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ARTICLE

Oospores of *Pustula helianthicola* in sunflower seeds and their role in the epidemiology of white blister rust

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Abstract: White blister rust (WBR) of sunflower caused by *Pustula helianthicola* is an important and often underestimated disease in many countries of the world. The epidemiology of the pathogen is not yet fully understood; particularly the role of oospores in primary infection and long distance dispersal. We analysed WBR severity in sunflower under natural conditions and found disease incidence of 97–99 % in fields where infected sunflower had first been observed *ca*. 8 yr ago. Besides the typical blisters of mitotic sporangia on leaves, large amounts of oospores were observed on the involucral bracts. Inoculation of sunflower seedlings with oospores from these bracts resulted in disease incidence of *ca*. 30 %, thus confirming their infectivity without a period of dormancy. Bracts of infected flower heads from the field were checked for oospores using a binocular microscope and seeds were checked by light microscopy. Oospores were found in all of the bracts and in up to 28 % of the achenes. Light microscopy revealed that oospores developed in the thin-walled, crushed parenchymatic cells of the inner layer and in the parenchymatic rays of the fibrous layer of the pericarp. Dried seeds were grown in soil to assess the occurrence of seed borne infection. Within 3 wk, up to 58 % of seedlings showed typical WBR pustules on cotyledons. Asymptomatic infections were confirmed in phenotypically healthy plants by using a PCR-based diagnostic test for *P. helianthicola*. The results showed the importance of oospores of *P. helianthicola* as the primary inoculum for WBR development in sunflower, and pointed to the potential role of contaminated seeds in the long distance transmission of the pathogen.

Key words:
field monitoring
Helianthus annuus
light microscopy
oospore seed contamination
PCR
seed-borne diseases

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INTRODUCTION

Sunflower is one of the economically most important oil seed crops in the world. Its cultivation is endangered by numerous fungal diseases, predominantly belonging to *Eumycota* (Gulya *et al.* 1997). Of the two diseases of sunflower caused by oomycetes, downy mildew caused by *Plasmopara halstedii* is regarded as the most serious, whereas the economic relevance of white blister rust (WBR), caused by *Pustula helianthicola* (syn. *Albugo tragopogonis*) (Thines & Spring 2005, Rost & Thines 2012) has often been questioned. *Pustula helianthicola*, which forms conspicuous white masses of spores on the lower surface of sunflower leaves, was first reported in South Africa (Verwoerd 1929). It has become a major disease of sunflower in the past 30 years, with losses of up to 80 % of the plants due to stem-breakage (van Wyk *et al.* 1995). Due to the increased production of sunflower, WBR has been reported from many other countries, predominantly in the Southern Hemisphere. Massive WBR infections of oil crop sunflower recently occurred in Argentina, where they were witnessed by O.S. in 2012, and also in areas with hot and dry summers in the USA and Russia (Zimmer & Hoes 1978). In Germany, *P. helianthicola* was first found in 2003 on cultivated sunflower in fields near Stuttgart (Thines *et al.* 2006) and quickly became important in the region because of severe losses in the production of ornamental sunflower. These flowering plants lose their economic value when the leaves are covered with chlorotic lesions.

The spontaneous occurrence of WBR in new areas suggests that seed transmission of the pathogen is responsible for its propagation. In fact, Viljoen *et al.* (1999) observed infection of sunflower heads, which led to the formation of *P. helianthicola* oospores on involucral bracts and in the pericarp and testae of seeds. Oospores are the most stable and durable stage in which the biotrophic pathogen can survive long enough to bridge the dormant phase of its host between two seasons (Spring *et al.* 2011). Seed transmission of oospores is established for various crop-infecting oomycetes of *Albuginomycetidae* (e.g. *Albugo candida*; Petrie 1975) as well as *Peronosporomycetidae* (e.g. *Peronospora manshurica*, Pathak *et al*. 1978; *Plasmopara halstedii*, Cohen & Sackston 1974; *Sclerospora graminicola,* Shetty *et al.* 1977). However, the role of seed-borne primary infection has been studied less frequently and sometimes with contradictory results. For instance, early attempts to raise sunflower from infected seeds resulted in symptomless

plants (Cohen & Sackston 1974), whereas it was shown subsequently that significant numbers of seedlings from contaminated seeds were infected in roots and hypocotyls (Spring 2001) thus contributing to soil-borne infections in later years. Similarly, oospores were formerly considered to play no role in disease transmission of *Peronospora arborescens,* until the seed-transmitted infection was shown to be effective in the field (Landa *et al.* 2007). Growing conditions apparently influence the degree of seed-transmitted infections, as was shown for *Albugo candida* in rapeseed (Barbetti 1981) and *Peronospora farinosa* in quinoa seeds (Danielsen *et al.* 2004). This shows the need to evaluate seed-transmitted infections for each pathogen species individually.

For sunflower, Viljoen *et al.* (1999) observed oospores in achenes, but did not test whether *Pustula helianthicola* oospores survived in seeds, or to what extent seed contamination could contribute to the dissemination of the pathogen. Hence, we address these questions by assessing the natural rate of infection of plants in a field where sunflower has been commercially grown for ornamental purposes for several years, and where soil contamination with infective spores of *P. helianthicola* has been shown in previous years (Spring *et al.* 2011). In particular, we quantified the rate of head infection and seed contamination with oospores. Primary infection through seed contamination was tested by raising seedlings from a naturally infected sunflower head under controlled conditions. To quantify infection with *P. helianthicola* we counted visible pustules and checked for asymptomatic infection with a PCR-based assay.

MATERIALS AND METHODS

Field data collection

Disease severity was recorded during the first two weeks of October 2012 in a commercial cut sunflower field near Stuttgart-Plieningen, Germany. WBR was first observed in this field in 2003 (Thines *et al.* 2006). Planting of sunflower was in small plots (*ca*. 30–80 m^2) every 2–3 wk from April to September to get fresh flowering plants throughout the season. For two plots containing plants both in bud and in flower, the number of infected plants showing typical white blisters on leaves was counted. Among the flowering plants the total number of capitula and number of visibly infected capitula was recorded.

Selection and harvest of oospore containing samples

One bract from each infected flowerhead was collected and used to detect oospores under a stereo-binocular microscope. In addition, 17 infected flowerheads in the late flowering stage (and one healthy capitulum as a control) were harvested and used for the detection of oospores in bracts and seeds. Seeds were removed from the flowerheads and 50 seeds of each sample were stored at 4 °C, whereas the remaining seeds were kept for approximately 3 wk in open petri dishes and stored at room temperature for drying and used for subsequent germination experiments.

Detection of oospores in bracts and seeds

Bracts and seeds from each sample obtained from the field were searched for oospores. On bracts, the presence of oospores could be observed through the epidermis using a binocular microscope. For the detection of oospores in achenes, the inner, cotton-wool like parenchymatic tissue of the pericarp was analysed as previously described (Spring 2001). From 50 seeds of each sample, the inner pericarp tissue was scratched out using a fine spatula and examined with a binocular microscope to assess the number of seeds with and without oospores.

Light microscopy of pericarp and seed tissues

Semi-thin sections were used to localize the site of oospore formation in achenes of infected flower heads. Nine achenes, not completely dried, were cut into halves with a razor blade and the basal parts (pericarp and seed tissues with the embryo) were fixed with 5 % glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) and stored at 4 °C until further preparation. It was not possible to get intact samples, because the soft tissues separated from the hard pericarp tissues during dissection. Samples were washed three times in the same buffer and dehydrated in an ethanol series (30 %, 50 %, 70 %, 90 %, 95 %, 100 %, 100 % (v/v)) for 2 h each. Afterwards, samples were infiltrated for 3–4 d in acrylate resin (Technovit 7100, Kulzer, Heraeus). The resin was allowed to polymerise for at least 2 h. Cross sections of pericarp and seed tissues of 5 µm thickness were made using a rotational microtome HM 340E (MICROM) with a metal knife. Serial sections were collected on Poly L-lysine coated glass slides. All sections were stained with 0.05 % aqueous Toluidine blue O (MERCK) for 20–25 min, washed with distilled water and mounted by Roti ®Histokitt (Carl Roth, Karlsruhe). Serial sections of three achenes of each of the samples nos. 10, 13, and 17 were investigated in bright field using a Zeiss Axioplan light microscope (Göttingen) coupled to a digital camera (Canon Powershot A640).

Primary infection with isolated oospores

Oospores were isolated from bracts of infected plants using the method recently described by Lava & Spring (2012). Oospores obtained from plants infected with the single spore strain (SLS 1000-SS-2-2010) and cultivated in a growth chamber were used as a positive control. Sunflower seedlings were inoculated with the isolated oospores according to the protocol described by Lava & Spring (2012). A suspension in water of approximately 400 oospores per plant was placed on the apical bud of 4–6 d old seedlings and the plants were kept at 100 % atmospheric humidity in darkness for 24 h. After this period, plants were grown under a photoperiod of 14 h light and 10 h darkness at 16 ºC in a growth chamber. Un-inoculated seedlings were used as a negative control.

Seed borne infections under controlled conditions

To observe naturally derived primary infections from oospore contaminated seeds under control conditions, 50 seeds of the 17 infected flower heads from the field were used in this study. These seeds were grown in pathogen free; heat

sterilised soil in trays and kept under a photoperiod of 14 h light and 10 h darkness each day at 16 ºC and 80 % humidity in a growth chamber. Uninfected seeds of the sunflower cultivar Giganteus were used for the control. One part of the control seeds was inoculated with isolated oospores (400 oospores per plant) of the single spore strain SLS 1000-SS-2-2010 as described above. The other remained un-inoculated and served as the healthy control. After 10 d, the majority of the seeds had germinated and produced seedlings with unfolded cotyledons. To avoid infestation of the soil, the pericarps and seed coats were removed when the seedlings had emerged. Infection of plants was checked daily in all samples until the formation of white pustules on leaves or cotyledons indicated the beginning of sporulation. Visibly infected plants were removed immediately to prevent secondary infection of healthy plants through fresh sporangia. The experiment was finished 4 wks post planting and the disease incidence was defined by counting the number of seedling with sporulation compared to the total number of germinated seedlings.

DNA isolation and amplification

DNA of sporangia of *P. helianthicola* and tissue of *H. annuus* was isolated according to the protocol described previously (Spring *et al.* 2011). To detect asymptomatic infections in the seed borne infection experiment, cotyledons of five seemingly uninfected plants of the samples #3, 10, and 15 were used for DNA extraction and screened for asymptomatic infection with *P. helianthicola* by means of a PCR-based diagnostic test. The samples #3, 10 and 15 had shown strong (58 %), medium (37 %) and no (0 %) visible symptoms in the seed borne infection assay. Leaf tissue of a visibly infected plant was used as a positive control and the cotyledon of an uninoculated plant served as the negative control.

The PCR experiments were carried out as described by Spring *et al.* (2011) using the specific primers COX2-HelF3 (TTAGAAACTTTTGTACAC) and COX2-HelR2 (AAATATCAGAATATTCATAT) for the amplification of the partial (190 bp) cox2 region from sunflower WBR. As a control for the function of the PCR, amplification of the partial region from the chloroplast $tRNA^{Leu}$ (UAA) was performed using the primers trnL-F (CGAAATCGGTAGACGCTACG) and trnL-R (GGGGATAGAGGGACTTGAAC) as described by Taberlet *et al.* (1991). The PCR was carried out in a volume of 12.5 µL containing 30 ng of total DNA. The amplification was performed in an Eppendorf Master cycler (Eppendorf, Germany) using the following conditions for both primers (COX2 HelF3/R2 and trnL-F/R): initial denaturation at 94 ºC for 4 min followed by denaturation at 94 ºC for 40 s, annealing 40 s at 52 ºC, elongation for 40 s at 72 ºC, 36 cycles (for COX2 HelF3/R2) or 40 cycles (for trnL-F/R) with repetition of the last three steps. A final extension step was carried out for 4 min at 72 ºC. The amplification products were analysed through microchip capillary electrophoresis (MultiNA, Shimadzu, Duisburg) according to the protocol of the manufacturer.

RESULTS

Natural infection in the field

In total, 1079 flowering plants and 795 younger plants (pre-flowering stage) were investigated, of which 1071 (99 %) flowering and 768 (97 %) preflowering plants were infected by *Pustula helianthicola* as assessed by the presence of white blisters with sporangia on leaves (Fig. 1C). Among the flowering plants, 1432 capitula were checked, out of which 167 were identified to be infected showing pustules of sporangia on the abaxial surface of bracts (Fig. 1A–B). One bract from each infected capitulum was collected and checked using a binocular microscope for the presence of oospores, which were visible through the epidermis (Fig. 1D). The oospores were thick walled, brown to blackish, and mostly in dense clusters in and around the pustules, in the tip region of the bracts, and all along the veins (Fig. 1E). Oospores were found in all bracts of the 167 flower head samples collected in the field. From the 17 infected flower heads harvested for the investigation of seed contamination, bracts and 50 seeds per sample were checked for the presence of oospores in the pericarp. Oospores were found in all 17 seed samples with a ratio ranging from 2–28 % (Table 1). The intensity of seed infestation was correlated with the number of oospores found on involucral bracts of the flower heads. In samples with fewer than ca. 200 oospores per bract, the average rate of oospore occurrence in seeds reached 5.6 % (SD +/-3.8; n = 5), whereas in samples with more than 5000 oospores per bract the average rate reached 19.4 % (SD ± 5.5; n = 7).

Localization of oospores in pericarp and seed tissues

Cross sections of the pericarp revealed the anatomy typical for achenes of sunflower: starting from the outside with the epidermis, followed by a multilayered cork hypodermis, a phytomelanin layer, a fibrous layer with rays of parenchyma cells, and an extended parenchymatic layer (Fig. 2A). The sclerenchyma fibers in contact with the phytomelanin layer showed thicker cell walls (Fig. 2A). Oospores were found in all achenes (n = 9), within the thin walled, crushed parenchyma cells of the pericarp, in the inner, extended parenchymatic layer (Fig. 2A–B). A few also were observed in the parenchymatic rays between the fibers (Fig. 2D). No oospores were found in the hard layers of the pericarp and the testa or other seed tissues, e.g. the embryo. No hyphae or mycelium were visible, but often the oospores were still encased by the oogonium, which was connected to an antheridium. In intact seeds, pericarp and seed coat were fused, but due to difficulties during preparation, the hard and stiff parts were torn from the soft part of the pericarp and the seed tissues. Therefore, oospores in these disruption zones might have been dislocated from within the parenchymatic tissue (Fig. 2D).

Infectivity of fresh oospores of *Pustula helianthicola* and seed borne natural infection

Freshly developed oospores from the field samples were shown to give raise to new infections, and caused symptoms of WBR. Pustules with white masses of sporangia occurred in 16 out of 60 seedlings within 14–18 d post inoculation. The

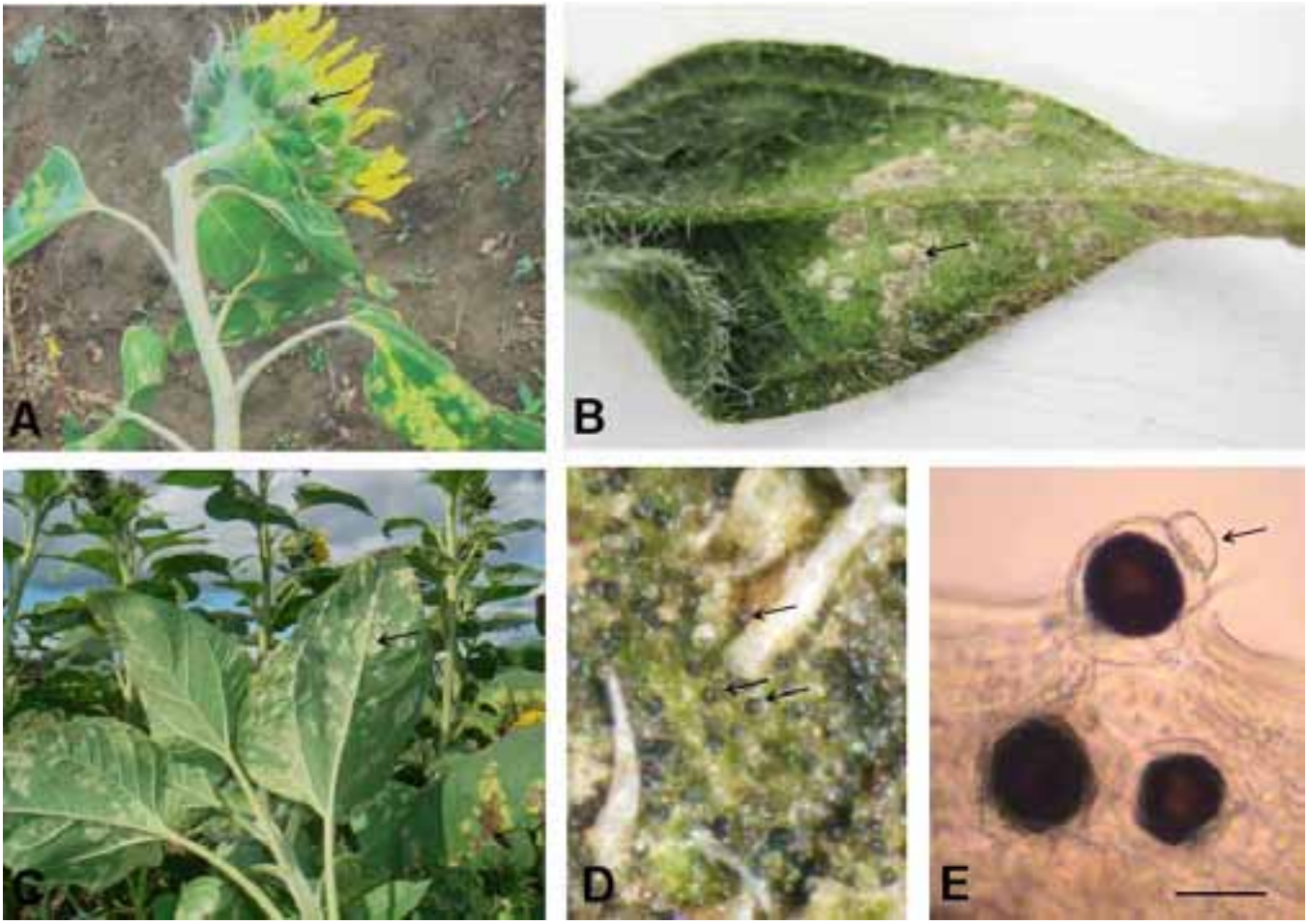

Fig. 1. Symptoms of white blister disease on leaves and bracts of sunflower and the presence of oospores in bracts. **A–B.** Head infection with involucral bracts showing pustules with sporangia (arrow). **C.** White blisters with asexually produced sporangia on the lower leaf sides (arrow) **D.** Blackish oospores (arrows) visible through the epidermis of the abaxial surface of a bract. **E.** Light micrograph of a fresh epidermal strip with black oospores still embedded in oogonium and antheridium (arrow). Bar = 50 µm.

Table 1. Occurrence of oospores in bracts and achenes of naturally infected sunflower from the field.

Sample number	Number of oospores in a bract (*n* = 5)[1]	Rate (%) of seeds with oospores (*n* = 50)
1	low	12
2	high	18
3	high	12
4	low	6
5	medium	10
6	high	16
7	medium	10
8	low	4
9	medium	10
10	high	22
11	low	4
12	medium	8
13	high	28
14	medium	6
15	low	2
16	high	16
17	high	24

[1]Low, medium, and high denotes approximately <200, <1000, and >5000 oospores per bract, respectively.

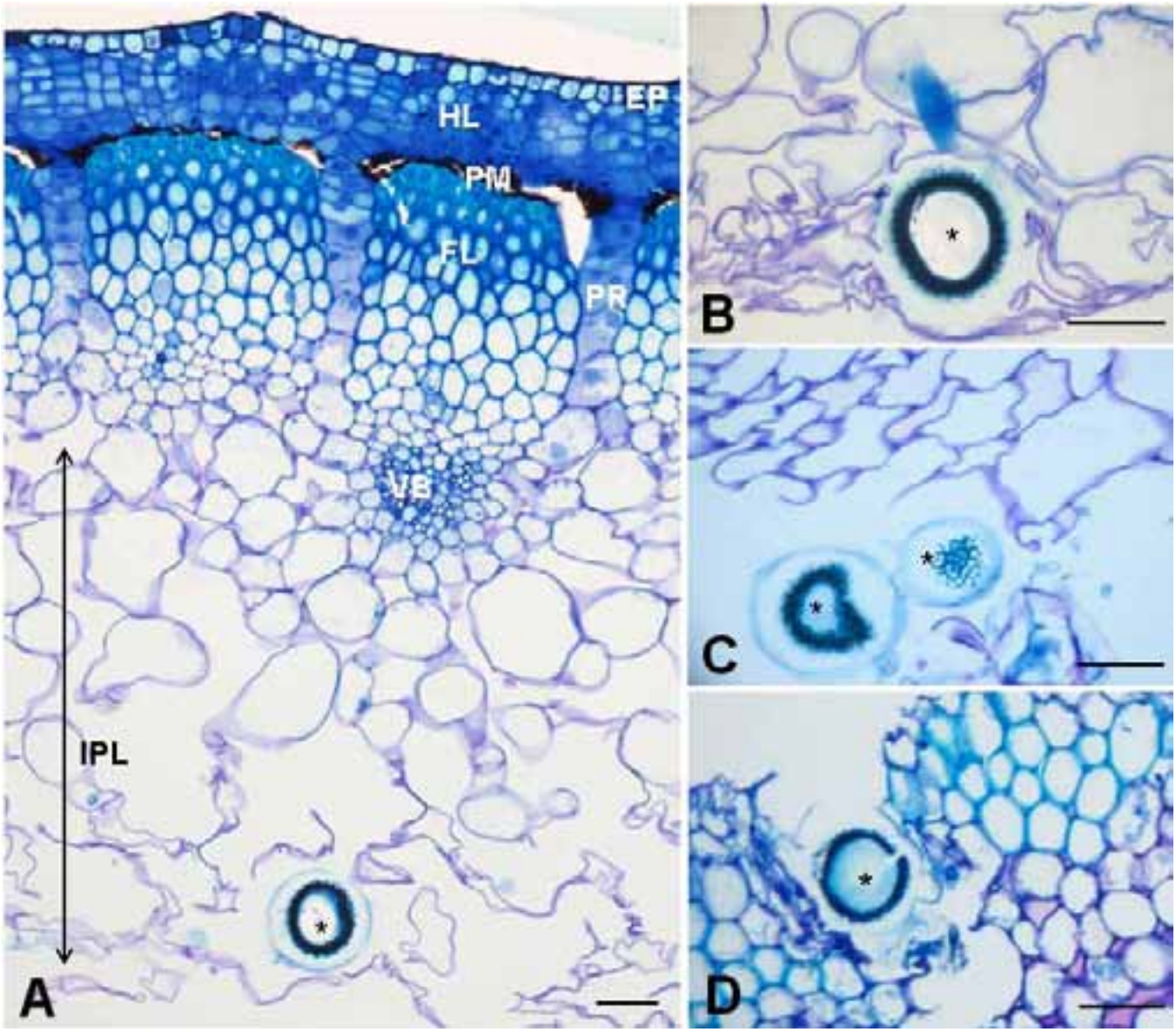

Fig. 2. Light micrographs of cross sections of seed pericarp stained with Toluidine Blue. **A.** Anatomy of the pericarp and location of an oospore (asterisk) in an oogonium. **B–C.** Details, oospores (asterisks) in the inner parenchymatic layer of the pericarp. **D.** Detail, oospore located in the parenchymatic ray of a fibrous layer of the pericarp. EP, Epidermis; HL, Cork hypodermal layer; PM, Phytomelanin layer; FL, Fibrous layer; PR, Parenchymatic ray; IPL, Inner parenchymatic layer; OS, Oospore; VB, Vascular bundle. Bars = 50 µm.

pustules appeared on the lower surfaces of the cotyledons and also at the tip of the primary leaves. When the disease was prolonged, pustules were also developed on the upper surface of the cotyledons. No infection occurred in un-inoculated seedlings of cv. Giganteus (negative control).

Naturally derived primary infections from oospore contaminated seeds of field samples were observed when grown under controlled conditions. Sporulation started 3 wks after sowing and reached 58 % of infection (sample 3, Table 2). A similar rate of 56 % infection was reached in a control where 6 d old seedlings were inoculated with 400 oospores per plant, whereas no infection occurred in un-inoculated seedlings of cv. Giganteus (negative control). Although seeds of 5 of the 17 field samples were immature and did not germinate, 10 of the 12 germinating samples showed infections ranging from 5–58 % with an average rate of 25 %. Samples with low rates of oospore-contaminated seeds (Table 1) showed no (sample 15) or low primary infection (sample 11), but the rate of infection was not always correlated with the observed rate of seed contamination (e.g. samples 5 and 16).

Asymptomatic infection of the pathogen

Asymptomatic occurrence of *Pustula helianthicola* in seedlings of the seed-borne-infection-test was found when DNA from five seemingly healthy plants of three samples (3, 10, and 15) was used for a pathogen specific PCR-based test (Fig. 3). PCR with COX2 HelF3/R2 gave amplification products of the expected size (*ca*. 190 bp) with DNA extracts of 10 out of the 15 seedlings. In sample #3, all five tested plants (S3/1–S3/5) showed amplification products for *P. helianthicola*, whereas in sample #10 only one plant (S10/4)

Table 2. Rate of infection in seedlings cultivated under controlled conditions from seeds of head-infected field samples.

Sample number	Number of seeds germinated out of 50	Rate of infection (%)[1]
1	36	36
2	6	17
3	36	58
4	0	n.m.
5	47	45
6	0	n.m.
7	0	n.m.
8	0	n.m.
9	50	25
10	50	37
11	40	5
12	41	0
13	40	25
14	0	n.m.
15	32	0
16	39	13
17	38	40
Control plants artificially infected with oospores	48	56
Uninoculated control plants	50	0

[1]n.m.: seeds were not mature enough for germination. Rate of infection (%) was determined by counting the total number of germinated seeds *versus* the number of plants showing pustules.

tested positive. In sample 15, four of the five plants tested positive (S15/1, S15/3-S15/5). The intensity of the bands varied considerably from very weak (e.g. S15/4) to strong (e.g. S15/5), resembling the intensity of the positive control obtained with DNA extracted from plant tissue with visible infection symptoms. No amplification product was obtained with DNA of a healthy control plant of cv. Giganteus.

DISCUSSION

Our field observations revealed that in field plots where infected sunflower had been detected in previous years, 97–99 % of the plants showed symptoms of WBR on leaves. In more than 11 % of the infected plants, the pathogen succeeded in invading the flowering parts and in all cases where symptoms were visible at the involucral bracts, oospores were found in achenes. Although this is contradictory to the observation reported by Viljoen *et al.* (1999), they did not describe their methods in detail. Interestingly, the rate of seeds with oospores in our trial was correlated with the number of oospores observed per bract. This may help to estimate the risk of seed-contamination by monitoring bracts of otherwise asymptomatic plants in the field.

Viljoen *et al.* (1999) described the presence of oospores in the pericarp of the achenes and in the seed testa, whereas they found that the embryo was not affected by the pathogen. This is in line with our results, except that we have not found any infective structures in the testa. The latter is difficult to observe and only visible in good semi-thin sections due to the fact that the testa is connected to the pericarp in the achenes of *Asteraceae*. Because the mycelium of the biotrophic oomycetes is usually limited to the intercellular space, it is unlikely that the dense testa can be penetrated. For that reason it was not surprising that we found oospores only in the parenchymatic parts of the pericarp, whereas the dense vascular bundles and fibrous layer were free of pathogen structures. For a second sunflower pathogen, *P. halstedii*, oospores were similarly located in the parenchymatic tissue of the inner pericarp (Delanoe 1972, Cohen & Sackston, 1974, Döken 1989, Spring 2001), and reports on the occasional presence in the embryo (Novotelnova 1963) or in the testa (Döken 1989) have not been confirmed.

Seed transmission requires not only the presence, but also the vitality of the pathogen. This was shown for the first time with sunflower WBR in this study. Seeds of the infected field plants gave rise to an unexpectedly high rate of infected seedlings. About 25 % showed symptoms as little as 3 wk after sowing. This was much higher than in most other seed-transmitted oomycetes where such tests have been conducted. In *P. halstedii*, seedlings from contaminated sunflower seeds remained mostly asymptomatic during the first few months of growth (Novotelnova 1963, Cohen & Sackston 1974, Spring 2001) and in quinoa, the rate of seed-borne infection with *Peronospora farinosa* ranged between 0.2–8.8 %, depending on cultivation conditions (Danielsen *et al.* 2004). Extremely high rates of infectivity were reported from *Hyaloperonospora camelinae* contaminated seeds of *Camelina sativa*, where 96 % developed symptoms (Babiker *et al.* 2012). Interestingly, the authors reported 3 % infectivity with seeds from asymptomatic plants. This indicates that in some cases, seemingly healthy plants can carry the pathogen and propagate it through seeds. Pathogen-specific PCR-based screenings, which have become increasingly available (e.g. Ioos *et al.* 2007, Landa *et. al.* 2007, Babiker *et al.* 2012) could limit the risk of such dissemination. Our own molecular detection system for *P. helianthicola* (Spring *et al.* 2011) helped to identify numerous additional asymptomatic infections amongst seedlings raised from seeds of infected plants (Fig. 3), thus indicating that the risk of infection for sunflower from contaminated seeds may exceed significantly the observed 25 % of early sporulating plants.

In conclusion, the results showed that oospores of *P. helianthicola* are frequently produced in achenes of infected plants. These oospores are highly infective, even in the absence of dormancy or any kind of temperature treatment. They are not only the primary source of infection in sunflower WBR disease in soil, but are a very important source for long distance transmission of the pathogen into new areas.

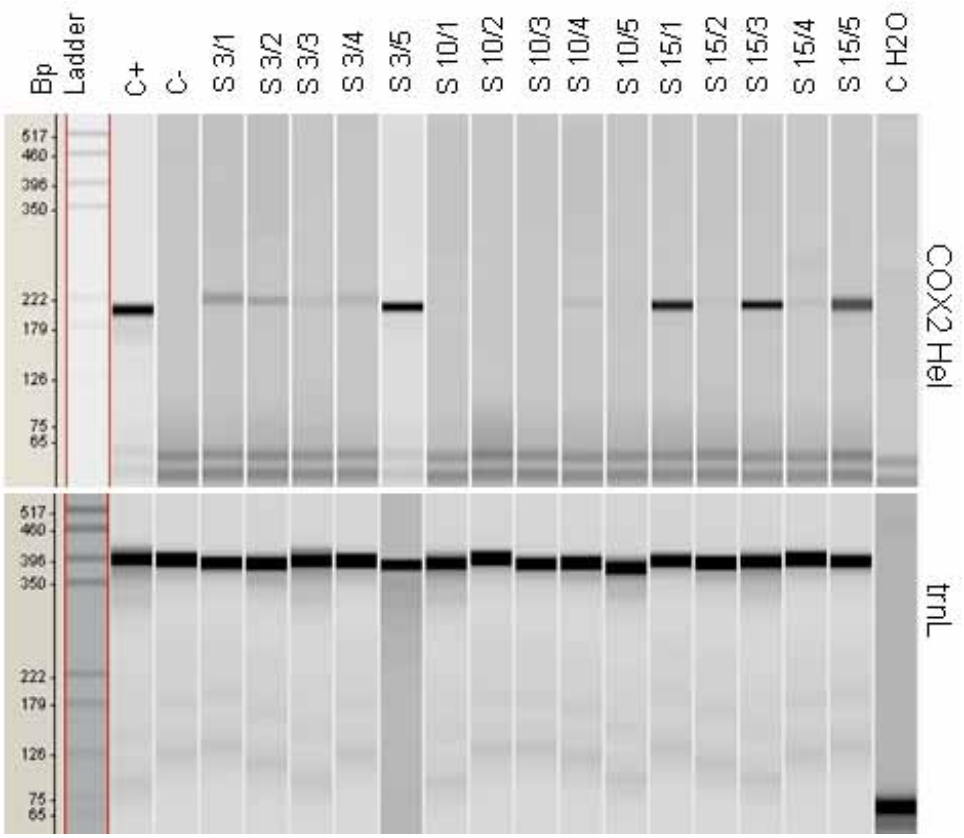

Fig. 3. Pathogen detection in asymptomatic host tissue through PCR with COX2 HelF3/R2. DNA samples: C+, leaf sample of infected plant (with pustules), C- leaf sample of healthy plant, S3 (1–5), S10 (1–5), S15 (1–5) leaf sample of five seemingly uninfected seedlings derived from the field samples S3, S10 and S15; C H2O, water control. PCR with primers for trnL-F/R served as control for the functionality of the DNA.

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