Local infection of opium poppy leaves by *Peronospora somniferi* sporangia can give rise to systemic infections and seed infection in resistant cultivars

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Abstract

Downy mildew (DM) of opium poppy (*Papaver somniferum*) caused by *Peronospora somniferi* is one of the most destructive diseases of this crop due to the systemic nature of infection as compared with local infections caused by *Peronospora meconopsidis*, the other downy mildew pathogen of this crop. We developed an inoculation method using *Peronospora somniferi* sporangia as inoculum and demonstrated for the first time that local infection of leaves by sporangia give rise to systemic infections in the plant as well as of seeds. Our results also showed that this inoculation protocol was very effective in reproducing disease symptoms and assessing the resistance response to DM in opium poppy genotypes under field conditions. More interestingly, results indicate that up to 100% of seed samples from some genotypes showing a complete (symptomless) resistant phenotype were infected by the pathogen when seeds were analyzed by a *P. somniferi*-specific nested-PCR protocol. This latter aspect deserves further attention while breeding opium poppy for resistance to *P. somniferi*.

Additional keywords: *Peronospora meconopsidis*; disease resistance screening; downy mildew; inoculum source; seed transmission.

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Introduction

Downy mildew (DM) caused by either of the biotrophic, obligate oomycetes *Peronospora somniferi* Voglmayr and *Peronospora meconopsidis* Mayor (Voglmayr et al., 2014) poses an important economic constrain on opium poppy (*Papaver somniferum* L.) crops worldwide (Kapoor, 1995; Landa et al., 2007; Scott et al., 2008). Attacks by the disease are also of social significance because opium poppy is the only source of codeine, morphine and thebaine drugs for the pharmaceutical industry, which are key components for alleviation of chronic pain associated with cancer diseases. Until very recently, *P. somniferi* and *P. meconopsidis* have been referred in the phytopathological literature as *P. arborescens* (Kapoor, 1995; Bajpai et al., 1999; Landa et al., 2005; Montes-Borrego et al., 2008; Dubey et al., 2009) and *P. cristata* (Scott et al., 2004), respectively. However, the taxonomic status of the latter two species was recently clarified by means of molecular analyses in conjunction with morphological characteristics (Voglmayr et al., 2014). In Spain, the incidence and severity of DM attacks caused by *P. somniferi* have steadily increased every year since it was first detected in 2005, thus making this disease the main limiting factor for opium poppy producers (Montes-Borrego et al., 2009a). In Tasmania (Australia), the largest world producer of poppy straw (INCB, 2014), DM caused by *P. meconopsidis* have affected opium poppy crops for decades (Scott et al., 2004). However, the recent introduction of the highly virulent *P. somniferi* in that area has caused total loss...
of entire paddocks in different opium poppy growing regions across Tasmania, which have led to increased growers’ concern and promptly triggered an industry-wide response (B. B. Landa, pers. obs.).

Although both \textit{P. meconopsidis} and \textit{P. somniferi} can establish local foliar infections in cultivated opium poppy, only \textit{P. somniferi} can systemically infect and colonize the entire plant thus conferring higher virulence to this species compared with \textit{P. meconopsidis}. According to findings from previous research, diseased plant parts, infested soil and infected seeds are the main sources of primary inoculum (oospores) for \textit{P. somniferi} that can give rise to systemic infection of the plant (Montes-Borrego et al., 2009a). Conversely, \textit{P. somniferi} sporangia formed on infected leaves are thought to be more important as secondary inoculum for leaf infections, with a primary role in the development of polycyclic DM epidemics (Navas-Cortés et al., 2009; Calderón et al., 2014), as it occurs for the other DM pathogen, \textit{P. meconopsidis}, in Tasmania (Scott et al., 2003; 2008). However, to our knowledge, the role of \textit{P. somniferi} sporangia as inoculum capable of establishing systemic infections in opium poppy resistant plants has not yet been demonstrated in spite of the significant risk that this may pose (Montes-Borrego et al., 2009b). Consequently, the objectives of this study were (i) to corroborate if \textit{P. somniferi} sporangia can establish systemic infection in opium poppy and give rise to seed infection in resistant varieties, and (ii) to evaluate the use of a new inoculation method for resistance assessment to DM in opium poppy genotypes under field conditions.

**Material and methods**

**Plant material**

A total number of 20 opium poppy genotypes provided by Alcaliber S.A. (Carmona, Sevilla, Spain) were used in the study (Table 1). Seeds were proven free of infection by \textit{P. somniferi} by molecular testing using a specific-PCR assay (Montes-Borrego et al., 2009a). Out of the 20 genotypes, nine (designated R1-R9) and five (designated S1-S5) genotypes had previously been characterized as potentially resistant and susceptible to \textit{P. somniferi}, respectively, in field experiments under natural inoculum pressure (Montes-Borrego et al., unpublished results), three genotypes (designated M8, EA and TAR) are commercial varieties, and the remaining three genotypes (designated F1-1, F1-2 and F1-3) are F1 progenies from crosses between TAR and R9 genotypes. All seeds were surface-disinfested in 1% NaOCl for 5 min, rinsed twice in sterile water, dried up under a flow of filtered air, and stored under sterile conditions until used for experiments.

Seeds of the 20 opium poppy genotypes were sown in 15 × 15 × 20 cm³ pots (four plants per pot) filled with a mixture of artificial substrate (Ahrens Erde Universal Pflanzerde®, Hawita Grupper GmbH, Vechta, Germany): loam (1:1) amended with 4 g Floranid® fertilizer (Compo, Barcelona, Spain) per kg of soil. Plants were grown in a mesh-covered shade house under natural environmental conditions. Plants were watered daily as needed and fertilized every week with 50 mL of Hoagland solution.

**Inoculum source and inoculation method**

Sporangia suspensions were prepared using sporulating opium poppy leaves that were collected from an DM-affected opium poppy field in Écija (Seville) and kept in plastic bags at 4°C until used within the next day. Sporangia and sporangiophores in affected leaves were scraped off with a sterile needle into 1 mL of ultrapure sterile (US) water with a drop of Tween 20 as a wetting agent (Sigma-Aldrich, Madrid, Spain) in 1.5-mL microcentrifuge tubes. Suspensions with sporangia and sporangiophores were vortexed for a few seconds, filtered through a 0.8-µM Millipore filter and the filter was washed three times with US water. Sporangia and sporangiophores retained onto filters were suspended in 1 mL of US water and sporangia concentration in the suspensions was adjusted with US water to 10⁴ sporangia/mL using a hemacytometer. Viability of sporangia in the water suspensions were check by observation under the compound microscope at 400 x.

A new ‘drop’ inoculation method was tested for using sporangia as inoculum. This new method was developed as an alternative to the spraying inoculation method previously tested (Landa et al., 2005) to reduce the amount of inoculum needed for inoculating plants under field conditions. Plants were first sprayed to run off with sterile distilled water, then one 25-µL-drop of the sporangia suspension was placed onto the adaxial surface of each of two leaves or at the junction of a leaf petiole with the stem using a micropipette. Plants similarly treated but using US water without sporangia served as controls. After inoculation, plants were sprayed with sterile distilled water again.

**Assessment of DM resistance in opium poppy genotypes and demonstration of systemic infection by leaf inoculation with sporangia**

Pot-grown plants of the 20 opium poppy genotypes referred above were inoculated at the stage of 6 to 8 true leaves using the ‘drop’ inoculation method described
above. Inoculated and control plants were incubated in a mesh-covered shade house under natural environmental conditions from mid-January to mid-June, 2011. Since the environmental conditions in the shade house were unusually warm that season, plants were inoculated four times, each other 10 days apart, to ensure infection by sporangia. Plants were sprinkler-irrigated daily to ensure high humidity and fertilized once a week. There were five replicated pots (four plants per pot) for each opium poppy genotype in a completely randomized design. Plants were allowed for capsules to form to determine if systemic colonization by the pathogen had occurred and led to infection of the capsule and seeds.

Seeds were collected from individual capsules after aseptically removing the peduncle at its bottom. Then, a composite seed sample was obtained combining seeds from the four plants per pot to ensure enough seeds for molecular assays and next season sowings. DNA was extracted from two independent composite samples and assayed for detection of *P. somniferi* using the nested specific-PCR protocol as described below.

### DNA extraction and specific PCR assays

Genomic DNA was extracted from opium poppy seeds (100 mg samples) using the G-SpinTM II Plant...
Genomic DNA extraction kit (Intron Biotechnology, Suwon, Korea) according to Landa et al. (2007). A two-step nested-PCR protocol with improved sensitivity for in-planta detection of \( \textit{P. somniferi} \) in symptomless opium poppy tissues was used for the detection of the pathogen in seeds and in plant tissues as previously described (Montes-Borrego et al., 2009a). Briefly, primer DC6 specific for species in the orders Pythiales and Peronosporales of the Oomycota and the universal ITS4 primer were used in the first round of a nested-PCR protocol. The resulting PCR product was diluted 1:10 and used as a template for a second round of amplification using primers OMPac7fw/OMPac7rv and PCR conditions described by Montes-Borrego et al. (2009a). Results of the nested-PCR were compared with those of single PCR using primers DC6/ITS4 and OMPac7fw/OMPac7rv only. For all the different PCR reactions appropriate positive and negative controls were used to tests accuracy of amplifications and the lack of PCR inhibitors, as described in previous studies (Montes-Borrego et al., 2009a,b; 2011).

Results and discussion

Infected seeds, and infested plant parts and soil harboring oospores were demonstrated the main sources of primary \( \textit{P. somniferi} \) inoculum for DM epidemics in opium poppy crops in Spain (Montes-Borrego et al., 2009a; Navas-Cortés et al., 2009). Thus, infection by \( \textit{P. somniferi} \) oospores were shown to give rise to systemic infection of opium poppy that in some circumstances may remain asymptomatic. On the other hand, sporangia of \( \textit{P. meconopsidis} \) and \( \textit{P. somniferi} \) formed on primary leaf infections are known to play a key role as secondary inoculum for the spread of the pathogen and development of DM epidemics under field conditions (Scott et al., 2003; 2008; Calderón et al., 2014). However, the ability of \( \textit{P. somniferi} \) sporangia to establish systemic infection in opium poppy and to reach the internal tissues of the capsule infecting the seeds of resistant varieties had not been demonstrated yet. Consequently, the study was aimed to determine if \( \textit{P. somniferi} \) sporangia can establish systemic infection in opium poppy plants and give rise to seed infection in opium poppy genotypes showing different level of disease resistance to \( \textit{P. somniferi} \) under field conditions.

Symptoms on plants inoculated with the ‘drop’ inoculation method using a sporangia suspension started to develop by mid-march, about 1 month after first inoculation, when plants reached the rosette stage. Those symptoms included generalized leaf chlorosis, bulky plant growth, tissue deformation (Fig. 1A) and abundant leaf sporulation of the pathogen. Thereafter, symptoms continued to appear until formation of the flower stalk, 1 month after appearance of the initial symptoms. In susceptible genotypes, sporulation was observed occasionally on sepalsof the flower stalk (Fig. 1B) and further chlorotic lesions and sporulation on the flower peduncle developed at the flowering stage (Fig. 1C) or capsule formation (Fig. 1D). Overall, the incidence of disease ranged from 0 to 5% in the nine R1 to R9 opium poppy genotypes considered as resistant to DM, whereas 100% disease incidence occurred in the five S1 to S5 opium poppy susceptible genotypes (Table 1). As expected, the three opium poppy commercial varieties showed moderate susceptible (M) to DM, with a mean incidence ranging from 35 to 60% (Table 1). Interestingly enough, the three F1 progenies of crosses between TAR (M) and R9 (R) showed no symptoms during all the growing season in spite of repeated inoculations (Table 1). This result indicated that the resistance to DM caused by \( \textit{P. somniferi} \) seems to be inherited as a dominant trait contrary to results from studies about DM resistance in India that let to conclude on a monogenic recessive inheritance of resistance (Dubey et al., 2009). Recent molecular analysis has shown that \( \textit{P. meconopsidis} \), but not \( \textit{P. somniferi} \), is the DM pathogen infecting opium poppy crops in India (Gupta et al., 2016). This may explain the different results obtained in our study.

Results of nested-PCR assays using seeds collected at the end of experiment from capsules displaying: (i) severe symptoms (genotypes S2, S4, S5, Table 1), (ii) some sporulation (genotypes S1, S3, M8, EA, TAR; Table 1), or (iii) no symptoms (genotypes R1 to R9 and F1-1 to F1-3; Table 1) (Fig. 1H,I), indicated that inoculation with sporangia can result in infection of capsules and give rise to infected seeds. All seed sampled from sporulating capsules were positive for \( \textit{P. somniferi} \) in the nested-PCR assay (Table 1). Moreover, the pathogen could be detected in samples of capsules showing severe (i.e., genotypes S2, S4, S5; Fig. 1E) or moderate (i.e., genotypes S1, M8, EA, TAR) symptoms (Table 1) by single round of PCR using primers OMPac7fw/OMPac7rv or DC6/ITS4 without the need of the second round of the nested-PCR (Table 1), although at a frequency lower than the latter. More interestingly, use of the nested-PCR assay allowed detection of \( \textit{P. somniferi} \) in 20 to 100% seed samples from symptomless capsules, as well as from capsules of all the genotypes rated as resistant due to the absence of DM symptoms. Comparatively, use of primers OMPac7fw/OMPac7rv in single PCR assays allowed detection of the pathogen in 50 to 75% of seed samples in only three of the nine resistant genotypes evaluated (Table 1).
Therefore, results show that the ‘drop’ inoculation of opium poppy leaves with a suspension of *P. somniferi* sporangia is efficient for establishing infection and reproducing the chlorotic and deformation syndrome frequently observed in DM affected-plants in commercial fields (Montes-Borrego *et al.*, 2009a). This ‘drop’ inoculation method would be particularly useful when assessing a large number of opium poppy varieties under field conditions, since the amount of inoculum needed is 40X times lower than that for the spray inoculation method used previously (data not shown). Also, results from this work clearly demonstrated that sporangia of *P. somniferi* formed on infected plants are effective in establishing secondary local infections that later may become systemic and give rise to infected seeds, thus confirming our early preliminary observations (Montes-Borrego *et al.*, 2009a). This result is of much importance for understanding the DM pathogenesis caused by *P. somniferi*, and indicate that the opium poppy/DM pathosystem is similar in that regards to other systemic downy mildew diseases such as sunflower DM caused by *Plasmopara halstedii* (Cohen & Sackston, 1974) and pea DM caused by *Peronospora viciae* (Mence & Pegg, 1971).

Management of DM of opium poppy with fungicide treatments is difficult because of a scarcity of registered fungicides for this minor crop and the systemic nature of infection by *P. somniferi*. Consequently, development of new opium poppy cultivars with increased resistance to DM is the best alternative to chemical control for DM management, which in addition can contribute to limiting pesticide use and lowering of production costs (Bajpal *et al.*, 1999; Gisi, 2002). Accurate screening for resistance to DM in opium poppy is difficult to conduct because low and/or irregular soil- and air-borne inoculum can make results unreliable under some circumstances. In addition, the obligate biotrophy of the pathogen makes availability of pathogen inoculum and its infectivity to vary and dependent on cropping seasons. Moreover, a strong influence of environment on symptom development makes disease reaction to depend on occurrence of favorable environmental conditions and vary during cropping season. Although assessing DM
resistance under controlled environmental conditions could circumscribe those inconveniences, flower, mature capsules and alkaloid content produced by opium poppy plants under growth chamber conditions differ much compared with those produced under field conditions. Thus there was a need to develop a DM inoculation method suitable for use under filed conditions. The inoculation protocol used in this study was proven simple, less inoculum-consuming, and effective in establishing infection, and more important it allowed the correct and accurate phenotyping and evaluation of opium poppy genotypes for their disease reaction to P. somniferi, thus facilitating the identification of sources of resistance to DM and at the same time assessing the agronomic and physiological characteristics of the lines under field conditions. 

In this present work we also demonstrated the usefulness of a molecular protocol for in-planta detection of P. somniferi (Montes-Borrego et al., 2009b; 2011) to validate results of the ‘drop’ inoculation method for phenotyping resistance to P. somniferi in opium poppy genotypes. Thus, all opium poppy genotypes characterized as resistant because of lack of DM symptoms during the entire plant growth were found to produce seeds infected by the pathogen (Table 1). Interestingly, the amount of P. somniferi in seeds formed by some of these genotypes (genotypes R1, R5 and R7) must have been very high since the pathogen could be detected by single-PCR assays using primers OMPac7fw/OMPac7rv, which is 10 to 100 x times less sensitive compared with the nested-PCR assays using the same primers (Montes-Borrego et al., 2009b). This latter aspect deserves further attention while breeding opium poppy for resistance to P. somniferi.

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