. viticola* (Viala, 1893; Gregory, 1915). Sketches of this mode by Viala (1893) (Fig. 1b) bear a striking resemblance to the directly germinated sporangia

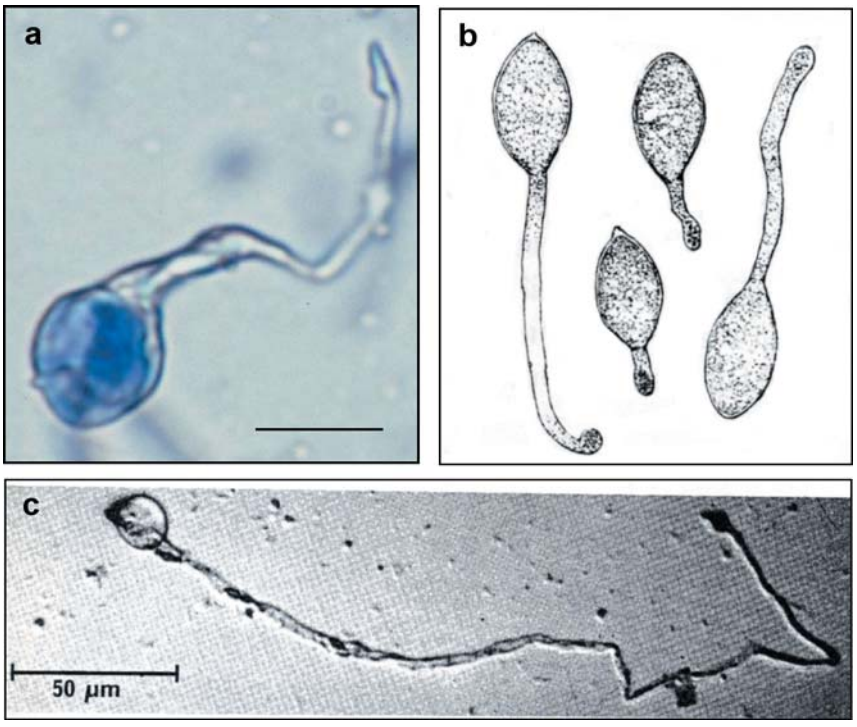

Fig. 1. Typical morphology of directly germinated *P. viticola* sporangia sourced from Western Australia (bar=10 μm); b) Germination of a *P. viticola* sporangia by "tube mycelium" (germ tube) as illustrated by Viala (1893); c) Long germ tube, devoid of cytoplasm, produced by a conidium of *Bremia lactucae* following germination *in vitro*. (Crute and Dixon, 1981). Reprinted from Crute and Dixon (1981) with the permission of the publisher.

observed in this study. Interestingly, the germinated sporangium in Fig. 1a is very similar in appearance, though the germ tube is not as long, to a directly germinating *Bremia lactucae* (lettuce downy mildew) conidium observed by Crute and Dixon (1981) (Fig. 1c).

On the control slides, zoospores were released within 1 h following the deposition of sporangia in water. There was no evidence of direct germination on these slides with nearly all (>90%) sporangia producing and releasing zoospores after 5 h incubation. Several sporangia had not germinated by the conclusion of the investigation.

It should be noted that the frequency of occurrence of direct germination was very low, with usually only one or two sporangia, among the 500 counted per slide, producing a germ tube. Often, there was no evidence of direct germination. A total of 108,000 sporangia were observed in this investigation. Of this total number, only seven sporangia exhibited a germ tube. Therefore, less than 0.0065% of *P. viticola* sporangia germinated directly. It was recognised in the earlier literature that any mode of germination other than the release of zoospores from *P. viticola* sporangia would be rare (Gregory, 1912). The findings from the current study confirm this. The rarity of the event has been recorded for other oomycete pathogens that typically produce zoospores. These include the downy mildews *Sclerospora graminicola* (Weston, 1924) and *Plasmopara halstedii* (Goossen and Sackston, 1968), and within the Pythiaceae, *Ph. cryptogea* and *Ph. infestans* (Colhoun, 1973, Erwin and Ribeiro, 1996).

Direct germination of *P. viticola* occurred under six of the 72 treatment conditions tested in this investigation (Table 1). Directly germinated sporangia were only observed on one of the three replicate slides assigned to each of the six treatment conditions. Of the treatment factors investigated, RH was found to have a significant influence ($P<0.05$) on direct germination. Sporangia which produced long germ tubes through direct germination were observed most often on slides which had been exposed to 57–58% RH. Direct germination was not observed on slides incubated in 97 or 30% RH, nor at 10°C.

The most significant finding in this study was that *P. viticola* sporangia had the capacity to germinate directly in the absence of free water, a factor previously thought to be essential for infection. When conditions do not favour the production and release of zoospores, *P. viticola* may be forced to employ alternative modes of germination to initiate the infection process in preparation for subsequent epidemics when weather conditions become conducive. This response is similar to those of *Ph. infestans*, where higher temperatures favour the formation of a germ tube instead of the release of zoospores, and *Ph. cryptogea*, where very wet or saturated soil conditions induce zoospore formation, whereas slightly drier conditions favour direct germination (Colhoun, 1973, Erwin and Ribeiro, 1996). Rather than perishing and/or becoming non-functional, *P. viticola* sporangia may use direct germination as a survival mechanism when conditions are not conducive for indirect germination which is commonly associated with noticeable epidemics.

Table 1. Treatment conditions under which *P. viticola* sporangia germinated directly via a germ tube. Sporangia were deposited without water onto the surface of glass slides and exposed to various combinations of temperature (10, 22 or 37°C), relative humidity (RH) (32.5–33.5, 57–58, 75–75.8 or 96–97%) and light (on or off) for 1, 3 or 5 h. Three replicate slides were allocated to each combination of the treatment factors. Numbers of directly germinated sporangia, out of the first 500 sporangia counted on each slide, were recorded. Directly germinated sporangia were only observed on one of the three replicate slides assigned to each of the treatments listed below.

Temperature (°C)	RH (%)	Light	Time (h)	No. of directly germinated sporangia	Germination (%)
22	57–58	on	1	1	0.2
22	57–58	off	3	1	0.2
37	57–58	off	3	2	0.4
37	57–58	off	5	1	0.2
37	57–58	on	5	1	0.2
37	75–75.8	off	5	1	0.2

Despite the lack of summer rainfall and hence a relatively dry growing season in WA, warm wet conditions can still occur interspersed in the season sufficient to favour the development of grape downy mildew outbreak. If such conditions occur at suitable frequency in relation to infection and the incubation periods, the disease can proliferate to epidemic proportions despite the otherwise dry conditions.

The infectivity of directly germinated *P. viticola* sporangia remains undetermined. In a study by Kortekamp and Zyprian (1999), the authors observed direct germination of *P. viticola* sporangia on *Vitis* spp. differing in leaf hair density. In that study, sporangia adhered to the leaf hairs well above the epidermis and as a result the germ tubes they produced were unable to bridge the distance to the leaf surface and infect the host. In future studies, the direct germination of *P. viticola* must be examined in a host-pathogen system, using a relatively glabrous cultivar of *V. vinifera*, to determine i) the influence of host factors on the germination process i.e. frequency and morphology and ii) the capacity of directly germinated sporangia to initiate infection.

Though direct germination of *P. viticola* appears to be a rare event, the contribution of directly germinating sporangia, if they are able to penetrate the stomata and infect the host, to the development of epidemics in WA and other climatically similar areas elsewhere should not be underestimated. It may require only a single propagule, whether it is a sporangium or a zoospore, to initiate an infection locus which could become the foundation for a future epidemic when conditions become conducive for the pathogen and the development of epidemics.

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