

The biology and epidemiology of sunflower white blister rust, *Pustula helianthicola*

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ABSTRACT

- **Background and aims.** Sunflower white blister rust is a major disease in many countries with intensive cultivation of the oil crop. The biology of the pathogen is poorly understood, particular in terms of sexual reproduction and primary mode of infection. However, this is a prerequisite for understanding the epidemiology and developing strategies for pathogen control. Therefore, we established a cultivation system for *Pustula helianthicola*, developed single zoospore strains, investigated the formation and germination of oospores and used a PCR-based test to trace the pathogen.
- **Methodology:** A micro-capillary technique was employed to select single zoospores for infection of sunflower seedlings. Sexual reproductive structures were traced by microscopy. Oospores were isolated and used for germination and infection studies. UV-irradiation was employed to test the longevity of sporangia. A PCR-based test was used to identify the pathogen in asymptomatic host tissue and soil samples.
- **Key results:** Single zoospores, released from zoosporangia of *P. helianthicola*, were used to generate unithallic, genetically homogenous infections. The infections resulted in mitotic sporulation in sub-epidermal blisters on cotyledons and true leaves within two weeks. One week later the formation of oospores started, hence indicating homothallic sexual reproduction. Isolated oospores germinated with a sessile vesicle like structure, germ sporangium or with germ tube alone. The ratio of germination reached ca. 40 % and did not depend on a resting period after oospore formation. Oospores applied to the above ground parts of sunflower seedlings led to infection within a time frame similar to that of mitotic zoosporangia. Inoculation of roots did not give rise to infections, neither with sporangia nor with oospores. PCR experiments revealed, that infection is often asymptomatic, unless mitotic sporulation in the typical white blisters becomes visible. Systemic invasion of leaf tissue was accompanied by profuse formation of oospores along leaf veins and in petioles. In flowering parts, *P. helianthicola* is known to form oospores within achenes. Hence, oospores are important for long-distance dispersal, hibernation and for primary infection at the beginning of the season. In contrast, mitotic sporangia serve for mass propagation during the season. They were shown to be short-living and highly sensitive to UV-irradiation. Fungicide treatment with metalaxyl M and dimetomorph reduced infection, whereas sunflower genotypes resistant to the downy mildew pathogen, *Plasmopara halstedii*, were fully susceptible for white blister rust.
- **Conclusions:** Single zoospore infection proves homothallic reproduction of *P. helianthicola* and explains the low genotypic and phenotypic variability observed in samples of different origin. Primary infection is initiated by zoospores from seed-borne or soil-borne oospores. Penetration seems to depend on the presence of stomata, similar as in secondary infection from zoosporangia. Resistance of sunflower towards downy mildew does not protect against white blister rust.
- **Contribution to the current knowledge:** This is the first report on the sexual life cycle and mode of primary infection of this important sunflower pathogen.

Key words: *Helianthus* – homothallism – Oomycetes – oospores – *Pustula* – sunflower

INTRODUCTION

In the past, incidences of epidemiologically relevant infections of sunflower with white blister rust have mainly been reported from areas with hot and dry summer such as South Africa (Van Wyk et al., 1995), Argentina (Delhey and Kiehr-Delhey, 1985), Australia (Allen and Brown 1980) and sporadically from the US (Gulya et al., 2002). Meanwhile, the pathogen occurs as well in temperate climate, as it is given in Germany (Thines et al. 2006) (Fig. 1). Former assumptions that the pathogen on sunflower is conspecific to *Pustula tragopogonis* (syn. *Albugo tragopogonis*), the white blister rust of *Tragopogon* spp., has been revised. The recent identification of distinctive phenotypic and molecular genetic characters (Rost and Thines, 2011) led to the description of the new species *P. helianthicola* C.Rost & Thines as the causal agent of sunflower white blister rust. The narrow species concept with high host specificity is supported by the fact that no cross-infection of sunflower with sporangia of *Pustula* from wild flowers such as goatsbeard has been reported yet. This raises questions on the origin of primary infections in sunflower fields which can not be answered unless principal aspects of the pathogen's biology (e.g. sexual and asexual reproduction, distribution of spores or sporangia, mode of germination and host penetration) are unravelled. The biotrophic nature of *P. helianthicola* impeded studies on the fungal development. Therefore, cultivation techniques on whole seedlings and detached sunflower leaves had been established (Kajornchaiyakul and Brown, 1976; Spring et al., 2011) to allow detailed analysis of the infection process and sporulation. We now employed these methods to study the formation of oospores and to unravel their role in the primary infection of sunflower. The role of oospores in comparison to zoosporangia for the survival and propagation of the pathogen is discussed.



Fig. 1. Sunflower infected with *P. helianthicola*. Left: natural infection in the field. Right: artificially infected cotyledone with typical white blisters.

MATERIALS AND METHODS

Zoosporangia of *P. helianthicola* C.Rost & Thines were harvested from naturally infected sunflower, found on a field near Plieningen, Baden Württemberg, Germany. A voucher (OS 1000) of the original material was deposited in the herbarium HOH. A permanent culture of the pathogen on sunflower seedlings, *Helianthus annuus* cv. giganteus, was established and maintained as described earlier (Spring et al., 2011). To obtain genetically homogenous strains we followed the protocol for single zoospore infections with sunflower downy mildew (Spring et al. 1998). Freshly harvested zoosporangia were spread on water agar plates and incubated in darkness at 16°C until zoospore discharge. Single zoospores were collected with a micro-capillary and transferred onto detached sunflower leaves or on the apical bud

of ca. five days old seedlings which had been raised on wet filter paper. After 24h at 16°C in darkness in petri dishes with saturated humidity, seedlings were potted. Further cultivation was performed in a climate chamber at 16°C, 80% humidity and 14h photoperiod. Spontaneous formation of subepidermal sporangia (the typical white blisters) indicated infection. Freshly developed sporangia were used for subsequent germination and infection studies and excess of sporangia was stored at -70°C for later use. Occurrence of oospores was detected microscopically. Clusters of oospores were excised from the host tissue. The material was chopped, suspended in water and sieved through different nylon filters (mesh 150, 100, 70, 50 µm). The residue of the 50µm sieve was suspended in water and checked for the purity and condition of oospores. The suspension was used for germination and infection tests with oospores. For the latter, ca. 400 oospores per plant were placed in between the cotyledons of ca. five days old seedlings, similarly as described above for the inoculation with zoosporangia. To study oospore germination we followed the technique described by Verma and Petri, 1975. The oospore suspension was diluted in 50-100ml sterile water and incubated at 200 rpm on a rotary shaker at 18°C for up to one week. Afterwards, the oospores were separated by filtering through nylon sieve (50µm mesh) and used for germination tests. These tests were carried out on water agar or on microscopic slides. Rifampicin (10 ppm) was added to prohibit bacterial growth. The agar plates and the microscopic slides were incubated in darkness at 16°C in saturated humidity and checked daily for oospore germination by microscopy. The formation of oospores was investigated in freehand sections of infected plant tissue using a Zeiss Axioplan microscope. Resorcin blue was used to stain fungal structures and fluorescence microscopy was carried out with a filter combination of FT 460/ LP470 barrier and UV 395-440nm excitation. The resistance of sporangia to UV light was tested by irradiating sporangia on water agar plates with UV light of 254 nm and 366 nm, respectively. The ratio of sporangia discharging zoospores within 3h after irradiation was counted with an inverse microscope.

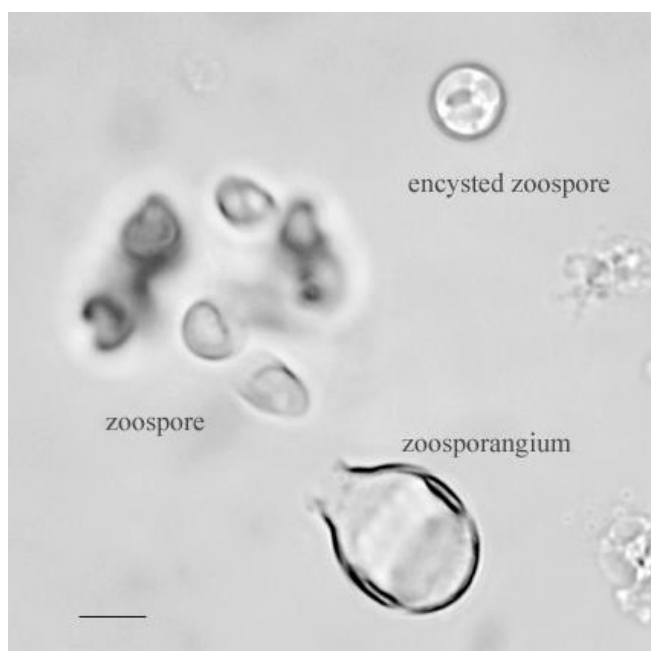


Fig. 2. Empty zoosporangium of *P. helianthicola* after discharge of zoospores. Bar equals 50 µm.

RESULTS AND DISCUSSION

Sporangia of *P. helianthicola*, when placed on water agar started to discharge zoospores after 60 to 90 min (Fig. 2). An average of seven zoospores per sporangium was released and we never observed the release of undifferentiated protoplasm, as it was describe by Kajornchaiyakul and Brown (1976). Only two out of 150 zoospores, collected one by one with a micro-capillary and transferred to the apical bud of a sunflower seedling, succeeded to infect. The infection became visible after two weeks, when sporulation started on leaves. The ratio of infection increased to 8%, when single sporangia instead of single zoospores were used as inoculum. The mitotically produced sporangia of the two single-zoospore-infected plants were used to establish genetically homogenous strains of the pathogen for future experiments. Within the two infected plants, the formation of oospores started approximately one week later. Antheridia and Oogonia with oospores of different developmental stage were frequently found in

the plant tissue along leaf veins and adjacent to the white blisters (Fig. 3). This showed that no mating partner is necessary for the sexual reproduction of *P. helianthicola*. Homothallism is known from some other Peronosporomycetes (Spring, 2000; Wong et al., 2001), but only few species have been investigated yet and we have not found any information for Albuginomycetidae. From the evolutionary point of view, the homothallic reproduction appears to be disadvantageous with respect to adaptation. However, for a biotrophic pathogen it can be beneficial to secure the survival between two growing seasons of its host through durable spores. Moreover, the occurrence of oospores of *P. helianthicola* in achenes of infected plants (Viljoen et al., 1999) enables the propagation of the pathogen together with its host and explains the long-distance dispersal to different continents. The homothallic sexual reproduction could also explain the low phenotypic and genotypic variability found in comparison of *P. helianthicola* samples from three different continents (Spring et al., 2005; 2011)

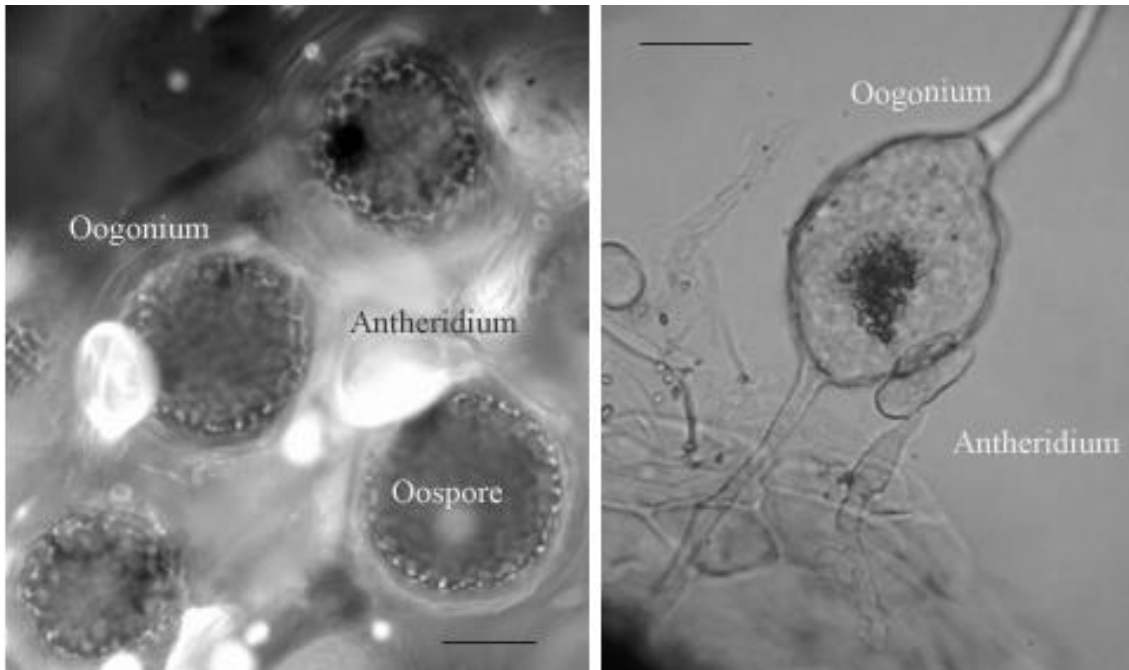


Fig. 3. Oospore formation in sunflower tissue. Left: clusters of oospores of different developmental stages still hulled by the oogonium and adjacent Antheridium (fluorescence microscopy after staining with resorcin). Right: young intercalary oogonium with attached antheridium. Bar equals 20 μm .

Oospores are assumed to be the source of primary infection for biotrophic oomycetes living on seasonal plants, but the mode of infection through oospores has not been investigated in many species. Studies on the germination of oospores in the Albuginomycetidae are yet limited to a single taxon, *Albugo candida* (DeBary, 1983; Vanterpool, 1953; Verma and Petri, 1975). Oospores of *P. helianthicola*, when placed in water on agar plates or on microscopic slides, formed vesicle-like structures, germ sporangia or in some cases just germ tubes (Fig 4). This process started after about 24h and within two weeks, approximately 40% of the oospores had germinated. The release of zoospores was not observed. Between oospore formation in the sunflower tissue and their germination, no dormancy or period of vernalization was necessary. The infectivity of the oospores was shown by placing them onto the apical bud of sunflower seedlings in the same way as described for inoculation with zoosporangia. The infection became visible after two weeks, when the typical pustules with zoosporangia developed on leaves. Attempts to infect seedlings through the roots failed. This parallels the results observed in infection experiments with zoosporangia and suggests, that both types of spores depend on stomata to enter the host tissue before establishing a primary haustorium. For the primary infection of sunflower in the field through soil-borne or seed-borne oospores we assume, that the inoculum must reach the above ground plant parts either at an early stage of seedling development or possibly through water splash when rain drops hit the soil. The site of primary infection is not necessarily close to the area on which first symptoms become visible through the formation of the typical blisters. Recent experiments employing a PCR-based detection system for *P. helianthicola* revealed that the pathogen can live asymptotically in areas outside the

chlorotic leaf lesions (Spring et al, 2011). The latter obviously develop only when mass propagation with mitotic zoosporangia starts.

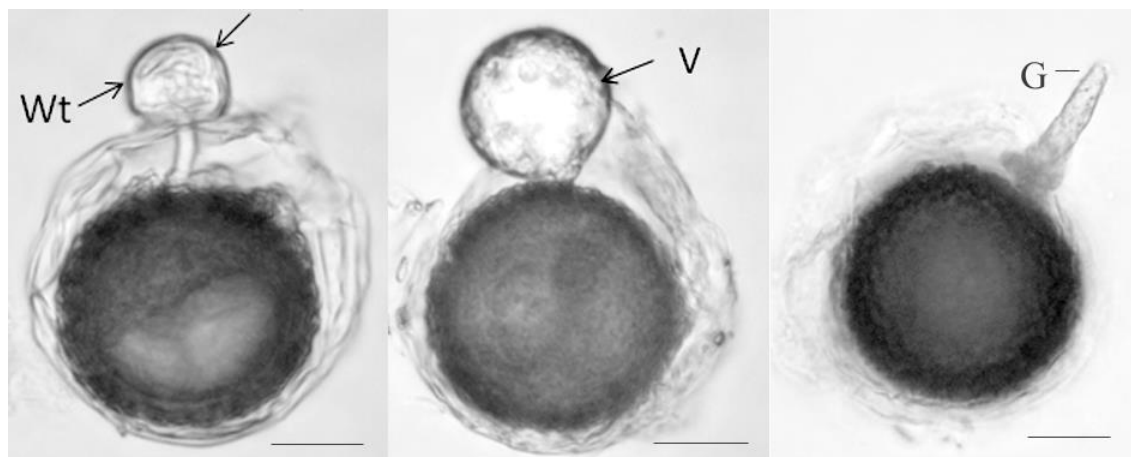


Fig. 4. Germination of *P. helianthicola* oospores with germ sporangium (left, Wt wall thickening), vesicle (middle, V) and germtube (right, G). Bar equals 20 μ m

In contrast to oospores, zoosporangia are short-living. Germination experiments with freshly collected zoosporangia showed a fast reduction of viability within only one week at 16°C. Moreover, treatment with short-wavelength UV light drastically reduced their viability. The irradiation of sporangia with UV of 254 nm at an intensity of 0.5 mW / cm² erased their germination ability within only 4 min, whereas a similar exposure to 366 nm did not affect their vitality at all. Both observations make it unlikely that zoosporangia of sunflower white blister rust contribute to long-distance dispersal, as it is assumed for some other oomycetes such as *Peronospora tabacina* or *Pseudoperonospora cubensis*, which yearly seem to migrate from south to north over thousands of km.

Current measures to protect sunflower cultivation from oomycetes include resistance breeding and fungicide treatment. The latter has been widely used to control the downy mildew pathogen *Plasmopara halstedii*, but fungicide resistance has developed in many countries (for ref. see Viranyi and Spring, 2011). The strains of *P. helianthicola* we collected from southern parts of Germany did not show any resistance against metalaxyl or dimetomorph. In contrast to sunflower downy mildew, no pathotypes of *P. helianthicola* were yet identified. Our infection tests using sunflower genotypes of different resistance as recommended for downy mildew pathotyping (Tourvieille de Labrouhe et al., 2000) showed that they were not protected against sunflower white blister rust. Even the line HA335, resistant to all currently observed field pathotypes of downy mildew, was readily infected. This was shown in laboratory experiments as well as under natural conditions in a field which contained oospores from infected sunflower of previous seasons. The preliminary results of infection studies indicate, that measures to control *P. helianthicola* are urgently needed to prevent the pathogen from further spreading and becoming a major disease, as it has been described from South Africa (Van Wyk et al., 1995).

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REFERENCES

- Allen, S.J., and J.F. Brown. 1980. White blister, petiole greying and defoliation of sunflowers caused by *Albugo tragopogonis*. Australas. Plant Pathol. 9:8-9.
- DeBary, A. 1883. Recherches sur le developpement de quelques champignons parasites. Annales des Sciences Naturelles Botanique 20:5-148.;
- Delhey, R., and M. Kiehr-Delhey. 1985. Symptoms and epidemiological implications associated with oospore formation of *Albugo tragopogonis* on sunflower in Argentina. P. 455-457. In: Proc. 11th Int. Sunfl. Conf., Mar del Plata, Argentina. Int. Sunfl. Assoc., Paris, France

- Gulya, T.J., F. Viranyi, J. Appel, D. Jardine, H.F. Schwartz, and R. Meyer. 2002. First report of *Albugo tragopogonis* on cultivated sunflower in North America. *Plant Dis.* 86:559.
- Kajornchaiyakul, P., and J.F. Brown. 1976. The infection process and factors affecting infection of sunflower by *Albugo tragopogi*. *Transact. Brit. Mycol. Soc.* 66:91-95.
- Rost, C., and M. Thines. 2011. A new species of *Pustula* (Oomycetes, Albuginales) is the causal agent of sunflower white rust. *Mycol. Progress*, in press.
- Spring, O., B. Rozynek, and R. Zipper. 1998. Single spore infection with sunflower downy mildew. *J. Phytopathol.* 146:577-579.
- Spring, O. 2000. Homothallic sexual reproduction in *Plasmopara halstedii*, the downy mildew of sunflower. *Helia* 23:19-26.
- Spring, O., K. Haas, S. Thurnhofer, and W. Vetter. 2005. The composition and taxonomic significance of fatty acid patterns in three white rust species: *Albugo amaranthi*, *A. candida* and *A. tragopogonis* (Peronosporales, Albuginaceae). *Mycol. Progress* 4:179-184.
- Spring, O., M. Thines, S. Wolf, and R. Zipper. 2011. PCR-based detection of sunflower white blister rust (*Pustula helianthicola* C.Rost & Thines) in soil samples and asymptomatic host tissue. *Eur. J. Plant Pathol.* 131:519-527.
- Thines, M., R. Zipper, D. Schäuffele, and O. Spring. 2006. Characteristics of *Pustula tragopogonis* (syn. *Albugo tragopogonis*) newly occurring on cultivated sunflower in Germany. *J. Phytopathol.* 154:88-92.
- Tourvieille de Labrouhe, D., T.J. Gulya, S. Masirevic, A. Penaud, K.Y. Rashid, and F. Viranyi. 2000. New nomenclature of races of *Plasmopara halstedii* (sunflower downy mildew). *Proc. of the 15th Internat. Sunflower Conference, Toulouse, France*; I-61-66
- Vanterpool, T.C. 1953. Oospore germination in *Albugo candida*. *Can. J. Botany* 37:169-172.
- Van Wyk, P.S., B.L. Lones, A. Viljoen, and I.H. Rong. 1995. Early lodging, a novel manifestation of *Albugo tragopogonis* infect on sunflower in South Africa. *Helia* 18:83-90.
- Verma, P.R., and G.A. Petri. 1975. Germination of oospores of *Albugo candida*. *Can. J. Botany* 53:836-842.
- Viljoen, A., P.S. Wyk, and W.J. Jooste. 1999. Occurrence of the white rust pathogen, *Albugo tragopogonis*, in seeds of sunflower. *Pl. Disease* 83:77.
- Viranyi, F. and O. Spring. 2011. Advances in sunflower downy mildew research. *Eur. J. Plant Pathol.* 129:207-220.
- Wong, F.P., H.N. Burr, and W.F. Wilcox. 2001. Heterothallism in *Plasmopara viticola*. *Pl. Pathol.* 50:427-432.