Zoosporogenesis and Differentiation of Grapevine Downy Mildew Pathogen *Plasmopara viticola* in Host-free System

Md. Tofazzal Islam and Andreas von Tiedemann

Division of Plant Pathology and Plant Protection, University of Göttingen, Grisebachstr. 6, D 37077 Göttingen, Germany

tofazzalislam@yahoo.com; Web: http://www.freewebs.com/tofazzalislam

Plant Pathology and Plant Protection Division
Department of Crop Sciences, University of Göttingen, Germany

Background and objectives

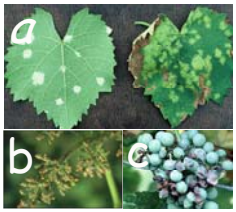

Fig. 1. Symptoms of downy mildew disease on leaves (a) and fruits (b & c) of grapevine

The downy mildew pathogen *Plasmopara viticola* is a major problem in vineyards all over the world. It spreads by extremely efficient cycles of asexual propagation. The success of this obligate biotrophic pathogen can be attributed in part to the speed of asexual differentiation to generate bi-flagellated motile zoospores from air-borne sporangia that target stomata of grapevine leaves through water films. Due to biotrophic nature, little is known about the underlying molecular mechanism of zoosporogenesis and following differentiation processes of *P. viticola*. Understanding molecular processes regulating and stimulating the asexual life stages of *P. viticola* is important for the future development of biorational approaches to control this pathogen. The objectives of this study were to-

- develop methods for production of copious number of zoospores, cystospores and germinated cystospores in host-free system;
- unravel mechanisms of asexual sporulation and early development of this phytopathogen.

Materials and Methods

Sporangia of *P. viticola* Pv-N was isolated from the infected leaves of *Vitis vinifera* cv. Müller-Thurgau (Fig. 1). This strain was maintained by regular culturing on the lower surface of young leaves of grapevine on petri dish containing 1.5% agar at 25 °C and 95% relative humidity. At day 6 of cultivation (Fig. 2a), the white powdery sporangiophores containing sporangia were harvested in an eppendorf tube by a micro-vacuum cleaner

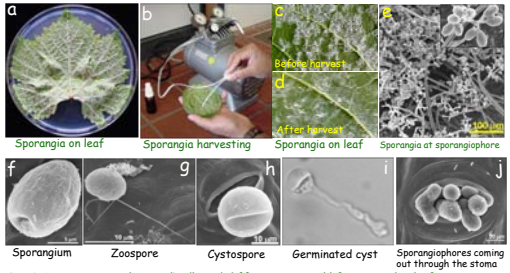

Fig. 2. Harvesting technique (b-d), and different asexual life stages (e-j) of *Plasmopara viticola* on grapevine leaves.

(Fig. 2b, 2c & 2d). Freshly harvested sporangia were washed twice and then incubated in sterilized water (*ca.* 10^5/ml) in the dark at room temperature (23 °C) for zoospore release. Quantification of time-course zoospore release and effects of pharmacological drugs on zoosporogenesis and encystment of zoospores were done as described previously [1,2]. Light and scanning electron microscopic analyses were done as described earlier[1].

Results and Discussion

Production of sporangia and zoospores: High density sporangia (1×10^{10}–10^{12}/leaf) were obtained by inoculating lower surface of excised grapevine leaf with sporangial suspension (1×10^4/ml) using a micosprayer followed by incubation at 25°C and 95% RH for 6 days (Fig. 2a-e).

Release of zoospores from the sporangia in sterile water was started from 2h after incubation and reached its peak (ca. 1×10^6/ml) at 6 h (Fig. 3). Nearly, 80% of sporangia gave birth motile zoospores (Fig. 2g). *P. viticola* zoospores swam fast with less turning. They became round cystospores (Fig. 2h) after 12-16 h of swimming and then immobilised. Light microscopy revealed that they were different in size and shapes. They showed geotaxis and did not show taxis to leaf extracts of host plant. Mechanical agitation of zoospore suspension by a vortex mixture for 40 s resulted 100% encystment.

Putative signal transduction pathways: We investigated mechanisms of asexual sporulation, motility and differentiation of *P. viticola* zoospores by using pharmacological tools (agonists/inhibitors) commonly used in cell biology.

The verapamil (inhibitor of calcium channels) and the trifluoroperazine (antagonist of calmodulin) inhibited zoosporogenesis and encystment (Table 1). The protein kinase inhibitors K-252a and KN-93 inhibited zoospore release and cyst germination, and K-252a significantly reduced motility of zoospores. Concentrations of actinomycin D (transcription inhibitor) did not affect zoospore release or encystment. The encystment of zoospores was triggered by the G-protein activator mastoparan and phospholipase C (PLC) in a dose dependent manner (Figs. 4 & 5). Primary and secondary butanol, which, like mastoparan have been reported to activate G-proteins, also induced encystment.

An inhibitor of phosphatidic acid generation, lisofyllene (18 μM) partially blocked mastoparan- but not PLC-induced encystment. Furthermore, U-73122 (PLC inhibitor) did not block mastoparan induced encystment but suppressed PLC induced encystment. Although Ca^{2+} did not directly induce encystment, but mechanically induced cystospores germinated (88±7%) in presence of 50 mM Ca^{2+}. Altogether, these results show that *P. viticola* contains a mastoparan- and butanol-inducible PLC and/or phospholipase D (PLD) pathways with a strong indication that PLC is involved in zoospore encystment. Gene expression profiling and the roles of G-proteins and PLD in asexual development of *Phytophthora* have been reported [3,4].

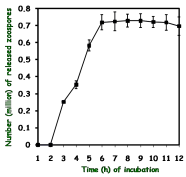

Fig. 3. Time-course release of *P. viticola* zoospores from sporangia incubated in sterilized water.

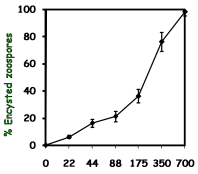

Fig. 4 Effect of mastoparan on encystment of *P. viticola* zoospores after 30 min of treatment.

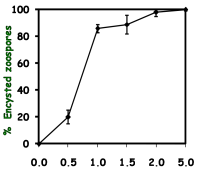

Fig. 5. Effect of PLC on encystment of *P. viticola* zoospores after 30 min of treatment.

Table 1. Occurrence of asexual developmental processes of downy mildew pathogen *Plasmopara viticola* in presence of inhibitors

Compounds (concn.)	Occurrence (%) of asexual developmental processes			
	Zoospore release[b]	Zoospores swimming[c]	Zoospore lysis[d]	Zoospore encystment[e]
Control[a]	100	100	0	0
Verapamil (0.8 μM)	95 ± 6	101 ± 3	0 ± 0	0 ± 0
Verapamil (1.6 μM)	58 ± 3	99 ± 5	3 ± 1	7 ± 2
Verapamil (3.2 μM)	0 ± 0	89 ± 4	9 ± 3	19 ± 4
Verapamil (6.4 μM)	0 ± 0	0 ± 0	98 ± 3	100 ± 0
Trifluoroperazine (0.08 μM)	100 ± 2	100 ± 1	0 ± 0	0 ± 0
Trifluoroperazine (0.16 μM)	95 ± 7	85 ± 6	0 ± 0	22 ± 5
Trifluoroperazine (0.32 μM)	5 ± 1	51 ± 11	3 ± 1	67 ± 8
Trifluoroperazine (0.64 μM)	0 ± 0	0 ± 0	96 ± 6	100 ± 0
K-252a (31 nM)	101 ± 4	82 ± 7*	0 ± 0	0 ± 0
K-252a (62 nM)	100 ± 2	73 ± 5*	0 ± 0	0 ± 0
K-252a (124 nM)	97 ± 5	64 ± 6*	0 ± 0	3 ± 1
K-252a (248 nM)	60 ± 7	10 ± 6*	5 ± 1	43 ± 5
KN-93 (16 nM)	103 ± 7	100 ± 0	0 ± 0	0 ± 0
KN-93 (80 nM)	89 ± 11	101 ± 2	0 ± 0	0 ± 0
KN-93 (400 nM)	78 ± 5	100 ± 0	0 ± 0	5 ± 1
KN-93 (2000 nM)	58 ± 9	97 ± 4	3 ± 2	23 ± 3
Actinomycin D (10 μg/ml)	104 ± 8	100 ± 0	0 ± 0	0 ± 0
Actinomycin D (100 μg/ml)	99 ± 3	102 ± 2	0 ± 0	0 ± 0
Actinomycin D (200 μg/ml)	97 ± 4	99 ± 3	0 ± 0	0 ± 0

[a]Values are standardized to 100% or 0% for solvent-only controls.

[b]Percentage was measured 6 h after placing sporangia in sterilized tap water at 23°C. An average of 81 ± 5% of sporangia released zoospores in controls (*ca.* 10^6 zoospores per milliliter). These and values in other categories show averages ± SE from three replication, with at least 300 spores counted for each sample. [c-e]Scored from samples kept at 23°C, about 30 min after addition of inhibitor.

Conclusions: We developed convenient methods to produce copious amounts of sporangia, zoospores, cystospore, germinated cysts of *P. viticola*. Our results show that *P. viticola* contains G-protein-mediated mastoparan-inducible PLC and/or PLD pathways with a strong indication that PLC is involved in zoospore encystment. Further proteomic and phospholipid analyses would provide precise information of signal transduction pathways of zoosporogenesis and differentiation of *P. viticola*.

References

1. Islam *et al.* (2003), Plant Soil 255, 131-142. 2. Islam *et al.* (2007), J Gen Plant Pathol 73, 324-329. 3. Latijnhowers et al. (2002), MPMI 15, 939-946; 4. Judelson et al. (2008), MPMI 21, 433-447.

Acknowledgements

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