UNIVERSITY *of* TASMANIA

Comparison of the biology and host range of downy mildew pathogens - *Peronospora somniferi* and *Peronospora meconopsidis* of opium poppy

by

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Statements and Declaration

Declaration of Originality

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Statement of co-authorship

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Abstract

Opium poppy is an economically important crop belonging to the family Papaveraceae. It is primarily grown for its opiate alkaloid compounds, including codeine, morphine, thebaine and noscapine, which are used in the manufacture of pharmaceutical products. The Australian state of Tasmania contributes more than 50% of licit global opiate production. Downy mildew, caused by infections with one of two distinct *Peronospora* spp., is a major threat to Australian opium poppy cultivation. *Peronospora meconopsidis* has been associated with poppy downy mildew in Tasmania since the 1990s. Infection by this pathogen is characterised by the production of angular localised lesions and sparse sporulation on leaves of infected plants. In recent years, a second species of poppy downy mildew has been found in Tasmania, *Peronospora somniferi*. This pathogen produces systemic host infection, with symptoms of chlorosis and stunting with profuse sporulation. This thesis has the broad aim of identifying the similarities and differences in infection and disease development of these two pathogens. This information is needed for the development of disease management strategies for these two pathogens.

The role of soil-borne oospores in the development of poppy downy mildew was examined for *Pe. somniferi* and *Pe. meconopsidis*. For both pathogens, oospores in infested soil were shown to initiate infection of host plant roots following germination and production of germ tubes and appressoria. This was initially asymptomatic both within and between cells before symptom expression was evident. Pathogen invasion from soil-borne infections was subsequently observed in leaf, root and stem tissues. The process of infection of both species were similar, although the rate and extent of symptom expression following inoculation with *Pe. somniferi* was faster than with *Pe. meconopsidis*. The study also identified the role of herbicide stress in stimulating expression of disease in asymptomatically infected plants. Foliar application of specific herbicides applied at sublethal levels to latently infected poppy plants stimulated more rapid expression of downy mildew symptoms reflecting what has been observed under commercial production.

The role of sporangial inoculation of two pathogens in the development of poppy downy mildew symptoms was examined. The study evaluated sporangial germination and growth, appressorium formation and infection dynamics following sporangial

inoculation to poppy leaves. Results showed that *Pe. somniferi* infective structures germinate, develop and invade the host faster than *Pe. meconopsidis*. Using a spectrophotometric assay, H_20_2 production was measured during foliar infection of both pathogens. The localisation of H_20_2 in the host leaf tissue was also visualised using a DAB staining method. H_20_2 production in opium poppy leaves was significantly higher following *Pe. meconopsidis* infection than with *Pe. somniferi* infections. This work suggests the host defence responses against *Pe. meconopsidis* may be more effective than *Pe. somniferi.* The results provided an insight into how *Pe. somniferi* is more aggressive than *Pe. meconopsidis* during the host-pathogen interaction.

Finally, the experimental host range of *Pe. somniferi* and *Pe. meconopsidis* within selected weed (*Pa. dubium*) and ornamental members (*Pa. nudicaule, Pa. orientale, Pa. commutatum, Pa. laciniatum, Pa. atlanticum Pa. rhoeas, Pa. paeoniflorum*) of the Papaveraceae family was investigated. Closely related Papaveraceae members including weed and the ornamental species, (*Meconopsis cambrica*) and a Papaveraceae control (*Pa. somniferum*) were challenged with both pathogens using both foliar-applied sporangia and soil-borne oospore inocula. Other than *Pa. atlanticum,* considered as putative weak host, all tested plant species were susceptible to both pathogens although expression of symptoms varied between species. The seeds collected from plants infected with either pathogen from several of the species *(Pa. somniferum, Pa. dubium, Pa. rhoeas* and *Pa. nudicaule)* was shown to carry the respective pathogen indicating infested seed could be an important means for the global spread of these pathogens.

These studies provide comparison and improve our understanding of the pathology of the two poppy downy mildew pathogens, *Pe. somniferi* and *Pe. meconopsidis* in opium poppy. While infection processes were similar for the two species, the rate of infection and symptom expression was faster for *Pe. somniferi*. This could have implications for disease management strategies, such as timing of fungicide applications. This study also identified alternative hosts that may be important as inoculum reservoirs or means of pathogen long distance spread.

Explanatory Note on Thesis Structure

This thesis has been written in the form of individual manuscripts for each chapter. As a result, some elements of replication between chapters may occur. Chapter 1 includes an introduction to the research work and the thesis overview. Chapter 2 reviews the available information on poppy downy mildew and the research gaps. Chapters 3, 4, and 5 are experimental chapters. Chapter 6 is a general discussion and future prospects.

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Chapter 1: General introduction

Opium poppy (*Papaver somniferum* L) is an annual crop, belonging to the family Papaveraceae and grown for its alkaloid compounds, including morphine, codeine, thebaine and noscapine (INCB, 2019; Thangavel, 2018). It is one of the most commercially valuable medicinal crops used by the pharmaceutical industry. Opium poppy cropping had its origin in middle Asia (Bernath, 2003; Kapoor, 1995) and spread to Europe and North Africa by the Greeks, Romans and Egyptians (Bernáth and Németh, 2009; Kapoor, 1995). The crop was later grown across all the temperate climatic regions of the world (Bernath, 2003). The first mixture of morphine and narcotine from opium was extracted from poppy capsules by J.F. Dorosne in the year 1803 (Kapoor, 1995).

Globally, only a few countries legally cultivate opium poppy for its pharmaceutical opiate compounds (INCB, 2019; Bernath, 2003). Production is regulated by the International Narcotics Control Board under a mandate from the United Nations. Australia is the world's largest producer of licit opium poppy (Frappell, 2010), with most of the Australian production from the state of Tasmania, due to its secured geographical location, suitable climate, political stability, natural resources, and modern agricultural practices (Miltenburg, 2018; Bradsher, 2014; Laughlin et al., 1998). Other major world producers of opiates include Turkey (23%), France (21%), Spain (4%) and India (INCB, 2019). According to the latest International Narcotics Control Board report, the Australian poppy industry supplied 220 tonnes of thebaine, 9.6 tonnes of codeine and 100 tonnes of morphine, on approximately 16,294 ha by 850 growers in 2018 (INCB, 2019).

Opium poppy is affected by several diseases, including poppy fire (*Alternaria penicillata, Alternaria papavericola*), collar rot (*Rhizoctonia solani*), bacterial soft rot (*Pectobacterium carotovorum*), Turnip mosaic virus (TuMV), Poppy mottle virus (PoMV) and Bean yellow mosaic virus (BYMV) (Woundberg et al., 2013; Sattar et al., 1988; Aranda et al., 2008; Mishra et al., 2013; Scott et al., 2004; Thangavel et al., 2016; Montes-Borrego et al., 2017; Kapoor, 1995, Yossifovitch, 1929). However, globally, downy mildew causes the most significant yield losses (Kapoor, 1995; Landa et al., 2007; Montes-Borrego et al., 2017; Thangavel et al., 2018). Downy mildew was first reported in Australia in the late 1990s (Cotterill and Pascoe, 1998) and it is considered the biggest disease constraint to the Australian poppy industry. The first report in Tasmania, identified the pathogen as

Peronospora arborescens (Cotterill and Pascoe, 1998) with the disease associated with localised angular necrotic foliar lesions with sparse sporulation. The pathogen nomenclature was later revised to *Pe. cristata* (Scott et al., 2004). A second form of downy mildew was reported in Australia in 2013 associated with systemic chlorosis, stunting and profuse sporulation (Thangavel et al., 2018). Following a recent taxonomic review, the pathogen identified causing the original localised form of downy mildew was reclassified as *Pe. meconopsidis* and the systemic form as *Pe. somniferi* (Voglmayr et al., 2014). *Peronospora meconopsidis and Pe. somniferi* are now known to occur in the poppy-growing regions of Tasmania, Asia and Europe (Landa et al., 2007; Montes-Borrego et al., 2008; Thangavel et al., 2016; Voglmayr et al., 2014; Gupta,2016; Alam, 2011).

Peronospora spp. are oomycetes and are obligate biotrophs that can only survive in a living host (Thines and Choi, 2016; Thines and Kamoun, 2010). This group of pathogens are identified by their morphological structures, including mycelium, sporangia and oospores by various microscopical techniques (Voglmayr et al., 2014), and by molecular techniques (Scott et al., 2004; Montes-Borrego et al., 2011; Thangavel et al., 2016). *Peronospora* spp. are known to produce oospores by sexual reproduction (Renfro and Shankara Bhat, 1981; Davis, 1987; Montes-Borrego et al., 2009; Gascuel et al., 2015; Gomez and Figueira-Duarte, 2012), with both *Pe. somniferi* and *Pe. meconopsidis* able to produce oospores (Voglmayr et al., 2014). The oospores of downy mildew pathogens have been recorded in leaves, root and stem tissue of infected plants (Gascuel et al., 2015; Gomez and Figueira-Duarte, 2012; Ei- Assiuty et al., 2019). Oospores are present in plant debris, seed, and in soil and are considered an important primary inoculum source for the disease cycle (Montes-Borrego et al., 2009; Thangavel et al., 2018). Reports from Spain have shown that oospores present in the soil can result in downy mildew infection of opium poppy (Montes-Borrego et al., 2009). It is not clear whether soilborne inoculum of both *Pe. somniferi* and *Pe. meconopsidis* can result in infected poppy seedlings nor how the pathogen invades systemically through the plant once infection has occurred to the aerial parts of the plant. This has been studied for other downy mildew pathogens (Ei- Assiuty et al., 2019; Kitz, 2008; Gascuel et al., 2015). Therefore, comparisons of infection and host invasion processes of *Pe. somniferi* and *Pe. meconopsidis* from the soil borne inoculum (oospores) is warranted to confirm the role of soil-borne inocula for each species in disease development and suggest approaches for disease management.

Asexual production of sporangia occurs for both species (Voglmayr et al., 2014; Thangavel et al., 2016). *Peronospora somniferi* produces profuse sporulation on undersides of leaves, whereas sparse sporulation on the abaxial leaf surface is observed for *Pe. meconopsidis* associated with vein-delimited, angular, localized necrotic lesions (Voglmayr et al., 2014; Thangavel et al., 2016). However, the differences in the infection and host invasion from a sporangial inoculum of each species have not been studied. This would be of research interest and important to further understand the comparative biology and pathogenicity of *Pe. somniferi* and *Pe. meconopsidis,* including why symptoms expression differs.

Alternative hosts play an important role in disease outbreaks. They can act as a green bridge for the pathogen survival between seasons and can assist long-distance dispersal of the pathogens with the movement of plant material and propagules. *Peronospora* spp. are known to cause downy mildew in a diverse range of weeds, horticultural and the ornamental crops (Choi et al., 2009; Crandall et al., 2018; García-Blázquez et al., 2008; Gaumann, 1918). Several weeds and ornamental *Papaver* species are found in the poppy-growing regions of Tasmania (Cotterill and Pascoe, 1998). For instance, weed *Papaver* spp. associated with commercial poppy crops in Tasmania include *Pa. dubium, Pa. rhoeas, Pa. argemone* and *Pa. hybridum* which have records of infection with *Pe. arborescens* (Cotterill and Pascoe, 1998; Garibaldi et al., 2003). Following taxonomic revisions, the host range of the two downy mildew pathogens is unclear, although Voglmayr et al. (2014) suggested a very limited host range for each species. Given the abundance and importance of weeds and ornamental *Papaver* spp. within or near commercial opium poppy production regions, and the likelihood of regular importations of seed, knowledge of the host status of these species with the two opium downy mildew pathogens will be important to determine potential risk or inoculum carryover and movement.

This PhD thesis compares the features and dynamics of the infection processes of the two important pathogens causing downy mildew of opium poppy and provides a greater understanding of the role of inoculum sources and alternative host species, which are important factors to consider when developing management strategies for the control of poppy downy mildew in Tasmania.

Overview of thesis structure

This thesis is formatted as individual chapters prepared for journal publication following university requirements. As such, some of the content, including literature review, general introduction and methods, is partially repeated between chapters. It should be noted that to avoid potential confusion between *Papaver* and *Peronospora*, the genus abbreviations *Pa*. and *Pe*. have been used, respectively, throughout this thesis rather than the standard *P*. for each.

Chapter 1: Provides an overview of this thesis, research area, major research questions and thesis structure overview.

Chapter 2: This chapter reviews relevant literature available on downy mildew disease and the pathogens and identifies the knowledge gaps to be addressed in this thesis.

Chapter 3: This experimental chapter examines the differences in infection and colonisation processes between the two downy mildew pathogens from oospore inocula present within infested soils. This chapter further investigates the role commercially used herbicides may play in induction systemic downy mildew symptoms.

Chapter 4: This experimental chapter examines the differences in infection and colonisation processes between *Pe. somniferi* and *Pe. meconopsidis* following inoculation of foliage with sporangia inocula. This study included an examination of the relative accumulation of reactive oxygen species associated with host defence in infected tissues by infections with the two species.

Chapter 5: This experimental chapter determines the host status and relative disease expression of a variety of weed and ornamental Papaver spp. for infections with *Pe. somniferi* and *Pe. meconopsidis* under glasshouse conditions. This was examined using both soil and sporangial spray inoculations.

Chapter 6: This general discussion chapter provides both summary and conclusions of the outcomes of this thesis and outlines future work of value identified from this project.

Chapter 2: Literature review

2.1 Opium poppy botany

Papaver somniferum, or opium poppy, is an annual herb belonging to the family Papaveraceae, which has its origins in the Mediterranean region (Egan, 2011; Labanca et al., 2018; Kapoor, 1995; Mahdavi-Damghani et al., 2010). The Papaveraceae family consists of 40 genera and 800 species (Egan, 2011). The term 'poppy' is used, generally, to refer to many species of the Papaveraceae family. 'Opium' refers specifically to the air-dried latex extract obtained from *Papaver somniferum* L.

The plants of this genus are characterized by the watery, milk-like latex in the vessels, in all their parts, except the seeds (Labanca et al., 2018). The opium poppy is characterised by a thick root and stem, intensely glaucous plant parts, and a light covering of hairs on the stem and the leaves (Kapoor, 1995). The development phases of opium poppy are seed germination, the formation of a rosette leaf, the hook, flowering, and maturation of the capsules. This is followed by drying and harvesting (Shuljgin, 1969; Mahdavi-Damghani et al., 2010).

In the early life stages, the opium plant is sensitive to variation in the photoperiod. As early as 4 days following emergence, it requires a minimum of four day and night cycles to initiate flowering (Wang et al., 1997). Flowering initiation is significantly affected by an extension of the photoperiod to 24 h light (Mahdavi-Damghani et al., 2010). The date of emergence of the flower hook is directly proportional to the duration of the photoperiod required to initiate flowering (Mahdavi-Damghani et al., 2010). Flower colour varies from white to red to purple with different genotypes, has four petals, and can measure up to 120 mm in diameter. Capsules are crowned with 12-18 stigmatic rays.

Growth and development of opium poppies are influenced by available water. High soil moisture is required for seed germination. Water stress during plant growth can result in up to 13 % reduction in biomass without any noticeable impact on capsule yield. The application of deficit irrigation before flowering can help reducing nitrogen leaching loss and may accumulate nutrients for the post-flowering phase (Mahdavi-Damghani et al., 2010). In contrast, excessive rainfall will enable the plants to grow taller and can results in crop lodging at maturity.

2.2 Global cultivation of opium poppy and its importance in Australia

Opium poppy is an ancient medicinal plant that remains the only commercial source of alkaloids such as morphine, codeine thebaine, and noscapine of great pharmaceutical importance (Calderón et al., 2014; Montes-Borrego et al., 2017; Thangavel et al., 2018; INCB, 2019). Poppy is also grown for the production of seed used in culinary processes. According to their commercial use, poppy genotypes are classified into three distinct groups: industrial, culinary, and industrial and culinary. The first group consists of the plants that are grown for alkaloid extraction from the capsule; the second, for those cultivated for seeds and oil production; and the final group, where the capsules are used for both above purposes (Labanca et al., 2018).

Australia is the world's leading licit producer of morphine, codeine and thebaine opiates, supplying approximately 50 % of global needs, with an annual farm gate value of AUD$100 million (Thangavel et al., 2018), followed by Turkey, France, Spain and India (INCB, 2019). Poppy cultivation in Australia occupies approximately 16,294 ha (INCB, 2019), with Tasmania being the leading producer due to its favourable climate, geographical location, and good crop security (Figure.2.1 A and B). Poppy production in Australia is conducted under contract to one of three commercial producers: Tasmanian Alkaloids, Sun Pharmaceuticals or Palla Pharma. The industry is regulated by the Poppy Advisory and Control Board which is administered by the Tasmanian State Government. The peak body representing the interests of poppy growers is Poppy Growers Tasmania.

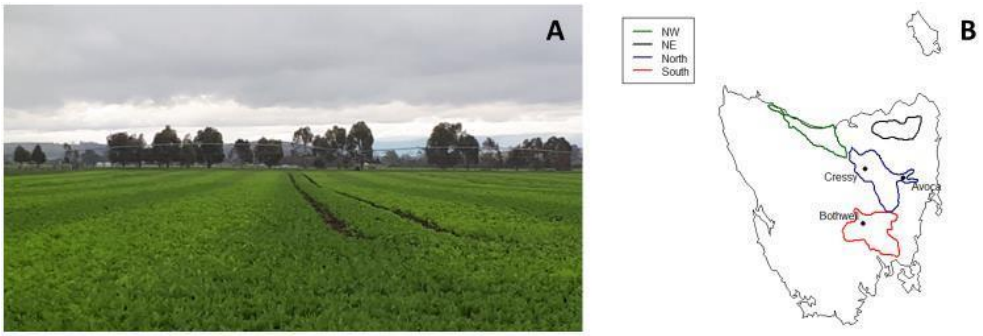

Figure 2.1: A- A commercial opium poppy field in Tasmania. B- Map of typical opium poppy growing regions in Tasmania, Australia.

2.3 Medical uses of opiates

The alkaloids extracted from *Pa. somniferum* capsules are prized for their medical and economic value (Tétényi, 1997; Labanca et al., 2018). However, while over 42 different alkaloids are found in *Pa. somniferum*, only a few are of direct pharmaceutical importance, including morphine, codeine, thebaine, papaverine and narcotine (Kapoor, 1995; Alam et al., 1996; Dittbrenner et al., 2012; Labanca et al., 2018). Their applications include use as muscular relaxants, analgesics, and antimicrobial agents (Ziegler and Facchini, 2008). While global consumption of morphine rose from between 5 and 8 Mg over the 13 years from 1981 to 1994, to 46 Mg in 2000 (Singh et al., 2003), the production demand has decreased since 2014, most likely due to oversupply in the world market (INCB, 2019).

2.4 Taxonomy *Peronospora* spp.

Oomycetes are diverse group of eukaryotic organisms, and no longer considered true fungi. They belong to the Phylum Oomycota, kingdom Straminipila (Dick, 2002; Beakes et al., 2014; Thines and Choi, 2016). More than 700 species of downy mildews have been assigned into 19 different genera, and these genera constitute three distinct monophyletic groups. These three major groups of downy mildew include (i) downy mildew with coloured conidia, (ii) downy mildews with pyriform haustoria and (iii) brassicolous downy mildews. The genus *Peronospora* belongs to a group containing coloured conidia (Thines and Choi, 2016). It was believed that the downy mildews originated from *Phytophthora*-like ancestors as they have been shown to be closely related to this genus (Göker et al., 2007; Choi et al., 2009; Cohen et al., 2014). However, phylogenomic analysis with available data suggests the independent evolution of *Phytophthora* and downy mildews (Matari and Blair, 2014; Thines and Choi, 2016).

The classification of *Peronospora* spp. is based on their morphology and molecular phylogeny. However, the practice of using morphometric methods to determine taxonomical classifications has been shown to be imprecise (Spencer-Phillips et al., 2002). This is mainly because of the influence of the environment on the morphology of the structure and due to the limited technical advances (Hall GS, 1996), which are reflected in the *Peronospora* taxonomy (Bary, 1863). Morphological characterisation of *Peronospora* spp. infecting poppy, does not provide enough

information to easily identify individual species as they share many common structural features. Therefore, molecular analyses have been necessary to identify and differentiate the species (García-Blázquez et al., 2008; Scott et al., 2004; Montes-Borrego et al., 2009; Montes-Borrego et al., 2011).

The highly conserved internal transcribed spacer (ITS) regions have been commonly used to provide specific sequence identities for oomycete species (Ristaino et al.,1998; Cooke et al., 2000). there have been disadvantages identified in the use or reliance on ITS sequences. This is due to the longer ITS region insertions by long repetitive repeats and exhibiting insufficient variability among the closely related species. (Choi et al., 2015). There may also be difficulty in differentiating very closely related species Due to these limitations, researchers have looked to additional conserved sequence regions for taxonomic comparisons. These have included the cytochrome oxidase COX 1 and COX 2 gene regions associated with mitochondrial DNA (Choi et al., 2015; Hudspeth et al., 2000) and are now widely used for the identification of oomycetes (Hudspeth et al., 2000, 2003; Cook et al., 2000; Voglmayr et al.,2014).

The first reported *Peronospora* pathogen of opium poppy was *Pe. arborescens* (Reid, 1969; Francis, 1981; Gustavsson, 1991; Landa et al., 2005). This was described from infected poppy plants in Tasmania (Cotterill and Pascoe, 1998), with the pathogen subsequently being reclassified as *Pe. cristata* (Scott et al., 2004). More recently, a comprehensive taxonomical revision of *Peronospora* spp. has been undertaken, which identified two pathogen species associated with poppy downy mildew, *Pe. somniferi* and *Pe. meconopsidis* (Voglmayr et al., 2014). Both these species are now known to be present within Tasmania (Thangavel et al., 2016).

2.5 Host range of *Peronospora* spp. associated with opium poppy.

With respect to plant hosts of *Peronospora* spp., there are both broad and narrow host ranges reported (De Bary, 1863; Gaumann, 1918; Yerkes and Shaw, 1959; Thines and Choi, 2016). For those species pathogenic on opium poppy (*Pa. somniferum*), infections caused by *Pe. arborescens* have been reported in *Pa. rhoeas* (Berkeley and Broome, 1846; Montes-Borrego et al., 2011), *Pa. dubium* (Cotterill and Pascoe, 1998; Cotton, 1929), *Pa. argemone* (Cotterill and Pascoe, 1998; Cotton, 1929), *Pa. nudicaule* (Alcock, 1933; Cotterill and Pascoe, 1998), *Pa. setigerum* (Behr, 1956), *Pa. alpinum*, *Pa.*

caucasicum Bieb. (Francis, 1981), *Argemone mexicana* (Maiti and Chattopadhyay, 1986), as well as many species of *Meconopsis* (Cotton, 1929; Alcock, 1933; Reid, 1969). Similarly, *Pe. cristata* has been recorded infecting *Pa. hybridum* (Constantinescu, 1991), *Pa. rhoeas* (Gäumann, 1923) *Pa. argemone* (Gäumann, 1923; Gustavsson, 1959), *Meconopsis cambrica* (Reid, 1969) and *Pa. somniferum* (Scott et al., 2004). However, some confusion remains as to the status of these reports following the revisions to the taxonomy of *Peronospora* spp. (Voglmayr et al., 2014). Following reclassification, *Pe. somniferi* was described as a pathogen of *Pa. somniferum* only (Voglmayr et al., 2014), and *Pe. meconopsidis* as a pathogen of two different species, *Pa. somniferum and M. cambrica* (Voglmayr et al., 2014). Given the conflict between previous reports of infection across *Papaver* and related spp. and the more recent narrow host definition there is a need to clarify the host status of alternative *Papaver* spp. to infection with the two poppy downy mildew pathogen species as many of these are common weed and ornamental species present in poppy growing regions that are potential pathogen reservoirs.

2.6 Global distribution of opium poppy downy mildew:

Downy mildew is a devastating disease of opium poppy reported wherever poppies are cultivated (Yossifovitch, 1929; Rathore, 1986; Kapoor, 1995; Landa et al., 2007). The first reported pathogen of this disease was *Pe. arborescens* (Reid, 1969; Francis, 1981; Gustavsson, 1991). Total crop yield losses were reported in Europe due to this disease (Yossifovitch, 1929). This disease was also reported as a major problem in India, Iran, South America and North America (Kothari and Prasad, 1970; Scharif, 1970; Francis, 1981; Tewari and Skorpad, 1981).

In Tasmania, downy mildew did not attract the attention of researchers until 1996, when the pathogen, *Pe. arborescens*, was first identified in local crops (Cotterill and Pascoe, 1998). In Spain, poppy crops, whose leaves showed similar symptoms (Landa et al., 2005; Landa et al., 2007) experienced a reduction in crop yield (Montes-Borrego et al., 2009). The species name of the downy mildew pathogen in Tasmania was later revised to *Pe. cristata* (Scott et al., 2004). In 2013, a new form of poppy downy mildew was reported in Australia associated with systemic chlorosis, stunting and profuse sporulation (Voglmayr et al., 2014; Thangavel et al., 2018).

According to the most recent taxonomical classification, the pathogen causing the localised lesion form of poppy downy mildew is identified as *Pe. meconopsidis* and that causing the systemic form as *Pe. somniferi* (Voglmayr et al., 2014). Occurrence of both *Pe. meconopsidis and Pe. somniferi* has been confirmed in the poppy-growing regions of Tasmania, Asia and Europe (Landa et al., 2007; Montes-Borrego et al., 2008; Alam, 2011; Thangavel et al., 2016; Voglmayr et al., 2014; Gupta, 2016). In Tasmania, the localized form of the disease was viewed as a serious but manageable threat to the poppy industry. However, the discovery of *Pe. somniferi* in 2014 (Thangavel et al., 2018; Voglmayr et al., 2014) in Tasmania, with the associated more severe symptom expression, represents a greater threat.

2.7 Downy mildew symptoms and signs

Downy mildews affect several economically important crops, and the characteristic symptoms of downy mildew infection are pale coloured hyphae and conidiophores on the lower surface of the leaves of the plant (Agrios, 2005; Spencer-Phillips et al., 2002). Other distinctive symptoms include leaf chlorosis, irregular localised lesions, leaf discoloration, dwarfing, and the formation of infertile flowers (Sackston, 1981; Singh, 1995; Lebeda and Cohen, 2010, Palti and Cohen, 1980). In general, the first symptoms to appear are small chlorotic leaf lesions, which can evolve into curled and thickened tissues that become deformed and necrotic as the disease develops (Oerke et al., 2006; Agrios, 2005; Cohen et al., 2013; Lebeda and Cohen, 2010). For example, *Sclerospora graminicola* in pearl millet causes chlorosis in the early stage of infection, followed by necrosis in the veins of leaves, which results in malformed plants (Thakur, 1992).

The symptomatic expression of downy mildew infection depends on the age of the plant, density of the inoculum and factors in the environment (Kothari and Prasad, 1970). In some cases, leaf discoloration extends to necrotic lesions (Savory et al., 2011), which were angular due to their being restricted to leaf veins (Savory et al., 2011). These symptoms can also be modified by the existing weather conditions, following leaf chlorosis, the formation of the necrotic lesions is significantly higher in hot, dry weather (Cohen and Rotem, 1971).

The symptoms of downy mildew in opium poppy (Fig 2.2 A and B) were delineated by causal species, *Pe. meconopsidis* or *Pe. somniferi,* in studies by Voglmayr

et al (2014) and Thangavel et al (2017). In the case of the former pathogen, they describe the symptoms as vein-delimited, angular, localized necrotic lesions, with sparse sporulation on the abaxial leaf surface. *Pe. somniferi* infections manifested as stunted and deformed plants, with chlorotic, distorted leaves that showed profuse abaxial sporulation. Although these symptoms are well described, the infection processes and mechanisms responsible for these differences are yet to be examined in detail.

Figure 2.2: Downy mildew disease observed in poppy-growing field of Tasmania. A) systemic infection caused by *Pe. somniferi*. B) Localized lesion caused by *Pe. meconopsidis.*

2.8 Disease cycle of *Peronospora* spp.

Peronospora spp. have both asexual and sexual reproductive phases (Figure 2.3) in their disease cycle. The sexual and asexual stages of the pathogen are well explained in the pathogens *Pe. parasitica. Pe. tabacina, Pseudoperonospora cubensis* (Donofrio and Delaney, 2001; Borras-Hidalgo et al., 2010; Savory et al., 2011). The main inoculum sources of downy mildew pathogens are classified as primary and secondary (Van der Gaag et al., 1993; Turkensteen et al., 2000; Montes-Borrego et al., 2009; Calderón et al., 2014; Ojiambo et al., 2015). An example of the former is carried by the seeds (Landa et al., 2007; Montes-Borrego et al., 2011; Cohen et al., 2014; Montes-Borrego et al., 2017; Thangavel et al., 2018). Microscopical examination of infested seeds found *Peronospora*

spp. like mycelia and oospores were found on the outer seed coat that penetrate the spermoderm tissues and confirmed the pathogen were both *Pe.somniferi* and *Pe. meconopsidis* based on species- specific PCR (Thangavel et al., 2018). These serve as vectors for spreading the pathogens globally and can initiate primary infections in developing plants. Sporangia that are produced from infected plants can act as secondary inoculum spreading the disease in the field by dissemination through the wind (Montes-Borrego et al., 2009; Cohen et al., 2012).

2.8.1 Sexual reproduction

Sexual reproduction occurs in all species of *Peronospora.* The process results in the production of oospores, (Montes-Borrego et al., 2009; Calderón et al., 2014). Oospores are formed when the male and female reproductive organs, the antheridium and oogonium, respectively, fuse in the host plant (Spencer-Phillips et al., 2002). The fusion of the two mating types, results in genetic recombination resulting in thick-walled oospores (Kandel et al., 2019). Two different mating systems are noted in oomycetes, homothallic and heterothallic mating. Homothallic mating can be completed by a single isolate of the pathogen, whereas the heterothallic mating system requires two distinct compatibility types (Gaumann, 1926; Dick, 2001; Judelson et al., 2009). For example, homothallic mating types are reported in *Plasmopora halstedii* and *Pseudoperonospora humuli*) (Gascuel et al., 2015; Gent et al., 2017), whereas heterothallic types are well documented in *Peronospora effusa*, *Bremia lactucae*, and *Plasmopora.viticola* (Judelson et al., 2009; Kandel et al., 2019). The mating system(s) of *Pe. somniferi* and *Pe. meconopsidis* have not been reported.

Within infected host plant tissues, oospores are formed which are then deposited into the soil, on or in plant debris, where they remain in the dormancy phase until conditions favour their germination (Gaumann, 1926; van der Gaag and Frinking, 1997; Lebeda and Cohen, 2010). The formation of oospores in or on the seeds have been reported for several downy mildew species, including opium poppy (Garibaldi et al., 2004; Landa et al., 2007; Kunjeti et al., 2016; Thangavel et al., 2018). While Behr (1956) identified the location of oospores in poppies in the epidermal layer of the leaves Alcock (1933) reported that they might originate within the capsule. Previous studies have reported the dormancy period of *Peronnospora* spp. oospores in the soil may range from 3 to 25 years (McKay ,1957; Gaag and Frinking 1997; Montes-Borrego et al., 2009). Thus,

they can act as an inoculum source for disease outbreaks for many seasons after their formation (Lai et al., 2004; Navazio et al., 2007; Montes-Borrego et al., 2009; Kandel et al., 2019; Zhang et al., 2012; Slagado–Salazar et al., 2018). The role of oospores as an inoculum source has been described for *Pe. arborescens*, *Peronospora effusa* (Montes-Borrego et al., 2009; Slagado–Salazar et al., 2018). However, the role of oospores in soilborne disease transmission by *Pe. somniferi* and *Pe. meconopsidis* remains unclear and needs to be addressed.

2.8.2 Asexual reproduction

The asexual stage of the *Peronospora* lifecycle results in the production of sporangia. The asexual spore is a defining characteristic of certain downy mildew genera. For example, the zoospore producing ability has been lost in the downy mildew genera *Bremia*, *Peronosclerospora, Hyaloperonospora* and *Peronospora* (Voglmayr et al., 2004; Göker et al., 2007; Thines et al., 2009; Thines and Choi, 2016). Sporangia, formed on sporangiophores, can either germinate directly or indirectly. Sporangia that germinate directly to infect new hosts are often called conidia to differentiate then from sporangia that germinate indirectly. Sporangia that indirectly germinate, release zoospores which then infect the host (Thakur and Mathur, 2002). Viable sporangia germinate via the germ tubes and penetrate the leaf tissue as reported in other Peronosporaceous members (Thines and Choi 2016; Kandel et al.,2019)

Sporangiophores that emerge out of the leaf stomata can then disseminate sporangia by wind and are responsible for the secondary spread of disease, an important factor in the poppy downy mildew epidemics (Scott et al., 2003; Calderon et al., 2014; Montes-Borrego et al., 2017). Oomycete sporangia are relatively short lived with persistence dependant on the environmental factors. For example, the lifespan of sporangia of *Peronospora destructor* was higher at a temperature of 10°C with the relative humidity 53 to 95 % and was lower at 33°C and 33% relative humidity (Bashi and Aylor, 1983). Similarly, the sporangial germination of the cucurbit downy mildew pathogen (*Pseudoperonospora cubensis*) has also reported to decrease under sunny days rather than cloudy days (Kanetis et al., 2010). Likewise, in *Peronospora effusa* causing spinach downy mildew, the sporangial germination was found to occur in the temperature range of 5 to 25°C and the rate decreased with an increase in temperature

(Choudhury and McRoberts, 2018). The above environmental conditions such as temperature, relative humidity is therefore important for sporangial germination.

2.9 Environmental factors associated with development of poppy downy mildew.

The epidemiological conditions and the disease progression of the two *Peronospora* species infecting poppy have not yet been thoroughly studied. What is known, however, is that the outbreak and proliferation of downy mildew is greatly influenced by environmental factors, such as, temperature, humidity (Scott et al., 2008), and contaminated soil (Montes-Borrego et al., 2009).

Air temperature plays a significant role in the infection and development of systemic infections by oospores of *Pe. arborescens*. A persistent temperature of 16°C was more advantageous for seedling infection than 20°C. Scott et al (2008) suggested air temperatures ranging from 2 to 26°C with prevalence of 96 % relative humidity were conducive for poppy downy mildew disease caused by *Pe. cristata*. Airborne sporangia of downy mildews are short-lived and may die within hours after release from sporulating lesions. However, those labile sporangia can be effective in inducing recurrent secondary infections because they develop in very high numbers and the damaging effects of suboptimal temperatures on germination and infection can be supplemented by extended periods of high humidity (Bains and Jhooty, 1976; Jeger et al., 1998; Aegerter et al., 2003, Montes-Borrego et al., 2009). The capability of airborne *Pe. arborescens* sporangia to incite local infections that can develop systemic colonization was recently validated under controlled-environment conditions (Montes-Borrego et al., 2017). Apart from this, initial studies in southern Spain implied that recurrent secondary cycles of infection by sporangia formed on plants systemically infected from oospore inoculum may result in epidemics under environmental conditions suitable for disease development (Navas-Cortés et al., 2009).

Temperature appears to be an important factor for downy mildew outbreaks. For example, a study from Spain found that when temperature was >18˚C, the incidence of poppy downy mildew increased (Montes-Borrego et al., 2009). Cohen and Rotem (1971) also observed in cucurbits, a positive relationship between the temperature and the rate at which they were infected by *Pseudoperonospora cubensis.* Temperature was

also found to be a factor in the induction of necrosis and sporulation of *Ps. cubensis*: the former occurred earlier at 25˚C, and the latter was enhanced when the plants were incubated at 20˚C during the day, and 15˚C, at night. Conversely, low temperatures slowed the appearance of the necrotic lesions. In downy mildews of cucumber caused by *Ps. cubensis*, a temperature of 10–15°C was found to be favourable for spore germination and mycelium growth and both declined when it exceeded 17°C (Cohen and Ben Naim, 2016; Cohen et al., 2017). Temperature is also an important factor in determining the transmission rate of *Peronospora* spp. It was observed that the transmission rate of *Peronospora documeti* was 35.9 % from seeds to the seedlings at 14^{o}C while it dropped to 1.3 % when the temperature was raised to 25^{o}C (Zimmer et al., 1992).

Another factor that influences the spread of downy mildew is humidity. A study by Shetty (1987) reported that *Peronospora* spp. grew vigorously in plants during wet and humid conditions. Still other studies have suggested that it may be the combination of temperature and humidity that is the key factor in the development of downy mildew disease. For example, Iliescu et al. (1977) reported that the optimal conditions for downy mildew in pearl millet is a temperature of 24-28°C, along with a relative humidity of more than 90 %, in the late evening and early morning. A similar condition was also observed in the epidemics of onion downy mildew caused by *Peronospora destructor* (Yarwood, 1943; Hilderbrand and Sutton, 1982). In general, infection and sporulation of oomycetes are clearly related to humidity and free moisture. In the case of downy mildew of cucumber caused by *Ps. cubensis,* it was observed that dew period duration and inoculum pressure compensated each other for the sake of successful completion of infection process (Cohen, 1977). In *Pe. tabacina*, the rapid increase in the sporulation was observed when the relative humidity ranged from 97 to 100%, while if the relative humidity was reduced below 96%, the sporulation was greatly decreased (Cruikshank, 1958). A similar study was also observed in other downy mildews such as basil and pearl millet (Su et al., 2004; Atri and Singh, 2019).

Light also plays a direct role in the sporulation of downy mildew pathogens (Cohen et al., 2013; Telli et al., 2020). For example, darkness greatly enhanced the sporulation of the cucumber downy mildew pathogen, *Ps. cubensis* (Rotem et al., 1978). Conversely,

sporulation was significantly reduced when the inoculation site was exposed to direct light in studies conducted with *Pe. tabacina* (Cruickshank, 1963). Similarly, the solar radiation and treatment with UV light reduced the viability sporangia produced *Peronospora destructor* (Rotem et al., 1985). Interestingly, the lethal effects were less severe to sporangia as long as they were attached to the sporangiophore (Rotem et al., 1985).

Thus, environmental conditions play a vital role in the progression of downy mildew. However, while a forecast system was developed for *Pe. cristata* outbreaks in Tasmania (Scott et al., 2008), further work is needed to clarify these relationships for *Pe. meconopsidis* and *Pe*. *somniferi*.

2.10 *Peronospora* spp. infection process

In general, these obligate biotrophic pathogens infect healthy tissue, by penetrating through the cuticle layer of the leaf (Sackston, 1981) and through the roots (Kamoun et al., 1999). These infection mechanisms function best when optimal environmental conditions, in terms of humidity and temperature favour the successful germination of the oospores and sporangia (Savory et al., 2011; Rotem et al., 1978). These then form a germ tube, appressorium and haustorium (Slusarenko and Schlaich, 2003).

The infection process by the oospores in some downy mildew pathosystems occurs through a series of events. The oospores in the soil germinate and penetrate the root epidermis by formation of an appressorium, followed by the colonization of the cells with an intracellular haustorium. This can then be followed by intercellular invasion with coenocytic mycelia of the vascular tissues and systemic movement throughout the plant (Kandel et al., 2019). Histological studies conducted by Milholland et al (1981), showed the inter and intracellular invasion of *Pe. tabacina* in xylem and phloem vessels, and parenchyma cells of tobacco plants.

Studies have shown that oospores play a direct causal role in seed-borne infection of germination seedlings (Landa et al., 2007; Montes-Borrego et al., 2009; Thangavel et al., 2018). Oospores can survive in a dormant state for long periods in the soil emphasising their potential importance as an inoculum source, however the infection processes from the oospore inocula of *Pe. somniferi* and *Pe. meconopsidis* remains poorly understood.

The sporangial infection process is well explained in a study by Donofrio and Delaney (2001). In this case, viable sporangia germinate on the host tissue and form a germ tube. The germ tube ends when an appressorium is formed and all the cytoplasmic contents of the sporangia are transferred (Gómez and Filgueira-Duarte, 2012). Following penetration of the host epidemis via an appressorium a haustorium is formed in the host mesophyll tissue which allows the oomycete to absorb its nutrition for survival (Gómez and Filgueira-Duarte, 2012; Koch and Slusarenko, 1990). A similar observation of haustoria formation in the parenchyma cells of leaves was observed in poppy downy mildew (Calderón et al., 2014) and in cabbage (Chou, 1970). The formation of haustoria in the epidermal cells or in stomatal apertures, constitute the most important phase in the development of downy mildews (Krober et al., 1979; Yeh and Frederiksen, 1980; Kamoun et al., 1999). Altering host susceptibility occurs by transporting effector proteins from the pathogen to the host (Judelson and Fong, 2019). The sporangiophores emerge out of the stomata, releasing the sporangia and forms abaxial sporulation as reported in other downy mildews such as, basil and sage (Cohen et al, 2013; Wyenandt, et al., 2015; Hoffmeister et al., 2020).

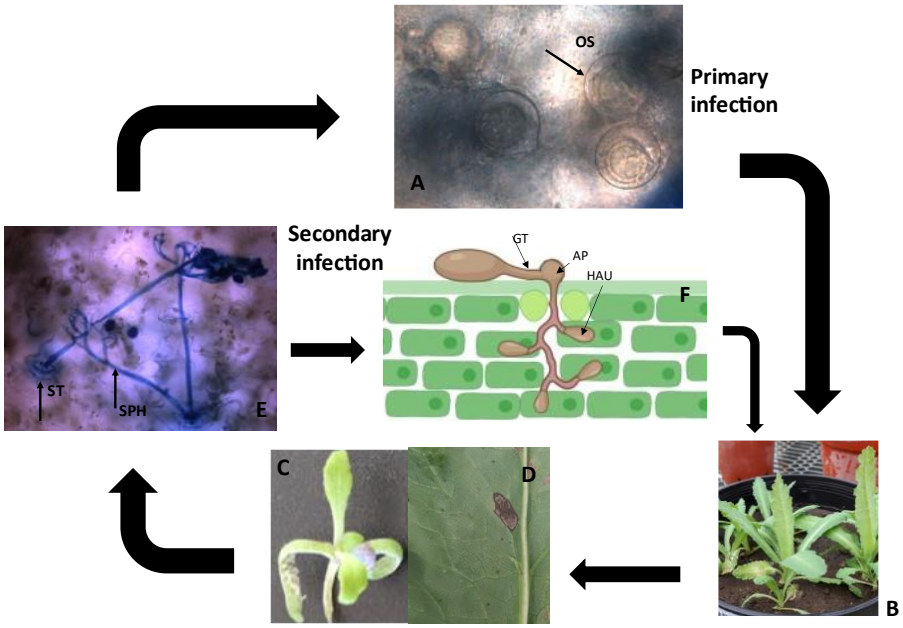

Figure 2.3: Infection processes of opium downy mildew pathogens. (A) Primary infections by oospores happen initially by the germination of oospores in the soil. (B) The host plants are colonized by a coenocytic, intercellularly growing mycelium which swells to fit the intercellular spaces, giving it an irregular appearance. The hypha extends its growth and forms feeding organs called haustoria into host cells for uptake of nutrition. (C) Profuse sporulation observed on the abaxial side of *Pe. somniferi.* (D) Localised sporulation observed on the abaxial side of *Pe. meconopsidis.* (E) Sporangiophores (SPH) emerge from stomata (ST) and bear sporangia, initiating secondary spread Oospores are formed on the mesophyll tissue of leaves by the fusion of male antheridium and female oogonium. (F) Secondary infection starts by germination of sporangia produced, which in turn forms a germ tube (GT) that ends in appressorium (AP). The intracellular haustoria (HAU) (feeding structures) are formed for the uptake of nutrition in the tissue.

Table 2.1: Infection process in various *Peronospora* spp. causing downy mildew.

Pathogen	Germ tube formation	Appressorium formation	Haustoria formation	Formation of Hyphae	Duration for sporangia formation	Duration for oospore formation	Reference
Peronospora parasitica (Arabidopsis)	within 24 h	2-3 DAI	2-3 DAI	3 DAI	4-6 DAI	6-8 DAI	(Slusarenko and Schlaich, 2003)
Peronospora sparsa (rose)	within 24 h	8-12 DAI	2 DAI	1-2 DAI	6-7 DAI	7 DAI	(Gómez and Filgueira-Duarte, 2012)
Peronospora tabacina (tobacco)	within 24 h				6-8 DAI		(Sukanya and Spring, 2013)
Pseudoperonospora cubensis (cucurbit)	within 24 h	5 DAI	4 DAI	6 DAI	5-7 DAI	7 DAI	(Lebeda and Cohen, 2010, Cohen, 1981)
Bremia lactucae (lettuce)	3 HAI	4 HAI	13 HAI	14 HAI	6 DAI	7-14 DAI	(Sargent, 1981, Crute et al., 1994).
Hyaloperonospora brassica	4 HAI	4 HAI	36 HAI	36 HAI			(Bahcevandziev et al., 2015)
Peronospora viciae (pea)	within 24 h				7 DAI	7 DAI	(Pegg and Mence, 1970)
Peronospora somniferi	NA	NA	NA	NA	NA	NA	NA
Peronospora meconopsidis	NA	NA	NA	NA	NA	NA	NA

NA – Not available

HAI = hours after inoculation

DAI = days after inoculation

2.11 Defence mechanisms and resistance development in plant pathogenic interaction:

The interaction between downy mildew pathogens and theirs hosts elicits a series of responses. Defence responses in plants involve signalling pathways that are known to comprise salicylic acid-, jasmonic acid- and ethylene-dependent pathways (Thakur and Sohal, 2013). The defence mechanism is triggered by a group of products called elicitors (Fig 2.4; Gómez-Vásquez et al., 2004). These may be physical, chemical or biological (Gómez-Vásquez et al., 2004). Oomycetes use specialised molecules to target signals, called effector proteins, within the host tissues (Kamoun, 2006, 2007; Schornack et al., 2009). These effector molecules enter the host and are responsible for altering the plant defence mechanism (Schornack et al., 2009). These effectors are called as avirulence (AVR) proteins and have been identified in several fungi and oomycetes (Allen et al., 2004; Shan et al., 2004; Armstrong et al., 2005). Efforts have been made to identify the effector genes in oomycetes by the advent of the developing techniques in genetic and bioinformatic strategies (Kamoun et al., 2007). To identify the type of elicitor, information concerning the interaction of the host cells and the elicitors is essential, however, little is known in this respect about poppy downy mildew.

The plant-pathogen interaction triggers several barriers in the host defence (Jones and Dangl, 2006). The initial defence mechanism is initiated by the recognition of the pathogen associated molecular patterns (PAMPs) or sometimes called Microbe associated molecular patterns (MAMPs) (Bent and Mackey, 2007). These PAMPs and MAMPs when recognised by the host plant, they become elicitors for an effective host pathogen interaction (Bent and Mackey, 2007).

Pathogen infection creates a biotic stress in their host plants (Neill et al., 2002; Kamoun et al., 1999; Torres et al., 2006). Plants respond to pathogen attack by activating an inducible immune response including production of reactive oxygen species (ROS). The production of reactive oxygen species (ROS), through the consumption of oxygen in a so-called oxidative burst, which is the earliest host responses following successful pathogen recognition. Apoplastic generation of superoxide ($O2^-$), or its resulting product hydrogen peroxide (H2O2), has been recognized following recognition of a variety of pathogens (Torres et al.,2006). The oxidative burst is a rapid, temporary, production of

significant amounts of reactive oxygen species (ROS), is one of the earliest observable characteristics of a plant's defence strategy (Wojtaszek, 1997).

A biphasic oxidative burst, resulting from a rapid release of reactive oxygen intermediates (ROI), has been concerned as an essential component of plant defense signalling during the early stages of pathogen infection (Lamb and Dixon, 1997). The first stage of oxidative burst has been detected as a response to infection in both resistant and susceptible hosts, whereas the second stage burst appears to relate only with disease resistance that is naturally associated with host– pathogen interactions (Grant et al., 2000).

Biotrophic pathogens activate the salicylic acid pathway (Thakur and Sohal, 2013) on encountering the host which will lead to stimulation of hydrogen peroxide and other reactive oxygen compounds. Salicylic acid (SA) is an important plant defence signal, identified in bio-trophic oomycete, *Hyaloperonosproa arabidopsis* (Sanchez et al., 2012). The entry of these pathogens stimulates the plant to produce SA, which then signals the pathways to take defensive action against pathogens (Glazebrook, 2005). Thus, this pathway plays an important role in the plant as an immune regulator (Vlot et al., 2009; Thaler et al., 2012). Salicylic acid is synthesised from chorismate, a precursor molecule of the amino acid tryptophan, by two pathways, namely the phenylalanine and isochorismate (Vlot et al., 2009).

The significance of SA in plant defence, with respect to downy mildew, is best understood in *Arabidopsis*. In this plant, Delaney et al (1994) found that low levels of SA failed to defend the plant against the pathogen *H. parasitica.* This is because such levels cause a shift from resistance to susceptibility towards the pathogen, even if these are avirulent (Mauch-Mani and Slusarenko, 1996). This defence mechanism was explained, at the molecular level, in a study on soybean downy mildew caused by *Peronospora manshurica* (Dong et al., 2018). They observed that the genes responsible for the salicylic acid, jasmonic acid and ROS pathways expressed differentially, in response to the pathogen. Some of the defence-related pathways for obligate pathogens are shown in Table 2.2 below. Once again little is understood about host defence responses against poppy downy mildew pathogens in opium poppy.

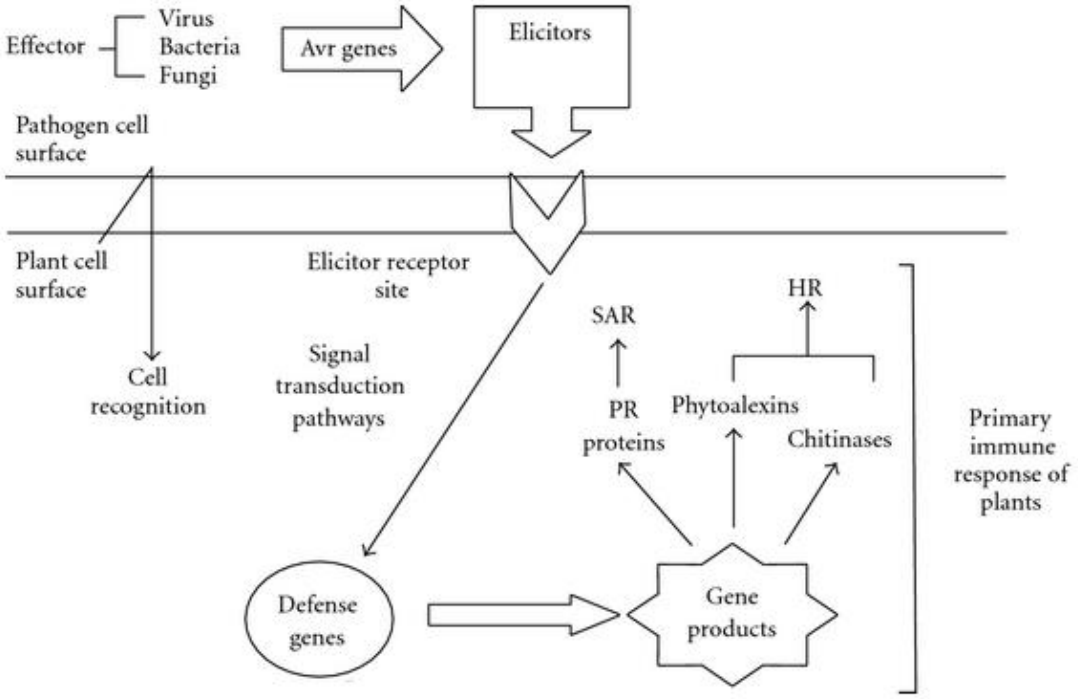

Figure 2.4: Immune response exhibited by the plants with response to plant-pathogen interaction (Image courtesy: Thakur and Sohal, 2013).

Table 2.2: Various defence related pathways identified in host pathogen interaction of *Peronospora* spp*,*

Pathogen name	Disease name and host	Defence related pathway	Reference
Plasmophora viticola	Downy mildew of grapes	JA, SA	(Trouvelot et al., 2014, Ishiai et al., 2016,),
Peronospora sparsa	Rose downy mildew	Abscisic acid, Phenolics	(Schulz and Debener, 2009, Kaufmann et al., 2012).
Bremia lactucae	Lettuce downy mildew	ROS, NO	(Lebeda et al., 2008),
Peronospora parasitica	Downy mildew in Arabidopsis thaliana	SA	(Donofrio and Delaney, 2001)
Peronospora manshurica	Soybean downy mildew	SA/ROS, JA, ET, BR	(Dong et al., 2018)
Peronospora tabaicana	Tobacco downy mildew	ROS, H2O2, PR proteins	(Wu et al., 2015)
Sclerospora graminicola	Pearl millet downy mildew	PR proteins	(Kulkarni et al., 2016)
Hyaloperonospora arabidopsis	Arabidopsis downy mildew	SA	(Wirthmueller et al., 2018)
Peronospora somniferi	Opium poppy downy mildew	Not identified	
Peronospora meconopsidis	Opium poppy downy mildew	Not identified	

SA- Salicylic acid, JA- Jasmonic acid, ROS- Reactive oxygen species, NO- Nitric oxide, PR- pathogenesis-related, ET- ethylene, H2O2- Hydrogen peroxide

2.12 Disease management

The knowledge on the disease management of opium poppy downy mildew is relatively limited compared to other downy mildew pathosystems.

2.12.1 Cultivar resistance

Developing a disease resistance cultivar can be a highly effective method to manage plant disease. For example, developing cultivar resistance in combination with the fungicides have been used to effectively manage spinach downy mildew (Correll et al., 2011; Klosterman 2016). Host resistance to downy mildew in poppy has been identified against *Pe. arborescens* (Mathur et al., 1994; Dubey et al., 2009; Manoj et al., 2011) but it remains to be definitively shown whether this resistance operates against either or both of the currently known poppy downy mildew pathogens. The task of producing a disease-resistant variety remains a major challenge for researchers and confounded by pathogen variation. Strain variation has been reported for poppy downy mildew caused by *Pe. arborescens* (Landa et al., 2007). Hence resistant cultivars may lose their disease resistance against the newly developing pathogens. Pyramiding resistance genes if identified could help in ensuring greater robustness of resistance, has been reported for other downy mildews affecting pearl millet, sunflower and grape (Hash et al., 2006; Qi et al., 2017; Saifert et al., 2018). Currently there are no available reports for commercial poppy cultivars for downy mildew resistance against *Pe. somniferi* and *Pe. meconopsidis*.

2.12.2 Chemical control

Fungicidal treatments can offer significant control against downy mildew disease in several susceptible crops. Effective fungicides belong to several important classes of chemicals such as benzamides, carbamates, cyanoacetamide-oxime, carboxylic acid amide, demethylation inhibitors, methyl benzimidazole, phynylamides, quinone inside inhibitors and quinone outside inhibitors (Ojiambo et al., 2010). Based on their activity, fungicides are categorised as protectants or preventive, curative, or eradicants/antisporulant (Balba, 2007). Protectant fungicides are effective as pre-infection treatment through inhibition of the initiation of disease cycle (Balba, 2007). These fungicides include chlorothalonil, mancozeb, phthalimides, and folpet (Gisi and Sierotzki, 2008). Curative fungicides however function to constrain fungal growth post infection, examples include triazoles (Balba, 2007), fosetyl-Al, trifloxystrobin, dimethormorh (Gent et al., 2015). Conversely, eradicants such as trifloxystrobin (Gent

et al., 2015), fluopicolide, Mancozeb and metalaxyl (Schilder, 2011) and metalaxyl (Wicks and Lee, 1982) act by preventing pathogen sporulation (Balba, 2007).

Another important criterion for fungicide selection in downy mildew control is topical activity. Based on fungicide topical activity they are either described as contact, localized penetrants, acropetal penetrants or systemic fungicide (Rouabhi, 2010). Whereas contact fungicides (e.g., dithiocarbamates, nitriles, phenylpyrolles, cyanoimidazoles.) do not get incorporated into the plant tissues but remains at the site of application as a protective shield, localized penetrants are taken up by leaves and travel only short distances within the treated leaf, example dicarboximides and all strobilurin except azoxystrobin and fluoxastrobin (Rouabhi, 2010) which have systemic activity (Adetutu et al., 2008). Systemic fungicides including metalaxyl (Wicks and Lee, 1982) are absorbed and redistributed throughout the plant through the xylem tissues, but acropetal penetrants ones absorbed by the root stem or leaves move only upwards through the xylem, example; benzimidazoles, triazoles, pyrimidines, carboximides, acylalanines (Rouabhi, 2010).

Systemic infection of downy mildew is common, thus often systemic fungicides are the preferred fungicide treatment for downy mildew control (Oros and Viranyi, 1987). This notwithstanding, the objectives of fungicide treatment application, whether it being preventative or curative, influences chemical selection, the observable effects of chemicals on the disease before infection, after infection, or symptom development referred to as fungicide's physical mode of action (Szkolnik, 1978) is also an important consideration (Gent et al., 2015).

Metalaxyl, a systemic fungicide with both curative and eradicant activity, has been used widely in many pathosystems. This has led to the development of resistant strains of various pathogens such as *Ps. cubensis* causal agent of cucumber downy mildew (Chen et al., 2019) resulting in decreased efficacy. Consequently, the use of metalaxyl in cucumber downy mildew endemic areas have either deactivated or restricted (Wang et al., 1996). To prevent or delay the development of fungicide resistance, alternate or mixed application of fungicides with different modes of action is strongly recommended (Ma and Wang, 2010).

Mancozeb, a contact carbamate and protectant-only fungicide are typically integrated with other systemic fungicide to broaden the spectrum of control and assist

in fungicide resistance management (Gullino et al., 2010). The direct effect of mancozeb on core enzymatic processes that interferes with various biochemical processes of the pathogen within the pathogen cytoplasm and mitochondrion inhibits spore germination (Wong et al., 2010; Wicks and Lee, 1982). Mancozeb's lack of curative activity renders it ineffective in post infection application (Gullino et al., 2010). Consequently, the time of treatment application is crucial to its effectiveness.

Considering chemical treatments have varying half-life, time and rate of application becomes essential to fungicide control of downy mildew. This makes disease forecasting essential in downy mildew management (Ma and Wang, 2010) particularly when the goal of disease control is prevention. Dimethomorph offers six to nine days pre-and post-infection protection against *Ps. cubensis* (Cohen et al., 1995; Johnson et al., 2000). The post-infection activity of phosphonate fungicides ranges between two to 14 days (Wicks et al., 1991). This imposes the requirement of repeated treatment application for effective downy mildew control depending on the fungicide and the susceptibility of the cultivar to the disease (Gent et al., 2015).

Methods that involve integrating good agronomic practices and the use of chemical fungicides, such as, benalaxyl, iprovalicarb and piraclostrobin, were effective in controlling downy mildew in grapes, while having the added benefit of increasing the fruit's aroma (González-Rodríguez et al., 2011). Other alternate methods of spore trapping and disease forecast models may help the growers to detect the airborne sporangia likely to be present in the field and to introduce appropriate fungicide (Koike et al., 2017). This has been already reported in other downy mildews of cucumber, spinach, and lettuce crops (Holmes et al., 2004; Klosterman et al., 2014; Dhar et al., 2020). In Tasmania, the localised form of poppy downy mildew has been managed for many decades by fungicide treatment and other integrated controls, however, the systemic form of poppy downy mildew has not been effectively controlled by the regular fungicidal programme (https://www.abc.net.au/news/rural/2015-09-03/tch-poppy-fungicide/6745872) raising industry concern and suggesting the need for greater investment in understanding differences between the two pathogens.

2.12.3 Biological control

Biological control of plant pathogens is an alternative disease management option in sustainable agricultural production. Biological mixtures such as *Trichoderma harzanium,*

Trichoderma viride and *Bacillus subtilis* may be used as antagonists to act against pathogen and thus reduce the reliance on fungicides for disease management. In a study conducted by Sadoma et al (2011), the above-mentioned biocontrol agents were used for the control of downy mildew of maize caused by *Peronosclerospora sorghi*. Bio priming of seeds and application to foliage with an isolate of *B. subtilis* was found to be effective in controlling pearl millet downy mildew (Atri et al., 2019). Kunene et al (1990) identified a reduction in the incidence of downy mildew in sorghum, of 58%, with the application of the biological control fungus, *Gaertneriomyces* sp. When added to soil, this fungus attacks the oospores of *Pseudoperonospora sorghi.* Similar results were also reported in studies by Salas and Diaz (1984). Likewise, the efficacy of biocontrol agents such as *B. subtilis* and thyme oil showed a significant control against basil downy mildew (Gilardi et al., 2013). However, this treatment is less effective with high rates of infection. For this reason, the usage of biocontrol agents has limited commercial and industrial viability. Despite of the available knowledge on the control of fungal oomycetes, no studies have made on opium downy mildew pathogens.

Chapter 3: Comparison of poppy seedling infection from soilborne oospore inocula of *Peronospora somniferi* and *Pe. meconopsidis* and determination of the role of herbicidal stress on the development of downy mildew of opium poppy

3.1 Abstract

Downy mildew of opium poppy is a major threat to the poppy industry globally. In Tasmania, this crop is grown for pharmaceutical alkaloid production, with the state producing more than 50% of world's licit opiates. Crop yield is limited by downy mildew, caused by either of two oomycete species: *Peronospora somniferi* and *Peronospora meconopsidis*. Identifying potential inoculum sources is an important step in the development of disease management strategies. This study compared the role of oospores as a source of soil-borne inoculum of the two pathogens for the development of poppy downy mildew. Results demonstrated that the presence of oospores in soil were an effective inoculum source for disease development for both pathogens. Subsequent histological studies confirmed that both pathogens infected the roots and that intra- and intercellular hyphae colonised the plant and formed sporangiophores which passed through leaf stomata to form sporangia. It was also demonstrated that foliar application of herbicides at sublethal levels to foliage of asymptomatically infected poppy plants can increase expression of downy mildew symptoms.

Key words: Downy mildew, *Peronospora* spp., oospores, herbicides

2. Introduction

Opium poppy *(Papaver somniferum)* is grown for its opiate alkaloid compounds, such as codeine, morphine, noscapine and thebaine (Kapoor, 1995; Yang et al., 2019; Zeigler et al., 2018; INCB 2019). The majority of licit poppy production occurs in Australia, France, Hungary, Spain and Turkey (INCB, 2019). Australia contributes more than 50% of the world's licit supply of opiates (INCB, 2019), followed by Turkey (23%), France (21%) and Spain (4%) (INCB, 2019).

Downy mildew is an important disease which is responsible for large economic losses in poppy production (Thangavel et al., 2018). The causative agent for this disease was first reported as *Peronospora arborescens* (Cotterill and Pascoe, 1998). This disease was characterised by localised angular necrotic foliar lesions with sparse sporulation. Later research suggested the causal agent was *Pe. cristata* (Scott et al., 2004). Following a recent taxonomical revision of the genus, the pathogen causing the localised downy mildew symptoms in Tasmania was classified as *Pe. meconopsidis* (Voglmayr et al., 2014). In 2013, a second more severe form of downy mildew appeared in Tasmanian poppy crops, characterized by plant stunting combined with chlorosis and profuse sporulation. This disease was later shown to be caused by *Pe. somniferi*, also considered a threat to the poppy industry (Thangavel et al., 2017).

Understanding the role of inoculum source(s) for a disease is important for effective management. The sexual oospores are a source of primary infection, mainly present in diseased plant debris, seed and infested soil (Montes-Borrego et al., 2008; Thangavel et al., 2018). Oospores can be an important part of the disease cycle in many downy mildew pathosystems (Lai et al. 2004; Navazio et al., 2007; Montes-Borrego et al. 2009; Zhang et al. 2012; Slagado–Salazar et al.2018; Kandel 2019), as they are robust and long-lived and promote genetic diversity (Gaag and Frinking 1997; Mackay, 1957; Corell et al., 2011; Kandel et al., 2019). Soil borne transmission of downy mildew to opium poppy seedlings has been reported in *Pe. arborescens* (Montes-Borrego et al., 2009). However, the soilborne transmission of *Pe. somniferi* and *Pe. meconopsidis* remains unclear and needs to be addressed. The infection process in other downy mildew pathosystems occurs through a series of events. Oospores in the soil germinate and penetrate the root epidermis, followed by the colonization of the cells by haustoria formation. This leads to inter- and intracellular invasion by coenocytic mycelia of the vascular tissues and systemic movement throughout

the plant (Kandel et al., 2019). Histological studies conducted by Milholland et al (1981), showed the invasion of inter and intracellular hyphae of *Peronospora tabacina* in xylem, phloem vessels, and parenchyma cells of tobacco plants. However, there are no comparable studies on the infection process from soil inoculum in poppy downy mildew.

Asymptomatic infection of opium poppy by *Peronospora* spp. is a well-known phenomenon (Montes-Borrego et al., 2009; Montes-Borrego et al., 2011). This raises the question of what triggers the switch from asymptomatic to symptomatic expression of the disease. This could be due to stress factor(s). Previous studies suggest that application of herbicides increased the disease severity of sorghum downy mildew, caused by *Peronosclerospora sorghi* (Craig et al., 1987). In general, herbicides can cause abiotic stress to crop and make them more vulnerable to disease attack (Bagavathiannan et al., 2017; Duke et al., 2006; Sanyal and Shrestha, 2008; Altman and Campbell, 1977; Lévesque and Rahe, 1992). In Tasmania there are several types of weeds belonging to the families Amaranthaceae, Liliaceae, Asteraceae commonly found in poppy crops. These are primarily controlled using chemical herbicides applied at the 4-6 leaf stage of the poppy crop, usually 3-4 weeks after emergence. These herbicides are non-lethal but will stress treated poppy plants. Anecdotally, observations from poppy crops surveyed in 2018-19 suggested that a rapid onset of systemic downy mildew symptoms due to *Pe. somniferi* occurred within 3-4 weeks post-emergence. This occurs after crops were sprayed with herbicides commonly used for weed control in poppy production. However, the role of herbicides and their interactive effect in stimulation of disease symptoms in latently infected plants has not been measured or tested experimentally.

Based on anecdotal observations and previously conducted studies, it is hypothesized that:

(i) Both *Pe. somniferi* and *Pe. meconopsidis* can initiate infection of opium poppy via oospores present in the soil and infested plant debris.

(ii) Downy mildew symptoms developing from soil-borne infection by *Pe. somniferi* and *Pe. meconopsidis*, occurred in a similar manner.

(iii) Foliar application of herbicides stimulates the expression of downy mildew symptoms.

3.3 Materials and methods

3.3.1 Plant material

For all experiments, a commercial *Papaver somniferum* seed lot, (SL 21) was used. This seed lot was kindly provided by the Tasmania poppy industry, the variety name is confidential and has been protected throughout the thesis, instead of seed lines names are provided with specified code (e.g., SL-21). Prior to sowing, all seeds used in the experiments were surface sterilised in a hypochlorite solution (2% available chlorine) for 5 minutes, followed by rinsing twice in sterile water and drying at ambient temperature in a laminar flow hood (Thangavel et al., 2020). The seeds were then stored in an airtight container at ambient room temperature in the dark until use.

3.3.2 Inoculum preparation

Diseased poppy leaves infected with either *Pe. somniferi* or *Pe. meconopsidis* based on characteristic symptomology were collected from commercial poppy-growing regions in Tasmania during the 2017-18 growing season. Leaves showing localised necrotic lesions (*Pe. meconopsidis*) or profuse sporulation (*Pe. somniferi*) were used to isolate separate inoculum sources for each pathogen. After collection, leaves were stored in a zip lock plastic bag generating humid conditions for 24 h to promote sporulation. Sporangia were brushed from infected leaves into sterile water (20 ml), adjusted to the concentration of 1.2 x 10^4 spores/ml and 10 ml was spray inoculated on to each healthy 4-week-old plants which were then covered in plastic bags for 24 h to maintain humidity. The plants were then maintained in the growth chamber at 20°C Day /15°C night temperatures at relative humidity 50% and with 16 h day/8 h night cycle, commencing in the dark cycle. Freshly produced spores from these infected plants were used to reinfect a new set of plants for inoculum maintenance every two weeks. To bulk the inoculum sources of each *Peronospora* sp. the same method was followed.

The presence of each specific *Peronospora* sp. within the inoculum samples was confirmed by species-specific PCR (Thangavel et al., 2018). DNA was extracted from 50 mg of leaf material using the Power Plant Pro DNA isolation kit following the manufacturer's protocols (QIAGEN, Chadstone, VIC, Australia). DNA extracts were quantified using Qubit 2.0 Fluorometer (Thermofisher, Australia) and 1.5 µl of 5 ng/µl DNA were then subjected to PCR amplification, using the COX 1-PSF-PSR and COX 2-PMF-PMR primer sets specific to *Pe. somniferi* and *Pe. meconopsidis,* respectively (Thangavel et al., 2018). The thermocycler conditions were set to a denaturation

temperature at 95˚C for 15 minutes and followed by 35 cycles of 95 °C for 30 s, 60˚C for 30 s and 72˚C for 30 s with a final extension period at 72˚C for 10 minutes. Amplified DNA was confirmed by electrophoresis in a 1 % agarose gel stained with 0.1 % of the SYBR Safe DNA gel stain (Life Technologies) and visualised under blue light with a 50 bp ladder (Bioline, Australia) as a molecular marker.

To prepare pathogen infested soil, sporulating leaves (100 leaves of each pathogen) were collected, air-dried at room temperature for one week and leaves with each were ground separately in a coffee grinder for 10 s (Model no: BCG200BSS, Breville, Australia). Three subsamples (10 mg) of the ground leaf material were mixed in 1 ml of sterile distilled water and stained with aniline blue and examined microscopically for the presence of oospores. The ground inocula (~50 g) of each pathogen were mixed with 5 L of autoclaved potting mixture containing sand, peat, and composted pine bark (10:10:80, pH 6.0) premixed with Osmocote 16-3.5-10 NPK resin coated fertiliser (Scotts Australia Pty Ltd.) and used as the inoculum source for all experiments. Infested soil was stored in the dark at 4˚C until further use, for a maximum of 3 months to protect the oospore survival (Van der Gaag and Frinking,1997a).

Prior to spiking the potting mixture with *Peronospora* inoculum, the presence of the intended *Peronospora* sp., within each infested soil and potting mixture were determined by species–specific PCR assay as follows. A 5 g sample of soil from each lot was ground using a coffee grinder for 10 s (Model no: BCG200BSS, Breville, Australia) and sieved firstly through a 50 µm and then through a 37 µm sieve (Greeburn laboratory sieves, Greer and Ashburner Pty Ltd, Australia). DNA was extracted from a 250 mg sample of the fine ground material using the Power Pro Soil DNA isolation kit (QIAGEN, Vic, Australia) following the manufacturer's protocol. DNA concentrations were quantified using a Qubit 2.0 Fluorometer (Thermofisher Australia). DNA extracts were then subjected to PCR amplification as outlined previously.

3.3.3 Experimental design

3.3.3.1 Comparison of *Pe. meconopsidis* and *Pe. somniferi* seedling infection from soil inocula:

Two glasshouse experiments (SM1 and SM2) were run to test the potential for seedling infection from soilborne oospores. Surface sterilised poppy seeds (30 seeds/pot) were sown into pots (27 cm (height) x 24 cm (top diameter) x 18 cm (base diameter)) containing the pathogen-free potting mixture that was layered with 10 mm of soil

infested with *Pe. somniferi* or *Pe. meconopsidis*. There were ten pots used for each treatment. Following germination, seedlings were thinned to 20 plants per pot. The plants were maintained under glasshouse conditions with temperature and humidity at 17-20˚C and 70-75 %**,** respectively. A single plant was harvested from each pot for histological assessment at 1, 2, 4, 6 and 8 weeks after emergence. The plants were examined microscopically for the presence of pathogen structures and tested by PCR to confirm species.

The ten remaining plants in each pot were assessed for visual disease symptoms at 1, 2, 4, 6 and 8 weeks after emergence. Visual disease was assessed by leaf and plant incidence: (a) leaf incidence refers to the proportion of leaves showing downy mildew symptoms in each plant out of the total number of leaves present per plant; and (b) plant incidence was estimated by the proportion of diseased plants which are showing symptoms of chlorosis, necrotic lesions and/or sporulation out of the total number of plants in the pot.

For histological examination, plants were dissected into three plant parts: root, stem and the leaves. The roots were washed in running tap water until all soil particles were removed. Each plant part was longitudinally dissected and placed on a glass slide, stained with 0.5 % of aniline blue and covered with a coverslip. Root size varied from 3-10 cm length at each examination. The slides were examined using a light microscope (Leica DFC 420, Germany) at 40 X magnification, and the images were captured using a Leica LAS software (Leica Microsystems Pty Ltd, Australia). Each dissected plant was assessed for the presence of oospores, haustoria, appressoria, sporangia and mycelia produced by the pathogens. The plants grown in pathogen free potting mixture were used as negative controls.

Dr Warwick Gill (TIA, New Town Research Laboratories) prepared plant tissue samples, sections and images following the following procedures. Thin sectioning and microscopic observation following the methods of Spurr (1969) was then used to provide more precise localisation of pathogen structures within infected plant parts. Infected seedlings were divided into eight different tissue types: root tip, mid-root, upper root, stem, cotyledons, lower leaves, middle leaves, and upper leaves. The individual tissues were cut into approximately 5 mm lengths and immersed in 2.5 % glutaraldehyde in 0.1 M PO_4 buffer, pH 7.2. The tissues were then fixed under vacuum

and stored overnight in fresh fixative at 4˚C. The samples were rinsed twice in distilled water and dehydrated through an ascending acetone series to 100 % acetone in 20 % increments. Following two changes of 100 % acetone, the tissues were infiltrated in 25 %, 50 %, 75 % and 100 % Spurr's resin in acetone (Spurr, 1969) and left at each concentration for 24 h at 4°C. Following a further 2 h in fresh 100 % Spurr's resin, the tissues were oriented and embedded in silicon moulds in fresh 100% Spurr's resin and finally polymerised at 70˚C for 12 h. Resin blocks were initially trimmed by a hand-held razor blade, then semi-thin sections averaging 5 µm thick were cut on a Reichert rotary ultra-microtome with glass knives. Sections were lifted from the knife with forceps and expanded in a droplet of distilled water on clean glass slides placed on a moderate hotplate. The sections were then gently heat-fixed to the slides by passing through a Bunsen flame. For morphological investigation, sections were stained in 0.5 % toluidine blue O in 0.1 M acetate buffer pH 4.5 for 5 minutes and rinsed in running water. Slides were air-dried and mounted in Euparal beneath a coverslip and examined under a light microscope (Leica DFC 420, Germany) with images captured using a Leica LAS software version 3.8.0 (Leica Microsystems Pty Ltd, Australia).

3.3.3.2 Effect of herbicidal stress in the development of systemic downy mildew:

To test the role of herbicide stress on downy mildew disease development, glasshouse studies were conducted using four different herbicides used for weed control in commercial poppy crops (Table 3.1). Ten poppy seeds were sown into 15 pots (27 cm (height) x 24 cm (top diameter) x 18 cm (base diameter)) filled with soil infested with *Pe. somniferi* as previously described and in a further 15 pots filled with uninfested soil. Seedlings were thinned to five plants per pot after emergence. The pots were maintained as outlined previously. Four weeks after emergence, the plant foliage was sprayed with three different concentrations of herbicides (Table 3.1) using a handheld spray bottle. For each herbicide, half, single and double strength of recommended concentration for weed control was applied to each plant (10 – 15 ml) with three pots sprayed with water only as an untreated control. The plants were then covered with polythene bags for 48 h to increase humidity. Plants grown in inoculum-free soil but sprayed with the various herbicide treatments or the water only control were used as inoculum-negative controls. There were three replicated pots for each treatment with

five plants per pot. Plants were observed for the development of downy mildew sporulation from 48 h after herbicide application for 7 days. For visual disease assessment, the total number of infected plants and sporulating leaves were determined by counting the number of plants showing sporulation out of the total number of plants within each pot, and by counting the number of leaves showing downy mildew sporulation out of the total number of leaves per plant. The experiment was repeated twice.

3.3.4 Data analysis

For all measured parameters in the experiments SM1 and SM2, the calculated proportion of disease incidence was plotted using the LOESS curve fit model with 95 % confidence interval and plotted using the ggplot2 package in R statistical language environment version 3.6.1 (R Core Team, 2019). To test for statistical differences between incidences of disease parameters, the area under the disease progress curve was estimated (Madden et al., 2009). Differences between pathogens, and host tissues in histological measurements, were then tested via ANOVA. Differences between experiments were tested for by including Experiment as a predictor factor in all analyses. Differences between individual treatment combinations were tested for using Tukey's honest significant difference comparisons.

In the herbicidal stress experiment the data were combined for the experiments as there was no significant difference between the trials. Differences between the experiments was included as a predictor in logistic regression (using R) for each response variable to test whether the experiments could be combined. The mean number of plants showing disease, sporulated leaves and seedling death was calculated, and statistical difference were analysed using one-way ANOVA and the means were separated using Tukey's honest significant difference comparisons.

3.4. Results

3.4.1 Comparison of *Pe. meconopsidis* and *Pe. somniferi* seedling infection from soil inocula.

Two distinct symptom types were observed following inoculation of poppy plants with *Pe. somniferi* or *Pe. meconopsidis*. Plants inoculated with soilborne oospores of *Pe. somniferi* displayed profuse sporulation on the abaxial leaf surface and associated with chlorosis upper side of the leaf. Plants inoculated with *Pe. meconopsidis* displayed

localised angular lesions on the adaxial side and a localised sparse sporulation on the abaxial leaf surfaces respectively (Figure. 3.1).

In the two soil inoculation experiments (SM1 and SM2), the symptom and sporulation incidence varied between *Pe. somniferi* and *Pe. meconopsidis.* Across the two experiments, symptom incidence in plants and leaves for *Pe. somniferi* and *Pe. meconopsidis* showed a significant increase from week 1 to week 6 that was statistically significant (P = 0.004). The highest symptom incidence in plants for *Pe. somniferi,* and *Pe. meconopsidis* were 0.31 and 0.11, respectively at week 6 (Figure. 3.2.A). There was also a rising trend in the symptom incidence observed in leaves of plants grown in soils inoculated by both pathogens. Leaf symptom incidence was observed from 2nd week after emergence in *Pe. somniferi* (0.02) and 6th week after emergence in *Pe. meconopsidis* (0.01) (Figure. 3.2.B). *Pe. somniferi* inoculum caused a greater incidence of both plant and leaf symptoms than *Pe. meconopsidis.*

Across the two experiments, sporulation incidence in plants and leaves inoculated with either *Pe. somniferi* and *Pe. meconopsidis* was significantly different (P ≤ 0.05) (Figure 3.2 C). Sporulation in the plants was first observed from the 2nd week in *Pe. somniferi* inoculated pots (0.09), whereas the plants started to show sporulation from the 4th week in *Pe. meconopsidis* inoculated pots (0.13). Overall, the sporulation incidence was greater for *Pe. somniferi* than *Pe. meconopsidis*.

Similarly, significant differences in the incidence of sporulation of each pathogen on individual leaves was found (Figure 3.2 D). Sporulation was first observed in *Pe. somniferi* from week 2 (0.04) and increased until week 6 (0.22). In *Pe. meconopsidis*, the sporulation incidence was observed from week 4 (0.04). Over their respective sporulation periods, both pathogens exhibited an initial increase in leaf sporulation incidence until week 6, followed by a decrease in week 8 (Figure 3.2 D).

The mean number of plants testing positive for each pathogen by PCR from weeks 2 to 8 differed significantly between the two pathogens (P ≤ 0.05). PCR positives plants in the pots infested with *Pe. somniferi* were observed from week 2 (0.37) to week 8 (0.80). However, in *Pe. meconopsidis,* the positive PCR detection was detected from week 4 (0.30) and increased gradually to week 8 (0.60). Across the experiments, PCR positives plants were greater in *Pe. somniferi* than *Pe. meconopsidis* inoculated pots (Figure 3.2 E)

Figure 3.1: Visual disease symptoms produced by the downy mildew pathogens of opium poppy plants in the glass house trials. A) chlorosis and B) profuse sporulation caused by *Pe. somniferi*; C) Local necrotic lesion on the adaxial side of leaf and D) sporulation on the abaxial side of leaf caused by *Pe. meconopsidis.*

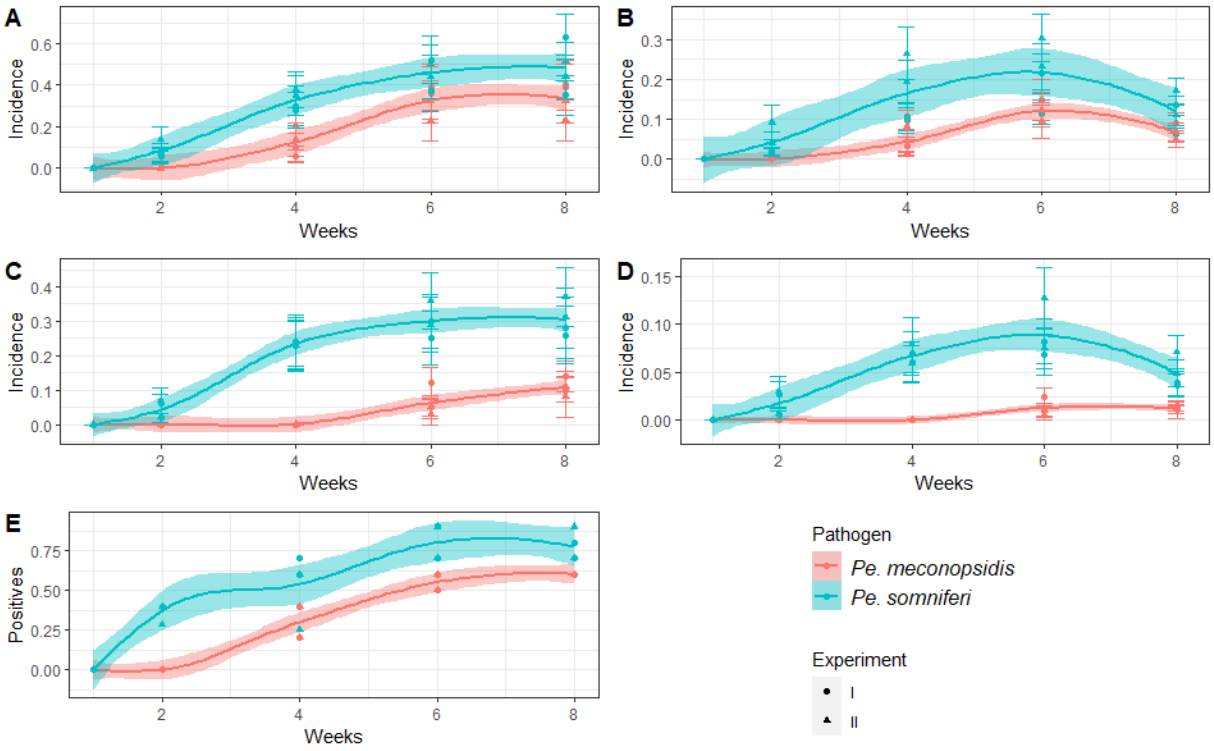

Figure 3.2: Comparison of disease incidence caused by *Pe. somniferi* and *Pe. meconopsidis.* The two experiments are shown in the figure by circles (SM1) or triangles (SM2). In each experiment, there were 20 plants for each pathogen. Incidence estimates of A) symptom incidence in plants; B) symptom incidence in leaves; C) sporulation incidence in plants (estimated by the proportion of diseased plants which are showing symptoms of chlorosis, necrotic lesions and/or sporulation out of the total number of plants in the pot); D) sporulation incidence in leaves (estimated by the proportion of leaves showing downy mildew symptoms in each plant out of the total number of leaves present per plant); and E) incidence of positive PCR detections in leaves. Each week after emergence, PCR testing was performed on 20 pots, each containing 20 plants. One leaf from each of the 20 plants in each pot was collected, to form 20 samples at each weekly interval. Error bars display the standard error (± SE).

3.4.1.1 Histological observations:

Following root infection, the formation rates of mycelia, appressoria, haustoria did not significantly differ between the two pathogens ($P \geq 0.05$), however, sporangia and oospore production did differ significantly ($P \leq 0.05$) (Figure 3.8 A-E). In both the experiments, the highest incidence of mycelia in *Pe. somniferi* and *Pe. meconopsidis* was observed in the root at week 6 (0.33 and 0.25), in stem at 8 weeks (0.60 and 0.55) and in leaf at 8 weeks after emergence (0.70 and 0.63) (Figure 3.8 A).

For both pathogens, the incidence of appressoria and haustoria formed in roots, stems and leaves (Figure 3.8 B-C) and the intra cellular haustoria was observed in the roots (Figure 3.3). The highest appressoria incidence in the roots, was observed in week 2 for both *Pe. somniferi* and *Pe. meconopsidis*, and was 0.10 and 0.13, respectively. While in the stems and leaves 2nd week after emergence, it was 0.23 for *Pe. somniferi*, and 0.20 (stems) and 0.15 (leaves) for *Pe. meconopsidis*. Thereafter, there was a general decline in the incidence of plants displaying these infective structures (Figure 3.8 B). Infective pathogen structures, intracellular haustoria and the intercellular mycelia were observed in the stem and leaf (Figure 3.5 and 3.7).

Similarly, the highest haustoria incidence in roots, was observed in week 2 for *Pe. somniferi* and *Pe. meconopsidis*, and was 0.12 and 0.1, respectively, while in the stems and leaves, it was 0.28. and 0.25 for *Pe. somniferi* and 0.25 for *Pe. meconopsidis.* Thereafter, there was a general decline in the incidence of plants displaying these infective structures (Figure 3.8 C). In the histological examination, haustoria were observed within cells and fungal hyphae were visible in the intercellular spaces of the xylem tissue, and in the collenchyma, parenchyma and cortical cells (Figure 3.4 and 3.5).

The differences in incidence of sporangia observed for the two pathogens were highly significant ($P \leq 0.05$) (Figure 3.8 D). Sporangia production by *Pe. somniferi* was greater (0.29) than *Pe. meconopsidis* (0.23) on average. Sporangial production was higher in leaves than on stem tissues and no sporangia were observed in root tissues with either pathogen (Figure 3.8 D).

The mean proportion of sporangia in the two experiments significantly increased from week 1 to 8 for *Pe. somniferi* in stem (0.35 and 0.65) and in leaf (0.10 and 0.55) tissues and for *Pe. meconopsidis* from week 1 to 8 in stem (0.23 and 0.53) and in leaf (0.00 and 0.55)

tissues (Figure 3.4 C). The infective structures of the pathogens in the stem and leaf are shown in Figure 3.4 and 3.6, respectively.

The differences in incidence of the oospores observed in *Pe. somniferi* and *Pe. meconopsidis* infected plants were highly significant ($P \leq 0.05$) (Figure 3.8 E). Oospores numbers were observed to be higher in the root and leaves followed by stem tissues for both pathogens. Overall, the oospore production in *Pe. somniferi* was generally higher (0.17) than in *Pe. meconopsidis* (0.12) on average. The incidence of oospores observed in the roots during the first week after emergence for *Pe. meconopsidis* and *Pe. somniferi* was 0.25 and 0.33, respectively. There was a constant and similar increase in oospore numbers, observed in stem (0.18 and 0.18) and leaf (0.38 and 0.35) tissues during eight weeks after emergence in *Pe. somniferi* and *Pe. meconopsidis* respectively (Figure 3.8 E).

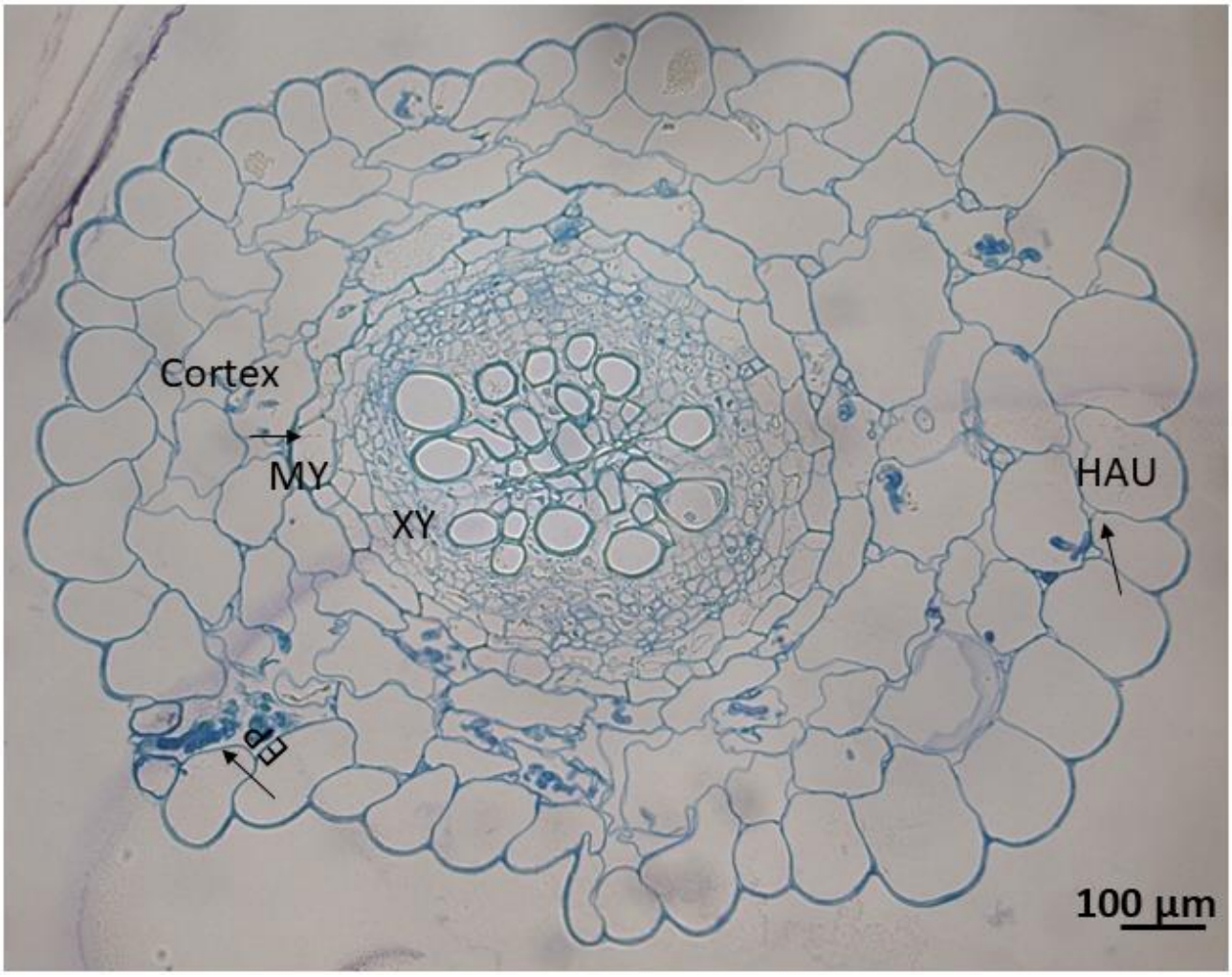

Figure 3.3: Representative image of *Pe. somniferi* showing the infective structures identified in the root region of *Papaver somniferum*: Transverse section of root tissue stained with 0.5% toluidine blue. Note the infection are observed in the epidermal layer (EP). Arrows indicate the intercellular hyphae (HY) in the parenchyma cells of the cortex, and the intracellular haustorium (HAU). Haustorium identified in the outer epidermal and cortex layer of the root region. Intercellular mycelium (MY) adjacent to the xylem layer (XY) of the root region (Image courtesy of Dr. Warwick Gill, TIA, New Town Research Laboratories).

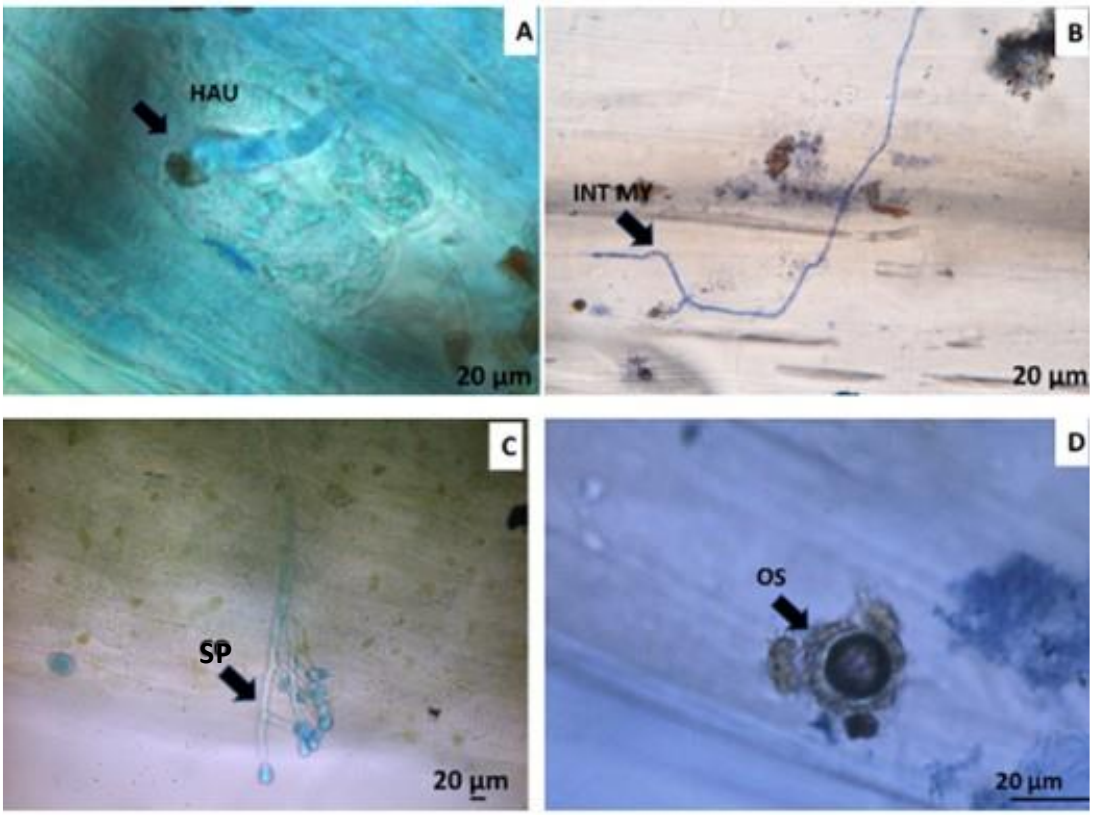

Figure 3.4: Infective structures of *Pe. meconopsidis* identified in the stem region of *Papaver somniferum* A) haustorium (HAU) like structures observed in the lenticels of stem region; B) intercellular mycelium (INT MY) identified in the parenchyma cells of the stem; C) mycelium bearing sporangia (SP); and D) oospore (OS) observed in the epidermal cells of the stem region.

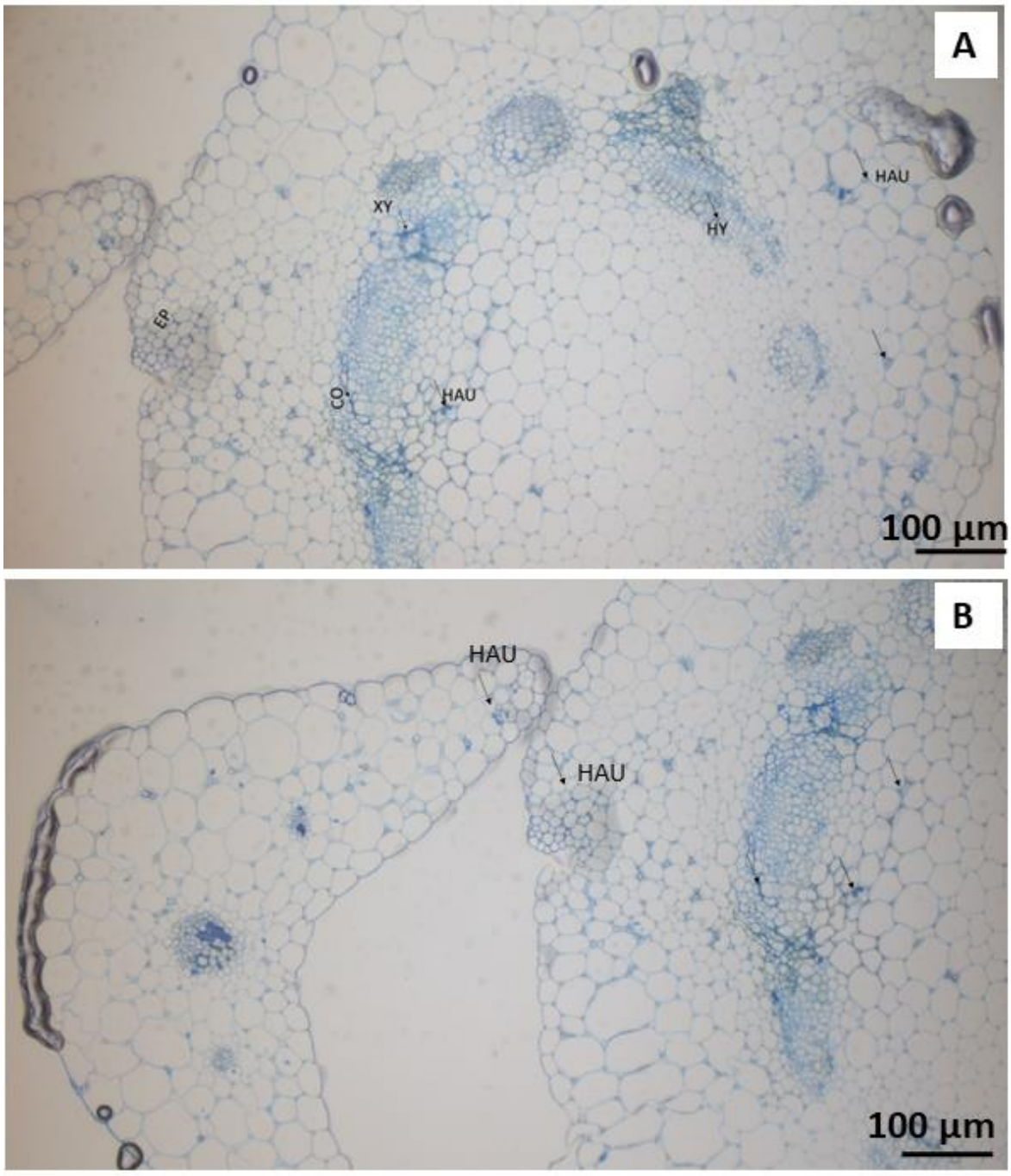

Figure 3.5: Transverse section of the stem of *Papaver somniferum* seedling infection with *Pe. somniferi* and stained with 0.5 % toluidine blue. Fungal hyphae are present in the outer epidermis (EP), and the inner vascular tissues (Xylem -XY). Note, also the hyphae (Hy) within the intercellular spaces of the collenchyma and the parenchyma cells of the cortex and the haustorium (HAU) in the cortical cells (Image courtesy of Dr. Warwick Gill, TIA, New Town Research Laboratories).

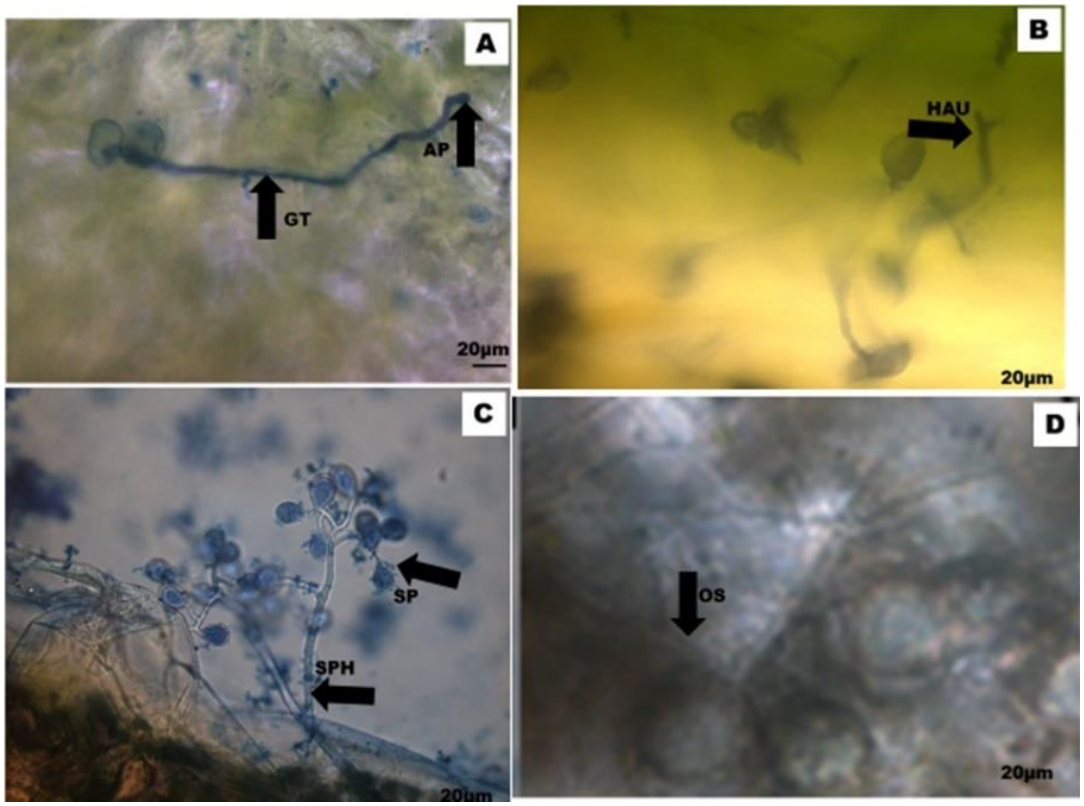

Figure 3.6: Fungal infective structures of *Pe.somniferi* in the leaves of *Papaver somniferum* due to secondary infection: A) germinated sporangia with a long germ tube, (GT) and appressorium (AP); B) haustorium (HAU); C) sporangiophore (SPH) and sporangia (sp) extension out of the leaves; and D) oospore (OS) formation in the mesophyll layer of the leaves.

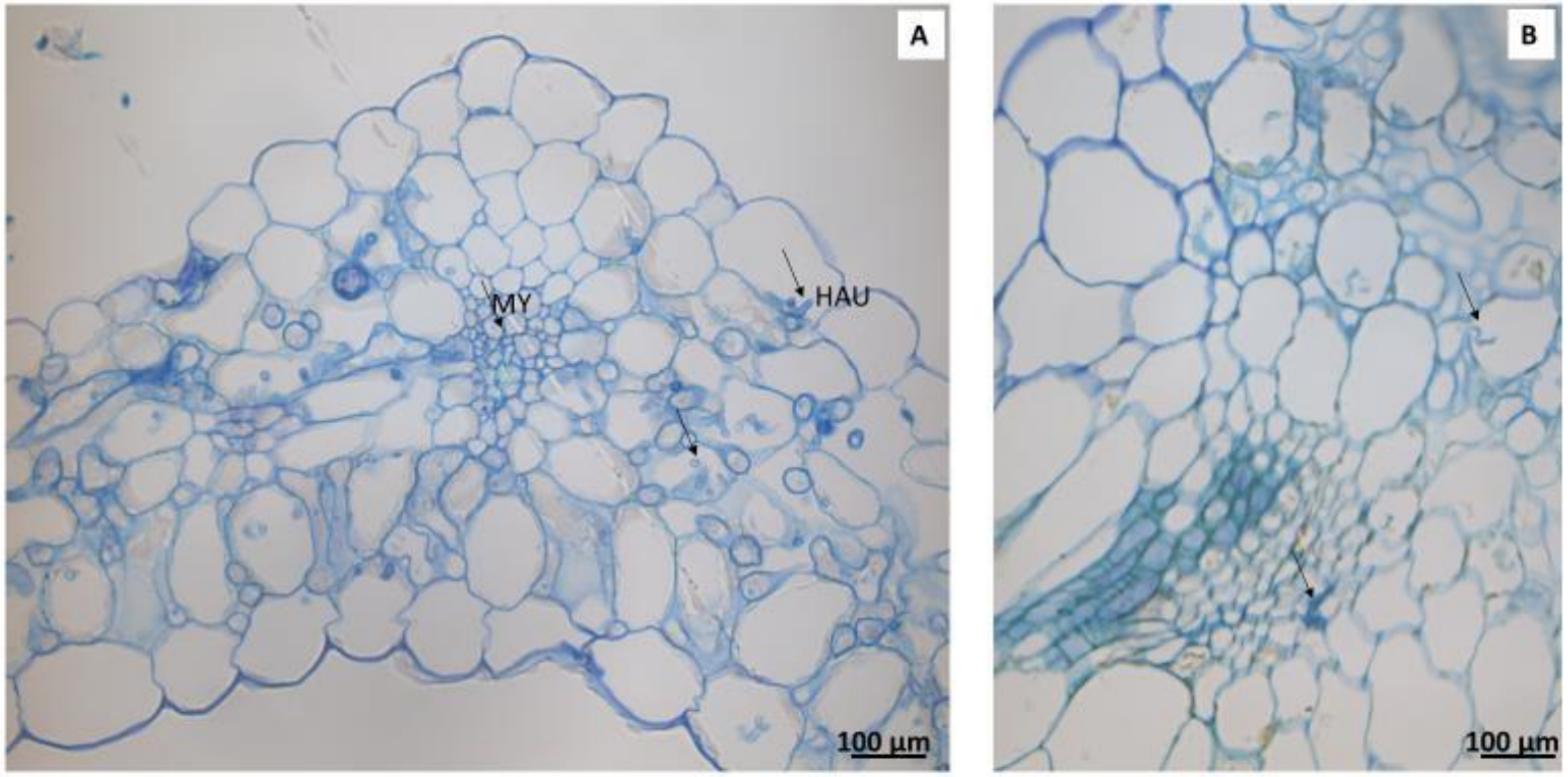

Figure 3.7: Fungal structures of *Pe. somniferi* detected in cross-sections of upper and lower leaves of 4-week-old *Papaver somniferum* (A and B) Transverse section of 4-week-old old plant stained with 0.5% Toluidine Blue. The mycelia (MY) within the intercellular spaces of the parenchyma cells and intracellular haustoria (HAU) on the parenchyma cells indicated by the arrows. Scale bars -100 µm (Image courtesy of Dr. Warwick Gill, TIA, New Town Research Laboratories).

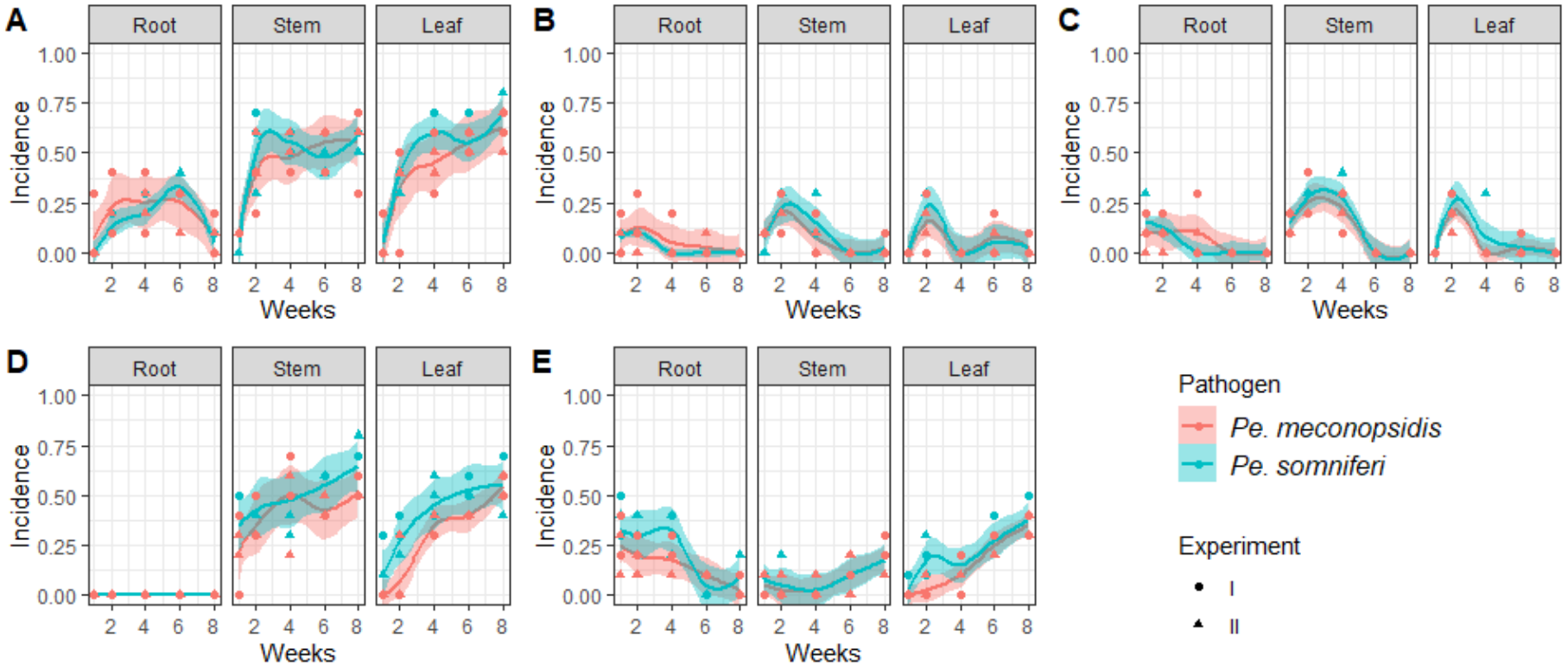

Figure 3.8: Histology of movement of *Pe. somniferi* and *Pe. meconopsidis*. Incidence of A) mycelia; B) appressoria; C) haustoria; D) sporangia; and E) oospores in plants infected with *Pe. somniferi* or *Pe. meconopsidis*. Ten plants observed at each week interval, for the presence of pathogen structures. The two experiments are shown in the figure by circles (SM1) or triangles (SM2). Trendlines indicate a localised estimate of sum of squares fit with coloured bands indicating the 95 % confidence intervals of the fit.

3.4.2 Effect of herbicidal stress on the development of systemic downy mildew

Application of certain herbicides such as metsulfuron–methyl and clopyralid was visually observed to exacerbate the symptoms of downy mildew infection (Table 3.2). Downy mildew symptoms in the plants varied with the different concentrations of the herbicides applied (Table 3.2). The highest mean number of plants that showed systemic infection (4.3) was observed following application of the herbicide metsulfuron-methyl at 2 X while the lowest (0.2) was observed in plants sprayed with imazamox at 0.5 X. Downy mildew symptoms in the water sprayed control plants with infested soil, showed a mean value of 0.8.

Similarly, the highest mean number of plants showing downy mildew sporulation was observed in plants treated with all the concentrations of the herbicide, metsulfuron- methyl. The lowest (0.40), was recorded in those treated with clomazone at 0.5 X and in the water sprayed control plants.

The highest mean number of seedling deaths (3.3) was recorded with the use of imazamox at 1 X and 2 X, followed by clomazone at 2 X (2.3) (Table 3.2). There was no seedling death observed in the clomazone sprayed plants with 0.5 X and 1.0 X. The herbicide sprayed plants with no infested soil, showed bleaching of leaves only (Figure 3.9 B) but did not show any sporulation or seedling death. No seedling deaths occurred among the water and herbicide sprayed control plants in uninfested soil.

Table 3.1: Type of herbicides used in the study.

Treatment	Product name and recommended rate	Active ingredient	Volume of herbicide solution applied per pot
control - water spray + soil inoculum	-	-	
Imazamox (0.5 X, 1 X, 2 X) + infested soil	Raptor ® WG, BASF Australia- 45g/ha	Imazamox 700 g/kg	10 - 15 ml
Clomazone (0.5 X, 1 X, 2 X) + infested soil	Clomazone 480 EC, FMC Australasia Pty ltd, QLD - 500 ml/ha	Clomazone 480g/L	10 -15 ml
Metsulfuron- methyl (0.5 X, 1 X, 2 X) + infested soil	Metsulfuron- methyl, Zelam Pty Ltd, Tullamarine, VIC; - 4.5ml/ha	Metsulfuron -methyl 28 g/L	10 -15 ml
Clopyralid (0.5 X, 1 X, 2 X) + infested soil	Clopyralid, Dow Agrosciences Australia limited, NSW 50 ml/ha	Clopyralid 600 g/L	10 - 15 ml

The herbicide concentrations 0.5 X, 1 X, 2 X of the herbicides used in the study are calculated from the original concentration applied to the commercial field crop.

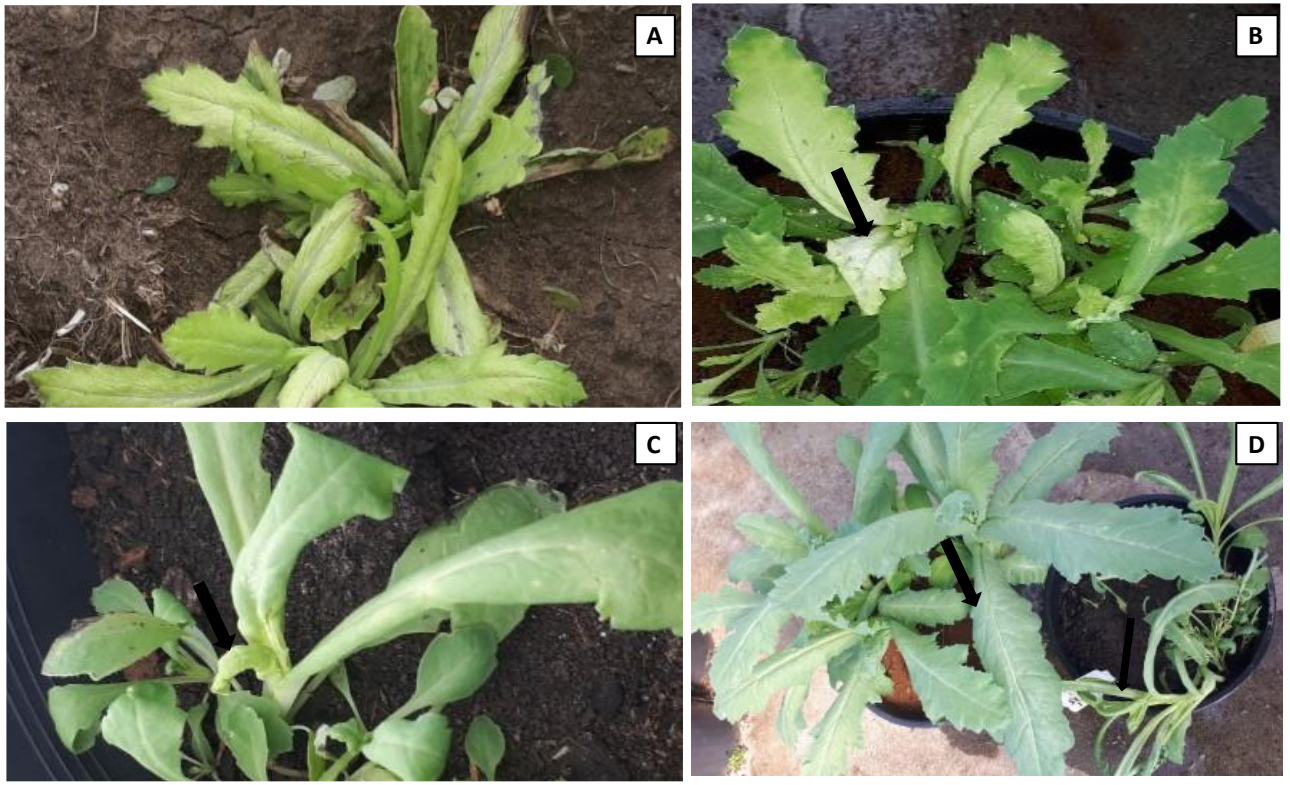

Figure 3.9: Effect of herbicide application in developing systemic downy mildew: A) plants showing systemic infection (systemic chlorosis) after herbicidal spray in a commercial poppy field; B) glasshouse trial (4 weeks after emergence) showing the leaf bleaching after herbicide application (Clopyralid 1X); C) systemic downy mildew expression in plants (indicated by arrow) that received herbicide application (metsulfuron- methyl); and D) morphological difference between the untreated control (healthy leaves) and those plants receiving herbicide application (metsulfuron-methyl 1X) .

Table 3.2: Effect of different concentrations of herbicide on the systemic downy mildew development

Treatment	Concentration of herbicide used (X)	Mean number of plants with downy mildew sporulation [a]	Mean number of sporulating leaves [b]	Mean number of seedling death [c]
Clomazone + infested soil	0.5	1.3 [cde]	0.4 [cd]	0.0 [b]
Clomazone + infested soil	1.0	2.3 [abcd]	0.7 [abcd]	0.0 [b]
Clomazone + infested soil	2.0	2.5 [abc]	0.9 [abc]	2.3 [ab]
Metsulfuron- methyl + infested soil	0.5	3.7 [ab]	1.2 [a]	1.15 [ab]
Metsulfuron- methyl + infested soil	1.0	4.0 [ab]	1.2 [a]	1.7 [ab]
Metsulfuron- methyl + infested soil	2.0	4.3 [a]	1.3 [a]	0.8 [b]
Clopyralid + infested soil	0.5	3.3 [abc]	1.0 [abc]	0.0 [b]
Clopyralid + infested soil	1.0	3.7 [ab]	0.8 [abc]	0.9 [b]
Clopyralid + infested soil	2.0	4.0 [ab]	1.1 [a]	0.9 [b]
Imazamox + infested soil	0.5	0.2 [de]	0.0 [d]	2.0 [ab]
Imazamox + infested soil	1.0	2.0 [bcde]	0.6 [abcd]	3.3 [a]
Imazamox + infested soil	2.0	3.2 [abc]	0.8 [abc]	3.3 [a]
Water + infested soil	0.0	0.8 [cde]	0.4 [bcd]	0.0 [b]
Clomazone control	0.5	0.0 [e]	0.0 [d]	0.0 [b]
Clomazone control	1.0	0.0 [e]	0.0 [d]	0.0 [b]
Clomazone control	2.0	0.0 [e]	0.0 [d]	0.0 [b]
Metsulfuron- methyl control	0.5	0.0 [e]	0.0 [d]	0.0 [b]
Metsulfuron- methyl control	1.0	0.0 [e]	0.0 [d]	0.0 [b]
Metsulfuron- methyl control	2.0	0.0 [e]	0.0 [d]	0.0 [b]
Clopyralid control	0.5	0.0 [e]	0.0 [d]	0.0 [b]
Clopyralid control	1.0	0.0 [e]	0.0 [d]	0.0 [b]
Clopyralid control	2.0	0.0 [e]	0.0 [d]	0.0 [b]
Imazamox control	0.5	0.0 [e]	0.0 [d]	0.0 [b]

Imazamox control	1.0	0.0 [e]	0.0 [d]	0.0 [b]
Imazamox control	2.0	0.0 [e]	0.0 [d]	0.0 [b]
Water control	0.0	0.0 [e]	0.0 [d]	0.0 [b]
Probability [d]		P < 0.05		

[a] Values are obtained from 30 plants (3 pots with 5 plants x 2 experiments), assessed after foliar spray of the herbicides at different concentrations, infested with *Pe. somniferi.* The data were combined for the two experiments and analysed via one-way ANOVA test.

[b] The mean number of sporulating leaves was calculated by number of sporulating leaves out of total number of leaves in the individual plants per pot.

[c] The mean number of seedling death was calculated by number of died seedlings out of total number of seedlings in the individual plants per pot post herbicide application after 7 days.

[d] Treatment differences were analysed via ANOVA. The mean values were recorded and mean within the same column followed by different letters are significantly different (P < 0.05) according to Tukey's honest significant differences.

3.5 Discussion

This study has demonstrated that both downy mildew pathogens can infect seedlings from infested soil. This has been previously reported for *Pe. arborescens* causing poppy downy mildew and with other downy mildew pathosystems (Montes-Borrego et al., 2009, Salgado–Salazar et al., 2018; Kandel et al., 2019). The two pathogens showed distinct symptoms with *Pe. somniferi* associated with profuse sporulation with chlorosis, whilst *Pe. meconopsidis* showed necrotic lesions with a localised sporulation. The mechanism underlying the variations in spread of sporulation by the two pathogens is not clearly understood. The rate of sporulation may be a consequence of species virulence, which needs to be studied in future.

Both pathogens formed similar infection structures such as appressoria, haustoria, mycelia, sporangia and oospores in infected host plant tissues. To the author's knowledge, this is the first experimental evidence providing information, of how these pathogens infect poppy plants through the roots and spread the infection by invasion with pathogen mycelium through the plant tissues. The results of the study are in accordance with other reported downy mildew pathosystems (Duduck et al., 2019; Ei-Assiuty et al., 2019; Kandel et al., 2019; Hoffmeister et al., 2020).

The infection process starts with the germination of the oospores by forming a germination tube. The germ tube penetrates the root epidermal layer by forming appressorium and then enters the host (Judelson et al., 2019). Direct germination of oospores has been previously reported in *Peronospora* and *Hyaloperonospora* (Grenville-Briggs and Van West, 2005). Appressoria and haustoria were observed within 2 weeks after plant emergence in this study. This indicates that soilborne oospores have germinated within this time frame, however determination of the exact time of oospore germination and the factors associated were beyond the scope of this experiment and not determined. This may be addressed in future research. Reports from other downy mildew pathosystems have demonstrated how germinating oospores attempt to penetrate neighbouring root tissues (Koch and Slusarenko, 1990; Mauch-Mani et al., 1989; Gascuel et al., 2015, Slagado–Salazar et al., 2018; Judelson et al.,2019). Following root penetration, intercellular coenocytic mycelium were observed between the parenchymal cells, adjacent to xylem tissues within the host. The mycelium spread from root tissue to the stem and from there to the aerial parts of the plants. The first formed

mycelium invades the host both inter and intra cellularly (Wehtje and Zimmer, 1978; Duduck et al., 2019; Ei-Assiuty et al., 2019; Hoffmeister et al., 2020).

During this invasive process, the downy mildew species also produced sporangia that emerged out from infected stem tissues. These could then facilitate secondary infection via movement of sporangia to the neighbouring tissues. Secondary infection by sporangia were not measured in this study. The mechanism of secondary infection by the sporangia are in accordance with the studies conducted by (Wehtje and Zimmer, 1978; Kitz, 2008; Borras-Hidalgo et al., 2010; El-Assiuty et al., 2019). A similar infection process has already been reported in other downy mildew infected crops such as, sorghum, pearl millet, sunflower, tobacco, quinoa (Ramalingam and Rajasab, 1981; Jeger et al., 1998; Borras-Hidalgo et al., 2010; Gascuel et al., 2015; El-Assiuty et al., 2019).

The timing of disease progression from soil inoculum differed for the two pathogens. Downy mildew sporulation and pathogen detection by PCR were observed in the pots inoculated with *Pe. somniferi* 2 weeks after emergence. However, it was not observed until 4 weeks after emergence for *Pe. meconopsidis.* This suggests that *Pe. somniferi* invades the host more rapidly than *Pe. meconopsidis*. Sporulation in young seedlings after infection from soil inoculum sources is important from an epidemiological viewpoint, as these can act as inoculum sources for secondary spread of the sporangia via wind dispersal. An appropriate disease management system including activities such as removal or burning of plant debris after harvesting, crop rotation, (Hannukkala et al., 2007) and for fungicide applications (Klosterman et al., 2014) could limit disease epidemics which has reported in other crops such as pearl millet and spinach (Thakur et al., 2003; Shetty et al., 2016; Slagado–Salazar et al. 2018; Kandel et al., 2019). These management practices must be advised for the growers to limit seedling infections and reduce secondary spread.

In the later stages of the infection, oospores are formed in the mesophyll tissue of leaves (Montes-Borrego et al., 2009; Gascuel et al., 2015; Lehtinen and Hannukkala, 2004; Wehtje and Zimmer, 1978). Under field conditions, infected leaves detach and fall on the ground as plant debris, and may act as a source of inoculum, in the next cropping season. This occurs by the overwintering process and is an important part of the disease cycle of *Peronospora* pathogens as well reported in spinach (*Peronospora effusa*),

sunflower *(Plasmophora halstedii*), (Cohen and Sackston, 1974; Gascuel et al., 2015), rose (*Peronospora rosa*) (Slagado–Salazar et al., 2018) sorghum, pearl millet (*Sclerospora graminicola*) (Jeger et al., 1998) and pea (*Peronospora viciae*) (Pegg and Mence, 1970).

This study also showed that foliar application of certain herbicides can act as an abiotic stress and lead to increased systemic downy mildew symptom expression. In previous studies, downy mildew incidence was increased due to the application of herbicide in sunflower and sorghum crops (Covarelli and Tosi, 2006; Craig, 1987). Herbicides applied to the crop may promote disease by altering host defence mechanisms or they may interact with the soil pathogens and supress them antagonistically (Altman and Campbell, 1977). Previous research shows that a sublethal doses of glyphosate inhibits the shikimic acid pathway that is involved in the release of aromatic amino acids. These are a mainly involved in the resistance of the plants towards plant pathogens (Duke et al., 2003; Duke et al., 2006). The full mechanisms of possible interactions between the plant susceptibility and the herbicides are yet to be understood. These results suggest selecting herbicides for the weed management could play an important role in the influence of disease expression in poppy crops. Further study is needed to increase understanding of the mechanisms that influence the host response and susceptibility to downy mildew infection.

In summary, this study has demonstrated that oospores produced by the pathogens *Pe. somniferi* and *Pe. meconopsidis* are involved in initiating the primary infection in opium poppy plant as has been previously reported (Montes-Borrego et al., 2009). However, it was presumed only the oospores were present in the infested soil, as the sporangia are relatively short lived as reported in other *Peronospora* species (Kandel et al.,2019). There is little documented data available on oospore's survival for *Pe. somniferi* and *Pe. meconopsidis*, however, it has been reported in other *Peronospora* species that this can range from 3 to 25 years (Mackay, 1957; Gaag and Frinking, 1997; Montes- Borrego et al., 2009). Both *Pe. somniferi* and *Pe. meconopsidis* follow a similar infection process from the infested soil into the host. However, rate of infection by *Pe. somniferi* and subsequent disease incidence was greater with *Pe. meconopsidis*. This is in accordance with the previous work suggesting *Pe. somniferi* is more virulent than *Pe. meconopsidis* (Montes–Borrego et al., 2017), The study also identified the role of

herbicides in exacerbate the expression of the downy mildew symptoms caused by *Pe. somniferi*. This has been previously reported for sorghum downy mildew caused by *Peronosclerospora sorghi* (Craig et al.,1987) and in *Rhizoctonia* spp. causing root rot (Altman and Campbell, 1977), damping off in *R. solani* (Heydari and Misaghi, 1998).

These studies have greatly increased our knowledge of poppy downy mildew infection processes by these pathogens through the oospores. However, a further study may be needed to understand the factors that initiate the oospore germination, such as temperature, nutrients, moisture and if any chemical signals produced from the host root system towards the pathogens. These factors would assist in further disease control and management strategies. The study will assist in systematic management through disease modelling and prediction, risk analysis, and developing disease resistance variety by breeding.

Chapter 4: Comparison of the sporangial infection process of the two downy mildew pathogens of opium poppy - *Peronospora somniferi* and *Pe. meconopsidis*.

4.1 Abstract

Downy mildew of opium poppy caused by *Peronospora somniferi* and *Pe. meconopsidis*, imparts a threat to poppy production worldwide. Understanding the infection process is an important key stage in the management of any disease. This study identified and compared the stages of infection of these two pathogens responsible for downy mildew of opium poppy. To study the infection process, sporangial inoculum suspensions of the two pathogens were applied to leaf disks or the foliage of intact plants. In *in vitro* studies, *Pe. somniferi* showed greater sporangial germination and germ tube development than *Pe. meconopsidis*. The processes involved in host infection were similar for the two pathogens. However, *Pe. somniferi* produced more numbers of appressoria, haustoria, sporangia and oospores than *Pe. meconopsidis* during host invasion. Analysis of induction of hydrogen peroxide (a reactive oxygen species associated with plant defence against pathogen attack) following infections with the two pathogens revealed greater H_2O_2 induction in *Pe. meconopsidis* than *Pe. somniferi* leaf infections.

4.2 Introduction

Opium poppy *(Papaver somniferum)* is an important commercial crop primarily grown for its alkaloid compounds, including as codeine, morphine, noscapine and thebaine (INCB, 2019). The largest commercial opium poppy growing countries are Australia, France, Hungary, Spain and Turkey (Kapoor, 1995; Yang et al., 2019; Saunders et al., 2001). Among these countries, Australia contributes more than 50% of the world's licit supply of opiates, with a farm gate value of over $100 million per year (INCB 2019). This is followed by Turkey (23%), France (21%) and Spain (4%) (INCB, 2019).

Downy mildew of poppy is a serious foliar disease that is responsible for considerable crop yield and revenue losses to the Australian poppy industry (Scott et al., 2003; Thangavel et al., 2017). Poppy downy mildew was first documented in Tasmania in 1996 with symptoms of localised angular necrotic lesions and sparse abaxial sporulation. Infections were attributed to *Pe. arborescens* (Cotterill and Pascoe, 1998) but was later reclassified as *Pe. meconopsidis* (Voglmayr et al., 2014). In 2013, a symptomatically distinct and more severe form of disease showing general chlorosis, stunting and accompanied by profuse sporulation was described. This newly identified form of the disease was subsequently determined to be caused by infections with *Pe. somniferi* (Thangavel et al., 2017).

In general, the lifecycle of *Peronospora* spp. has two stages (Spencer-Phillips et al., 2002): i) the sexual stage –the formation of oospores, which is a dormant structure formed in the later stages of the pathogen lifecycle; and ii) the asexual stage –the formation of the sporangiospores on the abaxial side of the leaf. The typical life cycle and biological infection processes of *Peronospora* spp., has been widely reported in many crops, including *Peronospora tabacina* (in tobacco), *Pe. sparsa* (in rose) and *Pe. effusa* (in spinach) (Borras-Hidalgo et al., 2010; Gómez and Filgueira-Duarte, 2012; Kandel et al., 2019). However, there is no information available on the infection process of *Pe. somniferi* and *Pe. meconopsidis* in opium poppy. Given the presence of two distinct pathogens with distinct symptom expression, a comparison of their infection process is warranted. Understanding the infection process of these pathogens will be helpful in the development of disease-resistant varieties and control measures against them.

Plant cells release reactive oxygen species (ROS) such as hydrogen peroxide as a physiological response to various biotic and abiotic stress, often leading to localised cell death (Camejo et al., 2016; Choudhury et al., 2017). ROS are commonly produced in plant cells as a defence reaction in response to plant pathogenic interactions. ROS production is termed an oxidative burst (Doke et al., 1996) and occurs in plant structures, such as chloroplasts (Camejo et al., 2006; Dietz et al.,2016), mitochondria (Vellosillo et al., 2010, 2013) and in peroxisomes (Gill & Tuteje et al., 2010). Host cell defence responses with the release of hydrogen peroxide has been recorded with in pearl millet downy mildew caused by infections with *Sclerospora graminicola* (Kumudhini et al., 2001). Given the two poppy downy mildew pathogens induce distinct disease symptoms with or without localised necrosis which may be associated with plant defence responses, it would be valuable to compare ROS production following foliar infections to determine whether there is evidence for differences in host defence responses *Pe. meconopsidis* and *Pe. somniferi* infections.

Based on available literature and previous studies, it is hypothesized that:

i) the two pathogens invade the host in a similar infective process, however, *Pe. somniferi* infection may progress more rapidly than *Pe. meconopsidis*; and

ii) host defence responses are greater following infection by *Pe. meconopsidis* than *Pe. somniferi* infection.

4.3 Materials and methods

4.3.1 Glasshouse conditions

For all experiments, a commercial seed lot of *Papaver somniferum*, kindly supplied by Tasmanian poppy industry was used. Due to commercial confidentiality requirements, the seed line used in this experimental chapter is specified with the code SL-21. This seed line was known to be susceptible to both downy mildew pathogens. Prior to sowing, seeds were surface sterilised in a hypochlorite solution (2% available chlorine) for 5 minutes, followed by rinsing twice in sterile water and dried at ambient temperature in a laminar flow hood (Thangavel et al., 2020). The seeds were then stored in an airtight container at ambient room temperature in the dark. Unless otherwise stated, poppy plants were grown in pots of 27 cm (height) x 24 cm (top diameter) x 18 cm (base diameter) size filled with a potting mixture containing sand, peat, and composted pine bark (10:10:80, pH 6.0) premixed with Osmocote 16-3.5-10 NPK resin coated fertilizer (Scotts Australia Pty Ltd.). Plants were grown under glasshouse conditions of 15-20°C and 50-60% relative humidity, monitored with a tiny tag data logger (Hastings data loggers, UK).

4.3.2 Inocula sources and culture maintenance

Diseased poppy leaves infected with *Pe. somniferi* or *Pe. meconopsidis* were collected from commercial poppy-growing regions in Tasmania during the 2017-18 growing season (Table 4.1). Leaves showing symptoms of distinct localised necrotic lesions or of profuse sporulation were used to isolate separate inoculum sources for each pathogen. After collection, leaves were stored in a zip-lock plastic bags generating humid conditions for 24 h to promote sporulation. Sporangia were brushed from infected leaves into sterile water (20 ml), adjusted to a concentration of 1.2 x 10^4 spores/ml as determined by counts using a haemocytometer, and 10 ml sprayed on to healthy 4-week-old plants which were then covered in plastic bags for 24 h to maintain humidity. The plants were then maintained in a growth chamber at 20°C Day/15°C night temperatures at relative humidity 50% and with 16 h day/8 h night cycle, commencing in the dark cycle. The viability of each sporangial sample was determined by plating 20 µl of the suspension onto 2% water agar media. The plates were then incubated at 15°C for 10 h and 20°C for 14 h. After 24 h incubation, the plates were examined under 400 X magnification for germ tube formation to confirm the viability of individual sporangia. Approximately 100 sporangia were examined on each plate within at least four

randomly selected fields of view. Sporangia were considered to be germinated when the length of the germ tube was twice the diameter of the sporangia.

For each pathogen, five opium poppy seeds were sown into pre-wetted Jiffy peat pots (Jiffy Products International AS, Norway; 44 mm in diameter) and thinned to two plants per pot at the 4-6 leaf stage. The plants were maintained in a growth chamber (Steridium, Brisbane Pty Ltd) at 20°C Day and 15 °C night temperature at a relative humidity of 50%. Sporangial suspensions of the two pathogens were sprayed onto the foliage of 4-week-old plants using a 20-ml spray bottle. Plants were then covered with plastic bags to increase the relative humidity to 100% for 24 - 48 h and placed in the growth chamber starting with the dark cycle. Freshly produced spores from these infected plants were used to reinfect a new set of 4-week-old plants for inoculum maintenance every two weeks.

4.3.3 Confirmation of pathogens by species-specific PCR

Infected leaves were collected from plants within the growth chamber and DNA was isolated from 50 mg of leaf material using the Power Plant Pro DNA isolation kit (Mo BIO Laboratories, Australia) following the manufacturer's protocol. DNA samples were subjected to PCR amplification, using the primer sets COX 1 PSF-PSR specific to *Pe. somniferi* and COX 2 -PMF-PMR specific to *Pe. meconopsidis* (Thangavel et al., 2016). All PCR reactions used a total volume of 20 µl, containing 10 µl of Hot Star Taq Plus Master Mix Kit, (QIAGEN, Australia), 0.75 µl each of the forward and reverse primers, 2 µl of CoralLoad dye, 5 µl of the RNAse free water and 1.5 µl of the 5 ng of DNA sample to be tested. The thermocycler conditions were set to a denaturation temperature at 95˚C for 15 minutes and followed by 35 cycles at 95˚C for 15 minutes, 60˚C for 30 s and 72˚C for 30 s, with a final extension period at 72˚C for 10 minutes. Amplified DNA was confirmed by electrophoresis in a 1 % agarose gel stained with 0.1 % of the SYBR Safe DNA gel stain (Life Technologies) and visualised under blue light with a 50 bp ladder (Bioline, Australia) as a molecular marker.

4.3.4 Comparison of germination rate and germ tube development

The germination rate and development of germ tubes of the two pathogens were compared by culture on 1 % water agar. Sporangia of *Pe. meconopsidis* and *Pe. somniferi* were harvested separately in sterile distilled water as previously described. Sporangial concentrations were adjusted to 1.2 x 10^4 sporangia/ml, and then 10 µl of sporangial suspension of each pathogen was independently placed onto a 1 % water agar block (1 cm^2) which were then placed onto a clean microscopic slide. To increase humidity for sporangial germination, slides were kept in closed Petri plates with a sterile cotton ball moistened with sterile water. Plates were incubated in a growth chamber set at 60% relative humidity with 20°C for 0, 2, 4, 6, 8, 10, 12, 16, 18, 20 and 24 h in darkness. Five agar blocks (n=5) per inoculum source were used at each time point. The slides were examined using a light microscope (Leica DFC 420, Germany) at 400 X magnification and the images were captured using a Leica LAS software. At each time point, at least 100 sporangia were observed and the proportion of germinated sporangia and germ tube length of each germinated sporangia were measured for both pathogens. The experiment was repeated five times.

4.3.5 Monitoring the infection process of two pathogens.

4.3.5.1 Leaf disk assays

The infection process and disease progression of the two pathogens, *Pe. somniferi* and *Pe. meconopsidis* were examined using a leaf disk assay. Leaf disks of 12 mm diameter were cut from fully expanded poppy leaves collected from the four-week-old plants using a sterile metal cork borer. Leaf disks were then placed with the adaxial surface facing upwards onto a 1 % water agar plate amended with 100 ppm of benzimidazole (SIGMA) (Angola et al., 2003). Five leaf disks were placed in each Petri plate. Sporangial suspensions of the two pathogens were adjusted to 1.2 x 10^4 spores/ml and 15 µl of the sporangial suspension of each pathogen was placed onto leaf disks which were then incubated in a plant growth chamber with a day setting of 20°C for 14 h at 90 % relative humidity and a night setting of 14 °C for 10 h at 90 % relative humidity. The leaf disks were then examined at 0, 3, 6, 12, 24, 48, 72, 96, 120, 144 and 168 h post-inoculation with five leaf disks observed at each time point and assessed for the mean number of germinating sporangia, germ tube length, appressoria and haustoria formed. The leaf disks were examined using a light microscope (Leica DFC 420, Germany) at 400 X magnification and the images were captured using a Leica LAS software. A sporulation

index score (0-3), 0- no sporulation, 1 – weak sporulation, 2- moderate sporulation, 3- dense sporulation) (Cohen et al., 2013) and the oospore index score (0-3), 0- no oospore formation, 1 – weak oospore formation, 2- moderate oospore formation, 3- dense oospore formation was used (Gómez and Filgueira-Duarte, 2012). The experiment was repeated three times.

4.3.5.2 Whole plant assays

To compare the foliar infection process of the two pathogens, a set of sporangial spray inoculation experiments were conducted on whole plants. Four-week-old poppy plants were used for the study. A sporangial suspension of each pathogen was prepared from the freshly sporulating leaves with sporangia numbers adjusted to 1.2 x 10^4 spores/ml for each pathogen. Each pathogen inoculum was applied as a foliar spray (10 ml per pot) to 30 plants (three pots with 10 plants per pot). The inoculated plants were incubated in separate growth chambers, ensuring no cross-contamination occurred between the two pathogens. Incubation commenced in the dark cycle maintained at 15°C for 10 h followed by 20°C for 14 h in light, with a relative humidity of 90 %. Plants were observed daily for up to 12 days post-inoculation (DAI) for the presence of disease symptoms. The total number of plants showing the downy mildew symptoms described above following infection with *Pe. somniferi* or *Pe. meconopsidis* was estimated visually, and the total number of individual plants showing downy mildew sporulation were recorded. The sporulation and oospore index were also determined as previously described. The experiment was repeated three times.

4.3.6 Quantitative and qualitative measurement of hydrogen peroxide production in infected tissues

The amount of hydrogen peroxide (H_2O_2) produced by the plants infected with *Pe. somniferi* and *Pe. meconopsidis* was determined based on the methods of Patel et al. (2016) and Velikova et al. (2000). For each pathogen, three opium poppy seeds were sown into pre-wetted Jiffy peat pots and thinned to one plant per pot following germination. At 4 weeks after emergence, sporangial inocula of each pathogen, adjusted to a concentration of 1.2 x 10^4 spores/ml, were sprayed (1-2 ml/plant) onto the foliage of the plants. Water only treated plants served as a non-inoculum control. Leaf samples were collected at 6, 12, 24, 48, 72, 96, 120, 144 and 168 hours after inoculation (HAI). A 0.5 g subsample of each leaf was homogenized in 3 mL of 1% (w/v) tri-chloroacetic acid (TCA) in a pre-cooled mortar and pestle. The homogenate was centrifuged at 10,000

rpm at 4°C for 10 minutes. Subsequently, 0.75 ml of the supernatant was added to 0.75 mL of 10 mM K-phosphate buffer (pH 7.0) and 1.5 ml of 1M potassium iodide. The concentration of H_2O_2 in the supernatant was evaluated by comparing its absorbance at 390 nm with a standard calibration curve, generated from a serial dilution of H_2O_2 in the range from 100 to 1000 µmol/ml. H_2O_2 concentration was expressed as µmol/g fresh weight leaf material. Absorbance was measured using a spectrophotometer (Thermo Scientific Spectronic 200E). The experiment was repeated twice and at each time point three plants were used per treatment.

The distribution of H_2O_2 accumulation in inoculated leaves was visually analysed following DAB (3,3-diaminobenzidine) staining of leaves (n=5), collected at 168 HAI, according to Thordal-Christensen et al. (1997). Control and inoculated leaves were incubated overnight in a solution of 1 mg/ml DAB solution (pH 7.5). Following overnight incubation, the DAB staining solution was replaced with bleaching solution (ethanol: acetic acid: glycerol in the ratio of 3:1:1). The slides were mounted on a clean microscopic slide, and the slides placed into a boiling water bath for 10 minutes until all the chlorophyll was removed. The interaction of the DAB and H_2O_2 produced a visible brown coloured precipitate within the cleared leaf. The leaves were then directly visualised under a light microscope at 400 X magnification (Leica DFC 420, Germany) with images captured using a Leica LAS software version 3.8.0 (Leica Microsystems Pty Ltd, Australia).

4.3.7 Data analysis

In the water agar assays, data comparing sporangia germination and germ tube length were analysed using one way ANOVA by the software statistical language framework v.3.4 (R Core Team, 2015). The cumulative germination and germ tube length of the sporangia across the experiments were fitted by a non - linear model for each pathogen. Analysis of the datasets across the five experiments indicated no significant differences and thus, the datasets were combined.

In the leaf disk and whole plant experiments, data were tested for normality, and the mean effects of each treatment were analysed using a one-way or two-way ANOVA. Where the mean P values showed a significant difference at the 5% significance level, a post hoc comparison of the treatment means were done using LSD for pairwise comparison.

For the measurement of hydrogen peroxide production in infected tissues, data were analysed by one-way ANOVA. Where the mean P values showed a significant difference at the 5% significance level, a post hoc comparison of the treatment means were done using LSD for pairwise comparison.

4.4 Results

4.4.1 Confirmation of the purity of pathogen inocula

The field inoculum sources of *Pe. somniferi* and *Pe. meconopsidis,* once passaged independently onto healthy poppy plants were shown to be the single species only by a positive reaction with PCR primers to the expected species and a negative reaction to the alternate using species-specific PCR testing. (Figure 4.1).

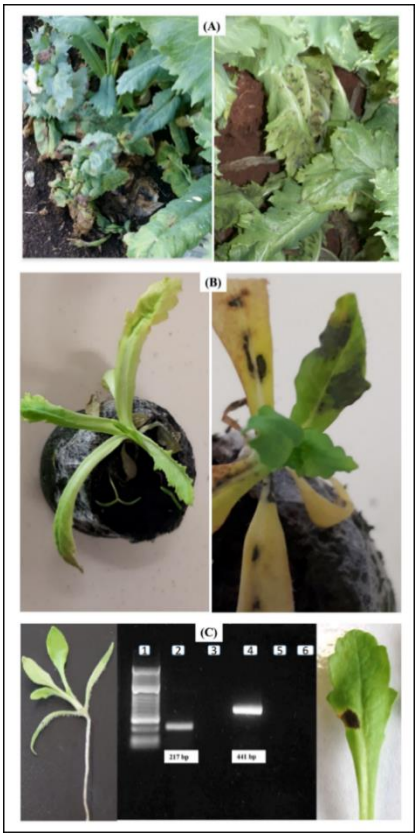

Figure 4.1: Pathogen sources. A) Examples of plants sampled from commercial poppy fields show the downy mildew symptoms of the two pathogens (Left – *Pe. somniferi* and Right – *Pe. meconopsidis)*; B) Pure cultured pathogens (left- *Pe. somniferi*, right- *Pe. meconopsidis*) under laboratory conditions; and C) species-specific PCR confirmation of the two pathogens, lane 1- 50 bp molecular marker, lane 2-*Pe. somniferi samples* testing positive to *Pe. somniferi* test (217 bp), lane 3- *Pe. somniferi* sample testing negative to *Pe. meconopsidis* test, lane 4- *Pe. meconopsidis samples* testing positive to – *Pe. meconopsidis* (441 bp), lane 5- *Pe. meconopsidis* test sample testing negative to *Pe. somniferi*, lane-6 non template control.

4.4.2 Comparison of germination rate and germ tube development

The proportions of germinated sporangia on 1 % water agar blocks significantly differed between the two pathogens at all time periods. The proportion of sporangial germination increased steadily for both pathogens from 0.01 to 0.06 with *Pe. meconopsidis* and 0.028 to 0.08 with *Pe. somniferi* (Figure 4.2A). The mean germ tube length for the two pathogens also increased with time. In *Pe. somniferi*, the length of the germ tube was to increase from 0 to 90 µm, and for *Pe. meconopsidis* from 0 to 42 µm between 0 – 24 h. Overall, *Pe. somniferi* showed greater germination and longer germ tube lengths from 0 - 24 HAI and which was statistically significant ($P<0.001$) (Figure 4.2A and B).

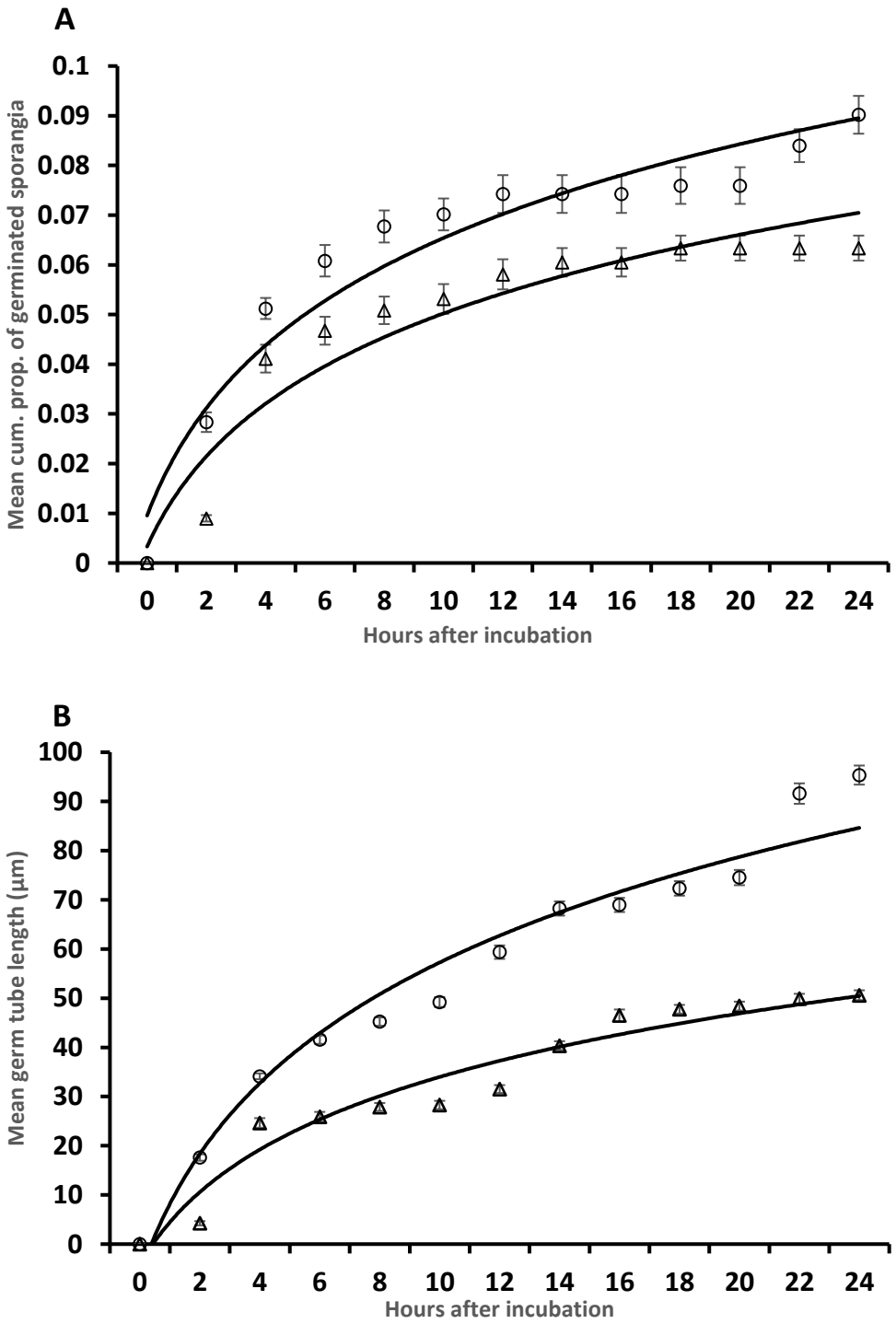

Figure 4.2: Sporangial germination on 1% water agar. Data from the five experiments were combined for analysis. A) Cumulative proportion of germinated sporangia; and B) mean germinated tube length was calculated for *Pe. somniferi*(Φ) and *Pe. meconopsidis* (Δ). The number of sporangia observed per block ranged from 15 - 68. Error bars are standard errors between the experiments (n=5).

4.4.3 Monitoring the infection process of the two-poppy downy mildew pathogens on leaf disks.

4.4.3.1 Leaf disk assays

Germinated sporangia were first observed at 1 HAI and ceased to be found after 72 HAI for *Pe. somniferi* with mean sporangial numbers per disk varying between 0.1 to 4. For *Pe. meconopsidis* germinating sporangia were first observed at 12 HAI with last observations at 120 HAI with mean numbers per disk varying from 0.5 to 1.8 (Table 4.1). Germ tube lengths of germinating sporangia increased from 4 to 118.4 µm from 1-72 HAI for *Pe. somniferi,* and from 29.9 to 42.6 µm from 12-120 HAI for *Pe. meconopsidis* (Table 4.1). Both pathogens were observed to form dual germ tubes over the course of time. The mean numbers of germinating sporangia and mean germ tube lengths significantly differed between the two pathogens at 3, 12, 24, 48, 72, 96 and 120 HAI and at 12, 24, 48 and 72 HAI respectively ($P \leq 0.05$).

The mean numbers of appressoria and haustoria also significantly varied between the pathogens at certain time points ($P \leq 0.05$). The appressoria, which develop from the germ tube by a small inflating structure, penetrating the epidermis (Figure 4.3 A), was first observed from 6 HAI for both pathogens. For *Pe. somniferi*, appressoria and haustoria were observed from 12 HAI until 168 HAI with the mean numbers ranging from 2.2 to 3.8 and 0.6 to 3.8 per disk, respectively (Table 4.1). For *Pe. meconopsidis*, appressoria and haustoria were found from 12 HAI or 24 HAI until 168 HAI, respectively, with mean numbers ranging from 0.1 to 0.6 (appressoria) and 0.1 to 0.6 (haustoria) (Table 4.1).

Sporulation was observed on leaf disks from 72 HAI in *Pe. somniferi* and 96 HAI in *Pe meconopsidis*, and oospores from 120 HAI in both *Pe. somniferi* and *Pe meconopsidis*. Mean sporulation and oospore indices varied between the two pathogens at 72 to 168 HAI and 144 to 168 HAI, respectively with *Pe. somniferi* having greater scores than *Pe. meconopsidis* at these time points (Figure 4.4 and 4.5)

Table 4.1: Relative comparison of asexual infection processes of *Pe. somniferi* and *Pe. meconopsidis* in opium poppy leaf disk.

Time (h)	Mean no. of. germinated sporangia per disk [a]		Mean germ tube Length per disk per disk (µm) [a]		Mean no. of. appressorium per disk [a]		Mean no. of. haustorium per disk [a]		Mean sporulation Index per disk (0-3) [b]		Mean oospore index per disk (0-3) [b]	
	Pe. mec	*Pe. som*	*Pe. mec*	*Pe. som*	*Pe. mec*	*Pe. som*	*Pe. mec*	*Pe. som*	*Pe. mec*	*Pe. som*	*Pe. mec*	*Pe. som*
0	0.0 ^{i}	0.0 ^{i}	0.0 ^{d}	0.0 ^{d}	0.0 ^{e}	0.0 ^{e}	0.0 ^{d}	0.0 ^{d}	0.0 ^{e}	0.0 ^{e}	0.0 ^{e}	0.0 ^{e}
1	0.0 ^{i}	0.1 ^{hi}	0.0 ^{d}	4.0 ^{d}	0.0 ^{e}	0.0 ^{e}	0.0 ^{d}	0.0 ^{d}	0.0 ^{e}	0.0 ^{e}	0.0 ^{e}	0.0 ^{e}
3	0.0 ^{i}	0.3 ^{gh}	0.0 ^{d}	8.1 ^{cd}	0.0 ^{e}	0.0 ^{e}	0.0 ^{d}	0.0 ^{d}	0.0 ^{e}	0.0 ^{e}	0.0 ^{e}	0.0 ^{e}
6	0.0 ^{i}	0.1 ^{hi}	0.0 ^{d}	5.1 ^{d}	0.0 ^{e}	0.0 ^{e}	0.0 ^{d}	0.0 ^{d}	0.0 ^{e}	0.0 ^{e}	0.0 ^{e}	0.0 ^{e}
12	1.0 ^{e}	3.4 ^{b}	29.9 ^{bc}	105.6 ^{a}	0.1 ^{e}	2.2 ^{b}	0.0 ^{d}	0.6 ^{c}	0.0 ^{e}	0.0 ^{e}	0.0 ^{e}	0.0 ^{e}
24	1.5 ^{d}	4.3 ^{a}	38.7 ^{b}	111.2 ^{a}	0.6 ^{c}	3.4 ^{a}	0.6 ^{c}	2.6 ^{b}	0.0 ^{e}	0.0 ^{e}	0.0 ^{e}	0.0 ^{e}
48	1.7 ^{cd}	4.0 ^{a}	39.1 ^{b}	115.2 ^{a}	0.6 ^{c}	3.8 ^{a}	0.6 ^{c}	2.6 ^{b}	0.0 ^{e}	0.0 ^{e}	0.0 ^{e}	0.0 ^{e}
72	1.8 ^{c}	4.0 ^{a}	42.6 ^{b}	118.4 ^{a}	0.5 ^{cd}	3.8 ^{a}	0.4 ^{cd}	3.8 ^{a}	0.0 ^{e}	0.3 ^{d}	0.0 ^{e}	0.0 ^{e}
96	0.7 ^{f}	0.0 ^{i}	14.7 ^{cd}	0.0 ^{d}	0.1 ^{de}	2.6 ^{b}	0.1 ^{d}	2.2 ^{b}	0.4 ^{d}	1.4 ^{b}	0.0 ^{e}	0.0 ^{e}
120	0.5 ^{fg}	0.0 ^{i}	11.2 ^{cd}	0.0 ^{d}	0.4 ^{cde}	2.6 ^{b}	0.3 ^{cd}	2.2 ^{b}	1.1 ^{c}	2.8 ^{a}	0.1 ^{de}	0.4 ^{cd}
144	0.0 ^{i}	0.0 ^{i}	0.0 ^{d}	0.0 ^{d}	0.2 ^{cde}	2.6 ^{b}	0.2 ^{cd}	2.2 ^{b}	1.1 ^{c}	2.8 ^{a}	0.2 ^{ce}	0.5 ^{c}
168	0.0 ^{i}	0.0 ^{i}	0.0 ^{d}	0.0 ^{d}	0.2 ^{cde}	2.6 ^{b}	0.2 ^{cd}	2.2 ^{b}	1.7 ^{b}	3.0 ^{a}	1.0 ^{b}	2.0 ^{a}

[a] Values are obtained from 15 leaf disks (5 disks at each time point each averaged across 3 experiments), assessed after inoculation of sporangial suspensions of each pathogen and observed for germinated sporangia, germ tube length, appressorium and haustorium from 0 to 168 hours after sporangial inoculation of *Pe. somniferi* and *Pe. meconopsidis.*

the downy mildew symptoms such as chlorosis with profuse sporulation (*Pe. somniferi*), polyangular necrotic lesions with localised sporulation (*Pe. meconopsidis*).

[b] Mean sporulation and oospore indices are measured from 0 to 168 h after sporangial inoculation of *Pe. somniferi* and *Pe. meconopsidis* in opium poppy plants. Treatments with the same letters under each measured parameter are not significantly different at $P \leq 0.05$ (n = 15).

Pe. mec- Pe. meconopsidis, *Pe. som – Pe. somniferi*

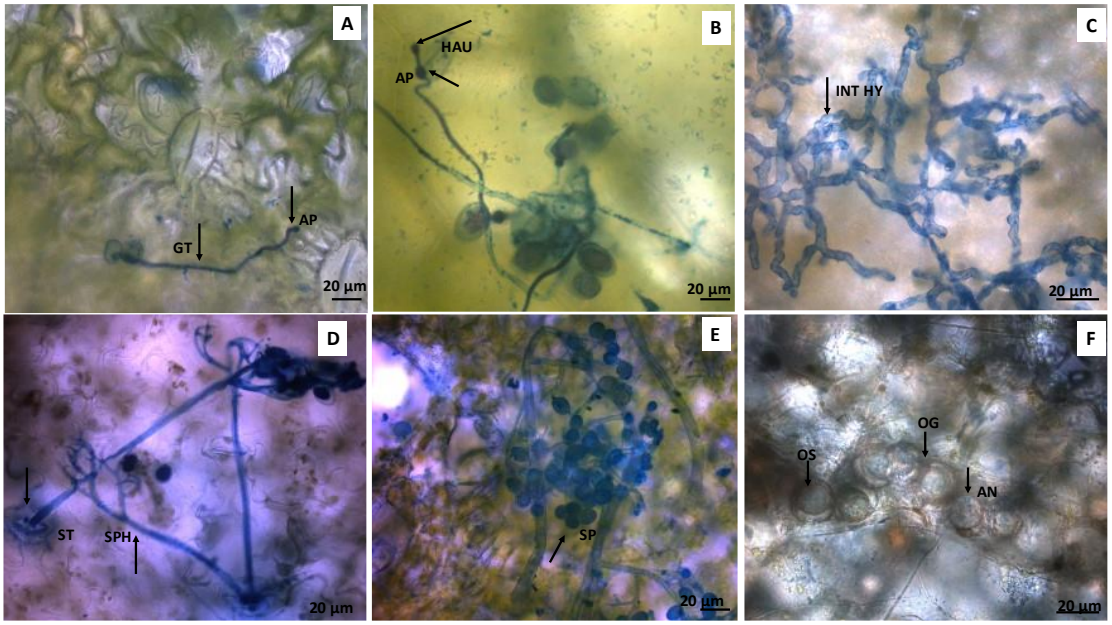

Figure 4.3: A snapshot of *Pe. somniferi* infection in opium poppy. A) Germination of sporangia at 12-24 hours after inoculation (HAI); B) Germ tube extends to form appressorium; appressorium (AP) and haustoria (HAU) formation within 24-48 HAI; C) Intercellular hyphae (INT HY) and the intercellular bifurcation of hyphae in the mesophyll cells of the host at 96 HAI; D) extension of sporangiophore (SPH) from stomata (ST); E) Sporangia (SP) are formed (initiating the sporulation) at 120 HAI and F) Formation of mature oospores (OS) at 168 HAI. Note the presence of antheridium (AN) and oogonia (OG) are also observed, indicated by arrows.

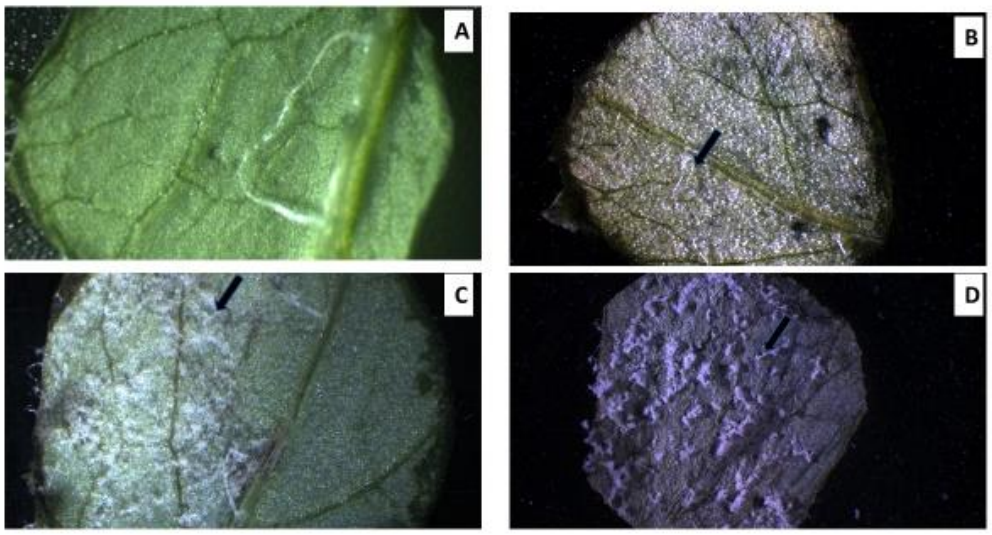

Figure 4.4: Sporulation index 0 – 3 scale: A) No sporulation (Score-0); B) Weak sporulation (Score-1); C) Moderate sporulation (Score-2); and D) Dense sporulation (Score-3)

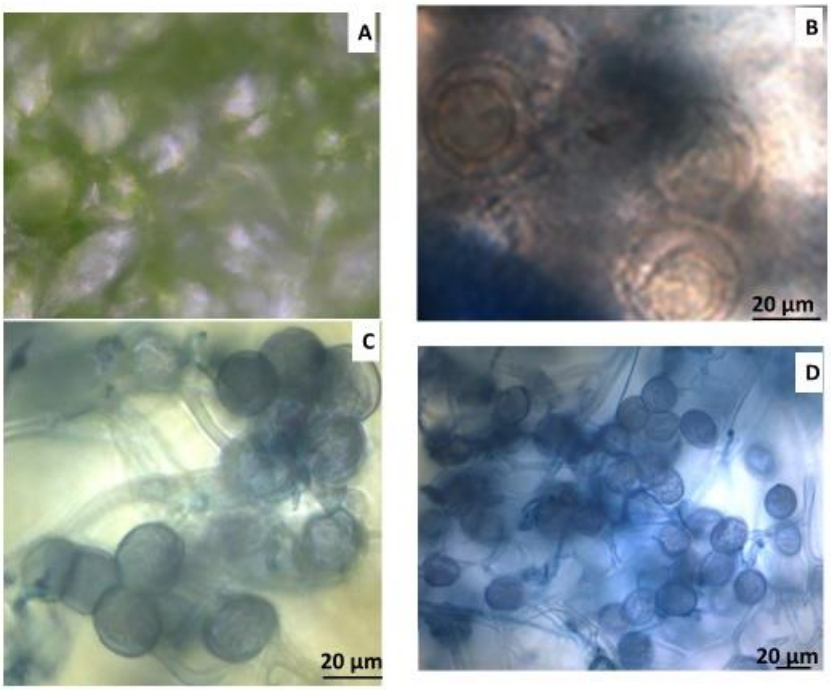

Figure 4.5: Oospore index 0 – 3 scale, observed in the mesophyll layer of the leaf tissue. A) No oospore (Score-0); B) Weak oospore formation (Score-1); C) Moderate oospore production (Score-2); and D) Densely produced oospores (Score-3)

4.4.2.2 Whole plant assay:

Challenge with sporangial sprays of the two pathogens onto the foliage of the host plant resulted in varied responses by *Pe. somniferi* and *Pe. meconopsidis* (Table 4.2). Following *Pe. somniferi* inoculation, the mean number of plants showing general chlorotic symptoms were observed from 4 DAI up to 12 DAI in the range of 2.0 to 2.4. Following *Pe. meconopsidis* inoculation, poly angular necrotic lesions were observed from 6 DAI to 12 DAI in the range of 0.7 to 1.3, with the mean necrotic lesion length ranged from 1.6 to 3.8 mm. The mean number of plants showing sporulation was significantly higher in *Pe. somniferi* than *Pe. meconopsidis* at each assessment from 4 to 12 DAI. The mean sporulation and oospore indices of also differed significantly between the two pathogens with a greater mean sporulation index for *Pe. somniferi* at 4, 10 and 12 DAI and mean oospores indices at 10 and 12 DAI compared to *Pe. meconopsidis*.

Table 4.2: Response of *Papaver somniferum* to foliar inoculation of sporangial suspensions of *Pe. somniferi* and *Pe. meconopsidis.*

Days after inoculation	Mean no. of plants showing polyangular necrotic lesion [a]		Lesion length (mm) [a]		Mean no. of plants with chlorosis [a]		Mean no. of sporulating plants [a]		Sporulation index (0-3) [b]		Oospore index (0-3) [b]	
	Pe. mec[c]	*Pe. som*[c]	*Pe. mec*	*Pe. som*	*Pe. mec*	*Pe. som*	*Pe. mec*	*Pe. som*	*Pe. mec*	*Pe. som*	*Pe. mec*	*Pe. som*
0	0.0 [c]	0.0 [c]	0.0 [c]	0.0 [c]	0.0 [b]	0.0 [b]	0.0 [f]	0.0 [f]	0.0 [e]	0.0 [e]	0.0 [c]	0.0 [c]
1	0.0 [c]	0.0 [c]	0.0 [c]	0.0 [c]	0.0 [b]	0.0 [b]	0.0 [f]	0.0 [f]	0.0 [e]	0.0 [e]	0.0 [c]	0.0 [c]
2	0.0 [c]	0.0 [c]	0.0 [c]	0.0 [c]	0.0 [b]	0.0 [b]	0.0 [f]	0.0 [f]	0.0 [e]	0.0 [e]	0.0 [c]	0.0 [c]
4	0.0 [c]	0.0 [c]	0.0 [c]	0.0 [c]	0.0 [b]	2.0 [a]	0.9 [e]	1.9 [d]	0.0 [e]	1.7 [c]	0.0 [c]	0.0 [c]
6	0.7 [b]	0.0 [c]	1.6 [b]	0.0 [c]	0.0 [b]	2.1 [a]	2.1 [d]	3.1 [c]	1.2 [c]	1.7 [c]	0.0 [c]	0.0 [c]
8	1.0 [ab]	0.0 [c]	3.2 [a]	0.0 [c]	0.0 [b]	2.2 [a]	3.4 [c]	4.4 [b]	2.3 [b]	2.3 [b]	0.6 [b]	0.6 [b]
10	1.3 [a]	0.0 [c]	3.7 [a]	0.0 [c]	0.0 [b]	2.3 [a]	3.6 [c]	4.7 [ab]	2.3 [b]	3.0 [a]	0.6 [b]	1.6 [a]
12	1.3 [a]	0.0 [c]	3.8 [a]	0.0 [c]	0.0 [b]	2.4 [a]	3.6 [c]	5. 1 [a]	2.3 [b]	3.0 [a]	0.6 [b]	1.6 [a]

[a] Values are obtained from 30 plants (3 pots with 10 plants each averaged across 3 experiments), assessed after foliar spray of the sporangial suspensions of each pathogen and observed for the downy mildew symptoms such as chlorosis with profuse sporulation (*Pe. somniferi*), polyangular necrotic lesions with localised sporulation (*Pe. meconopsidis*).

[b] Mean lesion length, sporulation and oospore indices are measured from 0 to 12 days after sporangial inoculation of *Pe. somniferi* and *Pe. meconopsidis* in opium poppy plants. Treatments with the same letters under each measured parameter are not significantly different at $P \leq 0.05$ (n = 90). No lesions, chlorosis, sporangia or oospore indices were observed in any control plants at any time point.

[c] Oomycete species: *Pe. mec* = *Pe. meconopsidis*; *Pe. som* = *Pe. somniferi*.

4.4.4 Quantitative and qualitative measurement of hydrogen peroxide production in leaf tissues infected with the two downy mildew pathogens.

The amount of hydrogen peroxide detected in infected foliage increased with time from 6 to 168 HAI with both pathogens (Figure 4.6). For *Pe. meconopsidis*, the amount of hydrogen peroxide ranged from 634.4 µmol/g at 6 HAI up to 2195.6 µmol/g at 168 HAI. For *Pe. somniferi,* the amount of hydrogen peroxide production varied from 407.4 µmol/g at 6 HAI to 1354.6 µmol/g at 168 HAI. The data from the two experiments was tested and shown to not significantly differ between the experiments and thus the datasets were combined from subsequent analysis. The mean level of hydrogen peroxide was greater in *Pe. meconopsidis* infected leaves than in *Pe. somniferi* infected leaves at all time points, but this difference was only statistically significant from 72 to 168 HAI. Accumulation of hydrogen peroxide in leaves infected by *Pe. somniferi* was only statistically greater than that in control plants at 120 and 168 HAI. The effect of necrosis due to the production and accumulation of hydrogen peroxide were observed in the mesophyll layers of the host tissue (Figure 4.7). In DAB staining, brown staining, appeared to be more intense and localized in *Pe. meconopsidis* infected tissues than in *Pe. somniferi* infected tissues.

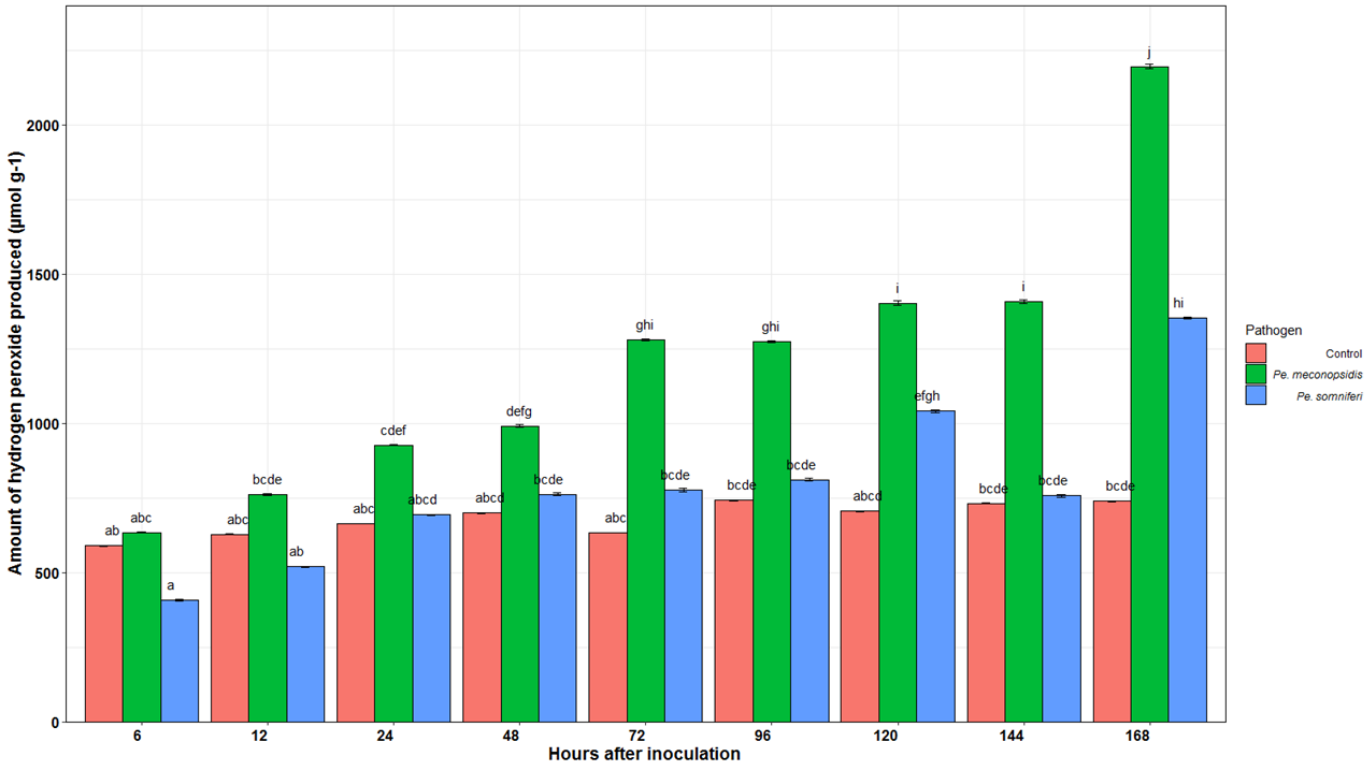

Figure 4.6: The amount of hydrogen peroxide produced within infected leaf tissues following inoculation with *Pe. somniferi* and *Pe. meconopsidis.* The experiment was repeated twice and expressed as the mean of three replicates in each experiment at each time point. The error bars indicate the standard errors between the replicates. Error bars with different letters are significantly different, calculated at $P < 0.05$.

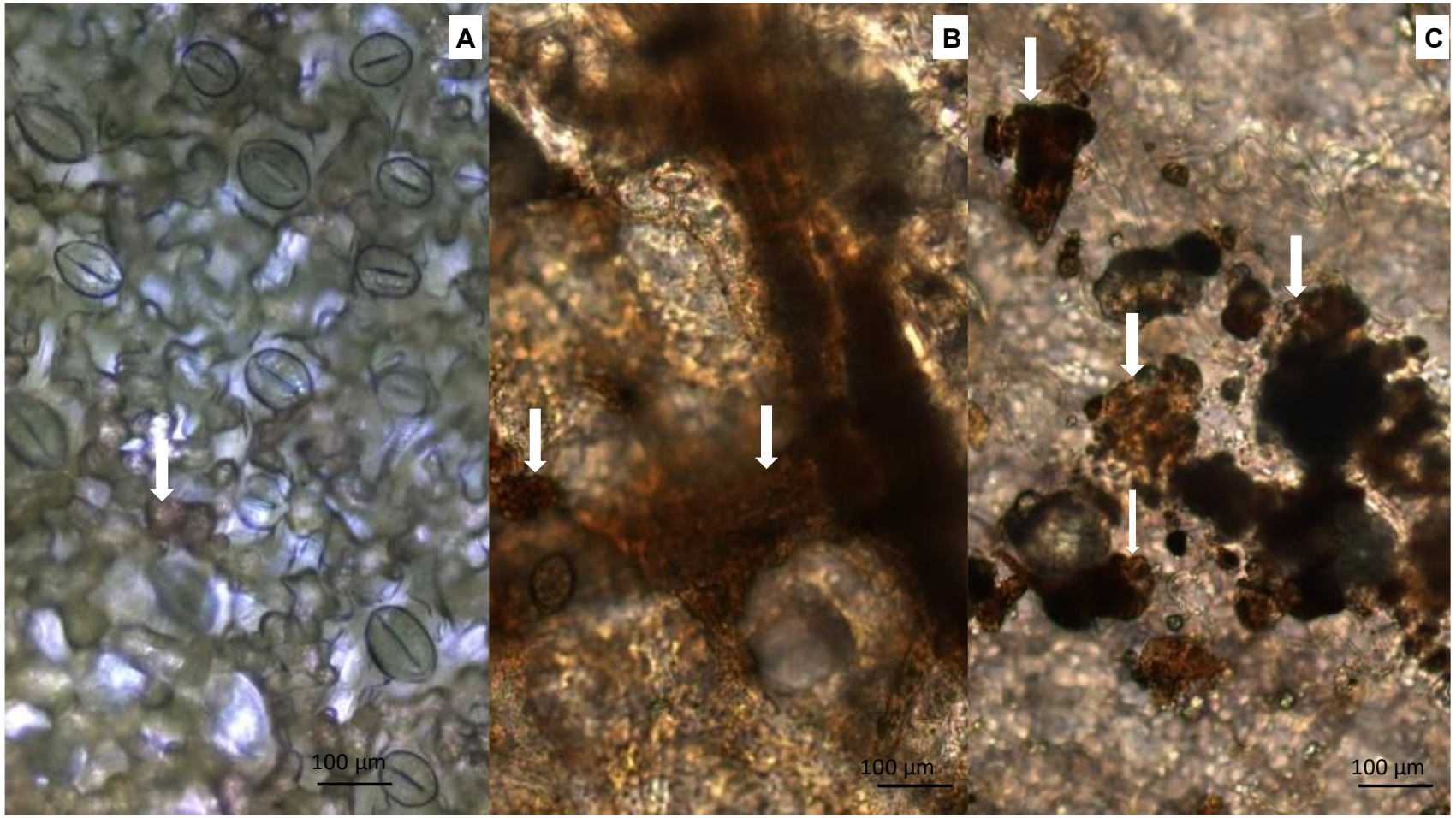

Figure 4.7: The localization of hydrogen peroxide by DAB staining observed after 168 hours post-inoculation. Brown spots in the images indicated by arrows represent the accumulation of H_2O_2 within infected leaf tissues following inoculation: A) control plant sprayed with water only; B) *Pe. somniferi* sprayed leaf, and C) *Pe. meconopsidis* sprayed leaf.

4.5 Discussion

Downy mildew of opium poppy is a worldwide issue and the basic knowledge on the infection process is limited. In the present study, the infection process of the two downy mildew pathogens *Pe. somniferi* and *Pe. meconopsidis* by sporangial inoculum was examined. The study has gained an insight on the events of infective process and will assist in developing effective disease management strategies.

The first examination of sporangial germination on water agar showed *Pe. somniferi* had a greater germination rate and faster germ tube development than *Pe. meconopsidis.* In comparison with other downy mildews such as rose and spinach (Gómez and Filgueira-Duarte, 2012; Choudhury and M^{c}Roberts, 2018), the germination percentage of these pathogens were found to be lower, however, it does not affect the infection process in the host.

The volume of the sporangia deposited onto the leaf disks were relatively same for both pathogens. Observation from the disks showed they had a lower germination rate, and in few others, there were no germination observed. On leaf disks, *Pe. somniferi* sporangia again produced longer germ tubes than *Pe. meconopsidis*. The maximal germ tube length attained by the pathogen provides the greater chance to take advantage of the host cell to establish its infection (Gómez and Filgueira-Duarte, 2012). The formation of dual germ tubes in course of time was observed, which has also reported in other downy mildews, such as *Bremia lactucae* in lettuce and *Pe. tabacina* in downy mildew of tobacco (Reuveni et al., 1986; Sargent, 1981). The divided germ tubes formed attempted to enter more than one site of the host tissue.

Following sporangial germination and production of a germ tube is the development of an appressorium to facilitate host penetration. Following penetration, the pathogen forms a haustorium within an infected cell as a means of deriving nutrition and suppressing host defence (Hahn and Mendgen, 2001; O'Connell and Panstruga, 2006; Borrás-Hidalgo et al., 2010). Further invasion may occur via intercellular hyphae as the pathogen grows within the infected plant. These infective structures (germ tube, appressorium, haustoria and intercellular mycelial) were observed for both pathogens during host invasion which corresponds with earlier studies on Peronosporaceous fungi (Channon, 1981; Michelmore,

1981; Tommerup, 1981; Gómez and Filgueira-Duarte, 2012; Cohen et al.,2017; Hoffmeister et al., 2020).

After infecting the leaf tissues, both pathogens colonized the mesophyll layer by intercellular mycelia, forming haustoria in the parenchyma cells leading to the formation of chlorotic symptoms, observed in *Pe. somniferi* and *Pe. meconopsidis*. This observation is in accordance with the earlier studies (Oerke et al., 2006; Calderón et al., 2014). Necrotic lesions which were observed in *Pe. meconopsidis* started to occur along the foliar veins in the later stages of the infection and started to expand in length. Similar symptomology was observed in the infection process with cucurbit downy mildew (Palti and Cohen, 1980; Lebeda and Cohen, 2010; Lebeda and Cohen, 2011). Abaxial sporulation was observed for both pathogens in the leaf mesophyll tissues. However, the *Pe. somniferi* spread was more expansive covering the whole leaf lamina, whereas in *Pe. meconopsidis* the spread and sporulation were localized.

As a general characteristic of obligate pathogens, they are relatively difficult to induce successful infections in their hosts under controlled condition. This has been found with most groups working with these pathosystems. The pathogens remain asymptomatic and hence the visual symptoms produced in this method were lower. The difference in the symptom expression of the two pathogens may be due to their variation in their infective ability, which needs a further understanding at molecular level. This could be achieved by the genome sequencing and assembly for these two important pathogens, as reported in other downy mildew pathogens (Derevnina et al., 2018; Klein et al., 2020; Natesan et al., 2020).

The production of oospores was observed for both pathogens; with, the mean numbers of oospores formed was greater in *Pe. somniferi* than in *Pe. meconopsidis*. These oospores formed in the leaves, can serve as primary inoculum sources when they reach the soil within plant debris, as has been reported in *Pe. arborescens* (Montes-Borrego et al., 2009) and well documented in other *Peronospora* spp. (Savory et al., 2011; Gómez and Filgueira-Duarte, 2012). Due to the insufficient evidence to support the mating system of these pathogens, the study remains inconclusive and a further work is needed.

In our study, *Pe. meconopsidis* induced more hydrogen peroxide in infected leaves than *Pe. somniferi*. The release of these molecules was first observed from 6 HAI. This represents recognition of pathogen effector molecules by the host to initiate a defence reaction

(Kamoun, 2009; Rezzonico et al., 2017). This was explained as the biphasic oxidative burst which are linked primarily to disease resistance that is naturally related with host–pathogen interactions (Grant et al., 2000).

Hydrogen peroxide is a reactive oxygen species produced by the host as part of the host plants pathogen defence processes that may result in programmed cell death (apoptosis) (Levine et al., 1994; Dangl et al., 1996; Hammond-Kosack and Jones, 1996). Expression of localised necrotic lesions by *Pe. meconopsidis* likely reflects this enhanced defence reaction of the host and provides an explanation for its relative limitation in invasive capacity. Conversely, *Pe. somniferi* spread leads to profuse sporulation in absence of localised lesions, suppressing the plant defence mechanism. A virulent pathogen produces a lower defence response than an avirulent or less virulent pathogen (Crute and Norwood, 1981; Crute et al., 1994).

The localisation of hydrogen peroxide within infected tissues was demonstrated by DAB staining which confirmed greater localised production in *Pe. meconopsidis* than *Pe. somniferi* infected leaves. This is the first study to observe the localisation of hydrogen peroxide in plants following infection with these poppy downy mildew pathogens. There is a need for further understanding of the signalling mechanisms, identification of pathogen effectors and host receptors as well as the involvement of signalling pathways important for the defence mechanism.

Results of this study indicate that both the leaf disk assay and whole plant inoculation methods were useful to understand and compare the infection process and symptom expression of the downy mildew pathogens. The leaf disk assay was suitable in monitoring infection process with over time as observed in other downy mildews (Gómez and Filgueira-Duarte, 2012). The results observed in this study showed, *Pe. somniferi* is more aggressive than *Pe. meconopsidis*. While anecdotal comparison of pathogenicity has been previously reported (Montes-Borrego et al., 2017), this represents the first direct comparison of pathogenicity of the two pathogens in the same host. This aspect deserves extra attention with transcriptomic studies to understand the expression of the defence genes in host-pathogen interactions at the molecular level as has been reported with other downy mildews (Garg et al., 2011; Savory et al., 2012; Kulkarni et al., 2016; Wallace and Quesada-Ocampo, 2017).

This disease is an important constraint for poppy growers in Australia and worldwide and an appropriate disease management strategy would control the severe outbreak of the disease in future. Understanding the differences between the two pathogens may assist in developing models to predict disease and control strategies to combat infection. For example, further elucidation of the defence responses associated with *Pe. meconopsidis* infection may enable selection of varieties that can more effectively recognise and restrict *Pe. somniferi* infections.

Chapter 5: Host range of *Peronospora somniferi* and *Pe. meconopsidis* within selected members of the *Papaveraceae* under controlled conditions

5.1 Abstract

Opium poppy, belonging to the family *Papaveraceae*, is grown for its alkaloid compounds of pharmaceutical value. Downy mildew caused by *Peronospora somniferi* and *Pe. meconopsidis*, substantially impacts crop production. The present study was conducted to identify the experimental host range of *Pe. somniferi* and *Pe. meconopsidis* within selected weed and ornamental members of the *Papaveraceae* family. Nine *Papaver* spp*., Meconopsis cambrica* and a non-host control (tomato, *Solanum lycopersicum*) were challenged with both pathogens under controlled glasshouse or laboratory conditions using infested soil or foliar applied sporangia as inocula. *Peronospora somniferi* and *Pe. meconopsidis* induced disease symptoms, including sporulation, in at least one trial for all tested species except for *Pa. atlanticum* and tomato. Species-specific PCR testing of foliage of challenged plants confirmed infections by both pathogen species of symptomatic plants, identifying these as hosts. Positive PCR tests were also obtained from *Pa. atlanticum* plants for both pathogens. However, in the absence of pathogen sporulation structures as further evidence of infection, the host status of *Pa. atlanticum* remains inconclusive. Testing of seeds collected from *Pe. somniferi* and *Pe. meconopsidis* infected plants of *Pa. somniferum, Pa. dubium, Pa. rhoeas and Pa. nudicaule* showed the presence of both pathogens, indicating likely ability for seed transmission in these species. We identified new hosts of these pathogens and discuss potential implications of these alternative hosts in pathogen survival, dissemination and epidemic initiation.

Key words: Alternative hosts, soil transmission, seed infestation.

5.2 Introduction

Opium poppy (*Papaver somniferum*) is an important crop grown for its pharmaceutically important alkaloids including morphine, codeine and thebaine (Fist and Chung, 2011; Kapoor, 1995; Landa et al., 2007). Opium poppy belongs to the family Papaveraceae which consists of 42 genera and 775 species (Christenhusz and Byng, 2016). They include annuals, perennials, and biennials which grow under various climatic conditions across Asia, Northern Africa and Europe (Carolan et al., 2006).

Downy mildew of opium poppy is a major concern for poppy production worldwide (Kapoor, 1995; Landa et al., 2007; Scott et al., 2004). In Australia, a localized necrotic form of the disease caused by infection with *Pe. meconopsidis* has affected crops since its first report in 1996 (Cotterill and Pascoe, 1998; Thangavel et al., 2017; Voglmayr et al., 2014). Since 2013, a second form of downy mildew has been found resulting from infection with *Pe. somniferi* that induces systemic symptoms distinct from the former and results in greater crop damage (Thangavel et al., 2017; Voglmayr et al., 2014). Systemic infections are characterized by stunted growth of the poppy plants with chlorotic leaves associated with profuse sporulation in the abaxial leaf surface (Thangavel et al., 2017).

Alternative hosts of biotrophic plant pathogens can play a major role as pathogen reservoirs between cropping seasons. They also provide inocula to initiate epidemics in new opium poppy crops. Reports of *Papaver* spp. infected by opium downy mildew exist, as *Pe. arborescens* or *Pe. cristata* (Landa et al., 2005; Scott et al., 2004). However, in light of the taxonomic revisions of the *Peronospora* genus the relevance of these reports is now in question. In defining *Pe. meconopsidis* and *Pe. somniferi*, Voglmayr et al (2014) noted that they were only associated with the hosts *Meconopsidis cambrica, Pa. pavonium* and *Pa. somniferum,* and with *Pa. somniferum*, respectively. Anecdotal evidence from the Australian poppy industry suggests that the host range of these species may be wider. This is of concern, as in addition to commercial plantings of opium poppy, there are several ornamentals and weed *Papaver* spp. that are present in Australia that could act as pathogen reservoirs. For example, the weed poppies commonly found in or near poppy crops include *Pa. dubium, Pa. rhoeas, Pa. argemone* and *Pa. hybridum* (Cotterill and Pascoe, 1998). *Papaver nudicaule* is also recorded as a host of *Pe. arborescens* in Italy (Garibaldi et al., 2003), although it is unclear which *Peronospora* species this refers to following taxonomic revisions (Voglmayr et al.,

2014). Here we test the hypothesis that the host ranges of both *Pe. meconopsidis* and *Pe. somniferi* are larger than currently recorded. We present results of testing a range of weed and ornamental species for their ability to host both opium poppy downy mildew pathogens, express disease and test the capacity of a selection of these hosts to carry these pathogens on their seed.

5.3 Materials and methods

5.3.1 Plant material

Nine *Papaver* species, the closely related *Meconopsis cambrica* and a non-host control (tomato – *Solanum lycopersicum*) were used for this study. Opium poppy (*Papaver somniferum*; seed lot SL-21) was used as a known host control. Seed of the weed species *Pa. dubium* was collected from plants growing near commercial poppy fields. The species identity of the *Pa. dubium* seed was confirmed by PCR amplification and sequencing of the ITS region, following the protocols of Carolan et al (2006). Seeds for ornamental poppies (*Pa. nudicaule, Pa. orientale, Pa. commutatum, Pa. laciniatum, Pa. atlanticum* and *M. cambrica*), were purchased from Frogmore Gardens, Victoria, Australia. Seeds for *Pa. rhoeas, Pa. paeoniflorum* and the tomato non-host were purchased from Southern Harvest, Tasmania. Collected seeds were stored at room temperature in the dark before use.

Prior to planting, all seeds were surface sterilized by soaking in a hypochlorite solution (2% available chlorine) for 5 minutes (Thangavel et al., 2020). Seeds were then rinsed twice with sterile water and air-dried within a laminar flow. Surface sterilized seeds were stored in the dark at room temperature until use. Subsamples of each seed lot were confirmed free of the two poppy downy mildew pathogens by species-specific PCR testing as described below.

5.3.2 Source and maintenance of downy mildew isolates

Inocula of the two pathogens were collected as infected foliage with pathogen sporulation from commercial poppy crops in Tasmania (Avoca - 41.796989” S, 147.708546” E; Bothwell - 42.369776” S, 147.034354” E; and Cressy - 41.68689” S, 147.07247” E). Foliage showing differential symptoms (systemic mildew or localized necrotic lesions) were used to establish independent cultures of each species. Leaves were stored in humid conditions for 24 hours to promote sporulation. Sporangia were brushed into 20 ml of sterile water, and sporangial suspensions of each pathogen separately sprayed onto leaves of 4-week-old opium poppy plants (five plants per pot per pathogen species) and covered in plastic bags for 48 hrs.

Following inoculation, plants were maintained in separate growth chambers at 20 ˚C Day at relative humidity (RH) 50% and 15 ˚C night temperature with RH 50 % at 16h day/8h night cycle, commencing in the dark cycle. Confirmation of species identity of the two pathogen maintenance cultures was provided by species-specific PCR (Thangavel et al., 2018) as detailed below, sequencing of the amplicons and comparison with sequences on the GenBank nucleotide database (NCBI; https://www.ncbi.nlm.nih.gov/BLAST/). Freshly produced spores were collected from infected leaves as previously described every two weeks and inoculated onto fresh plants for inoculum maintenance.

The previous studies conducted in our lab showed that the field soil had the presence of both pathogens, tested by PCR (unpublished data). Hence, to obtain pathogen-infested soils, leaves from freshly sporulating inoculum maintenance plants were collected and air-dried at room temperature. The dried leaves (*c.*100 leaves of each pathogen) were ground using a coffee grinder for 10 seconds (Model no: BCG200BSS, Breville, Australia). Samples (10 mg) of the ground leaf samples were mixed in 1 ml of sterile distilled water and stained with aniline blue and then examined under the microscope to confirm presence of oospores. Soil which had no prior history of poppy cultivation was sourced from the University Farm in Cambridge, Tasmania and autoclaved. The ground leaf samples of each pathogen were then mixed with 5 L of autoclaved soil and used as a soil-borne inoculum source for subsequent challenge experiments. This infested soil was stored in the dark at 4 ˚C until further use, for a maximum of 3 months. The presence of each specific pathogen within the infested soils was confirmed by species-specific PCR assay as described below.

For the sporangial inoculation experiments, inocula of each pathogen was prepared by brushing sporangia from freshly sporulating plants into a sterile Petri dish containing 20 ml sterile deionized water. Sporangial counts were made using a haemocytometer and sporangial concentrations adjusted to 5 x 10^5 spores/ml in sterile water. Prior to inoculation, the viability of sporangia was tested by plating 15 µl of sporangial suspension on 1 cm^2 2% water agar blocks followed by incubation at 20 ˚C for 2 hrs. The agar blocks were examined using a light microscope (Leica DFC 420, Germany) at 400X magnification for evidence of sporangial germination and germ tube formation. Positive germination was recorded when the length of the germ tube was twice the diameter of the sporangia (Figure 5.1). Following

confirmation of sporangial germination, the inocula was used for inoculation of experimental plants or detached leaves.

5.3.3 PCR testing for specific detection of the two poppy downy mildew species

Seed, leaf and soil samples were tested for the presence of *Pe. somniferi* and *Pe. meconopsidis* using species-specific PCR. Seeds (20 mg) or leaf materials (50 mg) were homogenized using a bead beater (Fast Prep-24, MP Biomedicals, USA) and DNA extracted using the Power Plant Pro DNA isolation kit following the manufacturer's protocols (Mo BIO Laboratories, Australia). For soil samples, 5 g of soil was subsampled from each lot, ground using a coffee grinder for 10 seconds (Model no: BCG200BSS, Breville, Australia), and sieved firstly through a 50 µm and then a 30 µm sieve (Greeburn laboratory sieves, Greer and Ashburner Pty Ltd, Australia), with 50 mg of the fine ground material used for DNA extraction using the Power Soil Pro DNA isolation kit (Mo Bio Laboratories) according to the manufacturer's protocols. DNA extracts were then subjected to PCR amplification following the methodologies described by Thangavel et al (2018). In brief, PCR amplifications were done with a 20 µl reaction mix containing 10 µl of Hot Start Taq Master Mix (QIAGEN, Australia), 0.75 µl of 10 µM forward and reverse primers (for *Pe. meconopsidis*: COX 2-PMF: CCGTGGTACATGGTGCTACTATC; COX 2-PMR: ATTGTCCATAAAAAACACCCTCT and for *Pe. somniferi:* COX 1 - PSF: ATCAGCCATTGTTGAATCAGGT; COX 1-PSR: CAAACAAATAAAGGTAATCTATGGAAG), 1.5 µl of 5 ng of DNA template, 2 µl of Coral Load dye (QIAGEN) and 5 µl of sterile water on a Mastercycler Gradient (Eppendorf, Germany) with a thermocycle of 95 °C for 15 minutes, followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, and a final extension at 72 °C for 10 minutes. PCR products (6 µl) were separated by electrophoresis in a 1 % agarose gel in Tris-acetate-EDTA buffer (pH 8.0) containing 0.1 % SYBR Safe™ DNA Gel Stain (Life Technologies) and visualized under blue light. The *P. somniferi* (COX 1-PSF, COX 1-PSR) and *P. meconopsidis* (COX 2-PMF, COX 2-PMR) amplified DNA fragments were 217 bp and 441 bp, respectively.

5.3.4 Infection from soilborne inoculum

Pots (27 x 24 x 18 cm^3) were ¾ filled with potting mix containing sand, peat, and composted pine bark (10:10:80, pH 6.0) premixed with Osmocote 16-3.5-10 NPK resin coated fertilizer (Scotts Australia Pty Ltd.). This was overlayed with a 10 cm layer of infested soil confirmed to

contain either *Pe. meconopsidis* or *Pe. somniferi* inoculum by PCR testing. Pots with no added infested soil were used as a negative control. Seed of each test species were sown into pots (two replicated pots per species, ten plants per pot for each inoculum source) and pots were placed in a glasshouse maintained at 17-20 ˚C with relative humidity 70-75 % monitored using Tiny Tag Plus 2 data loggers (Hastings data loggers, UK). Pots were hand watered daily to maintain soil moisture. Plants were observed weekly for 12 weeks following emergence for the symptoms of downy mildew infection. After 12 weeks, one leaf was collected from each plant and each leaf was individually tested for the presence of both pathogen species by PCR as previously described. Plants were grown to maturity. Any seed capsules that formed on plants that had been shown to be infected by PCR testing were harvested, air-dried at room temperature for a week and then the seeds carefully removed. Seeds from each species for each pathogen were pooled and a 20 mg sub-sample were used for the DNA extraction and tested for the presence of the respective pathogen by PCR as previously described. This experiment was repeated twice (giving a total of 40 plants per test species challenged with each pathogen). Experiments was included as a predictor in logistic regression (using R v.3.5; R Core Team, 2015) for each response variable (sporulation, necrosis, chlorosis and PCR testing) to test whether the experiments could be combined.

5.3.5 Infection by sporangial inoculum

Nine pots for each test species, with each pot containing ten plants, were grown in potting mix without infested soil under glasshouse conditions as previously described. At 4-6 weeks post-emergence, all foliage on the test plants were spray inoculated twice at 3-day intervals with 8-10 ml of sporangial suspensions (5 x 10^5 spores/ml) of *Pe. meconopsidis*, *Pe. somniferi* or water only as an uninoculated control (three pots of each test species with each spray treatment). Plants were covered in plastic bags for 48 hrs following each inoculation. Treatments were arranged in a completely randomized design within the glasshouse with growth conditions as described previously. Plants were hand-watered daily. The number of plants showing downy mildew symptoms (chlorosis and/or local angular necrotic lesions) and numbers of individual leaves showing sporulation were recorded 8 days post-inoculation. One leaf was collected from each plant, the ten leaves collected from each individual pot combined and then tested as a bulked sample for both pathogens by PCR as previously described. The experiment was repeated three times (giving a total of 90 plants per test

species challenged with each pathogen). Experiment was included as a predictor in logistic regression (using R) for each response variable to test whether the experiments could be combined.

5.3.6 Pathogen sporulation rate on detached leaves

Nine leaves were detached from 4-week-old plants grown under glasshouse conditions of each host species and placed on a sterile filter paper moistened with sterile deionized water in a Petri plate. Each leaf was then inoculated with a 15 µl volume of sporangial suspensions (5 x 10^5 spores/ml) of either *Pe. somniferi* or *Pe. meconopsidis* or water only as an uninoculated control (three leaves of each test species challenged with treatment). The Petri plates were then sealed and incubated in a plant growth chamber (Model: e1200, Steridium Pty Ltd, Brisbane, Australia) at 20 ˚C for 14 hrs and 14 ˚C for 10 hrs in day-night regime at RH 90 %. The leaves were observed 7 days post-inoculation and sporulation scored using the following scale: 0 - no sporulation, 1 – weak sporulation, 2 - moderate sporulation, 3 - dense sporulation (Cohen et al., 2013). The experiment was repeated three times (giving a total of nine leaves per test species challenged with each pathogen). Differences in sporulation rate between host plants were tested by ordinal logistic regression using R. Experiment was included as a predictor. There were no significant interaction involving Experiment (P >= 0.70), thus Experiment was excluded from the final model. Post hoc comparisons were conducted using the emmeans v1.2.3 (Lenth, 2016) using a Tukey correction for pairwise comparisons.

5.4 Results

5.4.1 Infection from soilborne inoculum

The PCR results of the infested soil inoculum showed only positive to the respective pathogens. In the two soil inoculation experiments *Pe. somniferi* and *Pe. meconopsidis* symptom expression and pathogen incidence varied among the ten test species. There were no symptoms observed and no pathogen detected by PCR on any uninoculated plants in either of the experiments. In all cases, the interaction terms of fitted models incorporating Experiment were non-significant (P > 0.05), and thus results across the two experiments were pooled for reporting (Table 5.1).

No symptoms or PCR detections of either pathogen were found in test plants grown in un-infested soil. In pots with soil infested with *Pe. somniferi*, foliar chlorosis was observed in *Pa. somniferum* (8 plants out of 40), *Pa. paeoniflorum* and *Pa. dubium* (6), *P. nudicaule, Pa.*

laciniatum (4), *Pa. commutatum* (1) and *M. cambrica* (1) (Table 5.1). Sporulation was observed on all species except *Pa. atlanticum*. The species with high numbers of plants exhibiting sporulation were *Pa*. *somniferum* (16 plants), followed by *Pa. laciniatum* (11), *Pa. paeoniflorum* (10), and *Pa. nudicaule* (8) (Table 5.1). PCR testing of seedling foliage indicated the presence of *Pe. somniferi* in all species, except the non-host control, and the absence of *Pe. meconopsidis. Pa. somniferum* showed the highest number of plants with PCR positives (21 plants out of 40) followed by *Pa. laciniatum* (13), *Pa. nudicaule and Pa. dubium* (11) and the least PCR positives was found in *Pa. atlanticum* (6).

In pots with soil infested with *Pe. meconopsidis,* only four species, *M. cambrica* (8 plants), *Pa. laciniatum* (3) *Pa. somniferum* and *Pa. dubium* (2), produced symptoms of infection (necrotic leaf lesions) (Table 5.1). The greatest number of sporulating plants observed were found in *M. cambrica* (12 plants), followed by *Pa. somniferum* (8), *Pa. nudicaule* (2), *Pa. dubium* and *Pa. laciniatum* (1). In addition to these species, the presence of *P. meconopsidis* was also detected in *Pa. orientale*, *Pa. rhoeas*, *Pa. commutatum*, *Pa. paeoniflorum* and *Pa. atlanticum* by PCR testing, suggesting asymptomatic infections (Table 5.1). No PCR detections of *Pe. somniferi* were found from any plants grown in *Pe. meconopsidis* infested soil.

Seeds were collected from plants infected by *Pe. somniferi* or *Pe. meconopsidis* from *Pa. somniferum, Pa. nudicaule, Pa. dubium* and *Pa. laciniatum.* PCR testing of harvested seeds showed positive seed infestation by their respective pathogen in all seed samples (Table 5.1).

Table 5.1. Response of Papaveraceae species to inoculation with *Peronospora somniferi* and *Pe. meconopsidis* from infested soil inoculum sources.

Species	*Pe. somniferi*				*Pe. meconopsidis*			
	No. plants with chlorotic lesions [a]	**No. plants with downy mildew sporulation [a]**	**No. plants PCR positive [b]**	**PCR positive seeds [c]**	**No. plants with necrotic lesions [a]**	**No. plants with downy mildew sporulation [a]**	**No. plants PCR positive [b]**	**PCR positive seeds [c]**
Papaver. somniferum	8 (20)	16 (40)	21 (52.5)	++	2 (5)	8 (20)	13 (32.5)	++
Pa. nudicaule	4 (10)	8 (20)	11 (27.5)	++	0 (0)	2 (5)	5 (12.5)	++
Pa. orientale	2 (5)	6 (15)	7 (17.5)	NA	0 (0)	0 (0)	6 (15)	NA
Pa. rhoeas	3 (7.5)	4 (10)	9 (22.5)	NA	0 (0)	0 (0)	1 (2.5)	NA
Pa. paeoniflorum	6 (15)	10 (25)	12 (30)	NA	0 (0)	0 (0)	2 (5)	NA
Pa. dubium	6 (15)	7 (17.5)	11 (27.5)	++	2 (5)	1 (2.5)	7 (17.5)	++
Pa. commutatum	1 (2.5)	7 (17.5)	10 (25)	NA	0 (0)	0 (0)	6 (15)	NA
Pa. laciniatum	4 (10)	11 (27.5)	13 (32.5)	++	3 (7.5)	1 (2.5)	6 (15)	++
Pa. atlanticum	0 (0)	0 (0)	6 (15)	NA	0 (0)	0 (0)	1 (2.5)	NA
Meconopsis cambrica	1 (2.5)	3 (7.5)	8 (20)	NA	8 (20)	12 (30)	10 (25)	NA
Solanum lycopersicum	0 (0)	0 (0)	0 (0)	NA	0 (0)	0 (0)	0 (0)	NA

[a] The number of symptomatic plants out of 40 inoculated plants summed across two independent experiments. Plants were assessed from 4-12-weeks for presence of downy mildew sporulation. Number in brackets is the derived % value.

[b] The number of 12-week old plants testing positive by pathogen species-specific PCR out of 40 inoculated plants. Number in brackets is the derived % value.

[c] Seeds collected from available capsules of the infected test species were tested for the presence of the pathogens by PCR; ++ = positive reaction and NA = non-availability of seed capsules.

5.4.2 Infection by sporangial inoculum

Challenge with sporangial sprays of the two pathogens onto the foliage of the test plants again resulted in varied responses between species. In all cases the interaction terms of fitted models incorporating Experiment were non-significant ($P > 0.05$), and thus data from the three repeated experiments were pooled for reporting (Figures 5.2 and 5.3; Table 5.2).

No symptoms or PCR detections of either pathogen was found in test plants treated with water only. Following *Pe. somniferi* inoculation, chlorotic symptoms were observed in all species except *M. cambrica* and the non-host control, with the total number of symptomatic plants varying from 20 out of a total of 90 (*Pa. nudicaule*) to 5 (*Pa. orientale*). Sporulation was observed in all species except *Pa. atlanticum* and in the non-host control (Figure 5.2). PCR testing confirmed the presence of *Pe. somniferi* within bulked samples of all test species except the non-host control (Table 5.2). No plants tested positive for *Pe. meconopsidis*.

Following *Pe. meconopsidis* inoculation, polyangular necrotic lesions were observed in all species except *Pa. nudicaule*, *Pa. commutatum, Pa. atlanticum* and the non-host control (Figure 5.3). Sporulation was observed in all species except *Pa. paeoniflorum, Pa. atlanticum* and the non-host control (Figure 5.3; Table 5.2). PCR testing confirmed the presence of *Pe. meconopsidis* within bulked samples of all test species except the non-host control (Table 5.2). No plants tested positive for *Pe. somniferi*.

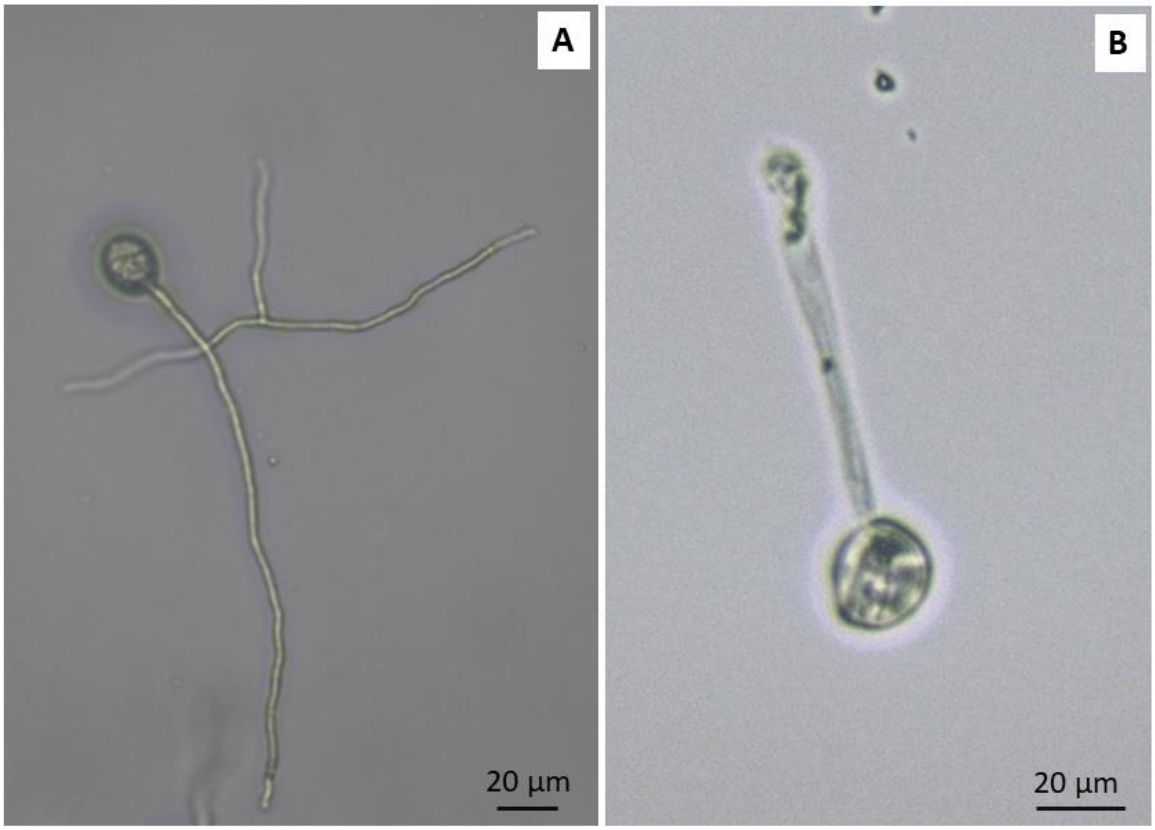

Figure 5.1. Germination of sporangia in 2% water agar at 2 hr. (a) *Pe. somniferi* (b) *Pe. meconopsidis*.

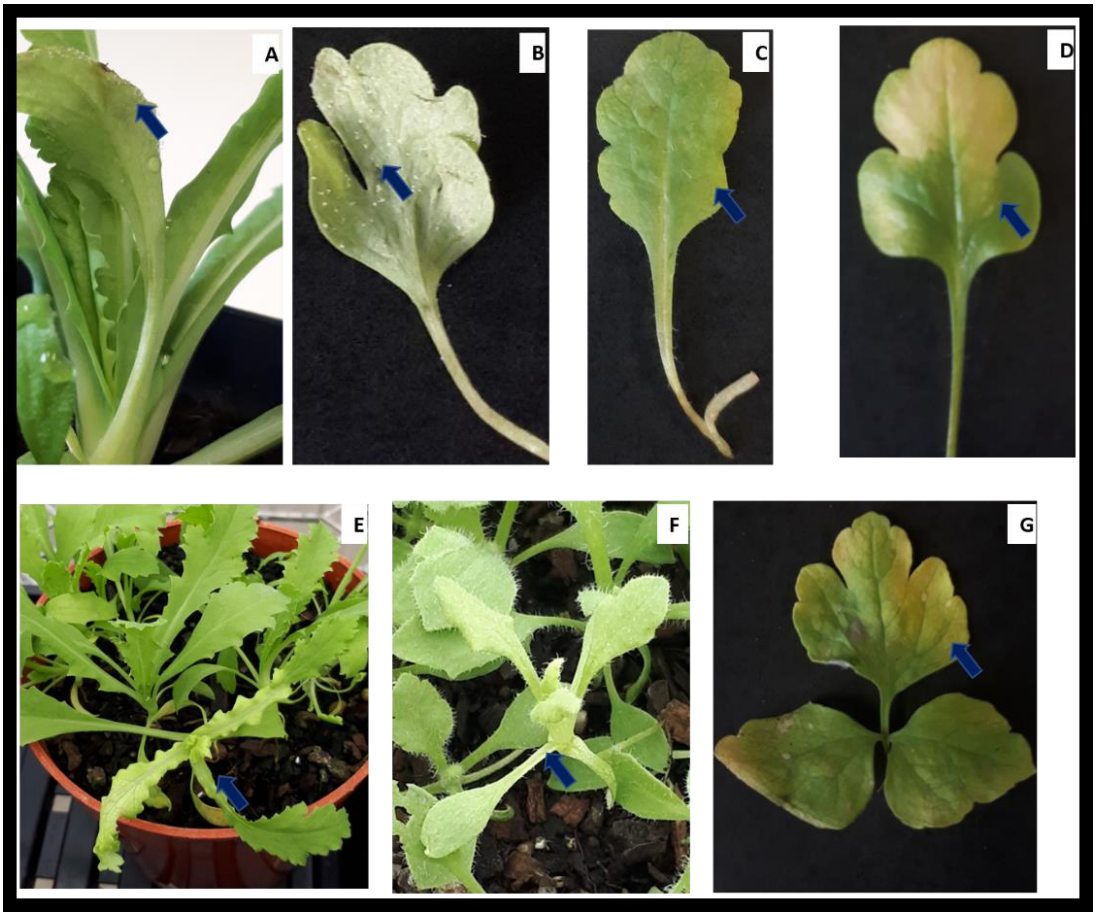

Figure 5.2. Downy mildew symptoms produced by *Peronospora somniferi* in Papaveraceae species. (a) *Papaver somniferum*, (b) *Pa. nudicaule*, (c) *Pa. rhoeas*, (d) *Pa*. *commutatum*, (e) *Pa. paeoniflorum*, (f) *Pa. laciniatum*, (g) *Meconopsis cambrica*. Sporulation shown in (a) and (b); (c -g) showing chlorosis.

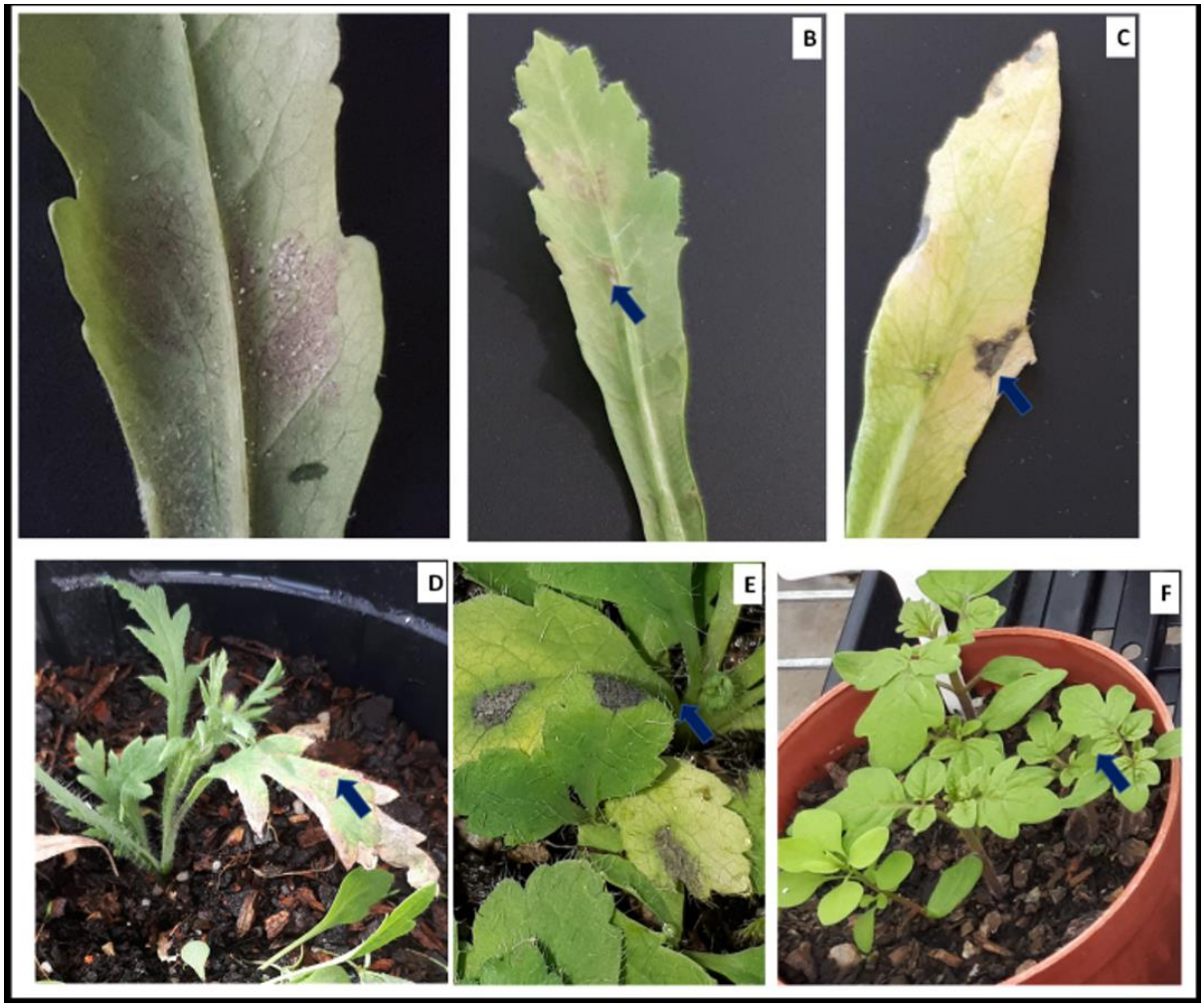

Figure 5.3. Downy mildew symptoms produced by *Peronospora meconopsidis* in *Papaveraceae* species. (a) *Papaver somniferum*, (b) *Pa. paeoniflorum*, (c) *Pa. laciniatum*, (d) *Pa. dubium*, (e) *Pa. rhoeas*, (f) *S. lycopersicum*. Localized sporulation shown in (a) and (b); (c-e) Showing the necrotic lesions; (f) non- host control with no infection.

Table 5.2. Response of Papaveraceae species to foliar inoculation of sporangial suspensions of *Peronospora somniferi* and *Pe. meconopsidis*.

Species	*Pe. somniferi*			*Pe. meconopsidis*		
	No. of plants with chlorosis [a]	**No. of plants with downy mildew sporulation** [a]	**No. of PCR positives from grouped testing** [b]	**No. of plants with necrotic lesions** [a]	**No. of plants with downy mildew sporulation** [a]	**No. of PCR positives from grouped testing** [b]
Papaver somniferum	12 (5; 13.3)	45 (9; 50)	9/9	20 (9; 22.2)	17 (9; 18.9)	9/9
Pa. nudicaule	20 (9; 22.2)	15 (8; 16.7)	7/9	0 (0; 0)	1 (1; 1.1)	5/9
Pa. orientale	5 (5; 5.6)	7 (6; 7.8)	5/9	6 (6; 6.7)	1 (1; 1.1)	4/9
Pa. rhoeas	12 (6; 13.3)	3 (3; 3.3)	3/9	11 (6; 12.2)	3 (3; 3.3)	3/9
Pa. paeoniflorum	8 (6; 8.9)	15 (7; 16.7)	5/9	9 (6; 10)	0 (0; 0)	3/9
Pa. dubium	3 (3; 3.3)	10 (7; 11.1)	8/9	6 (6; 6.7)	10 (8; 11.1)	5/9
Pa. commutatum	11 (6; 12.2)	16 (7; 17.8)	7/9	0 (0; 0)	2 (2; 2.2)	5/9
Pa. laciniatum	10 (7; 11.1)	18 (6; 20)	6/9	6 (3; 6.7)	6 (5; 6.7)	6/9
Pa. atlanticum	16 (7; 17.8)	0 (0; 0)	3/9	0 (0; 0)	0 (0; 0)	2/9
Meconopsis cambrica	0 (0; 0)	19 (8; 21.1)	8/9	3 (3. 3.3)	9 (6; 10)	7/9
Solanum lycopersicum	0 (0; 0)	0 (0; 0)	0/9	0 (0; 0)	0 (0; 0)	0/9

[a] The number of plants out of 90 (9 pots of 10 plants) exhibiting symptoms (chlorosis; *P. somniferi*, polyangular necrotic lesions; *P. meconopsidis*, and sporulation; both pathogens) assessed 8 days after inoculation (summed across three experiments). The numbers in brackets indicates the total number of pots (out of 9) observed to contain symptomatic; the derived % of total plants).

[b] Ten seedlings per pot were grouped and tested by species-specific PCR as a combined sample 8 days after inoculation.

5.4.3 Pathogen sporulation rate on detached leaves

No significant interaction involving Experiment (P >= 0.70) was found and thus Experiment had no significant effect on model predictions. Data from the three repeated experiments were therefore pooled for analysis and reporting (Table 5.3). Inoculation of detached leaves from the ten test species with sporangial suspensions produced sporulation for both pathogens on all species except *Pa. atlanticum* and the non-host control (Table. 5.3). The mean sporulation index varied between test species with the most profuse sporulation occurring on *Pa. somniferum* with inoculation of *Pe. somniferi* and *Pe. meconopsidis* (3.0), followed by *Pa. nudicaule* (3.0 and 1.0) and *Pa. paeoniflorum* (2.7 and 2.3). *Pa. commutatum* and *Pa. laciniatum* had a similar high sporulation index as *Pe. somniferi* (2.0) but varied in response to *Pe. meconopsidis* (1.3 and 2.7). *M. cambrica* and *Pa. dubium* had the same sporulation index score with *Pe. somniferi* (1.0) but differed with *Pe. meconopsidis* (2.0 and 1.0). Sporulation of the two pathogens has also found in *Pa. orientale* (1.7 and 1.0) and *Pa. rhoeas* (0.7 and 1.0), respectively.

Table 5.3. Sporulation index on detached leaves of *Papaveraceae* species following inoculation with sporangial suspensions of *Peronospora somniferi* and *Pe. meconopsidis*.

Species	Mean sporulation index (0-3) [a]	
	Pe. somniferi	***Pe. meconopsidis***
Papaver. somniferum	3.0 [d]	3.0 [e]
Pa. nudicaule	3.0 [d]	1.0 [b]
Pa. orientale	1.7 [bc]	1.0 [b]
Pa. rhoeas	0.7 [ab]	1.0 [b]
Pa. paeoniflorum	2.7 [cd]	2.3 [cde]
Pa. dubium	1.0 [b]	1.0 [b]
Pa. commutatum	2.0 [b]	1.3 [bc]
Pa. laciniatum	2.0 [c]	2.7 [de]
Pa. atlanticum	0.0 [a]	0.0 [a]
Meconopsis cambrica	1.0 [b]	2.0 [cd]
Solanum lycopersicum	0.0 [a]	0.0 [a]
Probability [b]	***<0.001***	***<0.001***

[a] Detached leaves from the test plants were inoculated with 15 µl of sporangial suspensions (5 x 10^5 spores/ml) of the two pathogens. A sporulation index (0 - no sporulation, 1 – weak sporulation, 2 - moderate sporulation, 3 – dense sporulation) was calculated (Cohen et al., 2013).

[b] Treatment differences were analysed via ordinal logistic regression. The mean values of sporulation index in the individual experiment were recorded and mean within the same column followed by different letters are significantly different ($P < 0.05$) according to Tukey's correction for multiple pairwise comparisons.

5.5 Discussion

Downy mildew diseases affect many horticultural crops and weed species (Choi et al., 2009; Crandall et al., 2018; García-Blázquez et al., 2008; Thines and Choi, 2016). *Peronospora* spp. are obligate biotrophs that require a susceptible host plant to proliferate. During the last decade, many emerging *Peronosora* species have been reported. For example, since 2014 *Peronospora somniferi* (infecting opium poppy; Vogylmayr et al., 2014), *Peronospora aquilegiicola* (*Aquillegia* spp. and *Semiaquiliegia* spp; Thines et al., 2019) and *Peronospora monardae* (*Monarda didyma* and *Agastache mexicana*; Salgado-Salazar et al., 2020) have been described as new species in the genus. As noted in the review by Thines and Choi (2016), downy mildews, with a few exceptions, generally have narrow host ranges. Thus, Thines and Choi (2016) suggest that emerging downy mildew pathogens on new hosts be treated as separate species until data contradictory to this assumption is obtained.

The opium poppy downy mildew pathogens *Pe. meconopsidis* and *Pe. somniferi* have few reported hosts; *Pe. meconopsidis* on *Pa. somniferum, M. cambrica* and *Pa. pavonium* and *Pe. somniferi* on *Pa. somniferum* (Voglmayr et al., 2014). However, there are reports of infections of various *Papaver* spp. by *Pe. arborescens* or *Pe. cristata*, previously thought to be causal agents of poppy downy mildew. In this study, all weed poppy and ornamental poppy species except *Pa. atlanticum* were confirmed to be experimental hosts of both *Pe. somniferi* and *Pe. meconopsidis* by the presence of disease symptoms, pathogen sporulation and species-specific PCR testing in at least one of the sets of experiments. Both pathogens were detected in inoculated plants of *Pa. atlanticum* by PCR, but we regard this as a tentative host until further confirmation of infection is obtained. Rates of infection and expression of disease symptoms varied between tested genotypes.

Previous records show ornamental varieties including *Pa. rhoeas, Pa. nudicaule, Pa. orientale, Pa. pavoninum, Pa. setigerum* were hosts of *Pe. arborescens* (Thines and Choi, 2016). Following the revisions to the taxonomy of the *Peronospora,* Voglmayr et al (2014) suggested that the redefined *Pe. arborescens* was limited to *Pa. rhoeas*. Conversely, *Pa. pavoninum* was considered a host of *P. meconopsidis* only. No *Peronospora* spp. pathogens of *Pa. orientale*, *Pa. nudicaule* or *Pa. orientale* were identified in the samples examined in that study. Here we suggest that the host range of *Peronospora* spp. infecting *Papaver* spp. is more complex with greater overlap than previously described. Based on the results obtained in this

study, we identified *Pa. dubium, Pa. nudicaule, Pa. rhoeas, Pa. paeoniflorum, Pa. laciniatum* and *Pa. orientale* as experimental hosts of both *Pe. somniferi* and *Pe. meconopsidis.*

The putative infections of *Pa. atlanticum* by both pathogens regardless of inoculum source and infections with *Pe. meconopsidis* of *Pa. orientale*, *Pa. rhoeas*, *Pa. paeoniflorum* and *Pa. commutatum* from soil-borne inoculum were asymptomatic. Similar asymptomatic infections have been reported for basil downy mildew (Farahani-Kofoet et al., 2012), spinach downy mildew (Kandel et al., 2019), and poppy downy mildew (Montes-Borrego et al., 2017; Thangavel et al., 2018). It is unclear whether asymptomatic systemic infections impact plant productivity, but they can have an impact on disease spread through association with the infested seed (Cohen and Sackston 1974). Considering the symptomless infection and the PCR testing of *Pa. atlanticum*, this could be a weak host for these pathogens, although results from this study are inconclusive.

We also detected *Pe. somniferi* and *Pe. meconopsidis* inoculum within seed collected from infected plants of *Pa. somniferum*, *Pa. dubium*, *Pa. nudicaule* and *Pa. lacinatum*. The presence of *Pe. somniferi* and *Pe. meconopsidis* in *Pa. somniferum* seed has been detected in Tasmania since 2017 and in the seed, lots tested from 1987 to 2016 respectively (Thangavel et al., 2018). However, seed-borne inoculum of downy mildew species in the other Papaver hosts tested here has not yet been reported. Association of these pathogens with seed from these species is of concern. The movement of ornamental poppy seed across the globe could be a potential means for the spread of downy mildew pathogens and strains into commercial opium poppy production regions. Testing of seed and seed treatments could be considered as one of the disease management strategies. (Thangavel et al., 2018; Umesha et al., 1998).

Furthermore, weed *Papaver* species in commercial poppy production are a significant concern for the industry due to plant competition and limitations in management strategies. The ability of such weeds to act as reservoirs for downy mildew inoculum further exacerbates this issue. Pathogen survival between cropping seasons can occur by association with their host's seed, persistence in the soil as dormant oospores or by passage to an alternative host species (Montes-Borrego et al., 2009; Scott et al., 2004; Thangavel et al., 2018). Alternative hosts can also be important in the initiation of new epidemics within crops through provision of external inocula (Dinoor, 1974). In poppy production, presence of weed poppy species in or near production areas is common. A range of ornamental poppy species are also commonly

grown in household gardens. If, as the results of this study suggest, these can act as pathogen reservoirs, then they may provide a green bridge for pathogen proliferation and could negate the benefits of crop rotation in reducing localized inoculum sources.

This study concludes that the pathogens causing downy mildew of poppy, *Pe. somniferi and Pe. meconopsidis,* were able to infect a moderately wide range of species within the Papaveraceae family. We acknowledge that the experiments reported in this study were all undertaken under controlled glasshouse and laboratory conditions and that infections in a natural ecosystem may not necessarily readily occur, as had been previously observed in studies with *Pseudoperonospora cubensis* (Runge and Thines, 2011). However, our study suggests pathogens infecting the ornamental varieties might be of concern for the effective disease management of poppy downy mildew. It is also known that plant species moved from their original habitat are exposed to additional pathogen challenges, which can lead to new diseases (Thines and Choi, 2016). This concept further needs attention and is of relevance to the opium poppy industry for their plant breeding programs for downy mildew resistance in crop varieties.

Chapter 6: General discussion and future prospects

The work presented in this thesis was designed to achieve the following objectives.

1. To characterise and compare the disease progression and symptom expression of *Pe. somniferi* and *Pe. meconopsidis* from infested soil inoculum sources.
2. To study the impact of herbicidal stress on downy mildew symptom expression.
3. To monitor and compare the infective processes of the two pathogens via sporangial infection.
4. To examine hydrogen peroxide quantity and its localisation in plants with response to pathogen infection.
5. To determine the host ranges of both *Pe. meconopsidis* and *Pe. somniferi* and test the capacity of these hosts to carry these pathogens on their seed.

The objectives of the thesis were achieved in the Chapter 3 (Objectives 1 and 2), Chapter 4 (Objectives 3 and 4), Chapter 5 (Objective 5).

6.1 Challenges faced during the research.

The greatest challenge of this research was maintaining the cultures of the two pathogens, *Pe. meconopsidis* and *Pe. somniferi*. This was because, the maintenance of obligate parasite cultures is both problematic and laborious. In relation to this research, two of the specific difficulties were faced: 1) mass producing inoculum; and 2) satisfying Koch's postulate experiments as both pathogens have morphological features, which are very similar. To overcome these challenges, we used a mist chamber (see Appendix I) to facilitate the pathogen inoculum bulking-up process and a species-specific PCR assay to confirm the presence and identity of each of the two pathogens (Thangavel et al., 2018).

6.2 Understanding the primary inoculum sources:

Spread of opium poppy downy mildew pathogens is known to occur via airborne sporangia (Scott et al., 2008) and contaminated seeds (Montes-Borrego, et al., 2011, Thangavel et al. 2018). While soil borne transmission has been demonstrated (Montes-Borrego et al., 2008), this was attributed to the *Pe. arborescens*, which is no longer considered a pathogen of opium poppy (Voglmayr et al., 2014). This study identified that *Pe. somniferi* and *Pe. meconopsidis* could move from soil borne inoculum sources into host plants. These results are in accordance with reports from downy mildew pathosystems, including floricolous,

quinoa, spinach and sage (Duduck et al., 2019; Ei-Assiuty et al., 2019; Kandel et al., 2019; Hoffmeister et al., 2020). Infection from the soil by *Pe. meconopsidis* was interesting as it only caused necrotic lesions on leaves. That it can spread from roots into leaves without inducing symptoms and the identification of the oospores produced are a novel finding of this study. However, when expressed, the difference in the downy mildew symptoms between *Pe. somniferi* and *Pe. meconopsidis* was noted in all the experiments conducted. While the current studies have significantly improved our knowledge of poppy downy mildew, considerable knowledge gaps regarding global population genetics, and management of this disease require further study.

Downy mildew infections caused by *Pe. somniferi* and *Pe. meconopsidis* may be symptomatic or asymptomatic, which was confirmed by species-specific PCR assays. This was also reported in other downy mildews such as basil, rose and spinach (Aegerter, et al., 2003, Gómez and Filgueira-Duarte 2012; Farahani-Kofoet etal., 2012; Kandel et al., 2019). Pathogen infection creates biotic stress in their host plants (Kamoun et al., 1999, Neill et al., 2002, Torres, 2006). However, this stress is not always sufficient to induce symptoms of disease. Abiotic stresses, such as those imposed by sublethal doses of herbicides, appear to exacerbate downy mildew symptoms in opium poppy. This was tested in the study conducted with herbicides applied to commercial poppy fields and found to increase the disease incidence. This is in accordance with studies conducted on sugar beet, sorghum, sunflower (Altman and Campbell, 1977; Craig, 1987; Covarelli and Tosi, 2006). A future study may be needed to identify the mechanism that makes the host susceptible to the pathogen. One of the important outcomes of this thesis is a careful consideration of selection and the dosage levels of the herbicides used and the time of application in the field. This information could help improve in the management of poppy downy mildew.

6.3 Infection process by the secondary inoculum

The infection process of the two pathogens were compared following sporangial infection. Histological and visual observations of two pathogens, confirmed their ability to establish infection in the host tissue. The pathogens spread the infection in the host tissue by the intercellular mycelium as previously reported in other downy mildews such as rose, sunflower and spinach (Gomez et al., 2012; Gascuel et al., 2015; Kandel et al., 2019). However, the spread of infection by *Pe. meconopsidis* is mitigated by host defence processes

and the infected tissues become necrotized. This is an important distinguishing feature between the infective events of the two poppy pathogens. From our results, the *Pe. somniferi* produced profuse sporulation and occupied the entire abaxial side of the leaf. Whereas, in *Pe. meconopsidis*, the sporulation is vein-delimited, angular, localized necrotic lesions with sparse sporulation on abaxial leaf surface. In this study, sporangial germination, infection and disease progression of the two pathogens was compared with *Pe. somniferi* having greater pathogenicity of the two, germinating and penetrating the host more rapidly, and stimulating a reduced defence response by the host.

Plants respond to pathogen attack by activating an inducible immune response including production of reactive oxygen species (ROS). Biotrophic pathogens activate the salicylic acid pathway (Thakur and Sohal 2013) on encountering the host which will lead to stimulation of hydrogen peroxide and other reactive oxygen compounds. A study was conducted to compare the production of hydrogen peroxide when plants were challenged by the two pathogens. This concluded that one of the pathogens, *Pe. meconopsidis* produced a higher level of the compound than the other. The results from the study showed only a small increase in the hydrogen peroxide level in the control relative to the pathogen inoculated leaves. These are in accordance with the studies reported (Patel et al., 2016; Ramezani et al., 2017). Hydrogen peroxide is a reactive oxygen species produced by the host as part of the host plants pathogen defence processes that may result in programmed cell death (apoptosis) (Levine et al., 1994; Dangl et al., 1996**;** Hammond-Kosack and Jones, 1996). Expression of localised necrotic lesions by *Pe. meconopsidis* likely reflects this enhanced defence reaction of the host and provides an explanation for its relative limitation in invasive capacity. The localisation of hydrogen peroxide confirmed by DAB staining was also higher in *Pe. meconopsidis* than *Pe. somniferi*. This type of host-pathogen interaction has been already reported in pearl millet downy mildew (Kumudhini et al., 2001). However, additional research is needed to further dissect variations in the host-pathogen interaction mechanisms of these two pathogens. Due to time constraints, the study only identified the first line of defence molecule, hydrogen peroxide and its localisation in the tissue by DAB staining technique. However, a further study to understand the pathways such as salicylic acid and Jasmonic acid pathway is warranted.

6.4 Host range and weed management strategies.

Based on the studies conducted on identifying the alternative hosts for the pathogens, the study concluded that these pathogens could infect many other weeds and ornamental Papaveraceae members. The studies conducted by Voglymayr et al. (2014) suggested that *Pe. meconopsidis* was only associated with *Meconopsis cambrica* and *Pa. somniferum,* whereas *Pe. somniferi* are associated only with *Pa. somniferum.* Here we identifed new hosts *Pa. dubium*, *Pa. nudicaule, Pa. rhoeas, Pa. paeoniflorum, Pa. laciniatum* and *Pa. orientale* for each of these downy mildew pathogens. Alternative hosts and the volunteer opium poppy plants may play an important role in carrying the disease from one season to the next (Dinoor, 1974). The weed poppies are grown in or near the commercial poppy production areas. For example, the weed *Pa. dubium* is commonly grown near the poppy growing regions, which could act as the reservoirs of both pathogens. The existence of these pathogens between the cropping seasons, could exist with the seeds, as dormant oospores in the soil or by mean of alternative host (Montes-Borrego et al., 2009; Scott et al., 2004; Thangavel et al., 2018). Therefore these act as a green bridge for the proliferation of the pathogens throughout the year. Our study also suggested the ornamental varieties might be of risk in the management of poppy downy mildew as their seeds could carry these pathogens. The evolution of new disease epidemics could arise due to the variations in the hosts within the closely related members of family.

6.5 Removal of regrown/volunteer crops during post harvesting.

The role of volunteer crops has found to act as the inoculum source in poppy downy mildew caused by *Pe. arborescens* (Cotterill and Pascoe, 1998). Surveys conducted in the poppy growing fields, after the harvest period noticed the presence of regrown poppies (data not shown). These crops were also shown to harbour systemic infection, with a profuse sporulation on the abaxial side of the leaves and were confirmed for the presence of *Pe. somniferi.* There were no symptoms of *Pe. meconopsidis* observed or detected in the regrown/volunteer crops. It is highly likely that the wet period during the winter and the early spring could enhance high moisture for a longer period in the field. This gives an insight that the pathogen overwinters from the soil borne or seed borne infections and survive throughout the year for spreading the infection. This is a critical factor should not be overlooked, and hence the growers are advised to remove all the plant debris from the field

after harvesting, to assist in managing primary inoculum in the region and in subsequent crops.

6.6 Conclusions and future research

The study demonstrated the capacity of both soil and foliar inoculum sources to lead to infection, suggested a role of herbicide stress in accelerating disease expression and identified alternative hosts within the Papaveraceae, responsible for the survival and the spread of both pathogens. The findings of this research could be used in improving the effectiveness of disease management. The project identified the infection process of both pathogens and explains a putative pathogen lifecycle. Based on our results, we can conclude that both pathogens can spread via asexual and sexual reproduction, as reported for other downy mildew pathogens such as *Hyaloperonospora parasitica, Peronospora tabacina, Pseudoperonospora cubensis, Peronospora effusa, Peronospora salviae-officinalis* (Donofrio and Delaney 2001; Borrás-Hidalgo et al., 2010; Savory et al., 2011; Kandel et al., 2019; Hoffmeister et al., 2020).

The main outcomes of this thesis could help in the development of disease management strategies. Soil borne oospores are identified as important primary inoculum sources for both poppy downy mildew species. Therefore, there is need for crop rotation in the field. Also, oospores in the soil may lead to population variation within the pathogen, which could cause a new disease epidemic. This concept has already been reported in spinach downy mildew (Corell et al., 2011; Kandel et al., 2019) Therefore, understanding the oospore viability, longevity and germination studies could assist in a long-term disease management. Our current study has not quantified the pathogen level in the experiments conducted. The quantification of the pathogens in the seed were already reported for these pathogens (Thangavel et al., 2018). However, an appropriate method needs to be developed to quantify the inoculum level in the growing area before the cropping season. The quantification of the pathogens would aim in a better understanding of the disease epidemiology and effective disease prediction and development of risk model. However, this might bring an issue with a larger sampling in the field and hence an accurate sampling method is needed. That weeds and ornamental poppy species also help carrying the pathogen between the seasons is a concern for poppy production. A proper weed control strategy and an effective seed treatment method are recommended to be employed.

Herbicide applications make the plants more vulnerable and its highly recommended to revaluate the need and the timing of herbicide application.

At present, the poppy industry in Tasmania, uses fungicides for the prevention of downy mildew, which may also lead to the development of fungicide resistant strains of the pathogens. There are reports associated with fungicide resistance observed in other downy mildew such as cucurbits, sunflower, basil, spinach caused by *Pseudoperonospora cubensis* (Urban and Lebeda, 2006; Molinero et al., 2008; Wyenandt et al., 2015; Kandel et al., 2019). However, there is no data available concerning the development of fungicide resistance reported in poppy downy mildew pathogens. Research efforts contributing to the development of sustainable management strategies, such as durable host resistance, are a priority to ensure the long-term viability of the economically important poppy industry.

Our study has demonstrated and compared some of the major events of the infection processes of the two pathogens within the poppy host. However, there are further aspects that deserve additional attention in future studies.

1. The taxonomic revisions of these downy mildew pathogens have redefined the species (Voglmayr et al., 2014). However, there is little research assessing the genetic or phenotypic variation within these species. Hence, there is a need for assessment of potential strain variation during every growing season of the crop.
2. The current study has not shown any data on how the alkaloid content of the crop is affected by the systemic mildew caused by the pathogens. It would be important to understand if the alkaloid content varies for the crops if infected by these pathogens (including those with apparent asymptomatic infections).
3. Oospores in the soil act as the primary source of inoculum for initiating the disease. However, the longevity of the oospores in the soil and the factors involved in the germination of oospores needs to be understood. For example, are there any phytochemical factors produced by the host, acting as germination stimulants to favour the oospore germination? In addition, application of biocontrol agents might reduce the oospore numbers and represent a possible control measure to reduce the disease severity as reported in impatiens downy mildew (Kloos, 2018).
4. An integrated approach of chemical and biological control could be suggested for an effective management of systemic downy mildew. Moreover, improved disease-resistant varieties are required as a more durable solution.

5. The recent advancements in the sequencing technologies such as next-generation sequencing may help in the identification of the differential expression of genes during the plant pathogenic interaction. For example, identifying the effector proteins released by these pathogens during infection and the defense genes expressed by the host such as ROS, will be useful. Understanding such plant-pathogen interactions is important for research interventions as it provides long-term management for minimizing the economic loss caused by the pathogens to the crop. This has been already reported for other downy mildew diseases on pearl millet, lettuce, cucurbits, hop, and spinach (Kulkarni et al., 2016; Stassen et al., 2012; Savory et al., 2012; Rahman et al., 2019; Kandel et al., 2020). Transcriptome analysis of pathogen interaction with the host is most warranted in poppy downy mildew infection.
6. The plants have evolved a complex network of immune complexes, when they undergo a specific or combination of stresses (Nejat et al., 2017). Recent studies demonstrate the production of ROS triggered by the pathogen interaction with the host. However, a future study could be focussed to understand the defence-related pathways such as salicylic acid, jasmonic acid, ethylene and abscisic acid, exhibited by the host in response to biotic and abiotic stress in poppy downy mildew.

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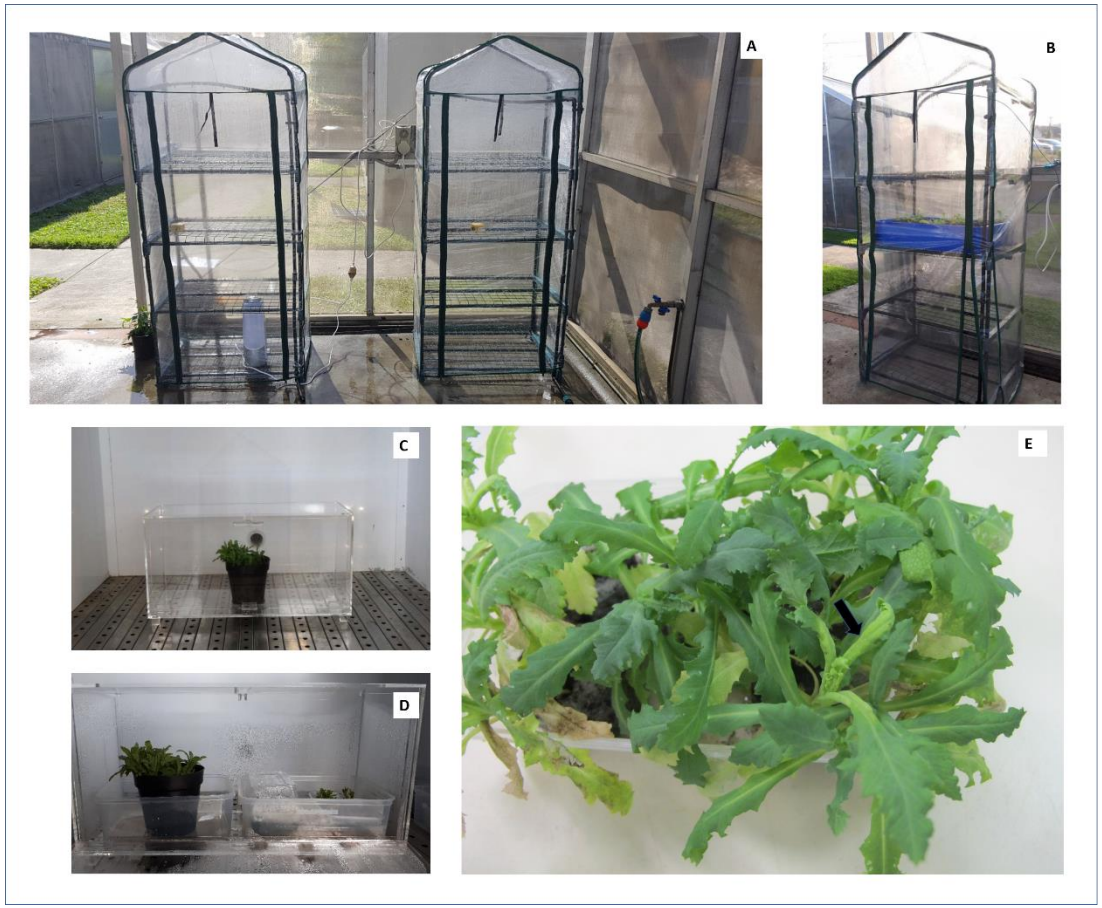
Appendix I: Mist house system used for poppy plant inoculation studies with the downy mildew pathogens:



A and B) Mist chamber used for mass production of inoculum sources, C and D) Customised mist chambers used in the growth chambers, E) Plants showing systemic infection, caused by Pe. somniferi.