Investigations into the infection biology, epidemiology and phylogenetics of sage downy mildew and its close relatives

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List of abbreviations

*cox*1: Cytochrome c oxidase subunit 1 gene

*cox*2: Cytochrome c oxidase subunit 2 gene

CRN: Crinkler proteins

CWDE: Cell wall degrading enzyme

*ef*1a: Elongation factor 1 alpha

EHM: Extrahaustorial membrane

EHMx: Extrahaustorial matrix

ER: Endoplasmatic reticulum

*hsp*90: Heat shock protein 90

ITS: Internal transcribed spacer

PAMP: Pathogen-associated molecular pattern

PRR: Pattern-recognition receptor

PTI: PAMP-triggered immunity

ROS: Reactive oxygen species

Abstract

Downy mildew of common sage (*Salvia officinalis*), caused by *Peronospora salviae-officinalis*, has become a serious problem in sage production worldwide. The causal agent of the disease belongs to the *Pe. belbahrii* species complex and was described as a species of its own in 2009. Nevertheless, very little is known about its infection biology and epidemiology. The aims of the current study were therefore to unravel the life cycle of this downy mildew and gain deeper insights into the epidemiology of the disease, as well as to clarify the species boundaries in the *Pe. belbahrii* species complex.

Infection studies showed that temperatures between 15 and 20 °C were most favourable for infection and disease progress. At 5 °C *Pe. salviae-officinalis* is still able to infect sage plants, but sporulation was only observed at higher temperatures. Furthermore, *Pe. salviae-officinalis* needs two events of leaf wetness or high humidity, a first one of at least three hours for conidial germination and penetration of the host, and a second one for sporulation. Additionally, contamination of sage seeds by *Pe. salviae-officinalis* was proven by seed washing and by PCR and DNA sequence comparisons, suggesting that infested seeds might play a major role in the fast spread of sage downy mildew, which is an important finding for phytosanitary or quarantine measures.

A protocol for fluorescence staining and confocal laser scanning microscopy was established and the whole life cycle of *Pe. salviae-officinalis* was tracked including oospore formation. The method was also used to examine samples of *Pe. lamii* on *Lamium purpureum* and *Pe. belbahrii* on *Ocimum basilicum* demonstrating the usefulness of this method for studying the infection process of downy mildews in general.

Peronospora species parasitizing *S. sclarea*, *S. pratensis*, *O. basilicum*, and *Plectranthus scutellarioides* were studied using light microscopy and molecular phylogenetic analyses based on six loci (ITS rDNA, *cox*1, *cox*2, *ef*1a, *hsp*90 and *β-tubulin*). The downy mildew on *S. pratensis* was shown to be distinct from *Pe. salviae-officinalis* and closely related to *Pe. glechomae*, and is herein described as a new taxon, *Peronospora salviae-pratensis*. The downy mildew on *S. sclarea* was found to be caused by *Peronospora salviae-officinalis*. The multi-gene phylogeny revealed that the causal agent of downy mildew on coleus is distinct from *Pe. belbahrii* on basil, and is herein described as a new taxon, *Pe. choii*.

Summary

With over 900 species spread throughout the world, *Salvia* (sage) is the largest genus of the Lamiaceae family. Several sage species provide antibacterial and antimicrobial activities and therefore are used as medicinal plants since ancient times. Furthermore, this aromatic herbs are applied as spices and flavouring agents in perfumery and cosmetics. Common sage, *Salvia officinalis*, is one of the economically most important sage species and has been used as medicinal plant since ancient times.

Over the past two decades several downy mildew diseases in medicinal plants and culinary herbs have been newly reported and led to economic losses. Prominent examples are *Peronospora somniferi* on opium poppy, *Pe. belbahrii* on basil and *Pe. saturejae-hortensis* on summer savory. Also the production of common sage has become affected by a downy mildew disease only recently, *Peronospora salviae-officinalis*, which belongs to the *Pe. belbahrii* species complex. To our knowledge downy mildew on *S. officinalis* has been officially reported for the first time in Florida in 1993. When the disease was first discovered it was considered to belong to the *Pe. lamii* species complex, and was described as a species of its own just in 2009. Until now the disease was reported from all over the world. Despite the fact that *Pe. salviae-officinalis* was described already a decade ago very little is known about its infection biology and epidemiology. It is also still unclear how the disease was able to spread throughout the world so quickly. It was speculated that downy mildew on *S. officinalis* has been distributed by contaminated seed lots, but the proof for this hypothesis is still lacking. Oospores sticking on seeds could be a potential source of contamination, as already proved for other downy mildews. However, oospores of *Pe. salviae-officinalis* have been observed just rarely and only from sage leaves.

So far, common sage is the only known host of *Peronospora salviae-officinalis*. From a phytopathological perspective it is important to clarify whether other potential hosts do exist that could serve as reservoirs of inoculum for the disease caused by sage downy mildew. Clary sage (*S. sclarea*) is another commonly cultivated sage species. Phylogenetic studies of Lamiaceae with a special focus on *Salvia* showed that *S. officinalis* and *S. sclarea* are closely related. Together with *S. pratensis* (meadow sage), they belong to the “Clade I” within the mint family. Because of their close phylogenetic relationship it seemed possible that these

two sage species could be alternative hosts and could play a role in the infection of sage fields, as host shifts of downy mildews are more likely between closely related host species.

Therefore, the main objective of this thesis was to elucidate the biology and epidemiology of *Peronospora salviae-officinalis*, and to clarify the phylogenetic relationships in the *Peronospora belbahrii* species cluster.

Chapter 1 (Hoffmeister et al., accepted) aimed to determine the impact of basic epidemiological factors like temperature and moisture on the speed and intensity of disease development to improve our understanding of the infection process. Furthermore, eight seed sample different origin were tested for potential contamination with *Pe. salviae-officinalis* by DNA extraction, ITS PCR and Neighbour Joining analysis, as well as by the *Tilletia* seed washing and filtration method.
The effect of temperature was determined for conidia germination and disease development. *In vitro*, conidial germination rate was highest at temperatures between 10 and 20 °C and was strongly reduced at temperatures above 25, but conidia were also able to germinate at 2°C. Temperatures between 15 and 20 °C were most favourable for infection and disease progress in infection experiments in climate chambers, with greatest sporulating leaf area observed at 15 and 20 °C, and highest symptomatic leaf area at 20 °C. *Peronospora salviae-officinalis* was still able to infect sage plants at 5 °C, but sporulation was only observed at higher temperatures. Oospores developed 14 days after inoculation at 15 °C, and 8 days after inoculation at 20 and 25 °C. At incubation temperatures of 5 and 10 °C no oospore formation was observed. The infection trials also showed that dark incubation is not a prerequisite for successful infection of sage. Furthermore, *Pe. salviae-officinalis* needs two events of leaf wetness or high humidity to complete its infection cycle: a first one of at least three hours for conidial germination and penetration of the host, and a second one at the end of the infection cycle for sporulation. Molecular biological investigations of sage seeds revealed that all eight seed samples tested were contaminated with DNA of sage downy mildew. In addition, three seed samples were also contaminated by some *Pythium* species. Seed washing revealed the presence of downy mildew conidia, conidiophores and oospores in seed lots of sage suggesting that infested seeds might play a major role in the fast spread of sage downy mildew, which is an important finding for phytosanitary or quarantine measures. These results are in line with observations from a monitoring survey conducted in

2016 and 2017 where *Pe. salviae-officinalis* was detected in all sage stands investigated throughout Germany, even in newly sown stands.

Chapter 2 (Hoffmeister et al., minor revisions) addresses the development of a robust method for confocal laser scanning microscopy (CLSM), including fluorescent staining, by which oomycete hyphae and plant tissue can be clearly discriminated. This was done based on the example of the previously unstudied infection process of *Pe. salviae-officinalis* in association with its host, *Salvia officinalis*. The presented staining procedure for CLSM of *Peronospora* species infective to Lamiaceae, involving bleaching with ethanol and KOH and staining with a 1:1 aniline-trypan blue mixture, allows detailed visualisation of downy mildew structures outside and inside the host tissue, as well as of callose deposition as an immune response of the plant. In addition, the application of CLSM bears the advantage that strong mechanical sectioning is not necessary as relatively big leaf parts can be investigated. Thus, less pressure is put onto the sample and natural structures are retained. In addition to the staining protocol a histological study of various infection events, from the adhesion of conidia on the leave surface until *de novo* sporulation was conducted. Also oospore formation within the host tissue was observed. The method was also used to examine samples of *Pe. lamii* on *Lamium purpureum* and *Pe. belbahrii* on *Ocimum basilicum* demonstrating the usefulness of this method for studying the infection process of downy mildews in general.

Chapter 3 (Hoffmeister et al., minor revisions) aimed morphological and molecular phylogenetic analyses on *Pe. salviae-officinalis* and closely related downy mildew species on other Lamiaceae. *Peronospora* species causing downy mildew on clary sage (*Salvia sclarea*) and meadow sage (*S. pratensis*), were studied in detail morphologically and by molecular phylogenetic analyses based on six gene loci (ITS rDNA, *cox*1, *cox*2, *ef*1a, *hsp*90 and *β-tubulin*). They were compared to closely related representatives of *Peronospora* species parasitizing Lamiaceae, with focus on species belonging to the *Pe. belbahrii* species complex, to evaluate species boundaries enabling predictions for potential host reservoirs. The downy mildews on clary and meadow sage were usually attributed to *Pe. swinglei*, or *Pe. lamii*, respectively. While *Pe. lamii* is clearly not an appropriate species name for downy mildews on sage, the application of the name *Pe. swinglei* to downy mildew pathogens of various species of sage seemed to be more plausible, because this taxon was originally described from *Salvia reflexa*. However, phylogenetic investigations revealed a very high degree of specialisation in *Peronospora* on Lamiaceae in general and on *Salvia* in particular.

Because of the close phylogenetic relationship between *S. officinalis*, *S. pratensis* and *S. sclarea* it also seemed possible that meadow and clary sage could be alternative hosts for *Pe. salviae-officinalis* described on common sage and could play a role in the infection of sage fields. In addition, *Peronospora* species causing downy mildew on members of the Mentheae, including basil, ground ivy (Glechoma hederacea) and coleus (Plectranthus scutellarioides) were studied. The downy mildew parasitizing coleus had initially been lumped within *Pe. lamii*, but was then relegated to *Peronospora belbahrii* s.l.. Nevertheless, downy mildew on coleus seems to be still having a restricted distribution, suggesting that it is not conspecific with *Pe. belbahrii* and thus representing another downy mildew pathogen posing a potential economic risk.
The downy mildew on *Salvia pratensis* is shown to be distinct from *Pe. salviae-officinalis* and closely related to *Pe. glechomae*, and is herein described as a new taxon, *Peronospora salviae-pratensis*. The downy mildew on *S. sclarea* was found to be conspecific with *Peronospora salviae-officinalis*. This is of phytopathological importance, because meadow sage thus does not play a role as inoculum source for common sage in the natural habitat of the former in Europe and Asia, while clary sage probably does. Furthermore, the multi-gene phylogeny revealed that the causal agent of downy mildew on coleus is distinct from *Pe. belbahrii* on basil, and is herein described as a new taxon, *Peronospora choii*.

In the present study we determined the impact of basic epidemiological factors like temperature and moisture on speed and intensity of disease development to improve our understanding of the infection process. Under favourable conditions *Pe. salviae-officinalis* grows rapidly and builds-up high amounts of inoculum. The findings towards the epidemiology and biology of *Pe. salviae-officinalis* gained in this thesis are a useful basis for developing forecast models and adjusting the combat strategies actually applied in sage production against sage downy mildew. The frequent contaminations of sage seed lots with *Pe. salviae-officinalis* found here could explain the observations from a monitoring survey conducted in 2016 and 2017 where *Pe. salviae-officinalis* was detected in all eight sage stands investigated throughout Germany, even in newly sown stands. The findings of Chapter 3 that *S. sclarea* most likely serves as a host for *Pe. salviae-officinalis*, complete the results of Chapter 1 concerning the epidemiology of the pathogen. Together with the proved seed transmitted character, two new factors influencing the spread of the disease were elucidated in this thesis.

This is the first study presenting a six-gene phylogenetic analysis of downy mildews on Lamiaceae. The combination of the used nuclear and mitochondrial gene regions gives a very good resolution. Our study also showed again that *cox*2 is a suitable barcoding marker for *Peronospora* species as (Choi et al., 2009a) and (Voglmayr et al., 2014) already proved. Also older herbarium samples were successfully amplified by the *cox*2 primer set, and *cox*2 sequences showed a sufficient resolution to distinguish between different close related *Peronospora* species within the Lamiaceae. Whereas ITS data are highly similar within closely related species of the investigated downy mildews and therefore do not allow for unequivocal distinction, the gene regions *cox*1, *EF*1a, *HSP*90 and *β-tubulin* are highly distinctive for the accessions from various hosts, and therefore are also suitable for species discrimination within downy mildews on Lamiaceae.

From a phytopathological point of view the results from this study showed that the wild sage species *S. pratensis* most likely do not play a role as primary inoculum for downy mildew epidemics in cultivated common sage. In contrast, clary sage, acts as alternative host for *Pe. salviae-officinalis* and is a potential inoculum source for the dissemination of the disease. Besides identifying a supplemental host for *Pe. salviae-officinalis* and consequently proving a broader host range for this pathogen, this study showed again that the host range of *Pe. lamii* and *Pe. swinglei* is not as broad as assumed before. Additionally, our results support the claim of Choi et al. (2009a), that the name *Pe. lamii* should be restricted to downy mildew parasitizing *Lamium* spp. or *L. purpureum* only because it is not tenable for downy mildews on sage. In our study it was confirmed again, that the narrow species concept by Gäumann (1923) reflects much better the diversity of downy mildews in Lamiaceae.

Zusammenfassung

Mit über 900 über die ganze Welt verbreiteten Arten, ist die Gattung *Salvia* die größte innerhalb der Familie der Lamiaceae. Zahlreiche Salbeiarten weisen antimikrobielle Eigenschaften auf und finden daher seit der Antike Anwendung als Medizinpflanzen. Außerdem werden diese Kräuter gerne als Gewürze und Aromastoffe genutzt und in Parfümen und Kosmetik verwendet. Der Echte Salbei, *Salvia officinalis*, ist dabei eine der wirtschaftlich wichtigsten Salbeiarten.

In den letzten Jahren haben einige Flasche Mehltau-Krankheiten an Arznei- und Gewürzpflanzen an wirtschaftlicher Bedeutung gewonnen, zum Beispiel *Peronospora somniferi* an Schlafmohn und *Pe. belbahrii* an Basilikum. Auch der Anbau von Echtem Salbei wird heute vom Auftreten eines Falschen Mehltaus, *Pe. salviae-officinalis*, beeinträchtigt. Nach unseren Kenntnissen wurde Falscher Mehltau an *S. officinalis* offiziell zum ersten Mal 1993 in Florida gemeldet. Die Krankheit wurde im Rahmen der Umsetzung des breiten Artkonzepts von Yerkes und Shaw (1959) allerdings zunächst der Sammelart *Pe. lamii* zugeordnet. Erst 2009 wurde *Pe. salviae-officinalis* als eine eigene Art beschrieben. In den letzten Jahren wurde Falscher Mehltau an Echtem Salbei auch in Israel, Italien, Österreich, Neuseeland, UK, Deutschland und Australien nachgewiesen und ist von zunehmender wirtschaftlicher Bedeutung.

Eine Infektion mit *Pe. salviae-officinalis* reduziert die Qualität des geernteten Salbeis, da Blätter infizierter Pflanzen chlorotische bis nekrotische Blattflecke aufweisen. Obwohl *Pe. salviae-officinalis* bereits vor gut zehn Jahren als eine eigene Art beschrieben wurde ist sehr wenig über die Infektionsbiologie und Epidemiologie des Erregers bekannt. Solche Informationen sind jedoch essentiell für die Entwicklung von Prognosemodellen und angepassten Bekämpfungsstrategien. Des Weiteren ist immer noch unklar, wie sich die Krankheit in solch kurzer Zeit über die ganze Welt ausbreiten konnte. Es wird angenommen, dass auch dieser Falsche Mehltau über kontaminiertes Saatgut verschleppt wurde, aber der Nachweis dafür steht noch aus. An Saatgut haftende Oosporen könnten, wie bei vielen anderen Falschen Mehltauen auch, eine mögliche Kontaminationsquelle darstellen. Oosporen von *Pe. salviae-officinalis* wurden bisher jedoch nur selten und nur an Salbeiblättern gefunden. Es bleibt außerdem zu klären, ob *S. officinalis* die einzige Wirtspflanzenart für *Pe. salviae-officinalis* ist, oder auch andere Salbeiarten gibt dem

Pathogen als Wirt dienen können. Neben Echtem Salbei wird auch Muskatellersalbei (*S. sclarea*) häufig angebaut. Eine weitere, zumindest in Deutschland relativ häufig vorkommende Salbeiart ist der Wiesensalbei (*Salvia pratensis*). Phylogenetische Untersuchungen ergaben, dass die drei genannten Salbeiarten sehr nah verwandt miteinander sind. Dies erhöht die Chancen, dass eine der Arten ebenfalls als Wirt für *Pe. salviae-officinalis* dienen kann und eine potentielle Inokulmumquelle darstellt, da Wirtssprünge innerhalb Falscher Mehltau eher zwischen nah verwandten Wirtspflanzen stattfinden.

Die hier vorgestellte Arbeit besteht aus 3 Kapiteln die sich mit folgenden Themen befassen:

Kapitel 1 (Hoffmeister et al., accepted) zielt darauf ab die offenen Fragen hinsichtlich der Infektionsbiologie und Epidemiologie von *Pe. salviae-officinalis* zu klären. Dazu wurde der Infektionserfolg des Erregers in Abhängigkeit von verschiedenen Klimafaktoren untersucht. Des Weiteren wurden acht Proben Salbeisaatgut verschiedener Herkunft mittels DNA-Extraktion, ITS-PCR, Sequenzierung und Neighbour Joining Analyse sowie einer Wasch- und Filtrationsmethode auf mögliche Kontaminationen mit *Pe. salviae-officinalis* getestet. Eine Dunkelinkubation in den ersten 24 Stunden nach der Inokulation erwies sich als nicht nötig für eine erfolgreiche Infektion von Salbei. *Peronospora salviae-officinalis* benötigt zwei Ereignisse von Blattnässe bzw. hoher Luftfeuchtigkeit zur Vollendung des asexuellen Entwicklungszyklus: ein initiale Blattnässedauer nach der Inokulation von mindestens 3 Stunden zur Konidienkeimung und dem Eindringen in das Wirtsgewebe, eine zweites Ereignis hoher Luftfeuchtigkeit am Ende des Infektionszyklus für die Sporulation. In-vitro war die Konidienkeimrate unter Inkubationstemperaturen von 10 - 20 °C am höchsten. Auch in in-vivo waren Temperaturen zwischen 15 und 20 °C am günstigsten für eine Infektion der Pflanze und den Krankheitsverlauf. Bei einer Inkubationstemperatur von 5 °C konnte *Pe. salviae-officinalis* die Salbeipflanzen noch infizieren, Sporulation war jedoch erst bei Erhöhung der Inkubationstemperatur zu beobachten. Bei Temperaturen von 15 °C wurden Oosporen 14 Tage nach der Inokulation, unter 20 und 25 °C 8 Tage nach der Inokulation im Blattgewebe beobachtet. Bei Inkubationstemperaturen von 5 und 10 °C konnten keine Oosporen beobachtet werden.
Die molekularbiologische Untersuchung von Salbeisaatgut unterschiedlicher Herkunft mittels molekularbiologischer Methoden ergab, dass alle acht Saatgutproben mit DNA von *Pe. salviae-officinalis* kontaminiert waren. Drei Saatgutproben waren zudem mit *Pythium* spp.

kontaminiert. Untersuchungen der Saatgutproben mittels Filtrationsmethode zeigten, dass sowohl Konidien und Konidienträger, als auch Oosporen an dem Saatgut hafteten. Belastetes Saatgut könnte somit die Ursache der schnellen weltweiten Verschleppung der Krankheit sein und auch die Ursache dafür, dass in unserem deutschlandweiten Monitoring sämtliche untersuchten Anbauflächen einen Befall aufwiesen, auch neu gesäte Flächen.

In **Kapitel 2** (Hoffmeister et al., minor revisions) wird die Entwicklung einer Methode zur Fluoreszenzfärbung und anschließender konfokaler Laserscanning-Mikroskopie (kLSM) dargestellt. Sie sollte dabei helfen den Infektionsprozess selbst im Detail zu untersuchen und eine histologische Studie zu erstellen. Mit dem in dieser Arbeit entwickelten Protokoll ist es möglich Strukturen von *Pe. salviae-officinalis* mit ausreichend Kontrast zu den ebenfalls Zellulose enthaltenden pflanzlichen Zellen zu färben und anschließend mittels kLSM zu untersuchen. Die Färbung mit einer 1:1 Mischung aus Anilin- und Trypanblau ermöglicht die Unterscheidung zwischen den Strukturen beider Organismen als auch die Visualisierung der pflanzlichen Immunreaktion in Form von Kalloseanreicherungen. Die Verwendung eines konfokalen Laser-Mikroskops bietet zudem den Vorteil, dass eine mechanische Sektionierung der Proben nicht nötig ist beziehungsweise relativ große Blattstücke untersucht werden können. Dadurch wird weniger Druck auf das Präparat ausgeübt und die natürlichen Strukturen bleiben erhalten. Um den zeitlichen und zellulären Verlauf des Infektionsprozesses von *Pe. salviae-officinalis* an *S. officinalis* zu beobachten, wurden zu verschiedenen Zeitpunkten Blattproben von inokulierten in-vitro Pflanzen genommen und für die kLSM gefärbt. Mittels kLSM wurde dann eine histologische Studie des Infektionsprozesses erstellt. Sowohl die asexuelle Entwicklung als auch Ausschnitte der sexuellen Entwicklung sind dargestellt. Die Methode wurde außerdem an Proben von *Pe. lamii* an *Lamium purpureum* und von *Pe. belbahrii* an *Ocimum basilicum* erfolgreich getestet.

Kapitel 3 (Hoffmeister et al., minor revisions) zielte darauf ab *Pe salviae-officinalis* und nah verwandte *Peronospora*-Arten an Lamiaceae mikroskopisch und phylogenetisch zu untersuchen und miteinander zu vergleichen. Hierfür wurden zum einen die Falschen Mehltaue an Muskatellersalbei (*S. sclarea*) und Wiesensalbei (*S. pratensis*) mikroskopisch und phylogenetisch analysiert um deren Identität zu klären. Beide *Peronospora*-Arten werden in zahlreichen Sammellisten zu Falschen Mehltauen genannt und im Zuge der Anwendung des breiten Artkonzepts den Arten *Pe. lamii* beziehungsweise *Pe. swinglei* zugeordnet. *Peronospora swinglei* findet zudem auch als Synonym für *Pe. lamii* Verwendung. Die

Zuordnung von Falschen Mehltauen an Lamiaceae zu einer dieser beiden Sammelarten hat sich in der Vergangenheit jedoch schon häufig als falsch erwiesen, wie viele phylogenetische Studien belegen. Mit unseren Analysen sollte zudem untersucht werden, ob Wiesen- und/oder Muskatellersalbei unter natürlichen Bedingungen Wirtspflanzen für *Pe. salviae-officinalis* darstellen. Des Weiteren wurden Proben eines Falschen Mehltaus an *Plectranthus scutellarioides* (Coleus), mikroskopisch und phylogenetisch analysiert. Dieser Falsche Mehltau war zunächst *Pe. belbahrii* zugeschrieben worden, aber auch hier gab es Zweifel hinsichtlich seiner Identität die es zu klären galt.

Bei der *Peronospora*-Art an *S. sclarea* handelt es sich weder um *Pe. lamii*, noch um *Peronospora swinglei*. Die morphologischen und molekularphylogenetischen Untersuchungen ergaben, dass der Falsche Mehltau an Muskatellersalbei (*S. sclarea*) konspezifisch mit *Pe. salviae-officinalis* ist und somit *S. sclarea* ein weiterer Wirt für *Pe. salviae-officinalis* darstellt. Die auf *S. pratensis* gefundene *Peronospora*-Art unterscheidet sich ebenfalls morphologisch als auch phylogenetisch deutlich von *Pe. lamii* und *Peronospora swinglei*. Der Falsche Mehltau an Wiesensalbei ist näher verwandt mit *Pe. glechomae*, beschrieben an *Gelchoma hederacea*, und bildet eine Schwestergruppe zu *Peronospora salviae-officinalis*. Bei dem Falschen Mehltau an *S. pratensis* handelt es sich um eine bislang unbekannte Art, die in dieser Arbeit als *Pe. salviae-pratensis* beschrieben wurde. Diese Ergebnisse legen nahe, dass der in Deutschland in bestimmten Regionen verbreitete Wiesensalbei keine Rolle als Inokulum für Echten Salbei spielt.

Die mikroskopischen Untersuchungen des Falschen Mehltaus an *P. scutellarioides* ergaben, dass diese Art sich morphologisch klar von dem Typusbeleg von *Pe. belbahrii* an Basilium unterscheidet. Es wurden morphologische Unterschiede in Konidienform, -größe und- farbe sowie in der Form der Endäste der Konidienträger festgestellt. Die *Peronospora*-Art an Coleus unterscheidet sich zudem phylogenetisch, als auch in ihrem Wirtsspektrum von *Peronospora belbahrii*. Die Daten legen nahe, dass es sich bei dem Falschen Mehltau an *P. scutellarioides* um eine eigen Art handelt, und dieses Pathogen nicht *Pe. belbahrii* zugeordnet werden kann. Der Falsche Mehltau an *P. scutellarioides* wurde daher im Rahmen dieser Arbeit als *Peronospora choii* beschrieben.

Mit den hier berichteten Einblicken in die Epidemiologie und Infektionsbiologie von *Pe. salviae-officinalis* ist eine Grundlage für die Entwicklung von Prognosemodellen und angepassten Bekämpfungsstrategien geschaffen worden. Die im Rahmen dieser Arbeit durchgeführten Untersuchungen ergaben, dass Infektionen mit *Pe. salviae-officinalis* an

Salbei unter günstigen Bedingungen schnell voranschreiten und große Mengen an Inokulum gebildet werden. Eine Infektion von Salbei kann selbst bei niedrigen Temperaturen noch erfolgen und führt zu einem latenten Befall der leicht übersehen werden kann. Wie die Saatguttestung gezeigt hat sind reguläre Saatgutproben häufig mit Falschem Mehltau kontaminiert. Die Übertragung des Pathogens mittels Saatgut in neue Regionen ist somit ein ernst zu nehmendes Risiko. Die hier erzielten Ergebnisse decken sich mit Beobachtungen aus einem in den Jahren 2016 und 2017 durchgeführten deutschlandweiten Monitoring im Salbeianbau. Muskatellersalbei scheint zudem einen weiteren Wirt für *Pe. salviae-officinalis* darzustellen, den es zu beachten gilt.

Diese Studie beinhaltet außerdem die erste phylogenetische Analyse von Falschen Mehltauen an Lamiaceae die auf sechs verschiedenen Genloki beruht. Die Kombination der verwendeten nuklearen und mitochondriellen Loki bietet eine sehr gute Auflösung zwischen nah verwandten *Peronospora*-Arten. In der vorliegenden Studie konnte erneut gezeigt werden, dass *cox*2 ein geeigneter Barcoding-Marker für *Peronospora*-Arten ist, wie es bereits Choi et al. (2009) und Voglmayr et al. (2014) vorschlugen. Auch bei älteren Herbarbelegen konnten *cox*2 erfolgreich amplifiziert werden und zeigte zudem eine gute Auflösung um zwischen verschiedenen nah verwandten *Peronospora*-Arten innerhalb der Lamiaceae zu unterscheiden. Eine ähnlich gute Auflösung wie mit *cox*2 konnte auch mit *cox*1, *ef*1a, *hsp*90 und *β-tubulin* erzielt werden. Im Gegensatz dazu hat sich wieder gezeigt, dass ITS-Sequenzen zwischen verschiedenen nah verwandten Falschen Mehltauen an Lippenblütlern sehr ähnlich sind und daher keine zweifelsfreie Artunterscheidung ermöglichen. Unsere Ergebnisse stützen die Forderung von Choi et al. (2009), dass die Anwendung des Artbegriffs *Peronospora lamii* mindestens auf *Peronospora*-Arten beschränkt werden sollte, die Arten der Gattung *Lamium* parasitieren und nicht auf andere Gattungen der Familie der Lamiaceae. Zudem ist zu betonen, dass es sich bei *Pe. lamii* und *Pe. swinglei* um zwei morphologisch und phylogenetisch unterschiedliche Arten handelt, die nicht synonym verwendet werden sollten. Die hier präsentierten Daten zeigen erneut, dass das Enge Artkonzept von Gäumann (1923) wesentlich besser die Artdiversität innerhalb der Falschen Mehltaue an Lippenblütlern wiederspeigelt als das Breite Artkonzept von Yerkes und Shaw (1959).

General introduction

1 Plant pathogenic fungi

The human interest in plant pathogenic fungi and fungi like organisms is a logical consequence of the important role these organisms play as devastating diseases of plants in agriculture and food production. Besides yield reduction, some fungal infections render crops inedible or poisonous for consumers by producing mycotoxins in the infected plant tissue (Ashiq et al., 2014, Freire & Sant'Ana, 2018). An infamous example for fatal crop losses or food spoilage is the potato late blight epidemic in the 1840s which was caused by *Phytophthora infestans* resulting in the Irish potato famine (Erwin & Ribeiro, 1996). All parts of *Solanum tuberosum* are susceptible and late blight can destroy a field of potatoes in a few weeks completely. In addition, tubers form infected stands are also a post-harvest problem, because late blight infections enable soft rotting bacteria to destroy potatoes during storage (Fry, 2008). One example for the dangerous effects of food posing is the ergot disease of wheat, which is caused by *Claviceps purpurea.* Within florets and grains of infected plants alkaloids are produced like the extremely hallucinogenic, illicit drug, lysergic acid diethylamide, also known as LSD (Florea et al., 2017).

The term pathogenic fungi refers to those species, which derive nutrients from plants while negatively influencing the plant health (Doehlemann et al., 2017). Fungi in general are eukaryotic organisms defined by non-motile mycelium formed on or in substratum, and heterotrophic nutrition (Gäumann, 1964, Ainsworth, 1973). The heterotrophic nutrition of fungi is based on a combination of extracellular digestion due to the activity of secreted enzymes followed by absorption of solubilised products (Dix & Webster, 1995). With their special ability to degrade plant polymers, fungi are perfectly adapted to plant substrates. Equipped with an arsenal of cell wall-degrading enzymes, phytopathogenic fungi can, for example, overcome the barrier of the plant cell wall to exploit their substratum (Kubicek et al., 2014). Their pathogenic lifestyles are diverse, with transitions between necrotrophic, hemibiotrophic and obligate biotrophic parasitism (Thines & Kamoun, 2010, Doehlemann et al., 2017, Judelson, 2017). While necrotrophs kill the host cells before colonization and feed from dead substratum (Oliver & Solomon, 2010), biotrophs take nutrients from living plant cells and depend on their host for the completion of their life cycle (Gäumann, 1951a, Judelson & Ah-

Fong, 2019). One example of the nearly perfect adaption of obligate biotrophic pathogen-plant associations are the downy mildews in the Oomycota.

2 Oomycota

The Oomycota (commonly referred to as oomycetes) are a monophyletic group in the kingdom of Straminipila (Dick, 2001), and are more closely related to golden-brown algae, the Ochrophyta, and some heterotrophic protists than to true fungi (Thines, 2014, Beakes & Thines, 2017). Although they are not true fungi (Eumycota), the Oomycota are still treated in mycological textbooks (Agrios, 2005), because they evolved fungus-like characters in parallel to true fungi. Some notable similarities are the formation of complex tip-growing filamentous hyphae, and osmotrophic absorption of nutrients (Beakes & Sekimoto, 2009, Beakes & Thines, 2017).

Oomycota differ from true fungi by aseptate hyphae, heterokont swimming stages, the so called zoospores, and a diploid or polyploid vegetative state (Dick, 2001, Beakes & Thines, 2017, Thines, 2018). A special feature of Oomycota is the formation of oospores, sexual resting spores, which are present in the majority of crown Oomycota (Thines & Choi, 2016). The cell walls of Oomycota mainly contain β-1,3, and β-1,6-glucan, and cellulose (Sietsma et al., 1969, Blaschek, 1992). The β-1,3-glucan structures of Oomycota are branched to variable degree (Yamada & Miyazaki, 1976, Fabre, 1984). In contrast to true fungi, chitin and its derivatives are not present in considerable amounts in most oomycete cell walls (Bartnicki-Garcia, 1968, Melida et al., 2013). Oomycota use soluble β-1,3-glucan called laminarin or mycolaminarin as storage polysaccharide (Judelson, 2017), while glycogen, the storage polysaccharide of fungi, has not been found in Oomycota (Clavaud et al., 2009). Their ability to produce secondary metabolites or digest toxic xenobiotics is reduced in comparison to true fungi. Most species, with the exception of downy mildews with coloured conidia, lack pigments or produce pigments only sparsely, so they have reduced protection from UV radiation (Thines & Choi, 2016, Judelson, 2017). Furthermore Oomycota differ from true fungi by their lysine biosynthesis pathway, which is similar to those of green algae, bacteria and vascular plants (Vogel, 1960).

2.1 Systematics and molecular phylogenetics of Oomycota

The majority of the currently known Oomycota are terrestrial organisms (Dick, 2001), the origins of the Oomycota are most likely marine (Sekimoto et al., 2008a, Sekimoto et al., 2008b). These heterotrophic filamentous organisms of the kingdom Straminipila consist of several lineages which have not been formally assigned to class level, yet, and of the two classes Peronosporomycetes and Saprolegniomycetes, which together form the crown group of the Oomycota (Dick, 2001, Beakes & Thines, 2017, Buaya et al., 2019). Early deriving lineages of the Oomycota are largely unexplored, and the vast majority of the known species belong to the Peronosporomycetes and Saprolegniomycetes (Thines, 2014, Thines & Choi, 2016). In the last years however, researchers undertook significant efforts to fill these knowledge gaps (Buaya et al., 2017, Buaya et al., 2019b, Vallet et al., 2019). Currently, it is assumed that there are at least seven basal lineages within the oomycetes including Atkinsiellales, Eurychasmales, Haliphthorales, Haptoglossales, Langenismatales, Pontismatales and Olpidiopsidales (Beakes & Thines, 2017, Buaya et al., 2019a). Basal lineages of oomycetes comprise mostly holocarpic and biotrophic pathogens of marine algae (*Eurychasma*, *Olpidiopsis*), nematodes (*Haptoglossa*, *Chlamydomyzium*), crustaceans (*Halodaphnea*, *Haliphthoros*, *Atkinsiella*), and molluscs (Sekimoto et al., 2008b, Beakes et al., 2012, Hatai, 2012, Thines, 2014). Most of Saprolegniomycetes are saprotrophs or parasites of animals, and only few of them are plant pathogens. With more than 1000 known species, the Peronosporomycetes comprise the highest number of plant pathogenic species within the oomycetes (Thines, 2014).

The majority of the plant pathogens within the Peronosporomycetes belong to the orders Albuginales and Peronosporales (Thines & Choi, 2016). The order Peronosporales contains several genera of saprotrophic, necrotrophic and hemibiotrophic species, as well as the obligate biotrophic pathogens known as downy mildews (Hulvey et al., 2010, Runge et al., 2011b, Beakes et al., 2012, Thines, 2014, Thines & Choi, 2016). Only obligate biotrophs are known within the white blister rusts of the order Albuginales (Thines & Choi, 2016). The order of the Peronosporales s.l. includes three families: Peronosporaceae s. lat. with the downy mildew genera and *Phytophthora*, Pythiaceae s.l., which are still under revision, and the saprotrophic Salispiliaceae (Beakes & Thines, 2017). With more than 700 known species in 20 genera, the downy mildews are the most species-rich group of Peronosporaceae (Thines & Choi, 2016, Beakes & Thines, 2017), and around 400 species have been described only in

the genus *Peronospora* (Constantinescu, 1991). Considering the high host specificity and the ongoing discovery of new downy mildew species such as *Pe. belbahrii* (Belbahri et al., 2005a), *Pe. salviae-officinalis and Pe. salviae-plebeiae* (Choi et al., 2009a), it can be assumed that the real number of species will further increase rapidly (Thines, 2018). Intriguingly, the total number of known species is estimated to only comprise about 10 % of all existing *Peronospora* species (Thines & Choi, 2016).

2.2 Ecology and distribution of Oomycota

Species of oomycetes are globally distributed and can be found in almost all ecosystems including marine, terrestrial and aquatic habitats (Beakes & Thines, 2017). The Oomycota are reported from temperate forests and agricultural ecosystems (Gessler et al., 2011, Sapp et al., 2019), tropical regions (Thines et al., 2015, Bennett, 2017), semi-arid regions and deserts (Mirzaee et al., 2012) and also from the arctic and antarctic (Johnson, 1971). Like true fungi, oomycetes became adapted to a wide range of different lifestyles. Certainly, the most known ones are the obligate biotrophic downy mildews, and the hemibiotrophic *Phytophthora* spp., such as *Phytophthora infestans* (Fry, 2008, Thines, 2014, Thines, 2018). The oomycetes include also necrotrophic pathogens of a wide range of plants (Dick, 2001, Voglmayr, 2008, Thines, 2014) and animals (Phillips et al., 2008), as well as saprotrophs (Thines, 2014). Some saprotrophs are important decomposers, infesting decaying plant and animal detritus, such as members of the genera *Halophytophthora* and *Salisapilia* (Newell & Fell, 1992, Dick, 2001, Riethmüller & Langer, 2005, Hulvey et al., 2010). Within the oomycetous species the greatest diversity is known from the plant pathogenic species (Beakes & Thines, 2017), but recent studies showed that the diversity of marine oomycetes have been greatly underestimated (Hulvey et al., 2010, Nigrelli & Thines, 2013, Bennett, 2017, Bennett & Thines, 2017).

With few exceptions, our view on oomycetes has been strongly influenced by species pathogenic to cultivated plants, farmed fish and Crustaceae, which attract attention due to their economic importance (Beakes & Thines, 2017). However, there are several species with beneficial features. Some oomycetous species produce long-chained polyunsaturated fatty acids in high amounts, which could be used as fish oil replacements (Domergue et al., 2005), but so far they have not been exploited for commercial usage (Beakes & Thines, 2017). Other

examples for oomycetes with for humans potentially beneficial characters comprises the nematophagous species *Nematophthora gynophila*, which parasitizes the eggs of plant parasitic nematodes (Kerry & Crump, 1980), and *Lagenidium giganteum*, which infects the larvae of mosquitos (Kerwin, 2007). Both have been explored as potential biocontrol agents (Beakes & Thines, 2017). Finally, due to their potentially devastating effects on crop plants some downy mildews such as *Pe. somniferi* on opium poppy and *Pe. tabacina* on tobacco were suggested as potential bioweapons to fight communities depending on those crops (Thines & Choi, 2016).

In contrast to Saprolegniomycetes, most oomycetes of the peronosporalean lineage are incapable of synthesizing sterols and therefore require exogenous sterols to complete their sexual life cycles (Kerwin & Washino, 1983). Downy mildews as well as white blister rusts – representatives of the Peronosporales and the Albuginales, respectively lost the genes for nitrate reductase, nitrite reductase, nitrate transporters (Judelson, 2017), sulphite oxidase and sulfate reductase (Baxter et al., 2010, Kemen et al., 2011). They are apparently unable to reduce inorganic nitrogen and sulfur, which is probably a result of the increasing specialisation to biotrophic plant pathogenicity (Thines & Choi, 2016, Judelson, 2017). Members of the peronosporalean lineage produce only zoospores of the secondary type, and mono-oosporic oogonia with a single oosphere, surrounded by a layer of periplasm (Dick 2001).

3 Downy mildews – Evolution and ecological interactions

It was suggested that downy mildews have evolved from grass-infecting *Phytophthora*-like ancestors, but evidence for this is still vague (Thines, 2009, Thines, 2014). Besides the notorious pathogens of the genus *Phytophthora*, downy mildews are some of the most economically important plant pathogens (Dick, 2001, Thines, 2018). The downy mildew lineage comprises endophytic, obligate biotrophs of several genera such as *Peronospora*, *Pseudoperonospora*, *Hyaloperonospora*, *Plasmopara*, and *Bremia* (Thines, 2014, Thines & Choi, 2016). They affect various crops, such as *Pl. viticola* on grapevine, *Pe. effusa* on spinach (Kandel et al., 2019), and *Ps. cubensis* on cucumber (Lebeda & Cohen, 2010), but also herbs or ornamental plants (Toppe et al., 2010, Gabler et al., 2012). Most downy mildews

are foliar pathogens, but there are also some species infecting the flowering parts (Thines & Kummer, 2012, Thines & Choi, 2016). During the evolution of downy mildews, several host-jump events are likely to have occurred leading to the colonization of new hosts (Voglmayr, 2003, Göker, 2004, Thines et al., 2009c, Thines, 2019). The majority of downy mildews are known to be parasitic to eudicots. Only a few species are known from monocots, *e. g. Pe. destructor* (Palti, 1989, Thines, 2014). Representatives of the genera *Graminivora* and *Peronosclerospora* are exclusively parasitic to Poaceae. The plant families infected with the highest numbers of known downy mildews are Asteraceae, Brassicaceae, Caryophyllaceae and Fabaceae. However, there are also several downy mildews known from Boraginaceae, Lamiaceae, Papaveraceae, Ranunculaceae, and Rosaceae (Constantinescu, 1991, Thines, 2014).

3.1 Disease cycle of downy mildews

The disease cycle of downy mildews involves both asexual and sexual reproductive phases. While within true fungi the asexual vegetative thallus is haploid and the sexual state is diploid (Piepenbring, 2015), in oomycetes it is the other way round. The vegetative state and its asexual spores are diploid or polyploid, while the sexual state is haploid (Dick, 2001, Beakes & Thines, 2017). Especially the asexual spores produced in enormous amounts contribute to epidemics in downy mildew diseases (Lebeda & Cohen, 2010, Sukanya & Spring, 2013, Cohen et al., 2017).

3.1.1 Asexual reproduction

The asexual reproduction cycle in downy mildews starts with an ovoid to ellipsoid multinucleate conidiosporangiocyst, generally referred to as conidium (Gäumann, 1926, Hardham, 2007). The ability to produce zoospores has been lost in several downy mildew species such as *Bremia*, *Hyaloperonospora*, *Paraperonospora*, *Peronosclerospora*, and *Peronospora* (Voglmayr et al., 2004, Göker et al., 2007, Thines et al., 2009a, Beakes & Thines, 2017). At least in some downy mildews this loss seems to be irreversible, as a recent finding indicated the complete absence of genes associated with the formation and function of a flagellum in the genome of *Hyaloperonospora* (Baxter et al., 2010). Thus, conidia of *Peronospora* spp. germinate by forming a germ-tube from the conidia, rather than by releasing motile zoospores (Thines & Choi, 2016). Germination is induced when free water

is available or humidity is high and can be triggered by plant signals. From *H. arabidopsidis* it is known that germ-tubes secrete sticky substances like proteins and fibrillary β-1,3-glucans to keep them attached to the substratum (Carzaniga et al., 2001). The germ-tube subsequently grows chemotropically towards a suitable penetration site (Hardham, 2007). Later it develops an appressorium by inflating its tip via a contact with hydrophobic surfaces *e.g.* the plant cuticle (Latijnhouwers et al., 2003, Judelson & Ah-Fong, 2019). Appressoria of oomycetes function in the penetration of the outermost, epidermal cell layers (Fawke et al., 2015). The level of differentiation and specialization shown by fungal appressoria is not apparent in oomycetous appressorium-like swellings, but the swellings of hyphae in oomycetes are commonly accepted as functional appressoria. Their formation is induced by factors similar to those that induce fungal appressoria (Hardham, 2007). From the appressorium a penetration hypha develops to enter the host tissue. Infection of the leaf tissue frequently happens through a stoma (Judelson, 2012), but epidermal cells can also be penetrated directly, likely by cell wall degrading enzymes (CWDEs) secreted at the hyphal apex (Hardham, 2007). Oomycetous hyphae are known to secrete a range of degradative enzymes, but studies concerning the role of CWDEs focused so far largely on *Phytophthora* and *Pythium*, while only sparse information is available about CWDEs in downy mildews (Kemen et al., 2011).

Oomycetes prefer to grow in moist environments, such as the apoplast of the host plant (Judelson, 2012). In downy mildews hyphae are exclusively formed intercellularly within the substratum. Only the conidiophores emerge from the surface of the substratum (Gäumann, 1926). In the intercellular space, a primary vesicle is formed (Spencer, 1981), from which hyphae spread between the mesophyll cells and develop first haustoria. Haustoria enter the host cell wall and invaginate the plasma membrane of the plant cell, thus becoming enveloped by a host membrane. Recent findings indicate that the extrahaustorial membrane (EHM) is not a simple invagination of the plasma membrane, but rather a novel host cell membrane resulting from reorganization of the cytoskeleton (Lu et al., 2012). This EHM separates the pathogen from the plant cell, and engulfs a carbohydrate-rich amorphous layer, the extrahaustorial matrix (EHMx), which is located between the EHM and a haustorium (Melida et al., 2013, Judelson & Ah-Fong, 2019). It is assumed that the EHMx is of plant and pathogen origin (Caillaud et al., 2014). Little is known about transport processes over oomycetous haustoria, but it was assumed that haustoria are implicated in nutrient uptake in a similar manner than those of true fungi (Lu et al., 2012, Fawke et al., 2015, Judelson & Ah-

Fong, 2019). However, the role of haustoria in transporting proteins to the EHMx was demonstrated, and also effectors are discharged by haustoria (Judelson & Ah-Fong, 2019). Haustoria generate an intimate association of the pathogens with their hosts. This activates plant defence responses, which often result in surrounding haustoria of downy mildews by a callose collar (Judelson & Ah-Fong, 2019). Older haustoria are often even surrounded completely by haustorial encasements, double-layered cupshaped callose-containing membrane structures (Lu et al., 2012, Melida et al., 2013). Those encasements are considered to restrict the nutrient uptake or effector translocation by the pathogen, or accumulate plant-derived antimicrobials (Judelson & Ah-Fong, 2019).

The colonization of the host tissue by downy mildews proceeds relatively quickly under suitable environmental conditions (Lebeda & Cohen 2011) and is completed with the production of new conidia. Conidia of downy mildews are usually formed on typical conidio-sporangiophores with distinctive branching morphologies, generally referred to as conidiophores, which emerge from the stomata (Gäumann, 1926, Beakes & Thines, 2017). The emergence of conidiophores bearing conidia give rise to the typical appearance of downy mildew diseases (Belbahri et al., 2005a, Lebeda & Cohen, 2010, Kandel et al., 2019). Conidia of downy mildews are separated from the conidiophores by a callose plug at their base (Hohl & Hamamoto, 1967). Mature conidia are then liberated and released actively to the air by twisting hygroscopic movements of conidiophores upon drying (Lange et al., 1989). Asexual spores of downy mildews have a short life span and after detachment are viable only for few hours to few days, if conditions are suitable (Cohen & Rotem, 1971, Lebeda & Cohen, 2010, Klosterman et al., 2014), but can be rapidly desiccated and transported by wind over long distances (Viranyi & Spring, 2010, Kandel et al., 2019).

3.1.2 Sexual reproduction

Besides asexual reproduction, downy mildews also undergo sexual reproduction and form of oospores which are formed within the host tissue, and survive adverse conditions after host death (Gäumann, 1926, Dick, 2001). In oomycetes two mating systems exist, the homothallic and the heterothallic mating system. In homothallic species the sexual reproduction can be completed by a single culture or isolate, whereas in heterothallic species two distinct sexual compatibility types, generally referred to as mating types, are required (Gäumann, 1926, Judelson, 2009). Thus, in heterothallic downy mildew species the gametes are formed by hyphae originating from two different strains, each possessing a distinct mating type.

Examples for heterothallic species are *Pe. effusa*, *Pl. viticola* and *B. lactucae* (Judelson, 2009, Kandel et al., 2019). Most of the species within the Peronosporomycetes are homothallic (Dick, 2001). With the start of the sexual reproduction gametes are formed by swellings of intercellular hyphae. These are the female ovoid oogonia containing one to several large eggs, also referred to as oospheres, and male antheridia containing donor gametangial nuclei (Gäumann, 1926, Dick, 2001, Beakes & Thines, 2017). Before formation of the gametes, gametangial meiosis takes place, therefore gametangia in Peronosporomycetes are also called meiogametangia (Dick, 2001). The female oospheral nuclei and the male antheridial nuclei are the only haploid stages in the life cycle of diploid oomycetes (Gäumann, 1926, Howard & Moore, 1970, Beakes, 1980). It is supposed that gametangium differentiation and therefore oospore formation in oomycetes is regulated and coordinated by nutritional and biochemical stimulants *e.g.* diffusible steroid hormones (McMorris & Barksdale, 1967, Dick, 2001, Beakes & Thines, 2017). However, for several species of the peronosporalean lineage it was shown that they are unable to synthesize sterols by themselves. They require these from external sources in order to be able to reproduce sexually (Kerwin & Washino, 1983).

Fertilization occurs when the gametes get in contact with each other (Dick, 2001). An oogonium usually penetrates the antheridium, and the amphigenous antheridium forms a collar-like structure around the base of the oogonium (Hemmes & Bartnicki-Garcia, 1975, Beakes & Thines, 2017). The antheridium stays attached to the oogonium and forms a fertilization tube to transfer one haploid nucleus into the oosphere in the case of downy mildews (Gäumann, 1926, Dick, 2001). Before oospores reach their maturity, the nuclei of the gametes fuse and the diploid state is restored. After the karyogamy, the oosphere matures into a thick-walled resting zygote known as oospore. The thin outer wall of an oospore, the epispore, is derived from the periplasm (Gäumann, 1926, Beakes & Thines, 2017). The inner oospore wall, the endospore, is rich in β-1,3-glucans which form a major storage reserve and is mobilized by glucanases just prior to germination (Judelson, 2009). Within the endospore the cyctoplasm is located containing often numerous lipid droplets. The centre of the oospores form the single zygotic nuclei and the ooplast, a large storage vacuole inside the oospore protoplast (Gäumann, 1926, Dick, 2001, Beakes & Thines, 2017). At the end of the oospore development, the oogonium is seperated from the supporting mycelium by a plug, probably to prevent reflux of cytoplasm (Hemmes & Bartnicki-Garcia, 1975, Judelson, 2009). Oospores are formed within the host tissue (Gäumann, 1926), and lead to

contamination of soil when infected leaf debris decomposes (Spring & Zipper, 2000, van der Gaag & Frinking, 2009, Lebeda & Cohen, 2010). For several downy mildew species oospore formation on seeds have been also observed (Garibaldi et al., 2004, Landa et al., 2007, Kunjeti et al., 2016). Oospores of *Peronospora* spp. germinate with a broad coenocytic germ-tube hyphae and the plasma of the oospore moves towards its distal end (van der Gaag & Frinking, 1996, Kandel et al., 2019). While in the genus *Peronospora* the germ-tube forms a mycelium within the host tissue, in the case of *Pl. viticola* or *Pl. halstedii* the germ-tubes ends with a single conidiosporangiospore (Gäumann, 1926, van der Gaag & Frinking, 1996, Spring & Zipper, 2000, Kunjeti et al., 2016, Kandel et al., 2019). For oospores a ‘‘ripening’’ period is typically required before germination can occur (Judelson, 2009). From several oomycetes it is known that oospores also go through a long period of constitutive dormancy that may last several years, potentially caused by self-inhibitors or barriers to nutrient entry (Sussman & Douthit, 1973, Spring & Zipper, 2000, Dick, 2001). Consequently, many oospores fail to germinate even in favourable environments (Judelson, 2009, Kandel et al., 2019).

3.1.3 Post infection – pathogen-plant interaction

Even though downy mildews are well prepared for the infection and colonization of their hosts, the penetration of the plant tissue will not remain unrecognized. Pathogen defence in plants is initiated by recognition of pathogen-associated molecular patterns (PAMPs) via plasma membrane-localized pattern-recognition receptors (PRRs), resulting in the activation of PAMP-triggered immunity (PTI) (Judelson & Ah-Fong, 2019). PAMPs are considered to be essential components in oomycetous cells, but are absent in higher plants as well as in most other classes of microbes, and elicit defence responses in attacked plants (Robinson & Bostock, 2015). The first PAMPs identified in oomycetes were components of the cell wall or plasma membrane such as β-1,3- and β-1,6-glucans, and arachidonic acid (Fawke et al., 2015, Robinson & Bostock, 2015). Besides the insoluble glucans, soluble β-1,3-linked glucans are present at various stages in the life cycle of oomycetes (Bartnicki-Garcia, 1968, Bartnicki-Garcia & Wang, 1983, Melida et al., 2013, Robinson & Bostock, 2015). With the method presented in Chapter 2, several downy mildew structures were visualized by staining glucan constituents of the pathogen, especially by aniline blue. In addition, for *Pe. parasitica* it was shown that germinating conidia secrete an adhesive layer containing glycoproteins and β-1,3-glucans that might contribute to gremlin attachment (Carzaniga et al., 2001). As β-1,3-glucans are elicitors for PTI of the host plant (Robinson & Bostock, 2015) a strong immune

response by sage should have been expected in the presence of *Peronospora salviae-officinalis*. However this phenomenon did not occur in the examined plant samples.

In the PTI against oomycetes both salicylic acid and jasmonic acid pathways are involved, as shown for *Ph. infestans* (Halim et al., 2009, Robinson & Bostock, 2015). The PTI of plants involves the induction of phytoalexins and elicit programmed cell death, characteristic for hypersensitive response and the generation of reactive oxygen species (ROS) (Robinson & Bostock, 2015). Defence molecules are delivered to the ATP-binding cassette transporters at the plant-oomycete interfaces when PTI is activated. Such defence molecules are pathogenesis-related proteins, toxic isoflavonoids or sesquiterpenes, callose for thickening the cell wall, and ROS. ROS are antimicrobial, and additionally help to strengthen cell walls by initiation of lignin polymerization (Judelson & Ah-Fong, 2019). Those responses are expanded when effector-triggered immunity is involved, often leading to hypersensitive cell death. It has to pointed out that PTI and effector-triggered immunity are not specific for oomycetes (Judelson & Ah-Fong, 2019).

Many oomycetes have developed counter defences against the PTIs that minimize the immune response and maintain host integrity (Judelson, 2017). Biotrophic oomycetes feeding from living cells resist host defence via cytoplasmic and apoplastic effector proteins (Judelson & Ah-Fong, 2019). Effectors are molecules, typically proteins, secreted by the pathogen that manipulate processes of the host cell for their own benefit within the host-pathogen interaction systems. While apoplastic effectors can be targeted to the space outside plant cell membranes, cytoplasmic effectors are translocated into the host cell (Bozkurt et al., 2012). Known cytoplasmic effectors of oomycetes are RxLRs and Crinkler (CRN) proteins (Bozkurt et al., 2012, Judelson & Ah-Fong, 2019). While RxLRs are restricted for Peronosporales, especially to *Phytophthora* and downy mildews, CRNs are ubiquitous in plant pathogenic oomycetes (Bozkurt et al., 2012). They defeat plant defence through many ways, including reprogramming of host gene expression, alterations in RNA metabolism, and binding to the host proteins, which are involved in signalling (Wang & Wang, 2018). RxLR effectors for example are thought to be predominantly secreted via haustoria (Bozkurt et al., 2012). RxLRs of *Ph. sojae* and *H. arabidopsidis* are localised to the plant plasma membrane and are considered to bind PRRs at the plant-oomycete interface (Caillaud et al., 2012, Yu et al., 2012). However, in resistant host genotypes, effectors addressed to support the growth and survival of the pathogen can become a burden for the pathogen. In resistant genotypes

disease resistant genes encode for receptors that help the plant to monitor the presence of pathogen effectors (Bozkurt et al., 2012). In the study discussed in Chapter 2, the presence of numerous glucan-containing structures obviously did not result in a strong PTI, as callose deposition and accumulation was only directed towards haustoria, leading to the assumption that effectors are involved in the infection and colonisation process of sage by downy mildew.

Most insights into the biochemistry of oomycetes are gained from genome mining, as oomycetes are less tractable to genetic manipulation than many true fungi. Therefore, our knowledge on their metabolism remain limited (Latijnhouwers et al., 2003, Baxter et al., 2010, Judelson, 2017). Despite the limitations, new findings were obtained especially for effectors of Peronosporaceae since *H. arabidopsidis* infecting the model plant *A. thaliana* became a tractable pathosystem for the study of effectors in the laboratory (Bozkurt et al., 2012, Caillaud et al., 2014, Fabro et al., 2011). Since common sage has not thoroughly been studied from this aspect, conclusions towards effectors produced by *Pe. salviae-officinalis* at the infection side can only be drawn from the research performed on other downy mildews, for example those on *Hyaloperonospora arabidopsidis* (Baxter et al., 2010, Fabro et al., 2011).

3.2 Species concepts and downy mildews on Lamiaceae

Downy mildew pathogens are usually highly host specific, as it has been shown by infection trials and phylogenetic studies (Gäumann, 1923, Voglmayr, 2003, Göker et al., 2007, Choi et al., 2009a, Voglmayr et al., 2014). The experimental outcomes of extensive infection studies coupled with detailed light microscopy led Gäumann (1918, 1923) to promote a narrow species concept which was also supported by Gustavsson (1959). Later, molecular phylogenetic studies generally supported the narrow species concept in most cases (Voglmayr, 2003, Göker, 2004, Thines et al., 2009a, Thines et al., 2009c, Choi et al., 2011). Yerkes & Shaw (1959) had questioned the narrow species concept of Gäumann, and promoted a broad species concept for downy mildews, such as the one defined by de Bary (1863). The broad species concept declares the species as the basic taxon, containing intraspecific taxa, considering downy mildews with overlapping morphological characteristics on different host species as one species (Yerkes & Shaw, 1959). Nevertheless, even if a large body of evidence shows that most downy mildew species have a narrow host range

there are exceptions to the rule: Examples are *Pl. halstedii*, the downy mildew of sunflower, which parasitizes around 100 host species in 35 genera of the Asteraceae (Leppik, 1966, Sackston, 1981), or the cucumber downy mildew *Ps. cubensis* with more than 60 reported host species in 20 genera of the Cucurbitaceae (Lebeda & Cohen, 2010, Runge et al., 2011a). These examples seem to support the broad species concept, but data accumulated in the last 20 years mainly by molecular phylogenetic studies evidence that the narrow species concept in most cases reflects the diversity of downy mildews much better (Voglmayr, 2003, Göker, 2004, Thines et al., 2009a, Thines et al., 2009c, Choi et al., 2011).

The broad species concepts by (De Bary, 1863) and (Yerkes & Shaw, 1959) complicated the taxonomy of the species, because it often pools several downy mildew species of one host family into collective terms (Thines & Choi, 2016). These collective terms are usually determined by the family of the infected host plants, for example the *Pe. lamii* species complex comprises *Peronospora* species infecting Lamiaceae (Belbahri et al., 2005a, Choi et al., 2009a, Thines et al., 2009a). The species *Pe. lamii* was first described from Lamiaceae in 1857 by Braun (Rabenhorst, 1857). Besides *Lamium purpureum* Rabenhorst also listed *L. album*, *L. amplexicaule*, and *L. maculatum*, in addition to species from other genera of Lamiaceae such as *Salvia pratensis*, *Stachys palustris*, *Calamintha acinos (syn. Satureja acinos)*, and *Thymus serpyllum* as potential host species (Rabenhorst, 1857). In 1866 Fuckel separated the downy mildew species on *S. acinos* from *Pe. lamii* and described it as *Pe. calaminthae* (Fuckel, 1866). In addition, Sydow described the *Peronospora* sp. parasitic to *S. palustris* as *Pe. stachydis* (Gäumann, 1923). (Gäumann, 1923) only listed *Lamium* species among the potential hosts of *Pe. lamii*. Additionally, Gäumann questioned the validity of *Pe. thymi*, a species split from the *Pe. lamii* species complex by Sydow (Sydow, 1887), because he recognised that the host plant on which *Pe. thymi* was found was *Calamintha acinos* and not *Thymus serpyllum* (Gäumann, 1923). These first efforts to dissolve the *Pe. lamii* species complex were interrupted when the application of the broad species concept received new support by the publication of (Yerkes & Shaw, 1959). From the second half of the twentieth century onwards, phytopathologists continued using the term *Pe. lamii* for all downy mildew species found on Lamiaceae, based on morphological similarities and host families. Examples are the downy mildews on basil (Martini et al., 2003), common sage (McMillan, 1993), and clary sage (Dudka et al., 2004). During the last years with the support of sequence data, again researchers continued to dissolve the *Pe. lamii* species complex promoting the narrow species concept.

Four new *Peronospora* species on Lamiaceae have been described so far: *Pe*. *belbahrii* on *Ocimum basilicum* and *Plectranthus scutellarioides* (Thines et al., 2009a), *Pe. salviae-officinalis* on *S. officinalis*, *Pe*. *salviae-plebeiae* on *S. plebeia* (Choi et al., 2009a), and *Pe. jagei* on *Stachys palustris* (Thines & Kummer, 2012). This work is not finished, yet, as for example the taxonomic placement of the *Peronospora* species on *P. scutellarioides* is still uncertain. (Thines et al., 2009a) defined *P*. *scutellarioides* (coleus) as a second host species for *Pe. belbahrii* in addition to basil, but they already suggested the downy mildew on coleus might represent a species of its own. Although they found morphological differences between the *Peronospora* samples from basil and coleus, they refrained from describing two different species. So far, the identity of the downy mildew species on coleus has not been unequivocally resolved. In order to implement quarantine measures for the *Peronospora* species on *P. scutellarioides* it is necessary to answer this taxonomic issue. Until now the downy mildew on coleus has been reported for USA (Palmateer et al., 2008, Daughtrey et al., 2006), Germany (Thines et al., 2009a), UK (Denton et al., 2015), Japan (Ito et al., 2015), and just recently from Brazil (Gorayeb et al., 2019). Considering the fact that the *Peronospora* species parasitizing coleus does not occur as widely distributed as *Pe. belbahrii* it is probably not too late to adopt appropriate measures to protect European ornamental plant production when species delimitations are solved.

Within Lamiaceae, the tribe Mentheae contains several *Peronsopora* species occurring on culinary herbs and medicinal plants (Dick, 2001, Choi et al., 2009a, Thines et al., 2009c). In addition to the sage downy mildews *Pe. salviae-officinalis* and *Pe. salviae-plebeiae*, downy mildews on *S. sclarea* (clary sage) and *S. pratensis* (meadow sage) have been noted in several checklists of micromycetes and Peronosporaceae in Europe and the British Isles. The downy mildew diseases were usually attributed to *Pe. swinglei* (Gaponenko, 1972, Mulenko et al., 2008), or *Pe. lamii* (Fuckel, 1866, Preece, 2002, Dudka et al., 2004, Müller & Kokes, 2008), respectively. *Peronospora lamii* was described primarily on *Lamium* spp. (Rabenhorst, 1857). Whereas, *Pe. swinglei* was first described from *S. lanceolata* by Ellis & Kellermann (1887), but later Constantinescu re-determined *S. reflexa* as the host species of the type specimen (Constantinescu, 1991). Furthermore, the taxon *Pe swinglei* is sometimes used as a synonym for *Pe. lamii* s. l. (Brandenburger & Hagedorn, 2006a). This shows that also the identity of the downy mildew species on *S. pratensis* and *S. sclarea* has not been definitely resolved, yet. Considering the narrow host specificity and the phylogenetic divergence within the *Peronospora* species on Lamiaceae, it seems unlikely that the downy

mildews found on clary and meadow sage are conspecific with *Pe. lamii* s. str. or *Pe. swinglei* s. str.. Additionally, it is unknown, if these two sage species can serve as supplementary hosts for *Peronospora salviae-officinalis*.

3.3 *Peronospora salviae-officinalis*

With over 900 species spread throughout the world, *Salvia* (sage) is the largest genus of the Lamiaceae family (Harley, 2004, Walker & Sytsma, 2007, Will & Classen-Bockhoff, 2014). Several sage species provide antimicrobial activities and have been used as medicinal plants since ancient times (Longaray Delamare et al., 2007). Furthermore, these aromatic herbs are applied as spices and flavouring agents, and are used for perfumery and cosmetics (Sulniute et al., 2017). Common sage, *S. officinalis*, is one of the economically most important sage species (Mirjalili et al., 2006, Raal et al., 2007). However, production of common sage has recently been reported to be affected by downy mildew. The causal agent of the disease belongs to the *Pe. belbahrii* species complex and was described as a new species, *Peronospora salviae-officinalis,* in 2009 (Choi et al., 2009a). To our knowledge the downy mildew on *S. officinalis* was officially reported for the first time from Florida in 1993, then as *Pe. lamii* (McMillan, 1993), as a consequence of the broad species concept advocated by (Yerkes & Shaw, 1959). Earlier reports of downy mildews on sage species mention the occurrence of *Pe. lamii* and *Pe. swinglei* on *S. pratensis*, *S. lanceolata* or *S. sclarea* but not on *Salvia officinalis* (Rabenhorst, 1857, Ellis & Kellerman, 1887, Gäumann, 1923, Kochman, 1970, USDA, 1960, Osipjan, 1967, Stanjavičenie, 1984). In recent years, downy mildew on common sage has been reported from Australia, Austria, Germany, Israel, Italy, New Zeeland, and UK (Gamliel & Yarden, 1998, Plenk, 2002b, Hill et al., 2004, Belbahri et al., 2005a, Liberato et al., 2006, Humphreys-Jones et al., 2008, Choi et al., 2009a). Considering all reports of *Peronospora* infections on *S. officinalis* since 1993 it is evident that the disease is of increasing economical concern and can be regarded as an emerging disease.

The disease caused by *Pe. salviae-officinalis* displays a broad range of symptoms, which depend on the sage cultivar, climate conditions and cultivation management. Leaves of infected plants show chlorotic to necrotic, polyangular leaf spots, which are clearly vein-limited in older leaves (Choi et al., 2009a). These chlorotic lesions are initially pale green to bold mustard yellow, and sometimes acquire a violet hue when aging, or become necrotic.

They are sometimes limited to a single spot or extend over larger parts of the leaves. Leaf spots reduce the quality of the crop, although there are no mycotoxins known to be produced in infected sage tissue by *Peronospora salviae-officinalis*. However, when used for medicinal purposes, zero tolerance for contaminations by pathogens or weeds in harvest products are acceptable (Bent, 2008, Ashiq et al., 2014). Very little is known about the infection biology and epidemiology of *Peronospora salviae-officinalis*. This knowledge is however crucial for targeted and efficient pest management strategies against downy mildew in sage production as well as for the development of forecast models.

When *Pe. salviae-officinalis* was described as a new species with common sage as the only known host in 2009 (Choi et al., 2009a), it was too late to implement quarantine measures, because it had already been reported from the USA, Israel, Italy, Austria, New Zeeland, UK, Germany and Australia (McMillan, 1993, Gamliel & Yarden, 1998, Plenk, 2002b, Hill et al., 2004, Belbahri et al., 2005a, Liberato et al., 2006, Humphreys-Jones et al., 2008). Whether common sage is the only host of this pathogen, or *S. pratensis* and *S. sclarea* can also serve as host species, remains uncertain. It was unclear how the disease was able to spread throughout the world so quickly, but it was hypothesized that downy mildew on *S. officinalis* might have been distributed by contaminated seed lots (Choi et al., 2009a). However, proof for this hypothesis was still lacking. Oospores that could play an important role in the spread have, to our knowledge, been reported only on sage leaves (Plenk, 2002b, Liberato et al., 2006). Whether common sage is the only host of this pathogen, or other sage species like *S. pratensis* or *S. sclarea* could potentially also serve as host species, was an open question.

3.4 Challenges in the control of downy mildew diseases

Infections with downy mildews decrease the quality of the harvested crops by prominent leaf spots visible on the upper leaf surface and the typical grey to brown spore masses on the lower leaf surface (Thines et al., 2009a, Lebeda & Cohen, 2010, Kandel et al., 2019). Downy mildew infections also often reduce the quantity of a crop due to a reduction of photosynthetically active leaf area, leaf deformation, and stunted plant habitus (Agrios, 2005, Lebeda & Cohen, 2010, Montes-Borrego et al., 2017).

Control of downy mildew diseases is challenging. Although different management methods are applied, such as good cultural practices, breeding for resistant cultivars, and chemical

control (Gisi, 2002), growers are still facing severe problems. Many crop varieties are not completely resistant (Djalali Farahani-Kofoet et al., 2014, Montes-Borrego et al., 2017), or the pathogen overcomes a formerly complete resistance (Gilles, 2002). For several speciality crop no attempts in resistance breeding are made against the respective downy mildew species, because the profit margin is not high enough to cover the breeding expenses (Gärber, 2019). Downy mildew pathogens often show a short period of latency following infection (Gilles, 2002). In addition, infected plants can be entirely asymptomatic and the pathogen can be transmitted vertically to the next host generation (Jacobson et al., 1998, Ploch & Thines, 2011). For *Pe. somniferi* it was even shown that seed samples of opium poppy genotypes rated as resistant due to the absence of symptoms of downy mildew were contaminated with downy mildew by a systemic infection (Montes-Borrego et al., 2017). The seed-borne character was also shown for different *Peronospora* species *e.g.* downy mildew on basil and spinach (Garibaldi et al., 2004, Landa et al., 2007, Kandel et al., 2019) suggesting that seed treatments are necessary to prevent the spread of these diseases.

Furthermore, populations of downy mildews can acquire resistances to the applied fungicides, and authorities as well as supermarket chains determine limits on the number of different fungicides applied during crop production and fungicide residues on the harvested crop (Gilles, 2002). Therefore, the remaining fungicides have to be applied at a timing where they take full effect. Disease forecasting systems, as those developed for *Ps. cubensis*, *Pl. viticola* and *B. lactucae* can help to predict the best time for the application of fungicides, leading to a more sustainable disease control due to reduced fungicide usage (Scherm et al., 1995, Gisi, 2002, Caffi et al., 2010, Arauz et al., 2010, Neufeld & Ojiambo, 2012). Besides the positive effect on the environment, a reduction in the amount of fungicides used reduces the selection pressure for resistant individuals in downy mildew populations (Gilles, 2002). However, to develop such forecasting models, or even to predict when crops are at risk of significant downy mildew development, knowledge about the infection parameters and the dispersal of the pathogen are needed. Forecast models are based on knowledge on the effect of climate factors on disease development, potential spore loads in an area and longevity of spores or other dispersal units (Scherm et al., 1995, Arauz et al., 2010, Caffi et al., 2010, Neufeld & Ojiambo, 2012).

Another major issue in controlling downy mildew diseases is certainly the insufficient knowledge of the species boundaries between distinct downy mildew pathogens (Thines &

Choi, 2016). As morphological features between closely related downy mildews are often very similar (Choi et al., 2005, Garcia-Blazquez et al., 2008, Choi et al., 2011, Runge & Thines, 2012, Choi et al., 2015a), species delimitation based only on morphological characters is in many cases not sufficient and should be coupled with molecular phylogenetics. Beyond doubt, most problems regarding the identification and control of downy mildew diseases occurred due to the application of the broad species concept advocated by (Yerkes & Shaw, 1959), as the downy mildews within the *Pe. belbahrii* species complex proof (Thines, 2014). Due to the high diversity within downy mildews findings towards epidemiology and host range are usually only known for those species with high economic importance (Thines & Choi, 2016), thus, making the establishment of control or even quarantine measures for downy mildew pathogen on niche cultures challenging.

4 Structure and aims of the doctoral thesis

The main objectives of this thesis were to elucidate the biology and epidemiology of *Peronospora salviae-officinalis*, the downy mildew on common sage (*Salvia officinalis*), and to clarify the phylogenetic relationships in the *Peronospora belbahrii* species cluster. In order to achieve these objectives, the following specific aims were addressed:

1. The epidemiological parameters for a successful infection of sage by *Pe salviae-officinalis* were assessed by testing
   1.1. the effect of dark incubation after inoculation
   1.2. the effect of leaf wetness and high humidity
   1.3. the effect of incubation temperature
2. Seed lots of sage were examined for downy mildew contaminations using a seed washing method in combination with light microscopy, as well as PCR-based detection methods
3. The infection process of *Pe. salviae-officinalis* on *S. officinalis* was tracked by confocal laser scanning microscopy (CLSM) with a new staining protocol developed in this study
4. Characterisation of the identity of the downy mildew species on *Plectranthus scutellarioides*, *Salvia pratensis* and *S. sclarea* by morphological and phylogentic investigations

This thesis is structured in three Chapters. Chapter 1 covers the infection studies with *Pe. salviae-officinalis* on *S. officinalis*, as well as the seed testing.

Chapter 2 compiles the development of a staining protocol for confocal laser scanning microscopy of downy mildews. It also presents a histological study of the complete asexual life cycle and the infection process of *Pe. salviae-officinalis*, and additional insights into the sexual reproduction of this downy mildew.

Chapter 3 studies the phylogeny of sage downy mildew and its closely related species on other Lamiaceae. This chapter includes the species description of the downy mildew on coleus, *Pe. choii* sp. nov. and of the downy mildew on meadow sage, *Pe. salviae-pratensis* sp. nov.. It also provides new phylogenetic evidence that the downy mildew found on *S. sclarea* is conspecific with *Peronospora salviae-officinalis*.

General discussion

This thesis was designed to gain deeper insights into the infection biology and epidemiology of sage downy mildew by unrevealing the complete life cycle of this species, and the climatic parameters promoting the disease process. In addition, the phylogenetic relationships within the *Pe. belbahrii* species cluster were studied to resolve species boundaries.

1 Epidemiology of *Peronospora salviae-officinalis*

In Chapter 1, several experiments were conducted to determine the climatic conditions needed for infection of *S. officinalis* by *Pe. salviae-officinalis* and further progress of the disease. The findings discussed in Chapter 1 laid the foundation for the establishment of the long-term maintenance of *Pe. salviae-officinalis* on its host under controlled conditions. The results of these experiments also facilitated the study of the complete infection process of the here discussed species on its host plant sage (see Chapter 2).

1.1 Effect of temperature and humidity on infection success

The results of Chapter 1 were largely consistent with the findings of other studies performing infection experiments with downy mildews. Conidia germinated *in vitro* at temperatures from 2 to 30 °C, and *Pe. salviae-officinalis* infected sage successfully at all temperatures tested, from 5 to 25 °C. For the closely related *Pe. belbahrii* conidia germination and infection of basil was observed at a similar temperature range from 5 to 28.5 °C (Djalali Farahani-Kofoet et al., 2014). While at 20 and 25 °C the symptomatic leaf area developed rather in parallel to the sporulating leaf area of sage infected with downy mildew, symptom development was delayed at incubation temperatures of 15 °C or lower. At 5 °C *Pe. salviae-officinalis* was still able to infect plants but did not sporulate. After infection it is apparently able to colonize the plant tissue at least partly and cause a latent infection, as no symptoms were visible. To induce sporulation, higher temperatures of at least 10 °C were necessary. Latent asymptomatic infections are also known from other downy mildew diseases as well as for the white blister rusts (Jacobson et al., 1998, Gilles, 2002, Montes-Borrego et al., 2009, Ploch &

Thines, 2011). Especially in natural host populations infested by downy mildews or white blister rusts, symptoms are often limited during sporulation (Thines & Kamoun, 2010). The factors triggering the onset of symptoms in asymptomatically infected plants are yet unknown, however it has been suggested that a combination of host genotype, biotic and abiotic factors are involved (Ploch & Thines, 2011, Thines & Choi, 2016).

Conidia of sage downy mildew germinate on wet surface, water agar or sage leaf, within two hours and penetrate into the epidermis within three to six hours as the *in vitro* and *in vivo* experiments presented in Chapter 1 showed. These results are very similar to those of some studies with *Peronospora belbahrii* (Djalali Farahani-Kofoet et al., 2014, Cohen & Ben-Naim, 2016). Furthermore, it was shown that sage downy mildew needs two events of leaf wetness or high humidity to infect the plant and complete the asexual life cycle. The first event of leaf wetness is needed for conidial germination and penetration of the host; and the second event of high humidity is necessary to promote sporulation at the end of the infection cycle. This had also been reported before for *Pseudoperonospora cubensis*, the causal agent of the cucurbit downy mildew (Cohen, 1977, Palti & Cohen, 1980, Lebeda & Cohen, 2010), and for *Pe. belbahrii* (Garibaldi et al., 2007, Cohen & Ben-Naim, 2016). Taken together, the results of the infection studies discussed in Chapter 1 confirm that leaf wetness is essential for infection and sporulation, while temperature determines the extent of the disease development. It can be concluded that in temperate regions *Pe. salvia-officinalis* is able to infect sage plants more or less over the whole year. Also in this respect *Pe. salviae-officinalis* behaves very similar to the closely related *Peronospora belbahrii* (Cohen et al. 2017).

The findings from the infection trials conducted in climate chambers can be transferred to the greenhouse and field, where the onset of a downy mildew disease depends on the availability of inoculum, leaf wetness duration, and temperature (Cohen et al., 2017, Thiemann & Blum, 2019). For basil downy mildew it was shown that the interruption of a dew period with a dry period of only 10 minutes reduces the sporulation significantly (Cohen & Ben-Naim, 2016). Thus, in greenhouse cultures the reduction of humidity by fanning is a reliable method to reduce leaf wetness and to suppress the development of downy mildew infections, as it was shown for basil production (Cohen et al., 2017). For sage cultivation in the field applying a wider row spacing is recommended to promote air circulation between the plants (Grunert, 2017). Furthermore, Elad et al. (2016) showed that passive heat treatment of basil plants by increased air and root temperature suppresses the development of downy mildew disease.

They concluded that the suppressive effect of high temperatures on *Pe. belbahrii* is rather an indirect positive high-temperature effect on the host than a negative effect on the pathogen itself (Elad et al., 2016). Cohen & Rubin (2015) also observed a suppressive effect of temperatures above 35 °C on basil downy mildew. During the infection studies with sage downy mildew (Chapter 1) a positive effect on plant growth was observed when sage plants were incubated at 25 °C. Plants seemed to be able to outgrow the infection and build up more biomass than plants incubated at lower temperatures (data unpublished). Therefore, it could be also recommendable to expose pot plants for sage production in the greenhouse to temperatures above 30 °C for some hours.

1.2 Sexual reproduction

So far, oospores of *Pe. salviae-officinalis* have been observed just rarely and only on sage leaves (Plenk, 2002b, Liberato et al., 2006, Choi et al., 2009a). During the infection studies discussed in Chapter 1, oospores were regularly formed within infected leaf tissue of sage at temperatures between 15 and 25 °C. Besides the role of temperature in the formation of oospores, other factors influencing oospore development in sage downy mildew have not been investigated so far. Factors influencing oosporogenesis are often related to the ecological niche occupied by the species (Judelson, 2009), as the process can be strongly affected by the composition of culture medium, temperature, light, and pH (Inaba & Morinaka, 1983, van der Gaag & Frinking, 1996, Spring & Zipper, 2000, Kandel et al., 2019). For *Ps. cubensis* it was reported that oospore formation is initiated at the end of the growing season, when the infected leaf tissue becomes necrotic (Bedlan, 1989). Oospores of *Pe. salviae-officinalis* were also primarily observed in those parts of the infected sage leaves that became necrotic. Nutrition can also play a role in oospore formation. For downy mildews and *Phytophthora* it has been shown that low carbon/nitrogen ratios, which are typical for infected hosts, can stimulate oospore formation (McMeekin, 1960, Leal et al., 1970). In general, conditions that favour sexual and asexual sporulation are often negatively correlated, and in some species asexual sporulation is repressed within mating zones (Inaba & Morinaka, 1983, Pegg & Mence, 1970). Furthermore, it was assumed that gametangium differentiation and therefore oospore formation in oomycetes is regulated and coordinated by diffusible steroid hormones (Beakes & Thines, 2017). However, for several species of the peronosporalean

lineage it was shown that they are unable to synthesise sterols. They require these as supplements in order to reproduce sexually (Kerwin & Washino, 1983, Judelson, 2009).

1.3 Tracking the infection process

Until now little is known about the infection biology of *Peronospora salviae-officinalis*. Chapter 2 presents a histological study of various infection events of downy mildew infection on common sage. Histological studies of oomycetes are challenging due to the lack of chitin in their cell wall. To overcome this hurdle, a method for staining downy mildews for CLSM was developed: A 1:1 mixture of aniline blue and trypan blue was found most suitable for staining of downy mildew and plant structures. This stain combination allowed a clear distinction between the oomycetous and plant structures, as well as the visualization of plant immune responses. The method was evaluated also on samples of *Pe. lamii* on *Lamium purpureum* and *Pe. belbahrii* on *Ocimum basilicum*, suggesting the potential of this method for other downy mildews.

The newly developed staining method facilitated to follow and to document the complete life cycle of *Peronospora salviae-officinalis*. As in other downy mildews, the infection process of *Pe. salviae-officinalis* starts with a conidium attaching to the leaf surface of *S. officinalis*. Infection of the leaf tissue happens through a stoma or by direct penetration of epidermal cells. In the intercellular space hyphae spread between the mesophyll cells and develop haustoria, which are essential for nutrient uptake. At the end of the asexual infection cycle conidiophores emerge through stomata, bearing numerous conidia on multiple ramified branches. Within chlorotic to necrotic host tissue oospores develop. With the new staining method, it was also possible to detect callose deposition and accumulation in the leave tissue of host plants, in response to downy mildew infection.

1.3.1 Haustoria and callose in downy mildew infections

As mentioned before, callose deposition and accumulation towards infection sites was also observed in the study discussed in Chapter 2. Callose deposition is a well-known and unspecific plant immune response towards mechanic and pathogenic injury (Kortekamp, 2005, Judelson & Ah-Fong, 2019). Encapsulation of haustoria by callose is known for several downy mildew infections, for example those of *Plasmopara viticola* on *Vitis vinifera*

(Kortekamp, 2005, Diez-Navajas et al., 2008), *Pe. parasitica* and *Hyaloperonospora arabidopsidis* on *Arabidopsis thaliana* (Donofrio & Delaney, 2001, Caillaud et al., 2012, Caillaud et al., 2014, Fabro et al., 2011). In studies concerning the resistance of crop cultivars towards downy mildew infections, the presence and absence of haustoria encapsulation is used to evaluate the degree of the resistance (Donofrio & Delaney, 2001, Diez-Navajas et al., 2008).

In order to harbour haustoria, reorganisation of the host cell and biogenesis of the extrahaustorial membrane (EHM) is necessary. It was shown that secretory vesicles and endosomal compartments surround haustoria of *H. arabidopsidis* and *Ph. infestans,* potentially delivering plant membrane material to the EHM as the haustorium develops (Lu et al., 2012). Host cell wall polarization was also shown for *H. arabidopsidis* and *Ph. infestans*, and involved reorganization of the cytoskeleton, aggregation of the endoplasmatic reticulum (ER), central accumulation of Golgi bodies and migration of the nucleus towards the penetration site (Schmelzer, 2002, Schutz et al., 2006, Takemoto et al., 2003).

In later stages of downy mildew infections, haustorial encasements are frequently observed (Donofrio & Delaney, 2001, Diez-Navajas et al., 2008). Haustorial encasements are double-layered and cup-shaped membrane structures, which are rich in callose. They often surround older haustoria as a part of host cellular defence. They apparently originate from secretory vesicles and exocytic compartments (Donofrio & Delaney, 2001, Meyer et al., 2009). It seems that plant proteins accumulated at haustoria of *H. arabidopsidis* are recruited from default vesicle trafficking pathways in pathogen defence. In compatible interactions between pathogens and their hosts, the encapsulation of haustoria is delayed and lags behind the spread of the pathogen (Lu et al., 2012). It is assumed that encasements restrict the nutrient uptake by the pathogen, impair effector translocation or concentrate plant-derived antimicrobials (Judelson & Ah-Fong, 2019). In the case of *Pe. salviae-officinalis* just a few of the numerous formed haustoria possessed a callose collar around the neck or were encapsulated by callose. For *H. arabidopsidis* it is reported that this pathogen is able to suppress the callose deposition of *A. thaliana* by several effectors (Fabro et al., 2011). Also for other downy mildews, *e.g. Pl. halstedii* parasitizing sunflowers it is known that they are able to mask PAMPs or they can actively down-regulate the plant immune response (Sharma et al., 2015). In the present study, relatively little callose was observed around haustoria. As no major differences were observed between infected leaf samples of sage from four to ten

days after the inoculation it can be suggested that this was rather the result of parasite-suppressed immune response, than an indicator for the juvenility of the haustoria. The low abundance of callose depositions, and the late formation of chlorotic or necrotic leaf spots in infected plants indicate that *Pe. salviae-officinalis* is well adapted to its host.

1.4 Contamination of sage seeds

Several downy mildews are distributed by oospores attached to seeds of their host plants. This was shown for example for *Pe. belbahrii* (Garibaldi et al., 2004, Farahani-Kofoet et al., 2012), *Pe. effusa* (Kunjeti et al., 2016) and *Pe. somniferi* (Landa et al., 2007, Montes-Borrego et al., 2017). Conidia can be produced in huge amounts and are the dispersal units for long distance transport, as it was shown for cucurbit downy mildew (Holmes et al., 2004). Nevertheless, conidia are short-lived and their ephemeral character is a big disadvantage for long-term survival of the pathogen (Beakes & Thines, 2017). Oospores as long lasting dispersal units, attached to the seeds of the host plants, could help downy mildews to overcome this shortcoming (Thines, 2014). Although, contamination of seeds by downy mildew agents is common (Garibaldi et al., 2004, Landa et al., 2007, Cohen et al., 2014, Kunjeti et al., 2016), transmission of downy mildew diseases by seeds is very rare (Cohen et al., 2014). For *Ps. cubensis* it was shown that only 1.6 % of contaminated seeds sown resulted in infected hypocotyls (Cohen et al., 2014), and for *Pe. belbahrii* the reported transmission rate ranged between 0.33 to 23 % (Garibaldi et al., 2004, Farahani-Kofoet et al., 2012). Other studies, however, were not able to show the transmission of *Pe. belbahrii* via basil seeds (Cohen et al., 2017, Falach-Block et al., 2019). In the present study several attempts to recover *Pe. salviae-officinalis* from sage seeds priorly tested positive for contamination with this downy mildew failed (unpublished data), despite optimal environmental conditions for downy mildew infection. Nevertheless, the seed-borne nature of downy mildew diseases poses a major risk regarding anthropogenic introduction of pathogens to new areas (Thines, 2014), as is evidenced by the examples of *Pe. belbahrii* and *Pe. somniferi*, and as the sage monitoring in Germany indicates (Thiemann & Blum, 2019).

Before the present study oospores of *Pe. salviae-officinalis* had been observed only on sage leaves (Plenk, 2002b, Liberato et al., 2006). In the present study the contamination of seed

lots of common sage by conidia and oospores of *Pe. salviae-officinalis* was demonstrated using PCR and DNA-sequencing and a seed filtration method as discussed in Chapter 1. Although contamination of sage seeds with *Pe. salviae-officinalis* could not be quantified with the applied methods, the results of the seed testing demonstrated that seeds carrying inoculum pose a potential risk for sage production. German sage growers usually use sage seeds obtained from their own stands. This propagation method bears the risk of introducing *Pe. salviae-officinalis* to new fields or areas, free from this pathogen so far. Regarding the contamination with downy mildew it is suggested that sage seeds should be tested for *Pe. salviae-officinalis* contamination before sowing and that seed treatments should be applied if necessary. This advice is supported by observations of a monitoring study conducted in 2016 and 2017 in sage stands throughout Germany. *Peronospora salviae-officinalis* was detected in all sage stands investigated, even in newly sown stands (Thiemann & Blum, 2019).

Vegetative dispersal units such as cuttings of infected sage plants, also pose the risk to introduce the disease to new areas (Thines, 2014, Montes-Borrego et al., 2009, Cohen et al., 2017). Pathogen-free *in vitro* plants obtained by heat treatment, like those used for the studies presented in Chapter 1 and 2, could help to establish healthy stocks of mother plant for seed or cutting production. In addition, oospores in infested soil or leaf debris are also an effective source for early infections through roots or cotyledons and leaves close to the ground, as shown by Montes-Borrego et al. (2009) for downy mildew infections of opium poppy. As infected leaf debris decomposes and oospores are released, inoculum in soil may accumulate over the years (Montes-Borrego et al., 2009). It can be assumed that this is also true for downy mildew infections on sage. Thus, particular caution is recommended concerning crop residues on the infected stands.

The role of conidia and oospores in epidemics of downy mildews is still a point for discussion. In some downy mildews the disease emerges in the new season from oospores in plant debris and soil, while others are of seed-borne nature with oospores attaching on crop seeds (Lebeda & Cohen, 2010). Especially for some seed-borne downy mildew infections, researchers are quite sure that oospores are the main source of primary inoculum causing downy mildew epidemics (Spring & Zipper, 2000, Viranyi & Spring, 2010, Montes-Borrego et al., 2017). In heterothallic oomycetes, sexual recombination leading to oospore formation can generate extensive genetic variation in a pathogen population and promote the development of new races (Kandel et al., 2019). For *Pe. somniferi* and *Pl. halstedii* it was suggested that

oospores formed on infected leaves as secondary inoculum for leaf infections are more important, and play a primary role in the development of polycyclic downy mildew epidemics (Spring & Zipper, 2000, Viranyi & Spring, 2010, Montes-Borrego et al., 2017). In other downy mildew diseases asexual spores are considered to be the primary and main infective unit (Lebeda & Cohen, 2010). For *Pe. salviae-officinalis* conidia as well as oospores seem to play an important role in epidemics as the infection studies and the monitoring show. Probably, it is the combination of both kinds of downy mildew spore types, giving rise to new infections and enabling the survival of the pathogen.

1.5 Host range of *Peronospora salviae-officinalis*

So far common sage was the only known host of *Peronospora salviae-officinalis*. Chapter 3 presents morphological and phylogentic investigations of sage downy mildew and closely related downy mildew species on other Lamiaceae. Based on molecular phylogenetic inferences the downy mildews parasitizing *Salvia sclarea* and *S. pratensis* were clearly distinct from *Pe. lamii*, but also from *Peronospora swinglei*. Phylogenenetics as well as the morphological investigations strongly support that the downy mildew from *S. sclarea* is conspecific with *Pe. salviae-officinalis*, thus adding this host for this species. Clary sage which is closely related to common sage (Will & Classen-Bockhoff, 2014) and is also cultivated as a medicinal plant, likely acts as alternative host for *Pe. salviae-officinalis* and is a potential inoculum source for the dissemination of the disease. In contrast, the wild sage species *S. pratensis* most likely does not play a role as primary inoculum for downy mildew epidemics in cultivated common sage as *Pe. salviae-pratensis* sp. nov. clusters as a phylogenetic group distinct from *Pe. salviae-officinalis*, but closely related to *Pe. glechomae* (see Chapter 3). These findings are important from a phytopathological point of view. In addition, basil downy mildew can be excluded from the list of pathogens potentially infecting common sage. Even though *Pe. belbahrii* was able to infect *S. eigii*, *S. fruticosa* and *S. pinnata* under laboratory conditions, it was not able to infect *Salvia officinalis* (Ben-Naim et al., 2019). This is in line with observations made in the present study, where basil downy mildew was never detected on samples of *S. officinalis*, not even if the samples originated from greenhouses harbouring basil heavily infested with *Peronospora belbahrii*.

2 Phylogeny and taxonomy of downy mildews on Lamiaceae

With more than 1.000 species, the Peronosporales are the largest order of the oomycetes (Thines, 2014). More than 400 species have already been described only within the genus *Peronospora* (Constantinescu, 1991), and it is suggested that the vast majority of species in this genus remains to be discovered (Thines & Choi, 2016). Especially the downy mildews of Fabaceae (Garcia-Blazquez et al., 2008) and Amaranthaceae (Choi et al., 2015a) seem to be highly diverse, but also for the Lamiaceae, several dozens of hosts have been reported (Constantinescu, 1991, Dick, 2001). Within Lamiaceae, the tribe Mentheae contains several *Peronospora* species occurring on culinary herbs and medicinal plants (Dick, 2001, Choi et al., 2009a, Thines et al., 2009c), and two species belonging to the *Peronospora belbahrii* species complex, *Pe. belbahrii* and *Pe. salviae-officinalis*, have proven to be particularly destructive as emerging pathogens in basil and common sage production, respectively. Chapter 3 presents the morphological and phylogenetic investigations of sage downy mildew and closely related downy mildew species on other *Lamiaceae,* with a special focus on the *Pe. belbahrii* species complex. The investigations revealed two additional downy mildew species on Lamiaceae, *Pe. salviae-pratensis* parasitizing *Salvia pratensis* and *Pe. choii* parasitizing *Plectranthus scutellarioides*, proving that the *Pe. belbahrii* species complex is still not fully disentangled and adding some of the expected 'missing' species diversity to the genus *Peronospora*.

The presented phylogenetic analyses of downy mildews on Lamiaceae were performed using six loci. The combination of four nuclear (ITS, *EF1a*, *HSP90* and *β-tubulin*) and two mitochondrial (cox1 and cox2) gene regions resulted in generally in highly-supported clades. Additionally, no supported discordance between mitochondrial and nuclear loci was observed. This is in line with previous studies, where also no discordance between mitochondrial and nuclear loci was observed (Choi & Thines, 2015, Choi et al., 2015a), but in contrast to the findings of a recent study on Peronosporaceae, where incongruences between nuclear and mitochondrial loci were recognised (Bourret et al., 2018).

ITS data were highly similar for closely related species of *Peronospora* investigated in this study, as previously shown for downy mildews on Amaranthaceae, Lamiaceae and Papaveraceae (Thines et al., 2009a, Voglmayr et al., 2014, Choi et al., 2015b). Even though ITS sequences showed sufficient resolution to distinguish *Pe. salviae-officinalis* from most of the other specimens of the *Pe. belbahrii* species complex with varying support, discrimination

between *Peronospora* species from *Elsholtzia*, *Glechoma,* and *Salvia* was not possible. Nevertheless, as the multi-locus phylogeny shows, the lack of ITS variability does not automatically confirm the conspecifity of those species. In contrast to ITS, *cox*2 resolved most of the lineages that were found by the six-gene phylogeny and thus qualified as a suitable barcoding marker for *Peronospora* species as demonstrated before (Voglmayr et al., 2014, Choi et al., 2015b). In addition, DNA extracted from older herbarium samples could be successfully used for amplification of the *cox*2 gene, which is in line with previous studies (Telle & Thines, 2008, Choi et al., 2015b). Voglmayr et al. (2014) also recommended *cox*1 as a barcode marker for *Peronopsora* species, even though they reported the same disadvantages observed in the present study as well as by Choi et al. (2015b), as amplification rates of *cox*1 are lower than those of *cox2*. Nevertheless, in the study presented in Chapter 3 *cox*1 also resolved most of the lineages that were found by the six-gene phylogeny, confirming the results of Voglmayr et al. (2014). While several studies discussing multi-locus phylogenies of downy mildews often use only ITS rDNA as nuclear gene region besides the two mitochondrial markers *cox1* and *cox2* (Choi et al., 2011, Voglmayr et al., 2014, Choi et al., 2015b), here also the nuclear markers *EF1a*, *HSP90* and *β-tubulin* were used. The suitability of those nuclear gene regions for the application in multi-locus phylogenies was shown before for downy mildews and *Phytophthora* species (Blair et al., 2008, Runge et al., 2011b, Choi et al., 2015a). In the study presented in Chapter 3 the *EF1a*, *HSP90* and *β-tubulin* genes performed as well as *cox1* and *cox2* in terms of phylogenetic resolution, and could also be amplified reliably after primer optimization. In general, trees obtained with the same methods but based on single, non-concatenated loci showed no conflicting topology with the multi gene analyses based on the concatenated sequences (data unpublished), which is in line with the study ofChoi et al. (2015a).

Based on phylogenetic analyses of the *cox*2 gene region distinct lineages within the *Pe. belbahrii* species complex are grouped according to their host genus indicating a high degree of host specialisation. The downy mildews *Pe. salviae-officinalis* on *S. officinalis* and *S. sclarea*, *Pe. salviae-plebeiae* on *S. plebeia* and *Pe. salviae-pratensis* on *S. pratensis* cluster in a yet unsupported '*Salvia*' group, also including *Pe. elsholtziae* on *Elsholtzia ciliata* and *Pe. glechomae* on *G. hederacea,* which form sister groups to *Pe. salviae-pratensis* in the *cox*2 phylogeny. *Peronospora belbahrii* on *O. basilicum* and *Pe. choii* on *P. scutellarioides* cluster separately also without support for their monophyly, however a common phylogenetic origin of both groups together is strongly supported. Interestingly, *Pe. swinglei* on *S. reflexa* does

not cluster with the downy mildews of the core '*Salvia*' group investigated here. Indeed, *Pe. swinglei* is more closely related to *Pe. lamii* (Chapter 3). This is in line with the studies of Choi et al. (2009a). A similar situation was observed in a phylogenetic study of *Bremia* species infecting Asteraceae. While several phylogenetically distinct lineages grouped according to their host genus or species, the species *Bremia tulasnei* showed a broad host range within three distinct tribes of Asteraceae (Choi et al., 2011). These findings suggest that instead of strict co-speciation, diversity of downy mildews might have been shaped by host shifts and subsequent radiation and specialization (Choi & Thines, 2015, Thines, 2019).

2.1 *Peronospora choii* sp. nov. and *Pe. salviae-pratensis* sp. nov.

In the present study, it was shown that the downy mildew species on coleus (*P. scutellarioides*) and *basil* (*O. basilicum*) can be reliably distinguished by differences in conidial shape, size and coloration, as well as in the shape of the ultimate branchlets of the conidiophores. In addition, phylogenetic analyses using four nuclear and two mitochondrial gene regions clearly resolved the downy mildew affecting *P. scutellarioides* as a highly supported monophyletic sister group to *Pe. belbahrii* s. str., and subsequently was described as *Pe. choii* in this study. Comparable to *Pe. belbahrii* and *Pe. salviae-officinalis* (McMillan, 1993, Martini et al., 2003) the downy mildew disease of coleus had initially been lumped within *Pe. lamii* (Daughtrey et al., 2006, Palmateer et al., 2008), but was then relegated to *Peronospora belbahrii* s.l. (Thines et al., 2009a).

It was also shown that the downy mildews parasitizing clary sage and meadow sage, respectively, were clearly distinct from *Pe. lamii*, but also from *Pe. swinglei* based on phylogenetic and morphological analyses (see Chapter 3). While the downy mildew on *S. sclarea* was conspecific with *Pe. salviae-officinalis*, the downy mildew parasitizing *S. pratensis* was considered to be a species of its own, *Pe. salviae-pratensis*, which forms a sister group to *Peronospora glechomae,* described on *Glechoma hederacea* (Oescu & Radulescu, 1939). Although the downy mildew accessions from *G. hederacea* and *S. pratensis* are closely related, there are subtle differences between the downy mildew found on meadow sage and those from *Pe. glechomae* based on the phylogenetic and in morphological data.

Thus, like the studies on *Pe. effusa* (Choi et al., 2007) and *H. parasitica* (Choi et al., 2003, Göker et al., 2007) already showed, and as the results of this study confirm, that the assumption of a broad host range is, with few exceptions, not appropriate for Peronosporales. It could be shown again that the taxon *Pe. lamii* should be restricted to the downy mildew parasitizing *Lamium* spp. or *L. purpureum* only (Choi et al., 2009a, Thines et al., 2009a). The present study confirms again that the narrow species concept proposed by Gäumann (1923) reflects the diversity of downy mildews in Lamiaceae much better. The tradition of relegating downy mildew species on Lamiaceae to the collective terms of *Pe. lamii* or *Pe. swinglei* should be rejected, also with respect to the establishment of quarantine regulations if new species emerge. In fact, the often-applied broad species concept leads to wrong conclusions in diagnostics and therefore high economic losses by refrained quarantine measures (Thines, 2014). As mentioned before, the taxa *Pe. lamii* and *Pe. swinglei* are sometimes used synonymously (Brandenburger & Hagedorn, 2006). However, as the results of the present study and earlier studies showed (Choi et al., 2009, Thines et al., 2009), the species *Pe. lamii* and *Pe. swinglei* are two separate species and should not be treated as synonyms.

3 Challenges in identifying plant pathogenic fungi

Many challenges are associated with the identification of plant pathogenic fungi or fungal like organisms. Despite numerous investigation methods, such as diverse microscopy techniques or DNA sequence comparisons, our knowledge of the diversity and biology of the pathogens is often insufficient. In several cases just a part of the life cycle is known. Prominent examples are asexual fungi, previously known as 'Fungi Imperfecti' or 'Deuteromycetes', an artificial group of true fungi comprising members of the Ascomycota or Basidiomycota for which only asexual structures are known so far (Piepenbring, 2015, Dyer & Kück, 2018). Nevertheless, species definitions for plant pathogens have considerable practical impact for plant protection and pathogen control. However, in many groups the delimitation of species is an infamous difficult taxonomic problem. This is particularly evident in the obligate biotrophic downy mildew genera, which display a considerable diversity with respect to genetic distances and host plants, but in many cases are morphologically rather uniform (Garcia-Blazquez et al., 2008, Göker et al., 2009). For the important and very common downy mildew pathogens on spinach and beet for example, a proper nomenclature and the identity of these pathogens

were published only recently (Choi & Thines, 2014, Choi et al., 2015a), although they have been reported for most countries cultivating those crops (Gäumann, 1923, Byford, 1967, Brandenberger et al., 1991, Choi et al., 2015a).

Many problems in identifying downy mildews certainly have their roots in the application of the broad species concept advocated by (Yerkes & Shaw (1959), as is evidenced by the downy mildews within the *Pe. belbahrii* species complex (Thines, 2014). Both, downy mildew on basil and downy mildew on common sage were initially classified as *Pe. lamii* (McMillan, 1993, Gamliel & Yarden, 1998, Belbahri et al., 2005a). With an assumed broad host range, precautions were not sufficiently taken and both pathogens were disseminated rapidly over the whole world (Gamliel & Yarden, 1998, Plenk, 2002b, Hill et al., 2004, Belbahri et al., 2005a, Liberato et al., 2006, Humphreys-Jones et al., 2008, Choi et al., 2009a, Wyenandt et al., 2010). Basil and sage downy mildew could not have spread as easily if it had been realised earlier that downy mildew pathogens of Lamiaceae are diverse and contain several distinct species (Constantinescu, 1991), as Gäumann (1923) already concluded. In this study again two downy mildew species on Lamiaceae were described. While *Pe. salviae-pratensis* parasitic to *S. pratensis* seems to be widespread within natural populations of meadow sage already (Gaponenko, 1972, Preece, 2002, Dudka et al., 2004, Mulenko et al., 2008, Müller & Kokes, 2008), *Pe. choii* parasitic to coleus was so far reported only for Japan (Ito et al., 2015), UK (Denton et al., 2015), USA (Daughtrey et al., 2006, Palmateer et al., 2008), and recently from Brazil (Gorayeb et al., 2019). Considering the fact that *Pe. choii* does not occur as widely distributed as *Pe. belbahrii* quarantine measures might still be useful to prevent the further spread of this disease throughout the world. Nevertheless, assuming a broad host range for downy mildew pathogens like *Pe. belbahrii* can also lead to an overestimation of a disease risk (Thines, 2014). In this case it could for example be deduced that basil downy mildew is a threat for crops of the Lamiaceae family. However, even though the potential host range of *Pe. belbahrii* under laboratory conditions comprises not only *O. basilicum* (Cohen et al. 2019), it can be supposed that under naturally occurring conditions basil downy mildew most likely does not parasitize other Lamiaceae than basil.

Determination keys for downy mildews are usually not only based on morphology, but rather incorporate the host species for species determination (Gäumann, 1923, Garcia-Blazquez et al., 2008). Although a narrow host range has been proven for many downy mildews (Gäumann, 1923, Choi et al., 2009a, Voglmayr et al., 2014, Choi et al., 2015a), accurate

species determination based on of the host plant only is not recommendable, as some downy mildew taxa do have multiple hosts (Crandall et al., 2018), such as *Ps. cubensis* (Leppik, 1966, Sackston, 1981) and *Pl. halstedii* (Leppik, 1966, Sackston, 1981). Furthermore, some host species are potentially parasitized by several downy mildews, e.g. it is suggested that *Cucumis sativus* can be infected naturally by *Ps. cubensis* and *Ps. humilii* (Runge & Thines, 2012). A special case which can make the identification of plant pathogens even more difficult is the infection of alien crops. Plant species not indigenous to an area can be infected by pathogens naturally occurring in this area (Thines, 2014). Thus, a pathogen known to be specific to one host species or genus in its native range suddenly occurs on a so far unknown host. One prominent example is maize, which originates from Mexico (van Heerwaarden et al., 2010), and is attacked by at least seven downy mildew species of the genus *Peronosclerospora* (Kenneth, 1981, Telle et al., 2011) originating from Asia or Australia (Spencer & Dick, 2002). Furthermore, the host range of downy mildews and other pathogens is not cast in stone and should be considered as a snapshot in time including some blind spots, as we will probably never find all downy mildew infections present in nature.

In this study with *S. sclarea* a second host was added for *Pe. salviae-officinalis,* which previously was only known from *Salvia officinalis* (compare Chapter 3). For *Ps cubensis* it is suggested that this downy mildew with more than 60 reported host species in Cucurbitaceae (Lebeda & Cohen, 2010, Runge et al., 2011a) is still actively radiating (Thines & Choi, 2016). This might be also true for the *Pe. belbahrii* species complex, rendering the discovery of new hosts for some of these species likely, especially if they occur outside their native range (Thines, 2019).

We should be aware that every species concept we postulate is an artificial one. The morphological and phylogenetic data gathered in this study suggest that species identification of *Peronospora* needs a holistic approach including morphological and DNA-based molecular studies. As morphological features of closely related downy mildews are often very similar (Choi et al., 2005, Garcia-Blazquez et al., 2008, Choi et al., 2011, Runge & Thines, 2012, Choi et al., 2015a), sequencing data should corroborate the results (Voglmayr, 2003, Göker et al., 2009). This becomes more important in view of the fact that the host matrix can influence the morphology of Peronosporales (Runge & Thines, 2010, Runge et al., 2012), and some downy mildew taxa have multiple hosts as mentioned before (Crandall et al., 2018). Additionally, in downy mildew collections from natural populations especially the length of the

conidiophores seems to be highly divergent (Choi et al. 2011 and Chapter 3). Furthermore, some studies reported that the age of the sample taken (Gäumann, 1923) and/or environmental conditions can influence morphological characters (Cohen & Eyal, 1977, Dudka et al., 2007). Implementing a two site approach in species delimitation based on morphological and phylogenetic analyses could help to improve plant protection strategies and pathogen control. In addition, infection studies with an extended range of potential host species could also improve our understanding of a plant pathogen.

4 Concluding remarks

The findings of this thesis are useful both in adjusting the combat strategies actually applied in sage production against sage downy mildew and to support further investigations into downy mildews on Lamiaceae.

In the present study we determined the impact of basic epidemiological factors like temperature and moisture on speed and intensity of disease development to improve our understanding of the infection process. Under favourable conditions *Pe. salviae-officinalis* grows rapidly and builds-up high amounts of inoculum. The finding that *S. sclarea* serves as a host for *Pe. salviae-officinalis*, complement the results of Chapter 1 concerning the epidemiology of the pathogen. Together with the proven seed transmission character, two new factors influencing the spread of the disease were elucidated in this thesis. The frequent contaminations of sage seed lots with *Pe. salviae-officinalis* found could explain the rapid worldwide spread of the disease.

This is the first phylogenetic analysis of downy mildews on Lamiaceae based on six nuclear and mitochondrial gene regions. The combination of the used loci resulted in a generally highly resolved topology. Our study also showed again that *cox*2 is a suitable barcoding marker for *Peronospora* species as the analyses of (Choi et al., 2009a) and (Voglmayr et al., 2014) had already demonstrated. Also older herbarium samples were successfully amplified by the *cox2* primer set, and *cox*2 sequences showed a sufficient resolution to distinguish between different closely related *Peronospora* species within the Lamiaceae. Whereas ITS data are highly similar within closely related species of the investigated downy mildews and therefore do not allow for unequivocal distinction, the gene regions *cox*1, *EF*1a, *HSP*90 and

β-tubulin are highly distinctive for the accessions from various hosts, and therefore are also suitable for species discrimination within downy mildews on Lamiaceae.

From a phytopathological point of view the results from this study showed that the wild sage species *S. pratensis* most likely does not play a role as primary inoculum for downy mildew epidemics in cultivated common sage. In contrast, clary sage, acts as alternative host for *Pe. salvia-officinalis* and is a potential inoculum source for the dissemination of the disease. Besides identifying a supplemental host for *Pe. salviae-officinalis* and consequently proving a broader host range for this pathogen, this study showed again that the host range of *Pe. lamii* and *Pe. swinglei* is not as broad as assumed before. Additionally, our results support again that the name *Pe. lamii* should be restricted to downy mildews parasitizing *Lamium* spp. , or even L. purpureum only (Choi et al., 200), and not for downy mildew on sage. In our study it was confirmed again, that a narrow species concept reflects much better the diversity of downy mildews in Lamiaceae.

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Appendix 1

Statement of Joint Authorship

On the publication: Epidemiology of sage downy mildew, *Peronospora salviae-officinalis*

Status: published (https://doi.org/10.1007/s10658-020-01967-1)

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Involved authors:

MH: Mascha Hoffmeister (PhD student)

DG: Doreen Gabriel

MT: Marco Thines

WM: Wolfgang Maier

What did the PhD student or the Co-Authors contribute to this work?

(1) Development and planning

PhD student MH: 70%

Co-Author MT: 10%

Co-Author WM: 20%

(2) Performance of the individual investigations and experiments

PhD student MH: 100%

(3) Preparation of the data collection and figures

PhD student MH: 80%

Co-Author DG: 20%

(4) Analysis and interpretation of data

PhD student MH: 50%

Co-Author DG: 35%

Co-Author MT: 5%

Co-Author WM: 10%

(5) Writing the manuscript

PhD student MH: 60%, corresponding author

Co-Author DG: 10%

Co-Author MT: 10%

Co-Author WM: 20%

Verification of the statements above:

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Date/ Place	Signature PhD student
_____	_____
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Date/ Place	Signature corresponding author

Chapter 1

Epidemiology of sage downy mildew, *Peronospora salviae-officinalis*

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Abstract

Downy mildew of common sage (*Salvia officinalis*), caused by *Peronospora salviae-officinalis*, has become a serious problem in sage production worldwide. The effect of temperature was determined for conidia germination and disease development. *In vitro*, conidial germination rate was highest at temperatures between 10 °C and 20 °C and was strongly reduced at temperatures above 25, but conidia were also able to germinate at 2 °C. Temperatures between 15 and 20 °C were most favourable for infection and disease progress in infection experiments in climate chambers, with highest sporulating leaf area observed at 15 and 20 °C, and highest symptomatic leaf area at 20 °C. *P. salviae-officinalis* is still able to infect sage plants at 5 °C, but sporulation was only observed at higher temperatures. Oospores developed 14 days after inoculation at 15 °C, and 8 days after inoculation at 20 and 25 °C. The infection trials also showed that dark incubation is not a prerequisite for successful infection of sage. Furthermore, *P. salviae-officinalis* needs two events of leaf wetness or high humidity to complete its asexual life cycle. First, a leaf wetness event of at least three hours is needed for conidial germination and penetration of the host. Second, high humidity of at least 90 % is needed at the end of the infection cycle for sporulation.

Seed washing revealed the presence of *P. salviae-officinalis* oospores in seed lots of sage suggesting that infested seeds might play a major role in the fast spread of sage downy mildew, which is an important finding for phytosanitary or quarantine measures.

Introduction

Common sage, *Salvia officinalis* (Lamiaceae), is the economically most important sage species (Raal et al., 2007, Mirjalili et al., 2006) and has been used as a medicinal plant since ancient times due to its antimicrobial activities (Longaray Delamare et al., 2007). Furthermore, these aromatic plants are used as culinary herbs and flavouring agents, and for perfumery and cosmetics (Sulniute et al., 2017).

Over the past two decades several downy mildew diseases in medicinal and spice plants have been newly reported and led to economic losses, for example *Peronospora somniferi* on opium poppy (*Papaver somniferum*) (Voglmayr et al., 2014), *P. belbahrii* on basil (*Ocimum basilicum*) (Thines et al., 2009b), and *P. salviae-officinalis* on common sage (Choi et al., 2009b). To our knowledge the downy mildew on *S. officinalis* has been officially reported for the first time in Florida in 1993, but had been initially attributed to *P. lamii* (McMillan, 1993). Earlier reports of downy mildews on sage species mention the occurrence of *P. lamii* and *P. swinglei* on *S. pratensis* (meadow sage), *S. lanceolata* (lance-leaf sage) or *S. sclarea* (clary sage) but not on *S. officinalis* (Rabenhorst, 1857, Ellis & Kellerman, 1887, Gäumann, 1923, Kochman, 1970, USDA, 1960, Osipjan, 1967, Stanjavičenie, 1984). In the meantime, downy mildew on common sage has been reported from Israel, Austria, Italy, New Zeeland, Australia, UK and Germany (Gamliel & Yarden, 1998, Plenk, 2002a, Hill et al., 2004, Belbahri et al., 2005b, Liberato et al., 2006, Humphreys-Jones et al., 2008, Choi et al., 2009b).

Despite the fact that *P. salviae-officinalis* was described a decade ago (Choi et al., 2009b) very little is known about its infection biology and epidemiology. This knowledge is, however, crucial for targeted and efficient pest management strategies against downy mildew in sage production as well as for the development of forecast models. It is also still unclear how the disease was able to spread throughout the world so quickly. It was hypothesized that downy mildew on *S. officinalis* might has been distributed by contaminated seed lots (Choi et al., 2009b), but proof for this hypothesis is still lacking and oospores, which could play an important role in the spread of the disease, have been reported only on sage leaves (Plenk, 2002a, Liberato et al., 2006, Humphreys-Jones et al., 2008).

The objectives of the present study were to examine the epidemiological parameters for successful infection of sage by *P. salviae-officinalis*. The effect of temperature on

conidia germination was determined *in vitro*. The effect of temperature and leaf wetness on infection and disease development was evaluated in climate chamber experiments *in vivo*. Furthermore, we screened for oospores during the infection trials to evaluate whether and how frequently this long-lasting inoculum source is produced. We also examined seed lots of sage for downy mildew contamination using a seed washing method in combination with light microscopy, as well as PCR-based detection, to determine if the pathogen is present in traded seed lots.

Material and Methods

Inoculum production and inoculation

Infection experiments were carried out using a *P. salviae-officinalis* population isolated from a common *s*age stand in Saxony (Germany). The downy mildew was cultivated and reproduced on *S. officinalis* under controlled conditions as described below. To ensure that the plants were healthy before inoculation, sterile-grown *in vitro* plants (Elsner pac Vertriebsgesellschaft mbH, Dresden, Germany) were used. After delivery, the plants were transferred from the cups with culture media to multipot plates (8 x 12 plots/ plate) filled with seeding compost and cultured in the greenhouse (20 °C, 16h light/ 8h dark, 60 % humidity) under a hood for about two weeks. After root formation the hood was removed and single plants were potted into containers (8 cm diameter, 190 ml, Pöppelmann GmbH & Co. KG., Lohne, Germany) with standard potting compost (Substrat 1, Klasmann-Deilmann GmbH, Geeste, Germany; chemical analysis [mg per 100 g]: N = 140, P = 100, K = 180, Mg = 100, S = 120; salinity 1.0 g/L; pH 5.5). For maintenance of *P. salviae-officinalis* and infection studies, inoculation using a spray bottle was conducted as soon as the plants had developed four mature leaves.

Plants were inoculated by spraying a conidial suspension (1 x 10^4 conidia ml^{-1}) on the upper and lower leaf surface until run-off. Inoculated plants were incubated under a hood placed in a plant growth chamber (Percival Scientific, Perry, IA, U.S.A) with 15 °C, 100 % relative humidity (rh.) and 12 h light/12 h dark for 7 to 8 days until sporulation. Sage leaves with mature conidia were harvested from the plants. For infection studies only fresh inoculum was used. Conidial suspensions were prepared by rinsing off conidia with deionised water. The conidial density was calculated using a microscope (Zeiss Axioskop 2 plus compound microscope, Carl Zeiss Microscopy

GmbH, Jena, Germany) and a haemocytometer (Fuchs-Rosenthal, 0.200 mm, 0.0625 mm², Glaswarenfabrik Karl Hecht GmbH & Co KG, Sondheim v. d. Rhön, Germany), and was adjusted to 10^4 conidia per ml by dilution with deionised water. For *in vitro* trials 4 ml of this conidial suspension was pipetted on the surface of 1.5 % water agar in a laminar flow hood. Moist sterile filter papers were inserted into the lids of the Petri dishes to preserve high humidity within the plates and to avoid drying of the suspension. Agar plates were also sealed with parafilm (Laboratory Film, Parafilm, Bemis, North America) and incubated under the conditions specified below. For each plate, germination of 100 randomly chosen conidia was assessed microscopically using an inverted microscope (Primovert, Carl Zeiss Microscopy GmbH, Jena, Germany). Damaged conidia or conidia without fully-developed germ tubes were not counted. In all experiments, one Petri dish represented a replicate. Sporulating and symptomatic leaf area was visually estimated by a single observer in percent with estimated values of 0, 1, 3, 5, 10 and increments of 10 up to 100 %.

Evaluating the effect of the light regime and relative humidity on disease development

The effect of an initial 24 h incubation in darkness (24h-dark) on disease development and disease severity was compared to direct incubation in a natural 12 hour day-night rhythm (12h-d/l) starting with the day phase. Plants were incubated at 15 °C and 100 % rh. The symptomatic and sporulating leaf areas were evaluated in percent leaf area at 7 dpi (days post inoculation) on 4 leaves per plant and 10 plants per treatment. The experiment was repeated twice resulting in 160 observations (4 leaves * 10 plants * 2 treatments * 2 replications in time).

The effect of the duration of leaf wetness directly after inoculation and relative humidity at the end of the infection cycle (5-7 dpi) on the disease development of *P. salviae-officinalis* on sage was tested. *In vitro* pre-trials on 1.5 % water agar were conducted to determine different developmental steps in the germination of conidia of *P. salvia-officinalis* during an incubation in the darkness at 15 °C. In *in viv*o trials inoculated plants were exposed for different time spans to 60 % or 100 % rh. to assess the effect of the leaf wetness duration after inoculation and high humidity at the end of the infection cycle. Plants were inoculated and incubated at 15 °C, 100 % rh. and a 12h-

d/n rhythm as described above. To manipulate leaf wetness duration the hood that ensured 100 % rh. was removed at different time points after the inoculation (3 hpi, 6 hpi, 12 hpi, 24 hpi, 48 hpi, and 5 dpi, Fig. 1) and plants were transferred to a growth chamber with 60 % rh. These time points were inferred from the results of the preceding *in vitro* experiment. From the optimisation of inoculum production it was known that *P. salviae-officinalis* starts to sporulate at 5 dpi when incubated at 15 °C. Hence, at the end of the infection cycle, i.e. at 5 dpi, half of the plants of each treatment were transferred again to the humid cabinet with 100 % rh. and covered with a hood (Fig. 1), while the other half was kept at 60 % rh. without a hood.

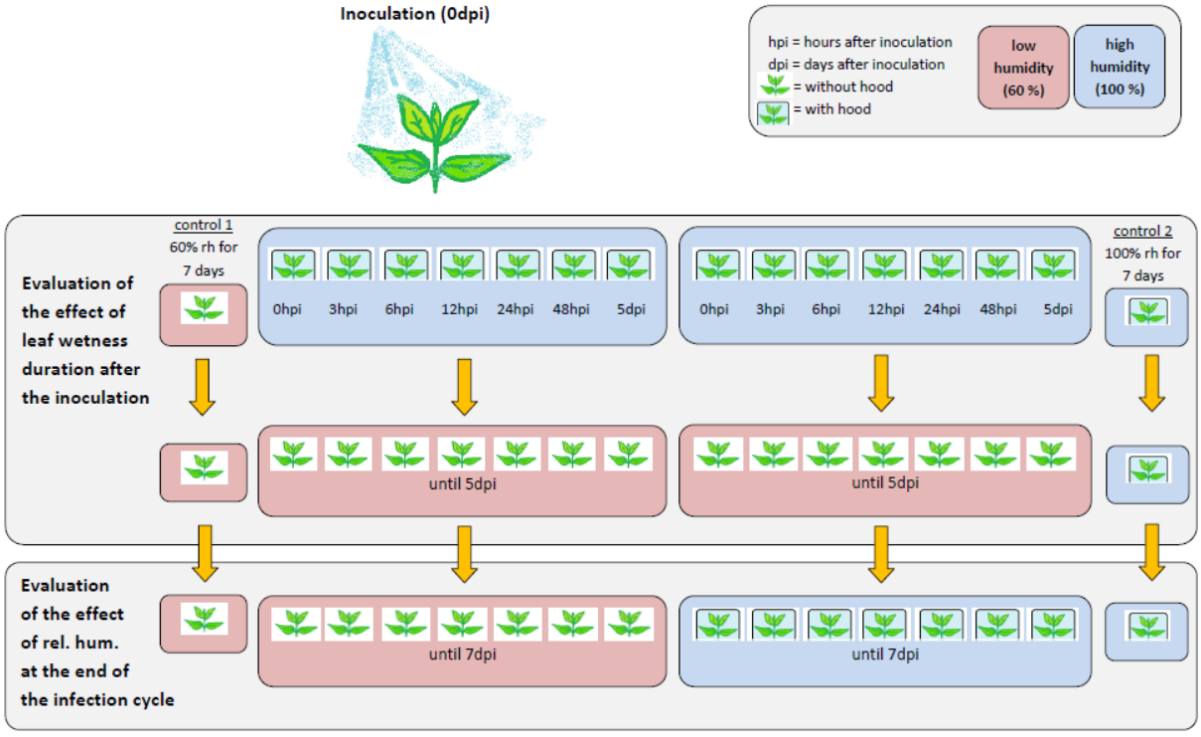

Fig. 1: Testing the effect of leaf wetness duration and relative humidity at the end of the infection cycle on disease development of *Peronospora salviae-officinalis* on *Salvia officinalis.* The diagram shows the different treatments. Control 1 was incubated over the whole time of the experiment at 60 % rh. Control 2 was incubated over the whole time of the experiment at 100 % rh

One control (control 1) was incubated at 60 % rh. directly after the inoculation until 7 dpi. A second control (control 2) was kept under a hood in the humid cabinet from inoculation until the end of the experiment at 7 dpi. Treatment "0h" was kept like control 1 until 5 dpi. Then the plants were transferred to 100 % rh. and covered with a hood until 7 dpi. Treatment "5dpi" was treated like the control 2 until 5 dpi, and then the plants were transferred to 60 % rh. and the hood was removed. At 7 dpi the

sporulating leaf area was evaluated in percent of total leaf area from 4 leaves per plant and 6 plants per treatment. The experiment was repeated twice, resulting in 672 observations (4 leaves * 6 plants * 14 treatments * 2 replications in time).

Evaluating the effect of temperature on conidia germination *in vitro* and disease development *in vivo*

The effect of temperature on germination of freshly harvested conidia was assessed *in vitro*. Petri dishes with 1.5 % water agar were inoculated as described above. Plates were incubated in the dark for 24 h and 48 h under the following temperatures: 2, 5, 10, 15, 20, 25, 30 and 35 °C, respectively. Germination rates were determined microscopically. Three replicates were carried out per treatment and the experiment was conducted three times.

To assess the effect of temperature on disease development and severity *in vivo* an experiment was conducted taking into account the results obtained from the *in vitro* germination study. Sage plants were inoculated and incubated at 5, 10, 15, 20 and 25 °C, respectively, 12h-d/n, and 100 % rh. in a plant growth chamber under controlled conditions as described above. The experiment was repeated three times and 16 plants (in experiments 1 and 2) and eight plants (in experiment 3), respectively, were inoculated per treatment. Sporulating leaf area and the percentage of symptomatic leaf area was evaluated in percent of total leaf area from 4 leaves per plant at 4, 5, 6, 7, 8, 9, 10 and 14 dpi.

All plants incubated at 5 °C did not show any signs of reaction to the inoculation. Hence, at 14 dpi they were transferred to 25 °C for another three days to test whether *P. salviae-officinalis* is able to infect sage at 5 °C, and to develop downy mildew once temperatures increase after the potential infection at 5 °C. The treatment at 5 °C was excluded from the statistical analysis. In total 2560 observations were included in the statistical analysis (4 leaves*16 plants*4 treatments*4 days of observation (8-14 dpi) = 1024 for experiment 1 and 2, 4 leaves*8 plants*4 treatments*4 days of observation (8-14 dpi) = 512 for experiment 3).

Seed testing for *P. salviae-officinalis*

Potential contamination of sage seed lots by *P. salviae-officinalis* was tested using eight seed samples produced by sage growers from different locations in Germany and Switzerland. This was done using both PCR-based and light microscopic detection methods, seed washing and spore filtration. For the PCR-based detection seed samples were first visually pre-checked for conspicuousness before PCR using a stereo-microscope. Single seeds showing whitish incrustation or lesions on the surface were used for DNA isolation. Samples were transferred individually to 2.0 ml reaction tubes and three tungsten carbide beads were added (3 mm diameter, Qiagen, Hilden, Germany). The samples were then cooled in liquid nitrogen and disrupted using a mixer mill (TissueLyser LT, Qiagen, Hilden, Germany) by shaking them twice at 50 Hz for 1.5 min with an intervening cooling step. Genomic DNA was extracted using the innuPREP Plant DNA Kit (Analytik Jena, Jena, Germany). ITS-PCR, specific for oomycetes, was conducted using the primers DC6 (Cooke et al., 2000) and LR-0 (Moncalvo et al., 1995). Amplification reactions were carried out in a reaction volume of 25 µl containing genomic DNA, 10 x Mango PCR Buffer, 0.2 mM dNTPs, 2 mM $MgCl^2$, 0.4 µM forward and reverse primers, and 1.5 units of MangoTaq Polymerase (Bioline GmbH, Luckenwalde, Germany). PCR conditions were as follows: an initial denaturation step of 95 °C for 3 min; 40 cycles of 95 °C for 30 s, 57 °C for 30 s, 72 °C for 1 min and 30 s, followed by a final extension of 72°C for 10 min. PCR products were separated by gel electrophoresis through a 0.8 % agarose gel (40 ml) stained with 2 µl SYBR safe DNA gel stain (ThermoFisher SCIENTIFIC, Dreieich, Germany) in TAE buffer. Visualization was done with UV light. PCR products were purified using the DNA Clean & Concentrator TM-5 Kit (ZYMO RESEARCH, Freiburg, Germany). Amplicons were sequenced at Eurofins Genomics (Eurofins Genomics GmbH, Ebersberg, Germany) using the same primers that were used for PCR. Forward and reverse strands were assembled and edited using Sequencher 5.4.1 (Gene Codes Corporation, Ann Arbor, Michigan, USA). Sequences obtained in this study were deposited in GenBank under accession numbers MN308034 - MN308053.

To identify the sequences obtained from the seeds that had tested positive in PCR for oomycetous contamination phylogenetic trees were inferred in MEGA 7 (Tamura et al. 2013) using Neighbour Joining analysis (NJ) (Saitou & Nei, 1987) and the Kimura 2-parameter model as substitution model. The robustness of the phylogenetic trees were

assessed on the basis of 1000 bootstrap (BS) replicates (Felsenstein, 1985). The data set consisted of sequences obtained from the positive PCR reactions, *P. salviae-officinalis* from infected sage leaves from this study, and several sequences of the closely-related *P. belbahrii* and other *Peronospora* species parasitic on Lamiaceae prepared by us. Also, sequences of *Pythium* species taken from GenBank selected according to BLASTn searches in GenBank were included in the analysis. A second NJ tree was inferred, using only the ITS sequences from *P. salviae-officinalis* and *P. belbahrii,* because of lacking resolution of these two species in the large data set.

A seed washing and spore filtration method developed for the detection of bunt (*Tilletia* spp.) in wheat seeds (Kietriber, 1984) was used to wash-off and trap potentially adhering spores from the surface of *S. officinalis* seeds. For the seed washing process, 5 g seeds per sample were added into a 250 ml Erlenmeyer flask with 80 ml of detergent solution (0.1% Tween 20 in deionised water) and were shaken for 20 min at 200 rpm. After shaking, the supernatant was immediately transferred to a second Erlenmeyer flask to separate it from the seeds. For filtration the supernatant was transferred into a Nalgene vacuum filtration system (MERCK, Darmstadt, Germany) with a moistened cellulose nitrate membrane filter (pore size 0.45 µm, diameter filter 50 mm, Sartorius AG, Göttingen, Germany). After filtration the filter was removed with tweezers and put into a small Petri dish until microscopic evaluation. Every seed lot was washed and filtered three times. The filter device was thoroughly cleaned after each seed sample to avoid carry-over of spores. For microscopy filter papers were cut in half. Each half was transferred to a microscopy slide in a 1:1 water-glycerine mixture and covered with a cover slip. Filters were screened under a compound microscope equipped with a Jenoptik ProgRes® digital camera. Nomarski Differential Interference Contrast (DIC) was used for observing, measuring, and taking pictures of morphological structures. Images were processed using CapturePro 2.8 (Jenoptic, Jena, Germany). Conidia and oospores detected on the filter paper were picked with an insect needle after the cover slip was removed, and transferred to a microscopy slide in water for better imaging.

Statistical analyses

All statistical analyses were performed using R version 3.6.1 (R Core Team, 2018) and various R packages. For generalized linear mixed effect models 'glmmTMB' was used (Brooks et al., 2017), 'effects' for displaying the effects from a fitted model (Fox, 2019), and 'emmeans' for computing estimated marginal means (EMMs) and performing post hoc tests (Lenth, 2019). Package 'dplyr' (Wickham, 2019) was used for data management and summary statistics. For graphics 'ggplot2' (Wickham, 2016), 'ggfortify' (Tang et al., 2016) and 'gridExtra' (Auguie, 2017), 'magrittr' (Bache, 2014) and 'ggpubr' (Kassambara, 2019) were used.

Data were analyzed using generalized linear mixed effect models, which model different probability distributions for the response, and estimate fixed and random effects. Disease development (sporulating leaf area (%)) and disease severity (symptomatic leaf area (%)) are both continuous response variables that vary between 0 and 100 %. Hence, we used beta regression mixed models with logit link since they provide predictions and confidence intervals limited to a range of >0 and <100 (Cribari-Neto & Zeileis, 2010). When measurements included 0 % or 100%, we transformed observations by either adding or subtracting a small constant x = 0.0000001 (Cribari-Neto & Zeileis, 2010).

The models for testing the effect of dark incubation on the sporulating leaf area (%) and the symptomatic leaf area (%) included the fixed effect incubation type (24h-dark and 12h-d/n) and the random effect leaf (1-4) was nested in plant ID (1-10), plant ID was nested within run of the experiment (1, 2). The random effect accounts for the hierarchy in design, i.e. the four measurements per plant and the three runs of the experiment. Similar models were fitted for testing the effect of leaf wetness duration (0 hours, 3h, 6h, 12h, 24h, 48h, 5 days to 7 days) on sporulating leaf area (%) and the symptomatic leaf area (%) for the treatment of high humidity (100 %) at the end of the infection cycle. The observations of the treatment with low humidity (60 %) at the end of the infection cycle were not included into the statistical analysis since only 2 out of 336 cases showed sporulation.

To describe the temperature response curve and find the optimum temperature for conidia germination *in vitro*, summary statistics on minimum, median and maximum of observed values are graphically displayed for each experiment. To evaluate the effect

of incubation temperature at different time steps after inoculation on disease development (sporulating leaf area (%)) *in vivo*, a beta regression mixed effect model was fitted with the fixed effects incubation temperature (10, 15, 20 and 25 °C) and days post inoculation (8, 9, 10 and 14 dpi) and its two-way interaction. Random effect was leaf (1-4) nested in plant ID (1-16/ 1-8) nested in treatment (temperature) nested in run of the experiment (1-3).

The significance of fixed effects was tested by likelihood-ratio chisquare test. Tukey tests were employed for multiple comparisons of estimated marginal means using the emmeans package ($P < 0.05$). Residuals were plotted against fitted values and visually checked for patterns in all models and no abnormalities were observed.

Results

The effect of dark incubation and relative humidity on disease development *in vivo*

Both sporulating leaf area (p = 0.503; estimated mean 24h-dark = 95.4 %; estimated mean 12h-d/n = 94.9 %) and symptomatic leaf area (p = 0.644; estimated mean 24h-dark = 6.0 %; estimated mean 12h-d/n = 5.2 %) did not differ significantly between the two treatments, 24h-dark and 12h-d/n (Fig. 2). For sporulating leaf area the medians and the upper quartile of both treatments were 100 %. For symptomatic leaf area the medians of both treatments were similar with 5 % for the 24h-dark and 0 % for 12h-d/n treatment. The lower quartile was 0 % for the symptomatic leaf area of both treatments.

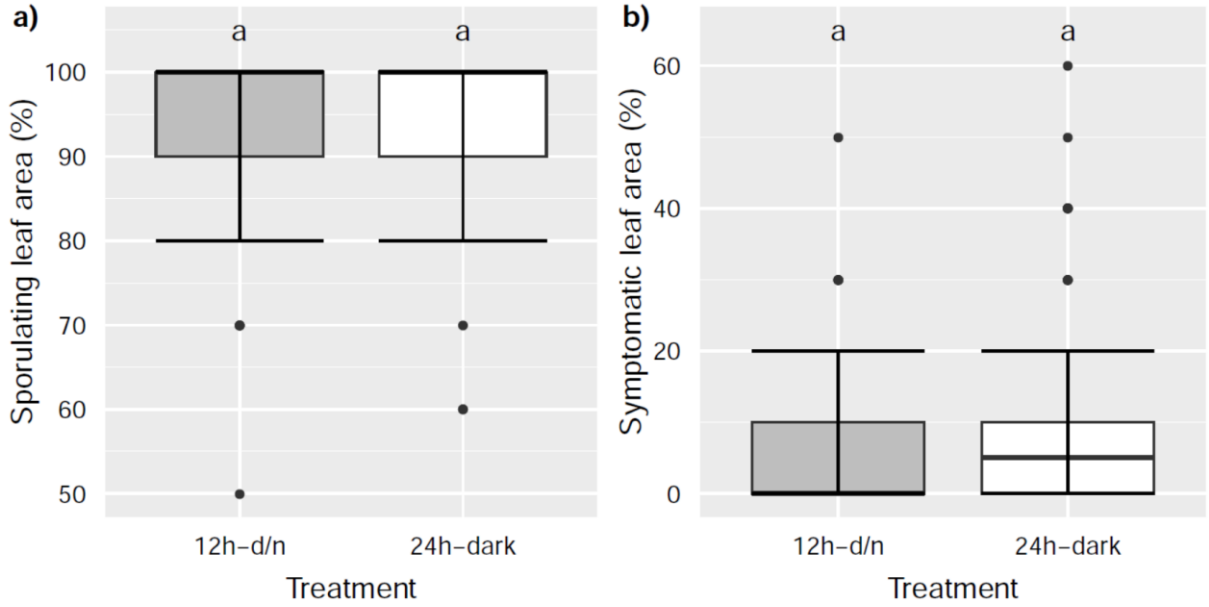

Fig. 2: Effect of 24 hour dark incubation (24h-dark) compared to direct incubation under a 12 hour day-night rhythm (12h-d/n) on a) disease development (sporulating leaf area %) and b) disease severity (symptomatic leaf area %) of *P. salviae-officinalis* infecting *Salvia officinalis*. Groups with a common letter are not significantly different at p = 0.05

In vitro pre-trials on 1.5 % water agar, with an incubation at 15 °C and in darkness, showed that germination started at 2 hpi (hours post inoculation) with the formation of a single, small, blister-like germ-tube primordium (Fig. 3). Six hours after inoculation, the germ-tube primordia started to stretch. Extended germ-tubes were observed 8 hpi. The germ-tubes ceased growth 12 to 24 hpi.

Only two out of 336 observations (one each in the 48h and 5d treatment) showed sporulation at low humidity at the end of the infection cycle (Fig. 4a). In contrast, in the treatment of high humidity at the end of the infection cycle, sporulating leaf area

increased with increasing leaf wetness duration (Fig. 4b). Sporulation was already induced when plants were incubated at 100 % rh. only for the first three hours after the inoculation. Sporulating leaf area was lower in treatments with 3h (median 10 %) than in treatments with 6 h (median 60 %) and longer leaf wetness duration (median ≥ 70% for 12 h, 24 h, 48 h and 7 d).

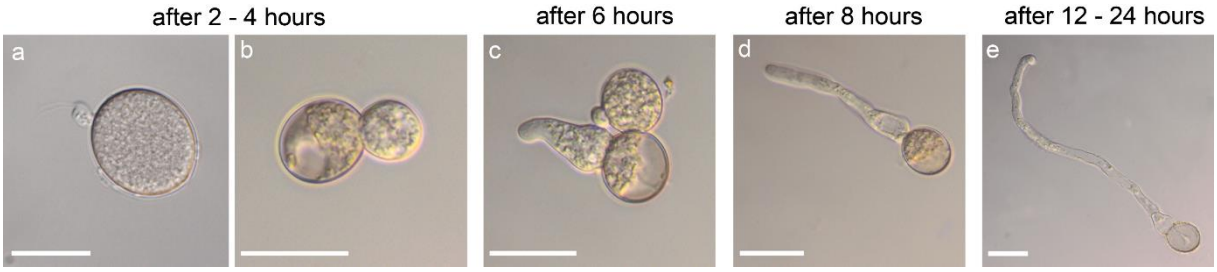

Fig. 3: Germinating conidia of *P. salviae-officinalis* on 1.5 % water agar at different incubation times, incubated at 15 °C and darkness. a, b: Conidia with a germ-tube primordium; c: Germ-tube primordia start to stretch; d: Conidia with young germ-tube; e: Conidia after 24 hours with a fully elongated germ-tube. Scale: 25 µm

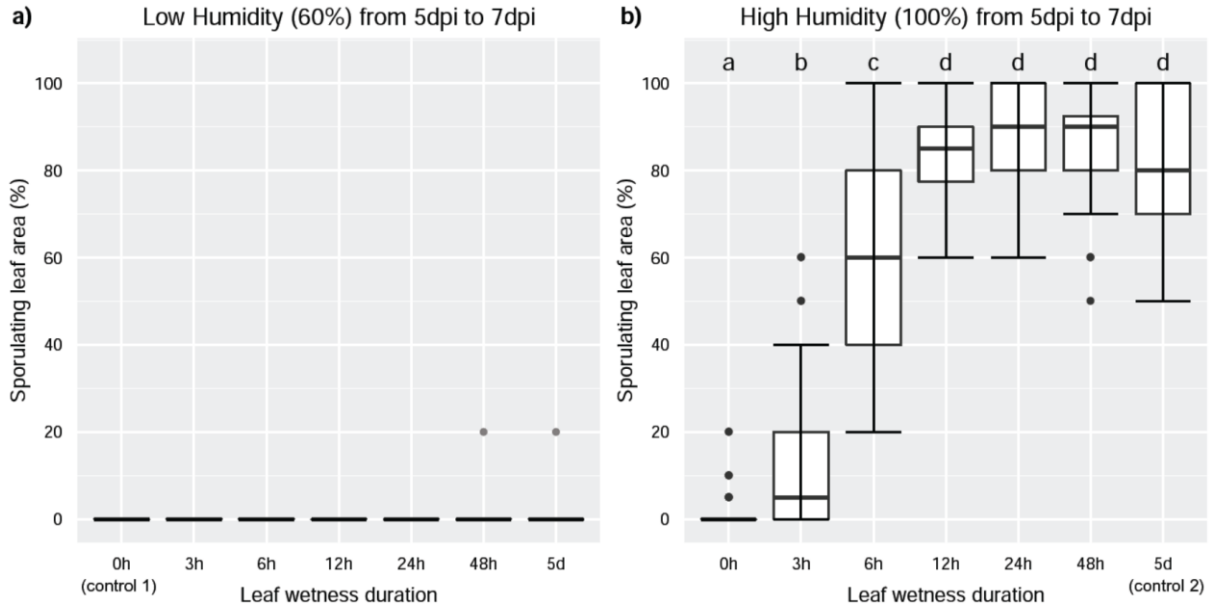

Fig. 4: Effect of leaf wetness duration after inoculation and humidity at the end of the infection cycle on disease development. Control 1 and control 2 refer to control 1 and control 2 described in the test scheme of Fig.1. Only for those treatments marked with letters a mixed effect model was fitted. The treatments at low humidity (Fig. 4a) were not statically analyzed because (almost) no sporulation was observed. Groups with different letters indicate significant differences in means at alpha 0.05

The effect of temperature on conidia germination in vitro and disease development *in vivo*

Peronospora salviae-officinalis did germinate *in vitro* over a temperature range between 2 and 30 °C. The temperature response pattern was similar across the three replicates/experiments but much lower germination rates were observed in experiment 1 compared to experiments 2 and 3. The optimum temperature for conidial germination *in vitro* was 20 °C in all three experiments (Fig. 5). At 20 °C, 82 out of 100 conidia germinated in the first 24 hours in experiment 2 and 3 and 43 out of 100 in experiment 1. The majority, i.e. more than 90 % of conidia, germinated in the first 24 hours in experiment 2 and 3 (Fig. 5).

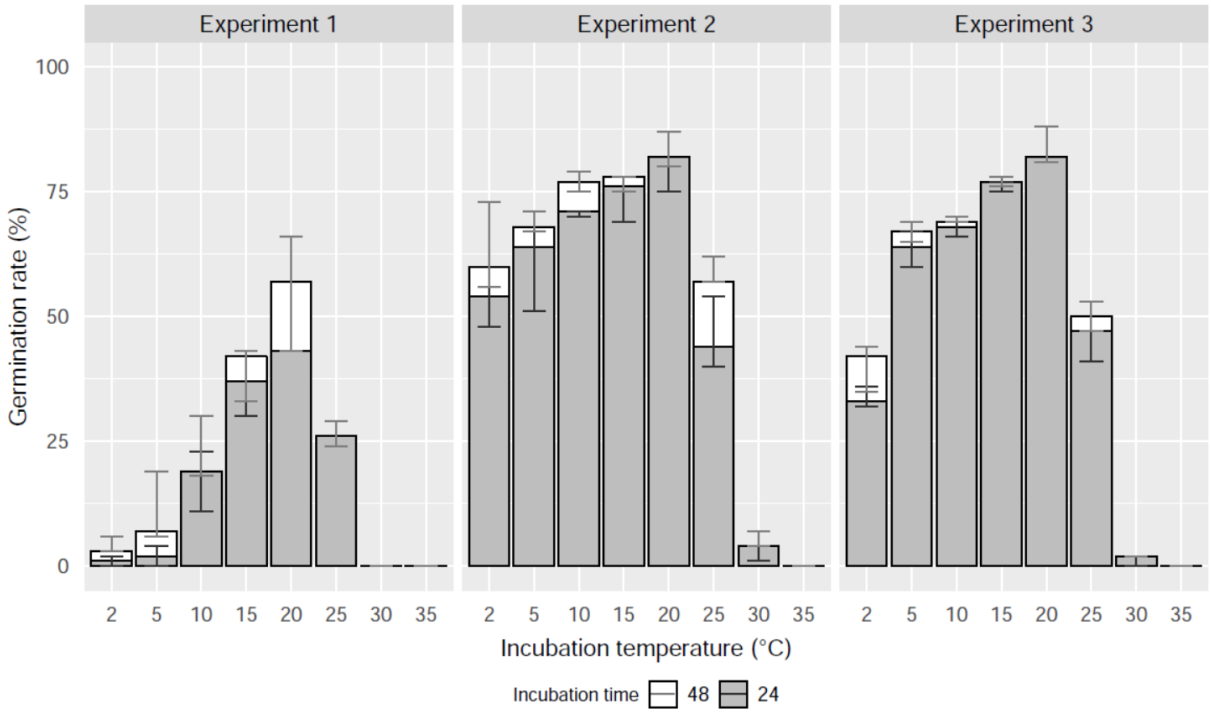

Fig. 5: Conidial germination rate (%) of *Peronospora salviae-officinalis in vitro*, incubated under different temperatures observed after 24 hours (grey bars) and 48 hours (white bars) in three replicated experiments. Barplots indicate the median and error bars the minimum and maximum germination rate

The highest sporulating leaf area *in vivo* was observed at inoculation temperatures at 20 degrees after 7 dpi (median at 80 %), and at 15 °C after 9 dpi (median at 80 %) (Fig. 6). At temperatures of 10 and 15 °C sporulation started at 8 dpi or 5 to 6 dpi, respectively, while at temperatures of 20 and 25 °C it started earlier at 4 dpi. At 5 °C *P. salviae-officinalis* did not sporulate until 14 dpi, but when plants were incubated at 25 °C for further three days, sporulation was observed (see 17 dpi in Fig. 6). The mixed model indicated a significant interaction between incubation temperature and time after

inoculation on sporulating leaf area (all p-values <0.001), which showed an increase in sporulating leaf area at 10 °C with increasing dpi, while at 15, 20 and 25 °C sporulating leaf area was already at high and constant level. This pattern was constant across all three experiments (Fig. S1).

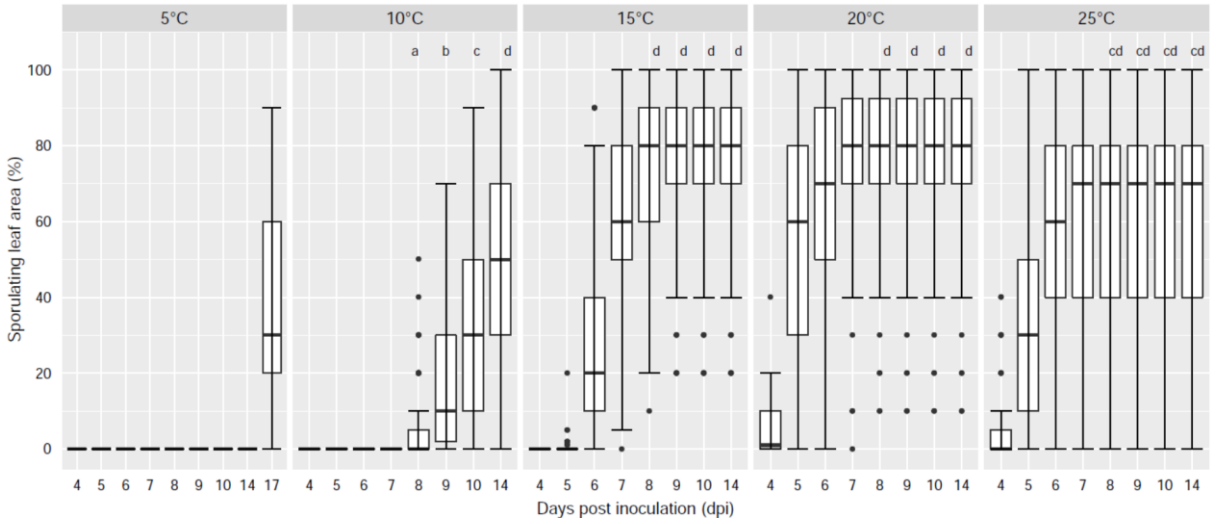

Fig. 6: Sporulating leaf area (%) of *Peronospora salviae-officinalis* on *Salvia officinalis* at different incubation temperatures and days post inoculation. A mixed effect model was fitted for treatments 10 - 25 °C for 8 - 14 dpi. Different letters indicate significant differences between the treatments according to Tukey post hoc test at significance level 0.05. The 5 °C-treatment was not included in the mixed effect model because no sporulation was observed during the whole 14 days of the experiment. Note: Level 17 dpi at temperature 5 °C represents the sporulating leaf area (%) after an increase in incubation temperature from 5 to 25 °C for three days after the end of the experiment

High temperatures expedite the appearance of symptomatic spots and hence increase symptomatic leaf area (Fig. S2). The highest symptomatic leaf area was observed at inoculation temperatures at 20 °C after 9 dpi (median at 80 %), and at 15 °C as well as 25 °C after 8 to 9 dpi (median at 70 %). At 20 and 25 °C first symptoms became visible at 5 dpi, at 15 °C at 6 dpi and at 10 °C at 10 dpi. At 5 °C no symptomatic leaf area was observed during the experiments. The mixed model indicated a significant interaction between incubation temperature and time after inoculation on symptomatic leaf area (all p-values <0.001), which showed an increase in symptomatic leaf area with increasing dpi, which differed in strength and maximum between temperatures (see suppl. Fig. S2). This pattern was constant across all three experiments (see suppl. Fig. S3).

In this study oospores of *P. salviae-officinalis* were observed in infected sage leaves after incubation at temperatures between 15 and 25 °C (Fig. 7). They were round and thick-walled, sometimes surrounded by a hyaline oogonium, and were formed close to the cuticle. The diameter of oospores of *P. salviae-officinalis* was between 26 and 41 µm. At 20 and 25 °C incubation temperature oospores developed at 8 dpi, at 15 °C at 14 dpi. No oospores were found in infected leaves of the 5 and 10 °C treatments during the time of observation.

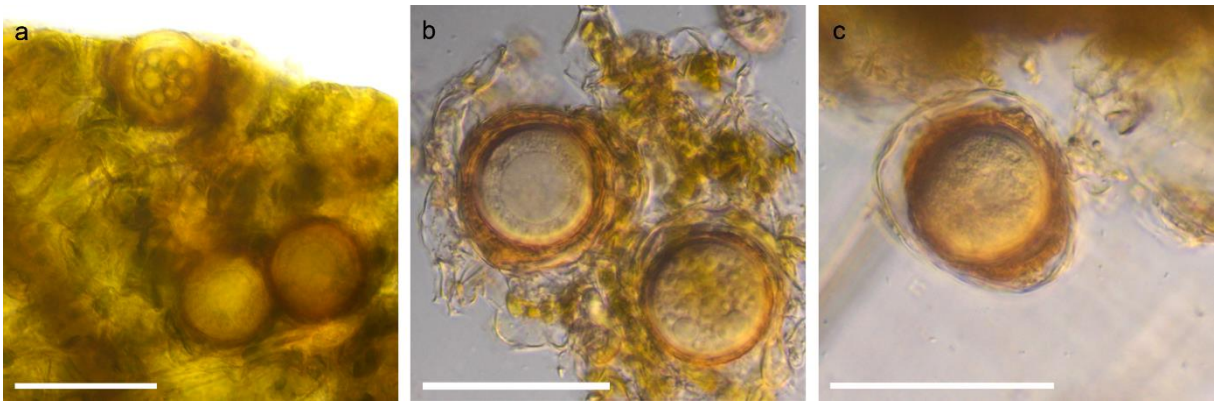

Fig. 7: Oospores of *Peronospora salviae-officinalis* in infected leaf material of *Salvia officinalis* 14 days after inoculation and incubation at 15°C. a: Section through an infected leaf reveals oospores between leaf mesophyll cells. b and c: Squeeze preparation of necrotic leaf spots of sage leaves showing oospores of *P. salviae-officinalis*. Scale: 50 µm

Seed testing for *P. salviae-officinalis*

All eight seed samples from *S. officinalis* tested carried DNA of oomycetes as revealed by PCR specific for the ITS-region of oomycetes (Fig. 8). The positive control for *P. salviae-officinalis* showed a clear and strong band around 1250 bp. Amplification products of samples 1.1-2.3, 3.3, 4.1, 4.3, 5.1, 5.2, 6.2, 7.1, 7.2, 8.1-8.2 were of the same size as the positive control and were sequenced for verification. BLASTn results of the obtained sequences in GenBank gave best hits with *P. salviae-officinalis* (1.1, 1.2, 2.1-2.3, 3.3, 4.1, 4.3, 5.1, 6.2, 7.1, 7.2, 8.1, 8.3) and *Pythium* spp. (1.3, 5.2 and 8.2), respectively. The 14 sequences, which gave best hits with *P. salviae-officinalis* were all identical among each other and clustered without any genetic distance together with *P. salviae-officinalis,* but also with *P. belbahrii,* in the phylogeny comprising a larger taxon sampling of *Peronospora* and *Pythium* species (Fig 9). If only *P. belbahrii* and *P. salviae-officinalis* were used in a neighbour joining analysis (Fig 10) they clearly clustered in two individual groups, which resulted from 2-3 distinct

substitutions. The small genetic distances caused by the substitutions were blurred in the larger data set because they were positioned at gap positions in the alignment that were introduced due to the larger and phylogenetically heterogenous taxon sampling. The sequences obtained from seed samples 1.3, 5.2 and 8.2 differed slightly (1.3 and 8.2) or significantly (5.2 from 1.3 and 8.2) among each other and clustered with different *Pythium* lineages, accordingly. The DNA sequence obtained from seed sample 5.2 clustered without any genetic distance together with *Pythium longisporangium* specimens, suggesting conspecificity. The sequences of the *Pythium* spp. found in seed samples 1.3 and 8.2 differed slightly among each other and could not be resolved based on ITS clustering within a heterogenous group consisting of sequences of *P. oligandrum*, *P. hydnosporum*, *P. ornamentum, P. amasculinum*. The final alignments obtained were deposited in TreeBASE and are available under http://purl.org/phylo/treebase/phylows/study/TB2:S25456.

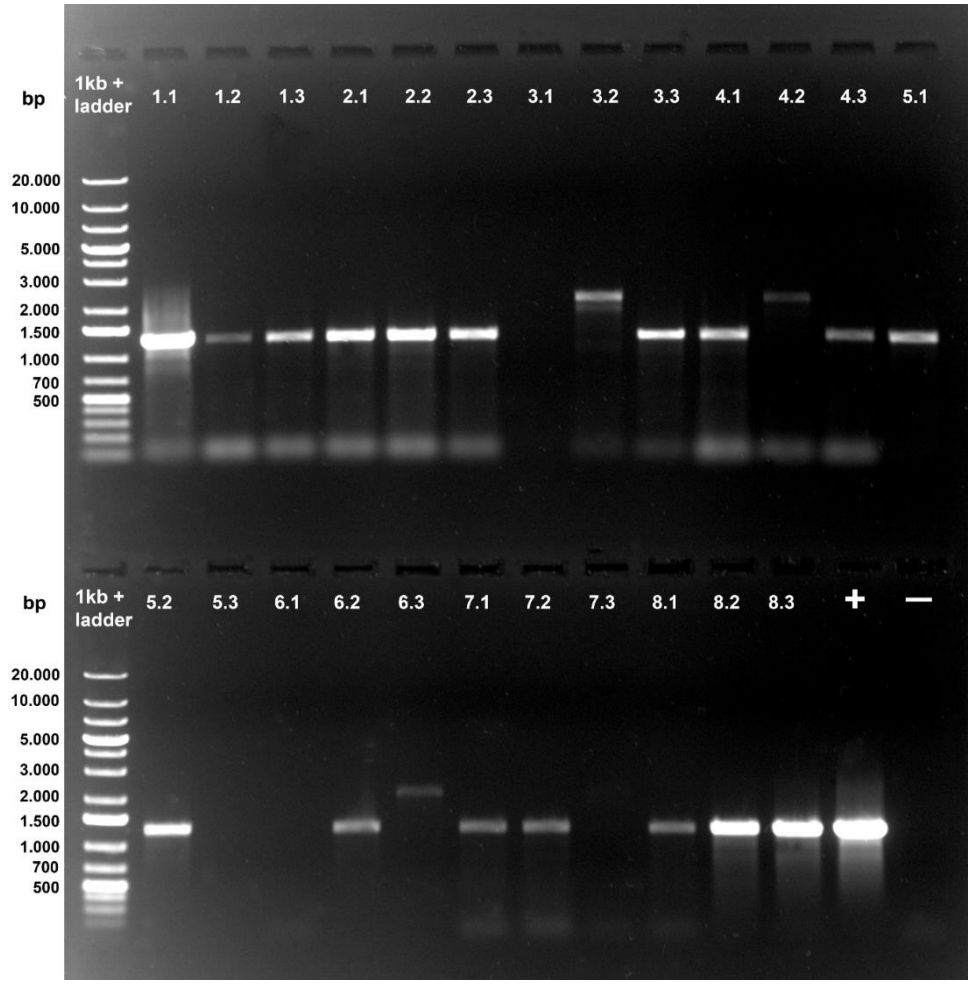

Fig. 8: Picture of an agarose gel of PCR products of the ITS rDNA of oomycetes extracted from seeds of *Salvia officinalis*. Fragment sizes are indicated to the left. DNA of *Peronospora salviae-officinalis* was used as positive control (+), a PCR mix without added DNA as negative control (-)

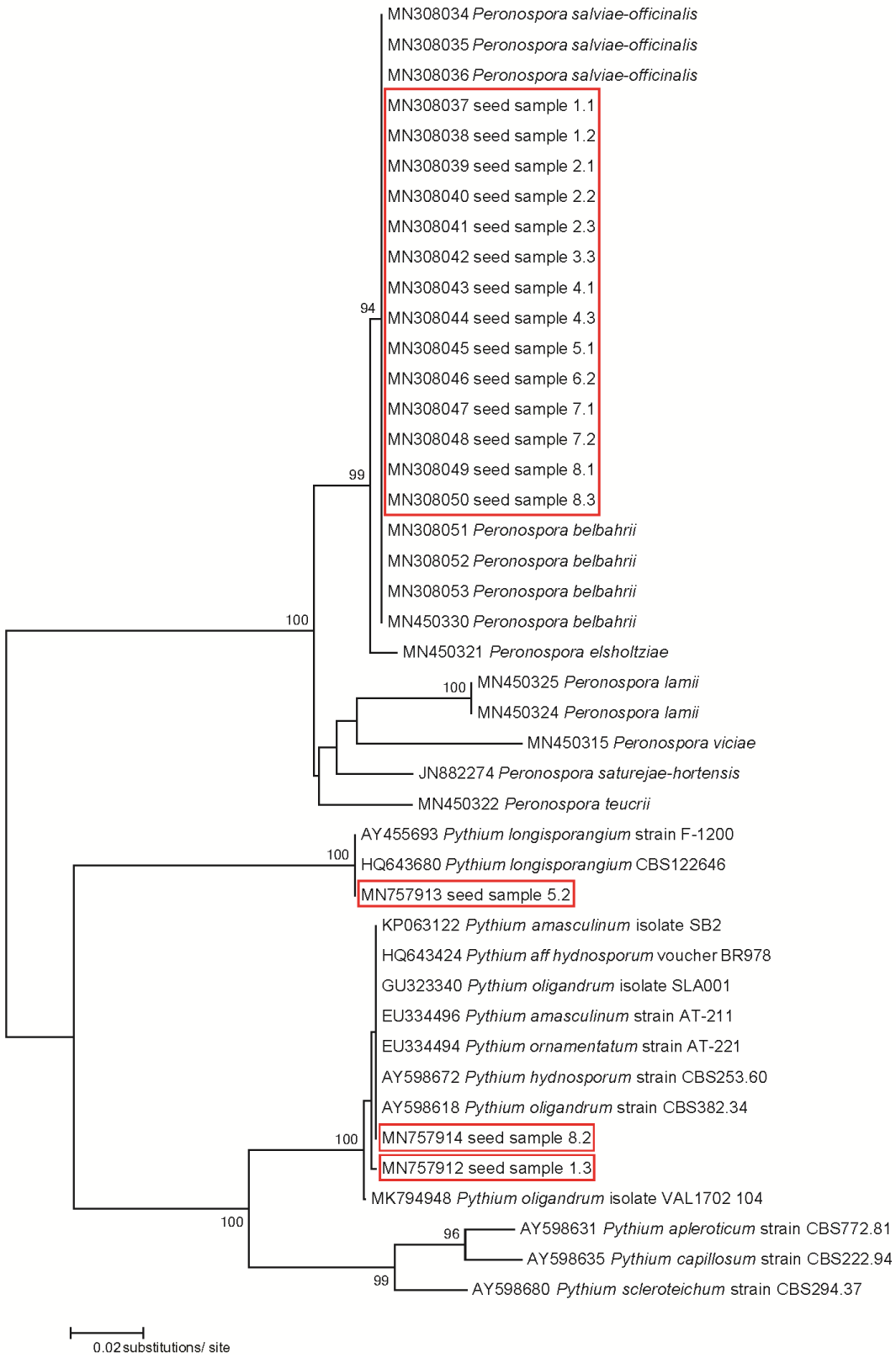

Fig. 9: Phylogenetic placement of the sequences obtained from sage seed samples (highlighted by a red box) based on ITS rDNA sequences using Neighbour Joining analysis with Kimura-2-parameter model within representative samples of *Peronospora* and *Pythium* species. Bootstrap values greater than 70 % are given above the branches. The number of nucleotide changes between taxa is represented by branch length and the scale bar represents the number of nucleotide substitutions per site. *Peronospora* samples acquired from seeds of *Salvia officinalis* cluster with sequences of *P. salviae-officinalis* and *P. belbahrii*. Scale bar indicates number of substitutions per site

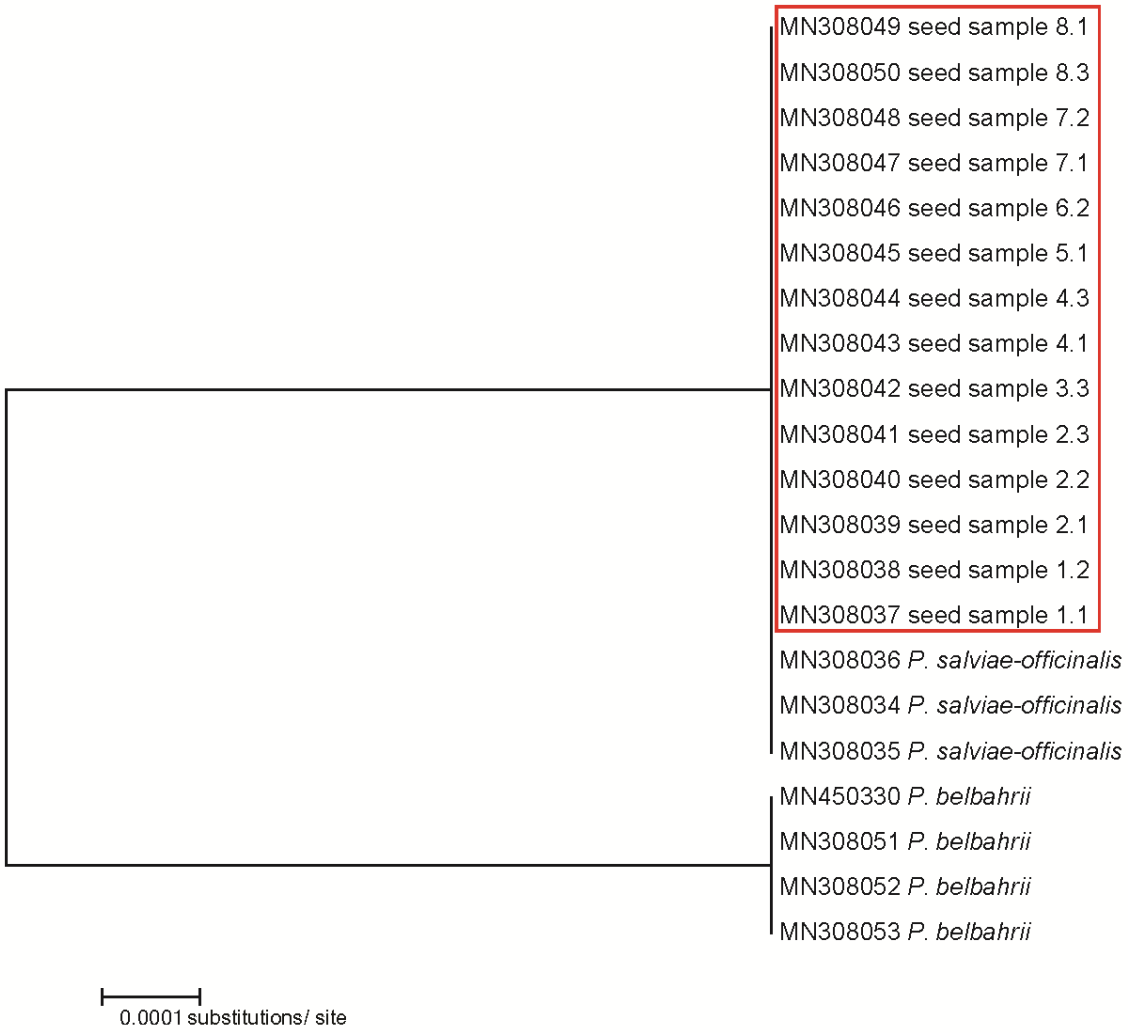

Fig. 10: Neighbour joining analysis of sequences from seeds tested positive for *Peronospora* (highlighted by red box) with ITS rDNA sequences of *P. salviae-officinalis* and *P. belbahrii*, respectively. Scale bar represents nucleotide substitutions per site. All *Peronospora* sequences obtained from seeds of *Salvia officinalis* cluster with *P. salviae-officinalis* and can be distinguished from the closely related *P. belbahrii*. Scale bar indicates number of substitutions per site

Microscopy of the filter papers obtained by the spore filtration method of the eight seed samples showed oospore-like structures on the membrane filter (Fig. 11), which were confirmed as oospores when observed in higher magnification in lactic acid. Shape and size of these oospores were consistent with shape and size of oospores found in leaves of *S. officinalis* infected with *P. salviae-officinalis*. The oogonium, was not detectable, and might have been disrupted during the seed washing process. Besides oospores, conidia and conidiophores that were characteristic of *P. salviae-officinalis* were detected on the membrane filters of two seed lots out of eight.

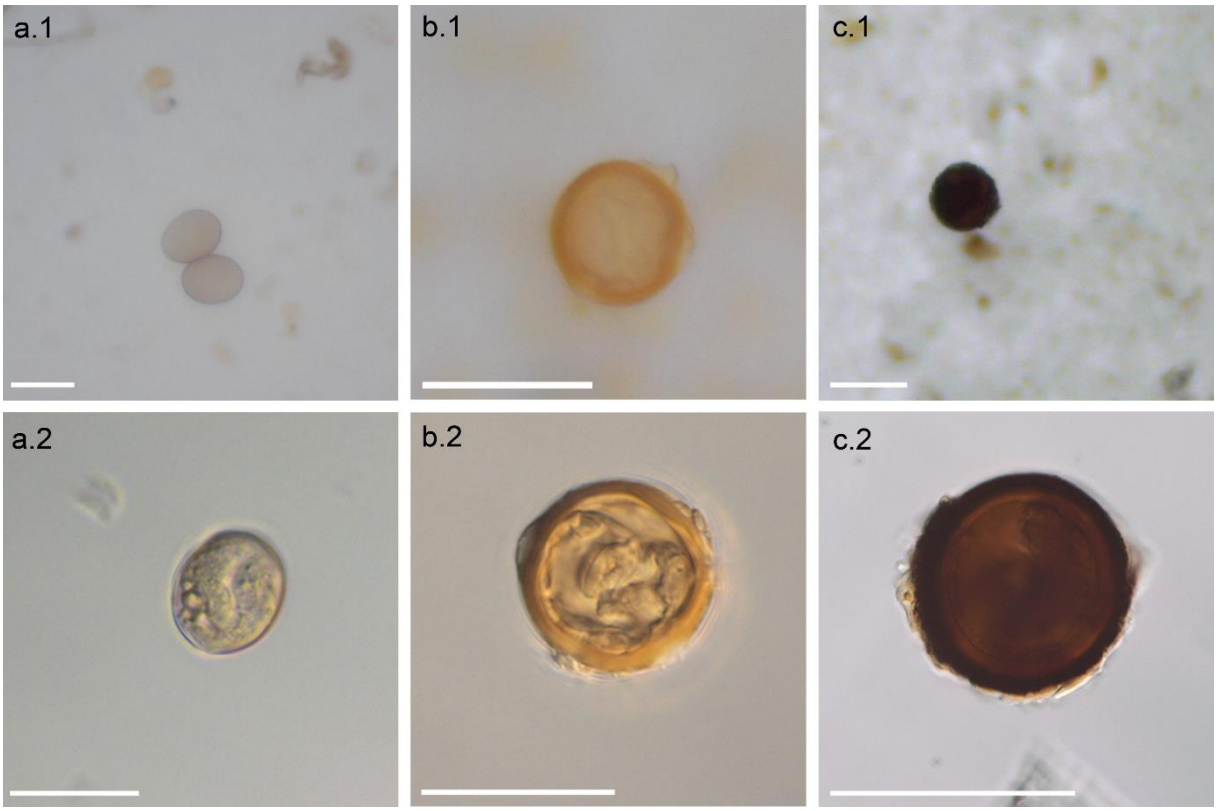

Fig. 11: Oospores and conidia collected from sage seeds by seed washing. a.1-c.1: Pictures of conidia and oospores on membrane filters after seed washing. a.2-c.2: Conidia and oospores after transfer to 60 % lactid acid on an objective slide. a.1 and 2: conidia. b.1, c.1, b.2 and c.2: Oospores. Scales: a.1 and 2: 20µm, b.1 and 2: 25µm, c.1 and 2: 50µm

Discussion

Peronospora salviae-officinalis, the downy mildew of common sage, is now widespread and impedes sage production worldwide while basic knowledge on the epidemiology of the disease was scarce so far. In the present study we determined the impact of basic epidemiological factors such as temperature and moisture on speed and intensity of disease development to improve our understanding of the infection process. We also screened for oospores *in planta* and in seed lots to gain insights into the potential avenues of spread of the disease that eventually could result in better containment of the disease.

Dark incubation over a prolonged time was not necessary for successful artificial inoculation and infection of sage downy mildew. In infection trials, downy mildews are usually incubated in complete darkness for the first 24 hours (Diez-Navajas et al., 2008, Lebeda & Cohen, 2010, Neufeld & Ojiambo, 2012, Djalali Farahani-Kofoet et al., 2014,

Kandel et al., 2019). Our findings suggest that the common practice of keeping downy mildews for 24 hours in dark after inoculation is not essential for the infection success. We also found that leaf wetness for three hours after inoculation is sufficient for infection. Similar times had been determined for other downy mildews such as *Pseudoperonospora cubensis* (Cohen, 1977, Neufeld & Ojiambo, 2012), *Plasmopara viticola* (Gäumann, 1951a), and *P. belbahrii* (Cohen & Ben-Naim, 2016). For sporulation, however, a second event of high humidity or leaf wetness, is necessary, which is probably the case for all downy mildew species (Gäumann, 1951b, Lange et al., 1989, Garibaldi et al., 2007). The timing of this second humidity event depends on incubation temperature. At 10 °C a humidity event no earlier than eight to ten days post inoculation will lead to sporulation, while at 25 °C high humidity between four to six dpi is a sufficient time span. These results are in line with observations made on cucumber downy mildew requiring an initial minimum duration of leaf wetness of two hours for successful infection (Cohen, 1977), and later a minimum of at least 90 % rel. humidity for sporangiophores production (Palti & Cohen, 1980, Lebeda & Cohen, 2010). In our studies temperatures between 15 and 20 °C were most favourable for disease progress, resulting in the highest sporulating and symptomatic leaf area. Similar temperature ranges were also found favourable for the infection of *P. belbahrii* on basil (Djalali Farahani-Kofoet et al., 2014) and the cucurbit downy mildew *Ps. cubensis* (Neufeld & Ojiambo, 2012). While leaf wetness is essential for infection and sporulation, temperature seems to be an important driver for the extent of disease development with highest disease outcomes at temperatures between 20 and 25 °C. Nevertheless, conidia of *P. salviae-officinalis* were able to infect sage plants at all temperatures tested between 5 and 25 °C. Even at 5 °C *P. salviae-officinalis* was still able to infect plants, but only sporulated at higher temperatures of at least 10 °C. This implies that *P. salviae-officinalis* can latently infect sage during long periods of the year in many climatic regions and is then able to sporulate quickly if temperatures rise.

In the present study oospores readily developed within 8 dpi at temperatures between 20 and 25 °C and 14 dpi at 15 °C. (Montes-Borrego et al., 2009) demonstrated that oospores of opium poppy downy mildew in infested soil or leaf debris are an effective inoculum for early infections of poppy seedlings through plant roots. As oospores were observed frequently at higher temperatures in infected sage plants in our infection trials particular caution is recommended concerning crop residues from infected stands. As

infected leaf debris decomposes and oospores are released, inoculum in soil may accumulate over the years if infested plants are not effectively removed from the field.

Contamination of sage seeds with *P. salviae-officinalis* were found in all seed lots investigated based on ITS-PCR and sequencing. Less frequently seed contamination with presumably *Pythium longisporangium* and one or two other *Pythium* species were detected. Applying a spore filtration technique to sage seeds the presence of oospores and conidia in seed samples could also be proven microscopically. However, it is difficult to distinguish between oospores of *Peronospora* and *Pythium* based on microscopy only. Oospores sticking on seeds of their host plants are very efficiently spread into new areas (Rennie & Cockerell, 2006). This was shown for example for *P. belbahrii* (Farahani-Kofoet et al., 2012, Garibaldi et al., 2004), *P. effusa* (Kunjeti et al., 2016) and *P. arborescens* (Landa et al., 2007). The frequent contaminations of sage seed lots with *P. salviae-officinalis* found by us could explain the observations from a monitoring survey conducted in 2016 and 2017 where *P. salviae-officinalis* was detected in all eight sage stands investigated throughout Germany, even in newly sown stands (Thiemann & Blum, 2019). Taken together, the reported findings on the epidemiology and infection biology of *P. salviae-officinalis* could serve as basis for the development of forecast models for the occurrence of downy mildew in sage production. Furthermore, our results may help to develop appropriate combat and containment strategies against sage downy mildew and hence support sage cultivation worldwide.

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Supplementaries

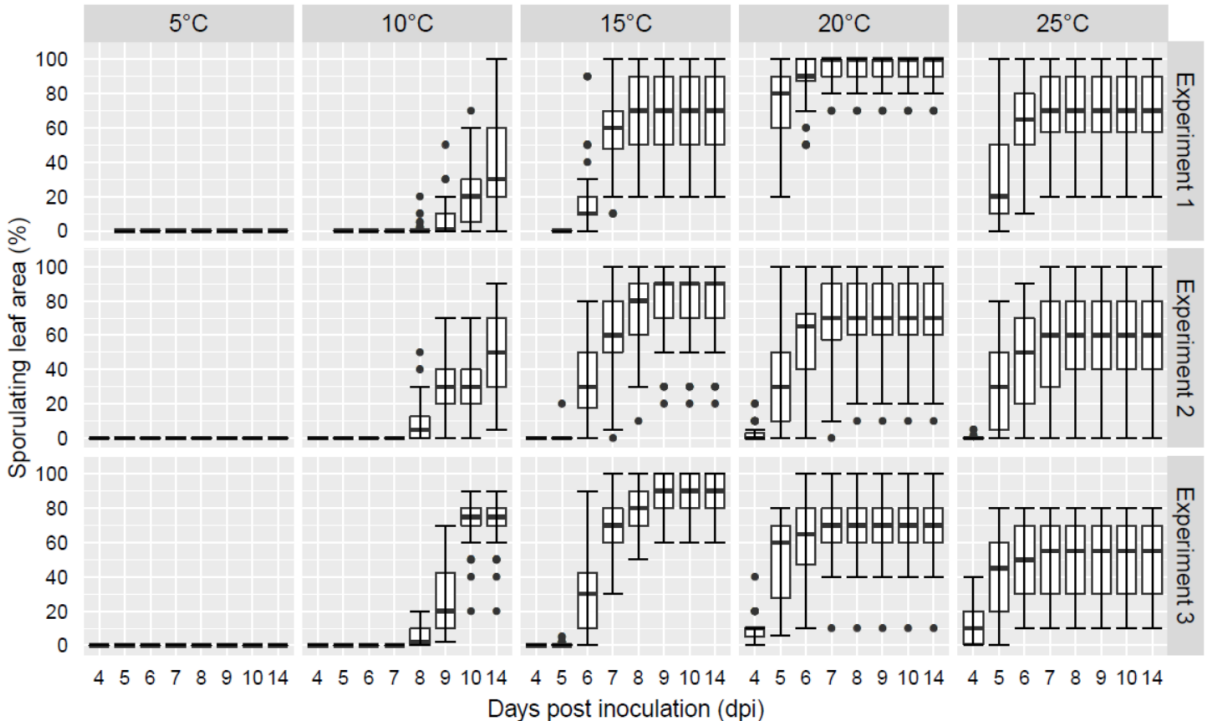

Fig. S1: Sporulating leaf area (%) of *Peronospora salviae-officinalis* on *Salvia officinalis* at different incubation temperatures and days post inoculation. The three experiments are displayed separately to show that the temperature response pattern was similar across all three experiments

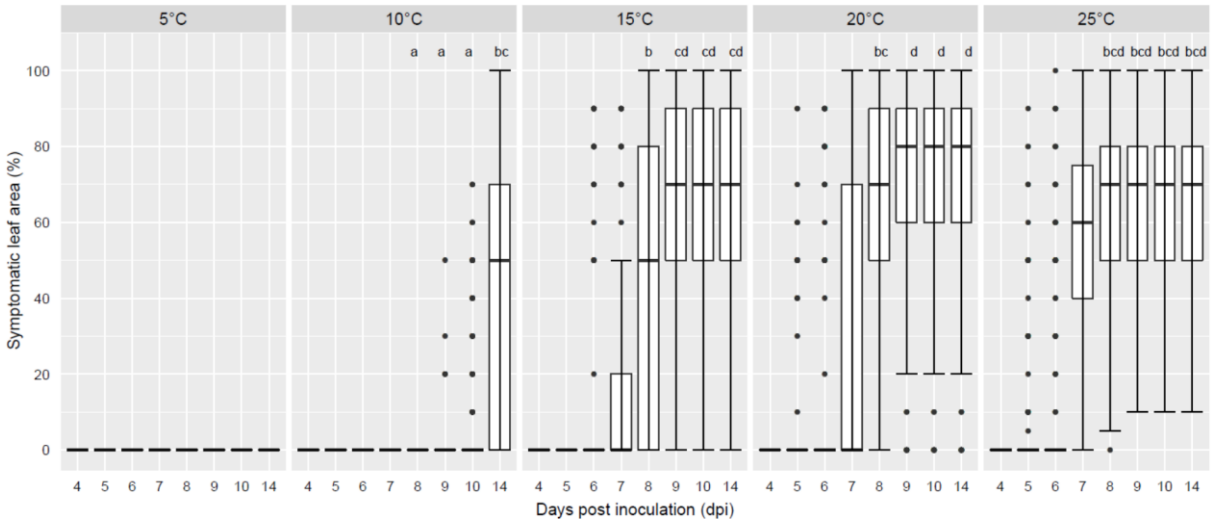

Fig. S2: Symptomatic leaf area (%) of *Peronospora salviae-officinalis* on *Salvia officinalis* at different incubation temperatures and days post inoculation. A mixed effect model was fitted for treatments 10 - 25 °C for 8 - 14 dpi. Different letters indicate significant differences between the treatments according to Tukey post hoc test at significance level 0.05. The 5°C-treatment was not included in the mixed effect model because no symptoms were observed during the whole 14 days of the experiment

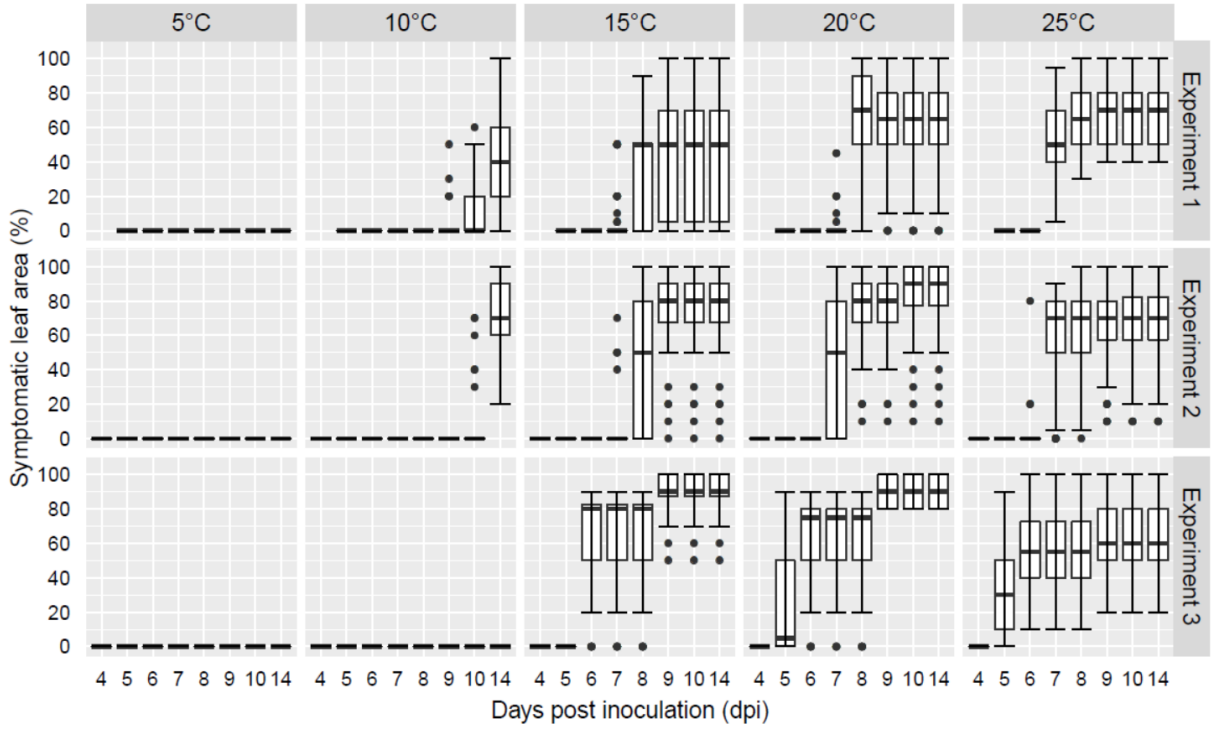

Fig. S3: Symptomatic leaf area (%) of *Peronospora salviae-officinalis* on *Salvia officinalis* at different incubation temperatures and days post inoculation. The three experiments are displayed separately to show that the temperature response pattern was similar across all three experiments

Data Availability Statement

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Compliance with ethical standards

This article does not contain any studies with human or animal subjects performed by any of the authors.

Conflict of interest

The authors declare that they have no conflict of interest.

Appendix 2

Statement of Joint Authorship

On the publication: Tracking host infection and reproduction of *Peronospora salviae-officinalis* using an improved method for confocal laser scanning microscopy (CSLM)

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Name of the journal: Plant Pathology

Involved authors:

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MT: Marco Thines

WM: Wolfgang Maier

YB: Yvonne Becker

What did the PhD student or the Co-Authors contribute to this work?

(1) Development and planning

PhD student MH: 60%

Co-Author WM: 10%

Co-Author YB: 30%

(2) Performance of the individual investigations and experiments

PhD student MH: 80%

Co-Author YB: 20%

(3) Preparation of the data collection and figures

PhD student MH: 80%

Co-Author YB: 20%

(4) Analysis and interpretation of data

PhD student MH: 60%

Co-Author YB: 35%

Co-Author MT: 5%

(5) Writing the manuscript

PhD student MH: 60%

Co-Author MT: 10%

Co-Author WM: 10%

Co-Author YB: 20%, corresponding author

Verification of the statements above:

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Chapter 2

Tracking host infection and reproduction of *Peronospora salviae-officinalis* using an improved method for confocal laser scanning microscopy (CSLM)

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Running head: Confocal microscopy of sage downy mildew

Keywords: aniline blue, Confocal Laser Scanning Microscopy, *Peronospora belbahrii*, *Peronospora lamii*, *Peronospora salviae-officinalis*, trypan blue

Abstract

Peronospora salviae-officinalis, the causal agent of downy mildew on common sage, is an obligate biotrophic pathogen. It grows in the intercellular spaces of the leaf tissue of sage and forms intracellular haustoria to interface with host cells. Although *P. salviae-officinalis* has been described as a species of its own already ten years ago the infection process remains obscure. To address this, a histological study of various infection events, from the adhesion of conidia on the leave surface until *de novo* sporulation is presented here. As histological studies of oomycetes are challenging due to the lack of chitin in their cell wall, we also present an improved method for staining downy mildews for confocal laser scanning microscopy. For this, we evaluate the potential of the autofluorescence of fixed non-stained samples. A 1:1 mixture of aniline blue and trypan blue was found most suitable and was used for staining of oomycete and plant structures, allowing a distinction between them and the visualization of plant immune responses. The method was also used to examine samples of *Peronospora lamii* on *Lamium purpureum* and *Peronospora belbahrii* on *Ocimum basilicum*. This shows the potential of the presented histological method for studying the infection process of downy mildews in general.

Introduction

The downy mildew *Peronospora salviae-officinalis* causes a severe disease on the medicinal plant *Salvia officinalis* (common sage, Lamiaceae). Since its first report as a *Peronospora* sp. on *S. officinalis* in 1993 (McMillan, 1993), the disease was reported from all over the world (Gamliel & Yarden, 1998, Hill et al., 2004, Belbahri et al., 2005b, Liberato et al., 2006, Humphreys-Jones et al., 2008). It is of increasing economical concern and can be regarded as a newly emerging disease. Although *P. salviae-officinalis* was described already ten years ago (Choi et al., 2009b) the infection process was never studied. Knowledge about the epidemiology and biology of this pathogen is however crucial for the development of forecast models, and appropriate combat strategies against it in sage production.

Downy mildews of the genus *Peronospora* are obligate biotrophs, which means that they are dependent on living host cells for nutrition (Thines & Choi, 2016). They belong to the *Peronosporaceae* within the phylum Oomycota of the kingdom Straminipila (Beakes & Thines, 2017). The cell-walls of oomycetes mainly contain (1,3)-β-glucan,

(1,6)-β-glucan and cellulose (Sietsma et al., 1969, Blaschek, 1992). The (1,3)-β-glucan structures of oomycetes are branched, to variable degree (Yamada & Miyazaki, 1976, Fabre, 1984). Previous studies have shown that the cell walls of Peronosporales contain up to 85% glucans, mainly glucopyranose and 1,4-linked glucosyl residues, derived from cellulose. In contrast to true fungi, chitin derivates are not present in considerable amounts in cell-walls of most oomycetes, especially in Peronosproaceae (Bartnicki-Garcia, 1968). Oomycetes also use (1,3)-β-glucan as storage polysaccharide, in the form of mycolaminarin, but not glycogen, which is the storage polysaccharide of fungi and animals (Clavaud et al., 2009). The similar cell wall structure of oomycetes and plants renders fluorescence microscopy challenging and the selectivity of fluorescent dyes to bind specific polysaccharides is low. Aniline and trypan blue, deployed in several experiments to detect oomycetes in plant tissue, (Hood, 1996, Donofrio & Delaney, 2001, Gindro et al., 2003, Huitema et al., 2003, Kortekamp, 2005, Diez-Navajas et al., 2007) are also used as dyes in plant histology (Smith & McCully, 1978). Aniline blue is used as a fluorochrome in fluoresence microscopy for a while to detect callose, a linear β-(1,3)-D-glucan, in plant cells (Stone et al., 1984, Eschrich & Currier, 2009), but it also binds to other β-(1,3) glucans (Wood & Fulcher, 1984) and cellulose (Mulisch & Welsch, 2015). All glucans are polysaccharides that consist of D-glucose, so the basis structure molecule is the same for all of them. Trypan blue is a benzidine-based, anionic azo dye (Combes & Haveland-Smith, 1982) that binds fungal chitin and glucans (Liesche et al., 2015), and reduces the intracellular auto fluorescence of NADH and riboflavine (Mosiman et al., 1997). Moreover, this dye binds to proteins located in the cytoplasm and in the plasma membrane of organisms (Avelar-Freitas et al., 2014).

Previous studies have shown that it is possible to recognize intercellular mycelium and haustoria in samples stained with aniline or trypan blue (Donofrio & Delaney, 2001, Diez-Navajas et al., 2007). A common problem of these methods is the often low visual contrast and resolution between plant and oomycete structures in thicker tissues, sometimes necessitating sectioning for a clear localisation of the haustoria within the plant tissue (Thines et al., 2007). Especially areas of close host-pathogen interaction, e.g. penetration hyphae that pierce the plant cell wall, are difficult to visualize. One of the advantages of CLSM is the optical sectioning it permits, reducing the need for mechanical sectioning (Czymmek et al., 1994). Because optical sectioning is not limited to a single focal plane confocal images can also be collected in vertical plane

by z stacks and combination thereafter allow to precise localisation of fungal structures within the host tissue.

Here we aimed at developing a robust method for confocal laser scanning microscopy by which oomycetous and plant tissue can be clearly discriminated. This was done based on the example of the previously unstudied infection process of *P. salviae-officinalis* in association with its host, *Salvia officinalis*. As a method published by (Becker et al., 2018) for visualization of endophytic fungi seemed to be especially promising for the purpose, it was adapted in this study for oomycetes.

Material and Methods

Plant and fungal material

Infection experiments were carried out using plants of *S. officinalis* inoculated with *P. salviae-officinalis.* Sage plants in the 4-leaf-stage were spray-inoculated with a suspension of freshly harvested conidia in deionised water (10^4 conidia ml^{-1}). Inoculated plants were incubated in a plant growth chamber (Percival Scientific, Perry, IA, U.S.A) under the following conditions; 15 °C, 100 % rel. humidity, 12 h light, and 12 h darkness. The downy mildew isolate used for the experiments was isolated from *S. officinalis* in Freital (Saxony, Germany) and is maintained in our lab on *S. officinalis* since 2017 at the Institute of Epidemiology and Pathogen Diagnostics of the Julius Kühn-Institut (Braunschweig, Germany). For comparison of the different staining solutions leave samples were taken seven days after inoculation when dense sporulation on the lower leaf surface could be observed. The staining method was also tested for *P. belbahrii* and *P*. *lamii* in a similar manner.

To study and document the infection process of *P. salviae-officinalis* on *S. officinalis* from conidial germination until sporulation, sage plants were inoculated and incubated as described above for 14 days. Leaf samples were taken at 1 day after infection (dai), 4 dai, 7 dai and 14 dai. For staining, leaf pieces of 0.5 x 1.5 cm, excluding the middle vein, were sampled for all specimens examined.

Fixation and Staining

To bleach infected leaf samples an ethanol series as described by (Bougourd et al., 2000) for *Arabidopsis* embryos was used: The leaves were successively incubated in 15 %, 50 %, 70 %, 95 %, 99 % ethanol for each 15 min at room temperature (RT), in 99 % ethanol over night at 4 °C; and afterwards successively in 95 %, 70 %, 50 %, 15 % ethanol, deionised water (2x) for 15 min at RT. After rehydration samples were incubated in 10 % KOH for 3 h at room temperature, washed 3 times in PBS buffer (pH 7.4; 0.1369 mol/l NaCl, 2.68 mmol/l KCl, 0.01 mol/l Na_2HPO_4, 1.76 mmol/l KH_2PO_4) and kept at 4°C. For staining, PBS was removed and samples were covered with the respective staining solution (ca. 300 µl/sample) listed in Table 1. A 1 % aniline blue stock solution was prepared by dissolving 0.01 g aniline blue diammonium salt (AB) (Sigma-Aldrich, Munich, Germany) in 1 ml deionised water. A 1 % trypan blue stock solution was prepared by dissolving 0.01 g Trypan Blue powder (TB) (Merck, Darmstadt, Germany) in 1 ml deionised water. Samples were stained by vacuum infiltration for 5 x 2 minutes in a glass desiccator (DWK Life Sciences, DURAN, Wertheim, Germany) as described by (Becker et al., 2018). To evaluate the autofluorescence of plant and downy mildew cells, bleached samples were not stained but analyzed by CLSM directly. Due to the high contrast offered by CSLM, destaining was not necessary.

Table 1: Composition of the different staining solutions

Staining solution	Composition of 1 ml staining solution
AB 20	20 µl aniline blue 1 %, 10 µl Tween 20 2 %, 970 µl PBS
AB 40	40 µl aniline blue 1 %, 10 µl Tween 20 2 %, 950 µl PBS
TB 20	20 µl trypan blue 1 %, 10 µl Tween 20 2 %, 970 µl PBS
TB 40	40 µl trypan blue 1 %, 10 µl Tween 20 2 %, 950 µl PBS
AB 20/TB 20	20 µl aniline blue 1 %, 20 µl trypan blue 1 %, 10 µl Tween 20 2 %, 950 µl PBS

CLSM

To determine the fluorescent properties of aniline blue and trypan blue and to define the autofluorescence of the samples lambda scans and CLSM imaging of unstained samples and samples stained with AB 40, TB 40 and AB 20/TB 20, respectively, were performed. Lambda scans were captured with photomultiplier tube PMT2. Lambda scan range properties are shown in Table 2.

Table 2: Lambda Scan range properties for UV Laser and for 561 DPSS

λ-Scan Range Properties for UV Laser			**λ-Scan Range Properties for 561 DPSS**	
Detection Begin	420 nm		Detection Begin	575 nm
Detection End	780 nm		Detection End	780 nm
Total Detection Range	360 nm		Total Detection Range	205 nm
Detection Band Width	30 nm		Detection Band Width	30 nm
No. of Detection Steps	67		No. of Detection Steps	35
λ-Detection Stepsize	5 nm		λ-Detection Stepsize	5 nm

Samples for confocal scanning laser microscopy (CLSM) were transferred from the staining solution to a droplet of 70 % glycerin (Carl Roth, Karlsruhe, Germany) on a microscope slide and covered with a cover slip. Fluorescence was recorded with a confocal laser scanning microscope (Leica TCS SP8, Leica, Wetzlar, Germany). Images were taken using the HC PLAPO CS2 20x/0.75 IMM objective and the Leica Application Suite X (LAS X) software for Leica microscopes.

AB and TB stained samples, as well as unstained samples, were excited with the 405 nm diode laser and the 561 nm diode-pumped solid-state laser (DPSS). The 405 nm laser was employed to visualize callose (□-1,3-glucan) through its UV light-induced fluorescence with the aniline blue fluorochrome as reported previously (Stone et al., 1984). The intensity of the 405 nm laser was set on 21.1 % for Lambda scans and dye evaluation. The 561 nm laser was used to visualize AB and TB stained glucans of cell walls and cytoplasm, as well as cytosolic proteins. The intensity of the

561 nm laser was set on 24.7 %. Laser intensities were adjusted for visualization of the infection process. The intensity of the 405 nm laser ranged between 15 % and 39 % and the intensity of the 561 nm laser between 8 % and 24.7 %. Two photomultiplier tubes (PMT2 and PMT4) and one hybrid detector (HyD3) were used to capture the emission fluorescence of the dyes and the autofluorescence of plant and fungal tissue. Pseudocolours were used to represent specific emission fluorescence. Blue pseudocolour (PMT2, 447 - 495 nm) was used to visualise aniline blue stained plant callose, UV-induced trypan blue fluorescence and autofluorescence. Green (HyD3, 593 - 625 nm) and red (PMT4, 673 - 749 nm) pseudocolours were used to visualise emission fluorescence from aniline and trypan blue and to capture plant autofluorescence. Multichannel images were produced by overlay of all three channels, in which overlapping green (HyD 3) and red (PMT 4) pseudocolours appear orange. Sequential acquisition in three different channels was carried out, to avoid cross talk. Images were generated by maximum intensity projection of confocal z-stacks. Z stack thickness was adjusted to the area of interest. Single images were taken every 1.5 nm. Adobe Photoshop CS2 was used to enhance both brightness and contrast by 33 % for the processed confocal z-stacks.

Results

Development of an aniline blue/ trypan blue based in planta downy mildew staining for CLSM

In the lambda scans of the fixed but unstained samples (compare Table 2) the excitation with the 405 nm laser showed a weak emission signal between 460 and 540 nm, as well as between 620 and 680 nm (Figure S1). Autofluorescence of the samples was strongest when excited with the 561 laser with emission between 575 and 720 nm, with a peak at 665 nm (Figure S2). To distinguish plant cells and intercellular hyphae by UV induced auto fluorescence very high laser intensities (over 50 %) had to be applied. High laser intensities lead to photo-bleaching. Therefore, microscopical analysis of bleached but unstained samples is not recommended. Using the 561 laser induced auto-fluorescence intercellular hyphae and haustoria could be recognized between plant cells. However, visual contrast and resolution were poor (Figure 1).

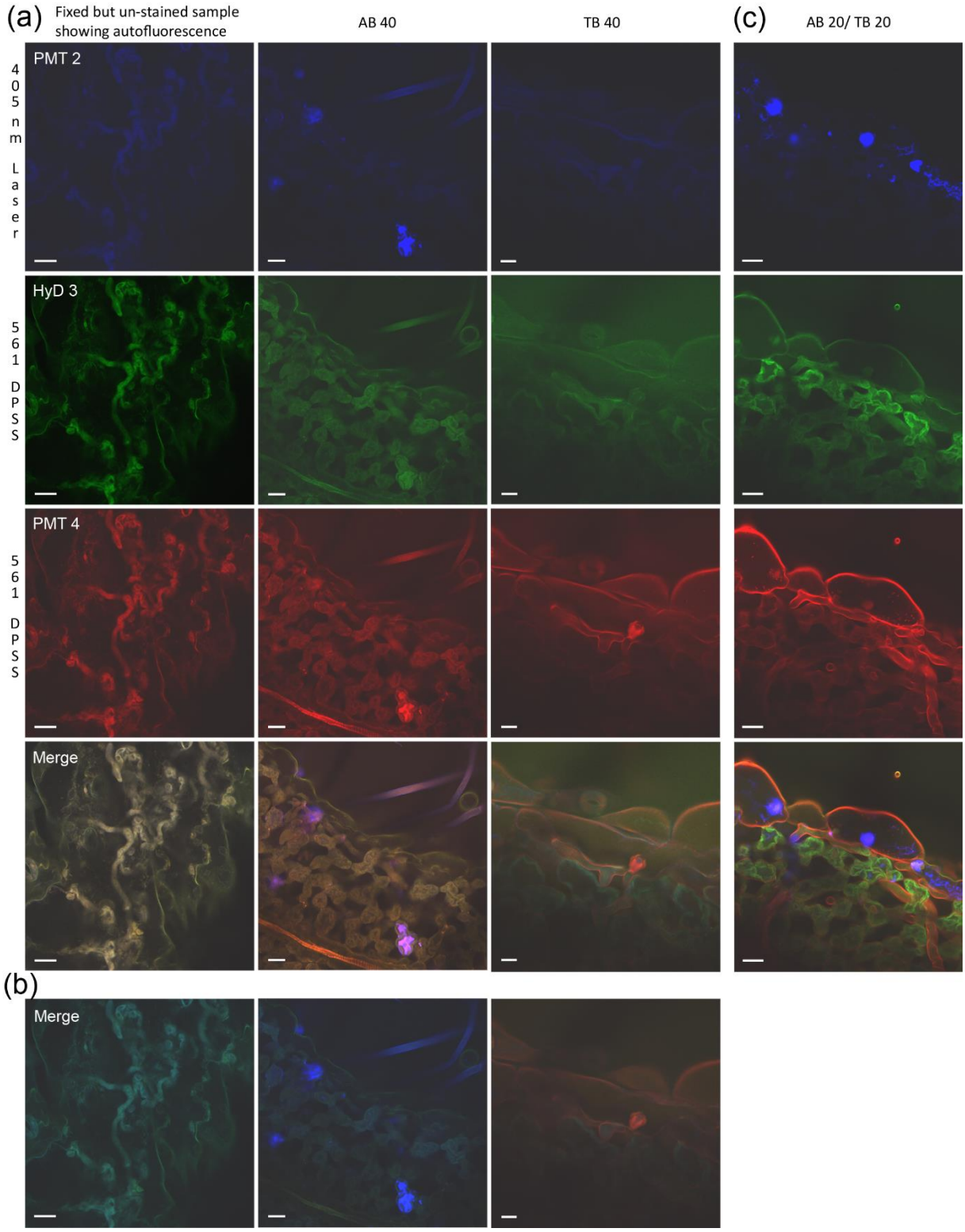

Figure 1: Comparison of different stains for visualization of *Peronospora salviae-officinalis* infections on *Salvia officinalis* and the resulting plant-pathogen interaction seven days after infection. To compare the sensitivity and performance of the different staining solutions (TB 40, AB 40, AB 20/TB 20) and sample auto fluorescence, the CLSM acquisition settings used for AB 20/TB 20 were applied. Sequential scans were captured with different detectors and merged images excited with the 405 nm laser and the 561 nm laser of fixed unstained samples, samples stained with aniline blue (AB 40), trypan blue (TB 40) and a 1:1 mixture of aniline and trypan blue (AB 20/TB 20) are shown. (a): To capture a signal for unstained, AB 40 and TB 40 stained samples the laser intensity of the 561 laser was increased (35-42 %) compared to 24 % for the samples stained with the AB 20/TB 20 mixture. (b): Merged images of the sequential scans of unstained and AB 40 and TB 40 stained samples, shown in (a), using the low laser intensity of

images shown in (c). (c): Sequential scans and merged image of the sequential scans of samples stained with AB 20/ TB 20 mixture. Laser intensity was 21 % for the 405 nm laser and 24 % for the 561nm laser. Scale bar represents 20 µm

Using staining solutions AB 20 and TB 20 containing 20 µl AB and TB, respectively showed low sensitivity, weak resolution, and weak visual contrast. AB 40 and TB 40, containing 40 µl AB and TB, respectively, enhanced the sensitivity. Several authors reported, that commercial aniline blue preparations from different companies differ (Bougourd et al., 2000, Smith & McCully, 1978). Therefore lambda scans were conducted. The lambda scan of a sample stained with AB 40 showed a strong 405 nm laser induced signal with a maximum at 450 - 480 nm and a faint signal induced with the 561 nm laser between 640 and 700 nm. The sequential acquisition of each channel revealed that PMT 2 (447 - 495 nm) visualized callose inside the plant tissue and callose accumulations around hyphae and haustoria. Detectors HyD 3 (593 - 625 nm) and PMT 4 (673 - 749 nm) visualized mainly the cytoplasm of plant and oomycete cells, but differentiation between cytoplasm and cell walls was difficult. Visual contrast and resolution provided by AB 40 was higher compared to the unstained samples, but not as high as with TB 40. Staining solution TB 40 showed a faint UV-induced fluorescence in the lambda scan, with a maximum between 640 - 700 nm. The PMT 2 captures only background noise in TB 40 stained samples, while the HyD 3 captured mainly cytoplasm of plant and oomycete cells and PMT 4 (673-749 nm) visualized mainly cell walls. Using TB 40 intercellular hyphae and haustoria are clearly distinguishable from plant tissue. Visual contrast and resolution provided by TB 40 was higher than with AB 40, but callose was not detected.

The lambda scan of the AB 20/TB 20 stained samples using the 405 nm laser showed a first strong emission signal with a maximum intensity around 460 nm and a second, fainter, emission signal around 680 nm (Figure S1). Using the 561 nm DPSS laser the detected emission in the lambda scan reached its maximum intensity at 670 nm (Figure S2). The first signal detected with the 405 nm laser was induced by the aniline blue part of the staining solution, while the second signal was induced by trypan blue, as lambda scans of AB 40 and TB 40 revealed. Using the 561 nm DPSS laser the detected emission of AB 20/TB 20 sample reached its maximum at 660 nm. This signal can be attributed to trypan blue. The AB 20/TB 20 treatment showed the highest image

resolution. Aniline blue stained callose depositions and accumulation, as visualised in blue pseudocolour (447 - 495 nm, PMT2). Plant and oomycete cytoplasm and plant chloroplasts are visualised in green pseudoclour (593 - 625 nm, HyD 3), cell-walls of host and oomycete were visualized in red pseudocolour (673 - 749 nm, PMT 4). Overall, the background noise of HyD 3 and PMT 4 was much lower using combined AB 20/TB 20, due to the quenching feature of trypan blue. The reduced auto fluorescence leads to a high resolution and a strong visual contrast. The usage of two detectors to capture emission from the 561 laser increases the contrast between cytoplasm and cell-wall of plant and oomycete. The overlay of green and red pseudocolours generates the pseudocolour yellow, resulting in a light to dark orange surface of intercellular hyphae. Cell structures of the downy mildew can thus easily be localised within the plant tissue. Early development of a callose sheath around the haustoria neck can be distinguished from the entire encapsulation of older haustoria (Figure 3). The emission wavelength range was extended to enhance the brightness of hyphae in thicker samples, without turning up laser intensity, to avoid photo-bleaching.

Histological study of *Peronospora salviae-officinalis* infection of sage

After optimisation, the CLSM visualisation method was applied for the histological study of *P. salviae-officinalis* on *S. officinalis*, documenting the infection process from the adhesion of conidia on the leaf surface until *de novo* sporulation. The infection process starts with an ovoid multinucleate conidiosporangiocyst, generally referred to as conidium attaching to the leaf surface of *S. officinalis*. The conidium displays the typical callose plug sealing at the base where it had been attached to the conidiophore (Figure 2a). Conidia of *P. salviae-officinalis* germinate with a germ-tube, which then develops an appressorium (Figure 2b) by inflating its tip, and from which a penetration hypha develops. Infection of the leaf tissue frequently happens through a stoma (Figure 2c), but epidermal cells can also be penetrated directly (Figure 2d.1), likely by cell wall degrading enzymes secreted at the hyphal apex (Hardham, 2007). In reaction to the penetration, the plant reinforces the cell wall by secreting callose at the point of entry (Figure 2d.1 and d.2, arrow). In the intercellular space, *P. salviae-officinalis* forms a primary vesicle (Figure 2d.2), from which hyphae spread between the mesophyll cells and develop first haustoria (Figure 2e and f, asterisks). Haustoria enter the host cell-wall and invaginate the plasma membrane of the plant cell.

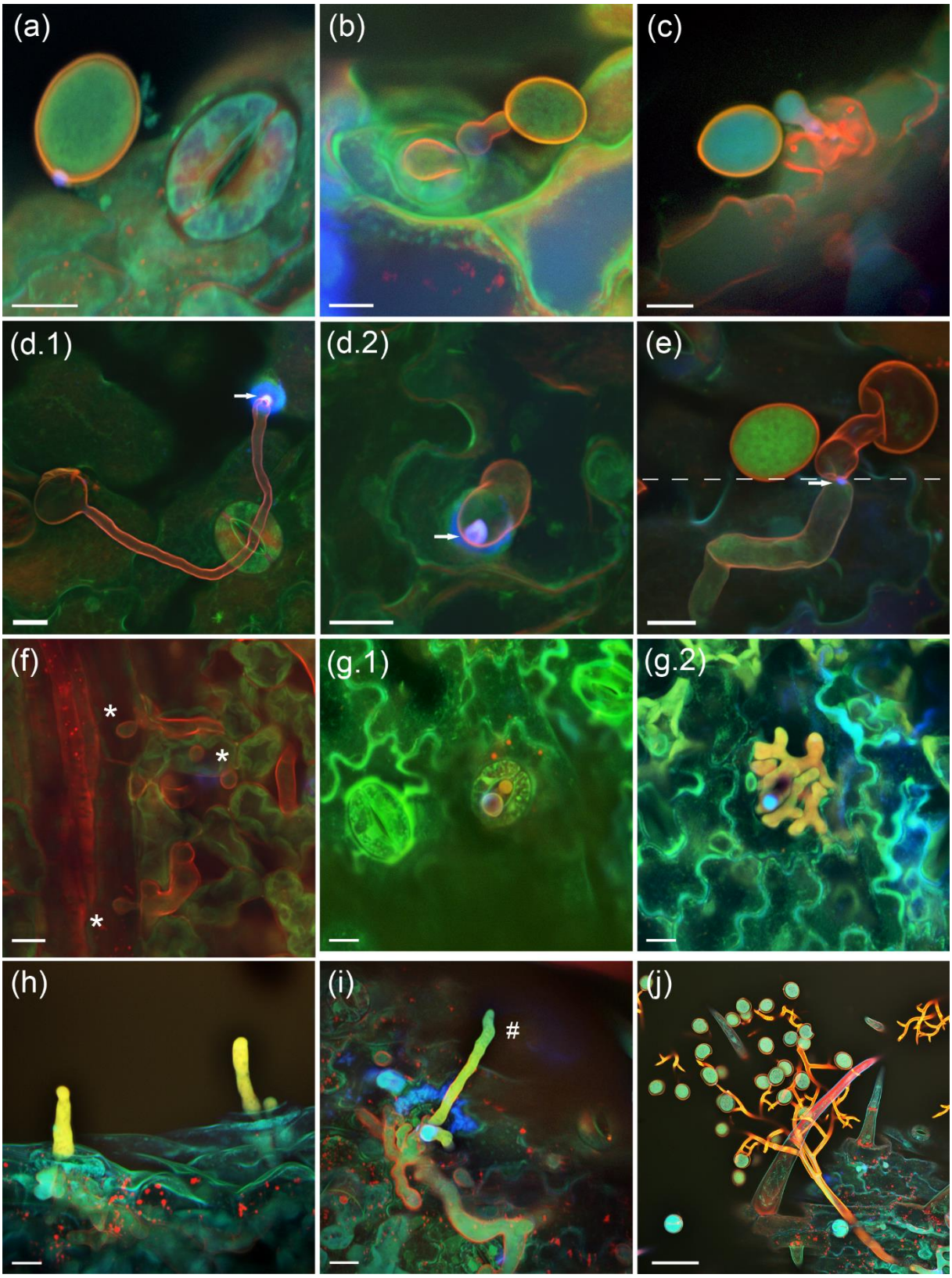

Figure 2: CLSM images of the asexual development of *Peronospora salviae-officinalis* on *Salvia officinalis* from one to seven days after infection, stained with a 1:1 mixture of aniline and trypan blue. (a): Conidium on lower leaf surface of common sage next to a stoma. The callose plug sealing appears blue, indicating accumulation of (1,3)-β-glucans. (b): Conidium germinated with germ tube and appressorium. (c): Side view of an appressorium entering a stoma with a penetration hyphae. (d.1): Conidium germinated with a germ tube, entering the leaf tissue directly via cuticula and epidermis. The plant accumulates callose around the penetration side, indicated by arrow. (d.2): After entering the leaf

tissue via the epidermis, *P. salviae-officinalis* builds a primary vesicle inside the plant tissue under the point of entry. (e): Side view of a germinated conidium, which enters the plant tissue directly via the epidermis. On the epidermis of the lower leaf surface (above the dotted line), the conidium forms a germ tube and penetrates the epidermis. Inside the leaf tissue (below the dotted line), directly under the epidermis, an intercellular hypha formed. (f): Haustoria, indicated by closed arrow heads, along leaf vein and in mesophyll cells, expanding in the plant cells from an intercellular hyphae. (g.1) and (g.2): Images of a developing conidiophore primordium, indicated by #, at two focal planes. (g.1): On the leaf surface the conidiophore primordium is visible. (g.2): Inside the leaf tissue strong hyphal ramification under the stoma is visible. (h) and (i): Young conidiophores expanding from stomata. (j): Conidiophore between leaf hairs and glandular hairs with glandular cells of the leaf. Scale: (a)-(f), (g.1)-(i): 10 µm; G: 20 µm; (j): 50 µm. The physical length of the z-stacks was the following for the single pictures: (a): 4.5 µm; (b): 1 µm; (d.1): 1 µm; (d.2): 1.5 µm; (e): 21.84 µm; (f): 5.2 µm; (g.1): 3.12 µm; (g.2): 11.4 µm; (h): 46 µm; (i): 21 µm and (j): 8 µm

In all observations, only a single haustorium was present per host cell. Occasionally an immune response of single plant cells was observed. Callose deposition and accumulation are found predominantly around haustoria, although most haustoria have little callose around them (Figure 3). Four days after inoculation intercellular hyphae were widespread between the mesophyll cells, and numerous intracellular globose haustoria inside the mesophyll cells could be observed (Figure 3) as well as first persistent conidiosporangiophores, generally referred to as conidiophores.

Before forming a conidiophore, the intercellular hyphae branch strongly beneath a stoma, grow into the stomatal grove and form a balloon-like structure, the conidiophore primordium, (Figure 2g.1 and g.2) which might help to keep the stoma open during initial conidiophore elongation. The conidiophore primordium develops into a conidiophore and emerges through the stoma. Usually one, but occasionally two to four conidiophores emerge from a single stoma (Figure 2h and i). Mature conidiophores bear numerous conidia on multiple ramified branches (Figure 2j). Unlike the majority of downy mildews conidiophores of *P. salviae-officinalis* can also be observed on the upper leaf surface, because leaves of *S. officinalis* are amphistomatic. Seven days after inoculation many conidiophores with conidia were observed on the lower leaf surface and also some on the upper leaf surface. Conidia are then liberated and released actively to the air by twisting hygroscopic movements of conidiophores upon drying (Lange et al., 1989). While sage leaf tissue is colonised extensively by intercellular hyphae of *P. salviae-officinalis* already four days after inoculation, chlorotic leaf spots can be observed on infected sage leaves seven days after inoculation at the earliest.

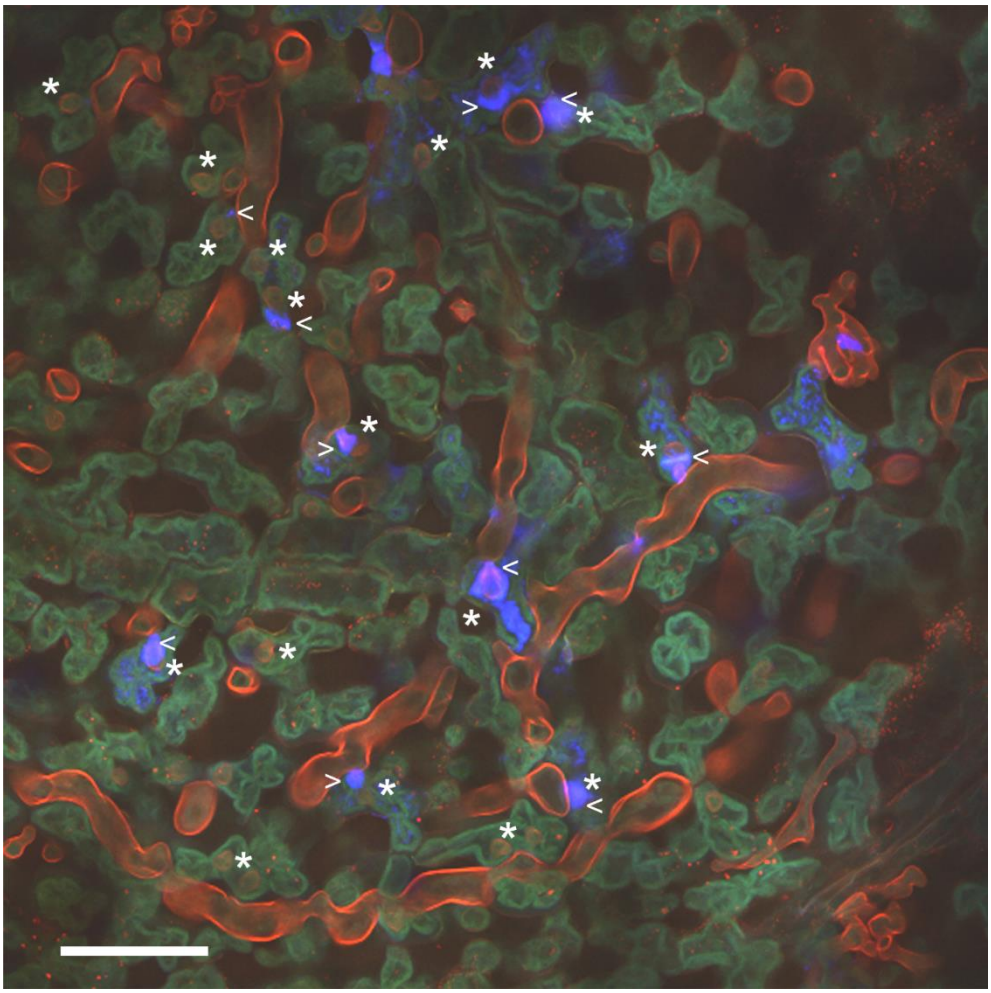

Figure 3: CLSM image of leaf tissue of Salvia officinalis infected by *Peronospora salviae-officinalis* four days after inoculation, stained with a 1:1 mixture of aniline and trypan blue. Intercellular hyphae are growing between leaf mesophyll cells. Strong ramification of the hyphae indicate points of conidiophore development. Numerous haustoria, indicated by closed arrow heads, are formed inside the mesophyll cells. Callose deposition towards the haustoria, as a form of plant immune response, has already started. Open arrow heads show callose accumulation around haustoria. Scale: 20 µm. Physical length of the z-stack: 1.5 µm

With the start of the homothallic sexual reproduction of *P. salviae-officinalis* intercellular hyphae form oogonia and antheridia. The antheridium forms a collar-like structure after the penetration through the oogonium (Figure 4a). The antheridium stays attached to the oogonium (Figure 4b-c) and forms a fertilization tube (Figure 4b, arrow) to transfer its haploid nuclei into the oosphere. Oospores are often formed directly under the cuticle, probably to promote their release after decomposition of the host tissue.

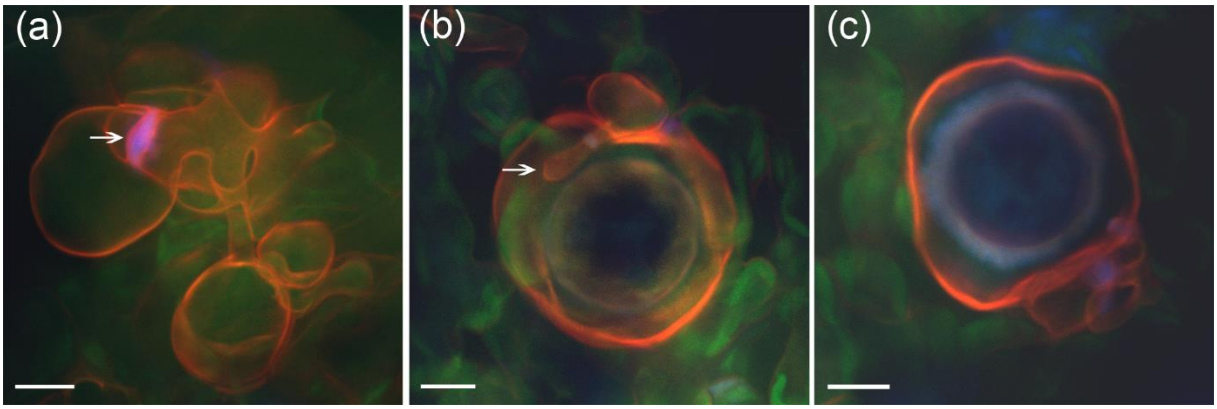

Figure 4: CLSM images of the sexual development of *Peronospora salviae-officinalis* on *Salvia officinalis*, fourteen days after infection, stained with a 1:1 mixture of aniline blue and trypan blue. (a): Oogonium, which had penetrated the antheridium. The antheridium formed a fertilization tube towards the oogonium (arrow). Oogonium and antheridium are formed by intercellular hyphae. (b): Oospore with attached antheridium and fertilization tube. (c): Mature oospore with attached antheridium. Scale bars: 10 µm. The physical length of the z-stacks was the following for the single pictures: (a): 7.3 µm; (b): 16.6 µm; (c): 5.2 µm.

Validation of the optimised staining method with samples of *P. lamii* on *L. purpureum* and *P. belbahrii* on *O. basilicum*

The optimised staining and CLSM visualisation method was also tested on *P. lamii* parasitizing *Lamium purpureum* (red dead-nettle) and *P. belbahrii* parasitizing *Ocimum basilicum* (basil) (Figure 5). All structures of *P. lamii* and *P. belbahrii* could be visualised with similar quality as shown for *P. salviae-officinalis* on *S. officinalis*. Consistent to the CLSM images taken from *P. salviae-officinalis* cell-walls of intercellular hyphae, conidia and conidiophores appeared red-orange, cytoplasm yellow to green and callose blue (Figure 5a, d). Also, basil and red dead-nettle deposited callose towards the haustoria and accumulated callose around haustoria. Like *P. salviae-officinalis* conidia of *P. lamii* and *P. belbahrii* have a callose plug at their base where they are attached to the end branches of the conidiophore, but in case of the latter two the callose plugs are very thick (Figure 5b, c, e, f). The strong blue signal indicates that the plug contains large amounts of β-(1,3)-glucans. Branches of conidiophores of *P. lamii*, from which the sporangia were already discharged also showed strong blue signals. The signal is probably a reflection of either sealing the conidiophore upon plasma retreat or a side-effect of drying.

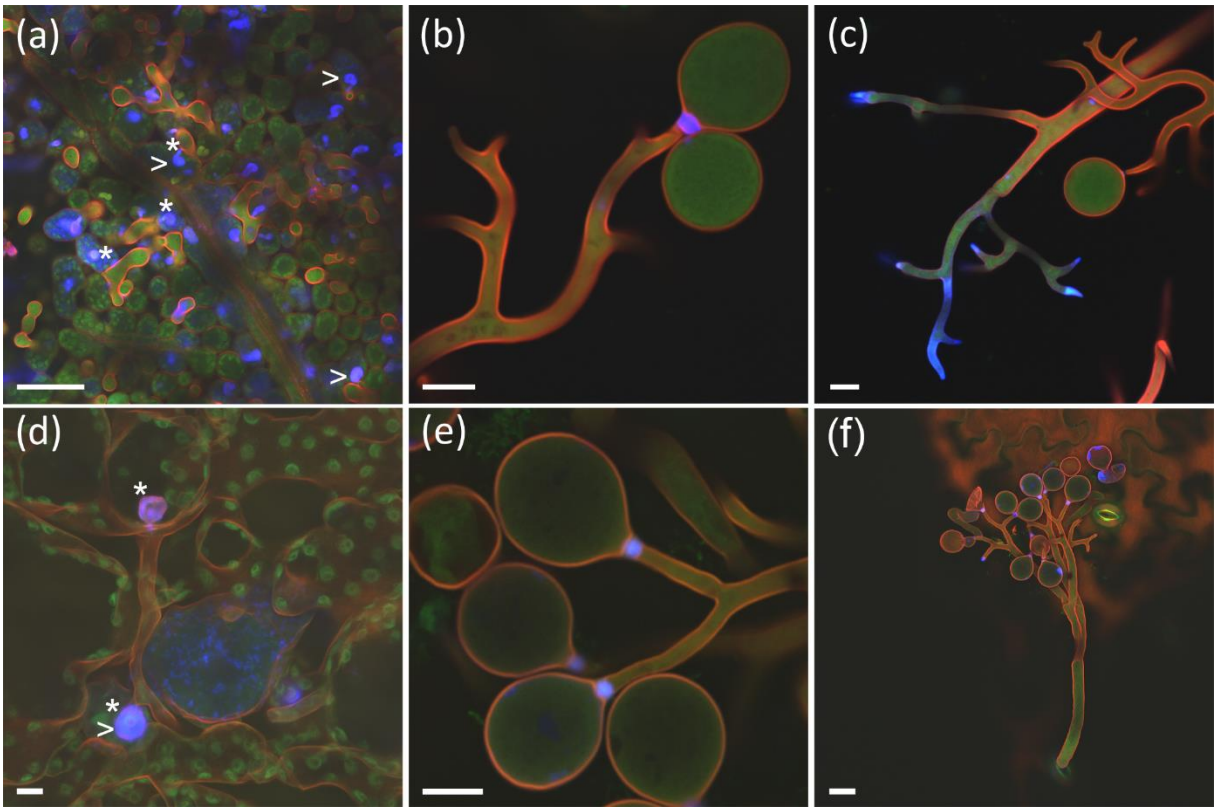

Figure 5: CSLM images of *Peronospora lamii* on *Lamium purpureum* and of *Perosnsopora belbahrii* on *Ocimum basilicum* stained with a 1:1 mixture of aniline and trypan blue. (a)-(c): *P. lamii on L. purpureum*. (a): Intercellular hyphae and haustoria. Callose is deposited towards haustoria and some haustoria are already encapsulated by callose. (b): Ultimate branchlets of a conidiophore with two conidia. A callose plug is formed between one conidia and the end branch at the abscission site of the conidia. The blue colour indicates that the plugs contain β-(1,3)-glucans. (c): Branches of a conidiophore. Older parts of the conidiophore with dense deposition of β-(1,3)-glucans at abscission sites. Younger parts without β-(1,3)-glucan depositions. (d)-(f): *P. belbahrii* on *O. basilicum*. (d): Intercellular hyphae between mesophyll cells with two intracellular haustoria. Callose deposition has started and one haustorium is encapsulated in callose. (e): Conidia of *P. belbahrii* on the ultimate branchlets of a conidiophore. Plugs with β-(1,3)-glucans are formed at the abscission site. (f): Conidiophore of *P. belbahrii* emerging from a stoma. Haustoria are indicated by a closed arrow head, callose deposition by an open arrow head. Scale: (a): 50 µm, (b)-(d): 10 µm. The physical length of the z-stacks was the following for the single pictures: (c): 6 µm; (d): 10 µm; (f): 10 µm.

Discussion

Fluorescence staining and imaging of oomycete-plant interactions is challenging due to the similarity in cell wall composition of both plant and microorganism with ß-1,3 bond glucose units as the dominating cell wall components. Because larger amounts of chitin are absent from most oomycete cell walls, staining protocols used for true fungi cannot be applied. The staining procedure for CLSM of *Peronospora* species infective to *Lamiaceae* presented here, involving bleaching with ethanol and KOH and staining with a 1:1 aniline-trypan blue mixture, allows detailed visualisation of downy

mildew structures outside and inside the host tissue, as well as of callose deposition as an immune response of the plant.

One of the major advantages of confocal microscopy is the non-invasive quality of optical sectioning (Czymmek et al., 1994). With our protocol, embedding or fixation of samples is not necessary, preserving a natural tissue structure. While pure TB staining was frequently used and resulted in enough resolution for the visualisation of oomyceteous hyphae in the thin cotyledons and leaves of *Arabidopsis thaliana* (Huitema et al., 2003, Robinson & Cahill, 2003, Takemoto et al., 2003), it resulted in blurred pictures of the oomycetous structures within sage leaves. The method developed here overcame this problem and can also be used in thicker and more complex tissues. Callose deposition within infected leaf tissue was not observed in studies where only TB was used for staining (Takemoto et al., 2003, Huitema et al., 2003). However, it had been shown that callose encasements around haustoria can be detected when AB was applied (Rumbolz et al., 2002, Dong et al., 2008). This is the first study where AB and TB are used simultaneously. This increases the resolution of oomycetes and plant structures significantly compared to studies where AB and TB had been employed, however in separate staining approaches (Donofrio & Delaney, 2001, Robinson & Cahill, 2003). The dual staining with AB and TB enabled the clear visualisation of oomycete and plant structures, such as the downy mildew infection structures and callose deposition as a result of host defence responses against pathogen infection (Figure 3). With AB alone, infection structures as well as callose deposition are also visible, but it is more difficult to distinguish between plant and oomycete structures. In contrast, callose cannot be visualized with TB, but it is easier to distinguish structures of *Peronospora* from plant cells compared to AB. Also in the bleached but unstained sample, oomycete structures inside the plant tissue could be observed. However, for the detection of intercellular hyphae or haustoria in bleached but unstained sage tissue ample experience in microscopic observation of these structures is necessary.

Besides the combination of AB and TB, in this study three detectors were used to capture the emission fluorescence of plant and oomycete tissue: the photomultiplier tubes PMT2 (447 - 495 nm) and PMT4 (673 - 749 nm) and the hybrid detector HyD3 (593 - 625 nm). The signals were displayed in different pseudocolours: PMT2 in blue, HyD3 in green and PMT4 in red pseudocolour. The merged images show an overlay

of all three channels. The overlay of green and red pseudocolours generates the pseudocolour yellow, resulting in a light to dark orange surface of intercellular hyphae. The intensity and the grade of the signal can vary, depending on the angle the image is taken and the number of overlying stacks. These additional pseudocolour grades improve the visualisation of different structures. Cell structures of embedded downy mildew pathogens can be readily visualised for their localisation. For example, developing, thin encapsulations of haustoria can be distinguished from the entire encapsulation of older haustoria, because the red-coloured structure of the haustorium itself shines through the blue-coloured callose layer, whereas the haustoria cannot be seen through the thick callose layer after complete encapsulation.

With the optimised method developed herein, the infection of *S. officinalis* by *P. salviae-officinalis*, was tracked from adhesion of conidia on the leaf surface until sporulation. This is the first study picturing the whole asexual, as well as the sexual developing cycle of *P. salviae-officinalis*. Moreover it was also possible to detect callose deposition and accumulation by the plant in downy mildew infected leaf tissue of sage, basil and red dead-nettle. Callose depositions are a well-known, unspecific immune response of plants towards mechanic and pathogenic injury (Judelson & Ah-Fong, 2019, Kortekamp, 2005). Encapsulations of haustoria by callose are known for several downy mildew infections, like those of *Plasmopara viticola* on *Vitis vinifera* and *Bremia lactucae* on *Lactuca sativa* (Sedlarova & Lebeda, 2001, Kortekamp, 2005, Diez-Navajas et al., 2008), *P. parasitica* and *Hyaloperonospora arabidopsidis* on *A. thaliana*. Evaluation of callose depositions around haustoria have been used evaluate the performance of an effector, or to determine host defence responses as a degree of resistance in a crop cultivar towards downy mildew infections. For this aniline blue staining of callose depositions is often used (Donofrio & Delaney, 2001, Diez-Navajas et al., 2008, Fabro et al., 2011, Caillaud et al., 2012). Haustorial encasements are double-layered, with callose-containing membrane structures that often surround older haustoria as part of cellular host defence. In compatible interactions the encapsulation of haustoria is delayed and lags behind the spread of the pathogen (Lu et al., 2012). It is assumed that encasements restrict the nutrient uptake by the pathogen, impair effector translocation, or concentrate plant-derived antimicrobials. Oomycetes, on the other side, have developed counter defences against this plant defence system to secure their nutrient source (Judelson & Ah-Fong, 2019). We found that in case of *P. salviae-officinalis* just a few of the numerous formed haustoria possessed a callose

collar around the neck or were encapsulated by callose. For *H. arabidopsidis* it is reported that this pathogen is able to suppress the callose deposition of *A. thaliana* by several effectors (Fabro et al., 2011). Also for other downy mildews, like *Pl. halstedii* parasitizing sunflowers, it is known that they are able to mask pathogen-associated molecular patterns, or are capable of down-regulating the immune response (Sharma et al., 2015). If the rather low amount of callose around haustoria in our study was due to the juvenility of these haustoria or if this was a result of a suppressed immune response needs further investigation. But the low abundance of callose depositions, and the late formation of chlorotic or necrotic leaf spots in infected plants indicate, that *P. salviae-officinalis* is well adapted to its host.

Acknowledgments

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Data Availability Statement

The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

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Supporting Information

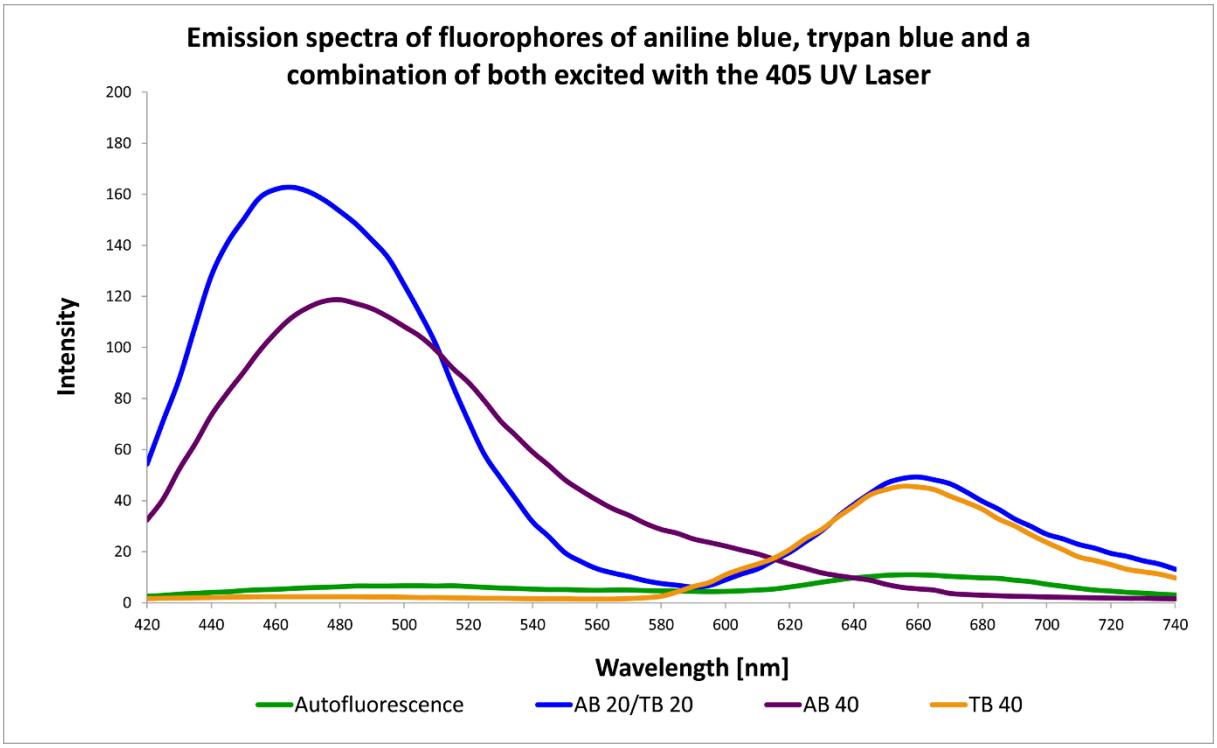

Figure S1: Lambda scans of the emission spectra excited with the 405 laser between 420 and 740 nm. Green line: emission spectrum of unstained, auto fluorescing sample. Blue line: emission spectrum of a sample stained with a combination of aniline and trypan blue. Purple line: emission spectrum of a sample stained with aniline blue. Orange line: emission spectrum of a sample stained with trypan blue.

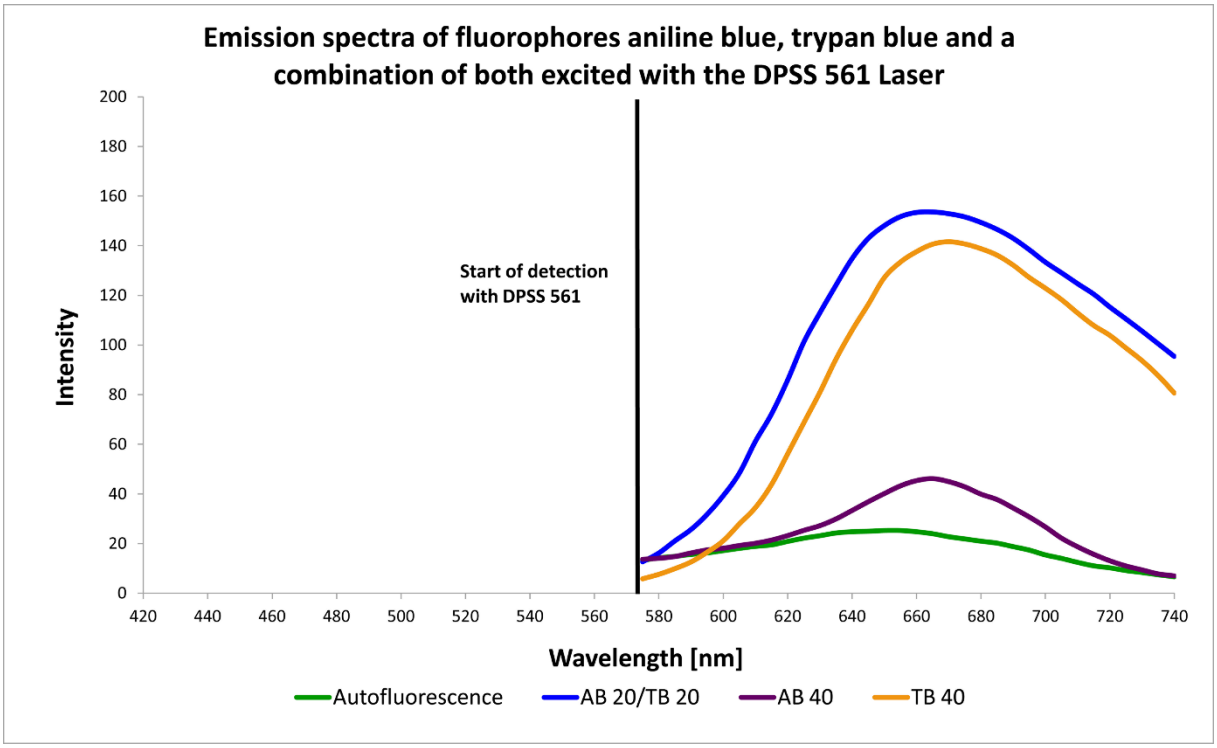

Figure S2: Lambda scans of the emission spectra excited with the 561 nm laser between 575 and 740 nm. Green line: emission spectrum of unstained, auto fluorescing sample. Blue line: emission spectrum of a sample stained with a combination of aniline and trypan blue. Purple line: emission spectrum of a sample stained with aniline blue. Orange line: emission spectrum of a sample stained with trypan blue.

Appendix 3

Statement of Joint Authorship

On the publication: *Two new species of the Peronospora belbahrii species complex, Pe. choii sp. nov. and Pe. salviae-pratensis sp. nov., and a new host for Pe. salviae-officinalis*

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What did the PhD student or the Co-Authors contribute to this work?

(1) Development and planning

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(2) Performance of the individual investigations and experiments

PhD student MH: 100%

(3) Preparation of the data collection and figures

PhD student MH: 60%

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(4) Analysis and interpretation of data

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Date/ Place	Signature Supervisor
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Chapter 3

Two new species of the *Peronospora belbahrii* species complex, *Pe. choii* sp. nov. and *Pe. salviae-pratensis* sp. nov., and a new host for *Pe. salviae-officinalis*

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Key words: coleus, downy mildew, multi-gene phylogeny, new host report, new taxa, *Peronosporaceae*

Abstract

The downy mildew species parasitic to *Mentheae* are of particular interest, as this tribe of *Lamiaceae* contains a variety of important medicinal plants and culinary herbs. Over the past two decades, two pathogens, *Peronospora belbahrii* and *Pe. salviae-officinalis* have spread globally, impacting basil and common sage production, respectively. In the original circumscription of *Pe. belbahrii*, the downy mildew of coleus (*Plectranthus scutellarioides*) was ascribed to this species in the broader sense, but subtle differences in morphological and molecular phylogenetic analyses using two genes suggested that this pathogen would potentially need to be assigned to a species of its own. In the present study, *Peronospora* species causing downy mildew on members of the *Mentheae*, including clary sage (*Salvia sclarea*), meadow sage (*S. pratensis*), basil (*Ocimum basilicum*), ground ivy (*Glechoma hederacea*) and coleus (*Plectranthus scutellarioides*) were studied using light microscopy and molecular phylogenetic analyses based on six loci (ITS rDNA, *cox*1, *cox*2, *ef1a*, *hsp*90 and *β-tubulin*) to clarify the species boundaries in the *Pe. belbahrii* species complex. The downy mildew on *Salvia pratensis* is shown to be distinct from *Pe. salviae-officinalis* and closely related to *Pe. glechomae*, and is herein described as a new species, *Pe. salviae-pratensis*. The downy mildew on *S. sclarea* was found to be caused by *Pe. salviae-officinalis*. This is of phytopathological importance, because meadow sage thus does not play a role as inoculum source for common sage in the natural habitat of the former in Europe and Asia, while clary sage probably does. The multi-gene phylogeny revealed that the causal agent of downy mildew on coleus is distinct from *Pe. belbahrii* on basil, and is herein described as a new taxon, *Pe. choii*.

Introduction

Over the past two decades several downy mildew diseases in medicinal plants and culinary herbs have been newly reported and led to economic losses. Prominent examples are *Peronospora somniferi* on opium poppy (Voglmayr et al., 2014), *Pe. belbahrii* on basil (Thines et al., 2009a) and *Pe. salviae-officinalis* on common sage (Choi et al., 2009a). The latter two species are closely related and belong to a clade we refer to as *Pe. belbahrii* species complex. Apart from the two mentioned species, it is known to contain *Pe. elsholtziae* and *Pe. salviae-plebeiae* (Choi et al., 2009a). Of the species in the complex, especially *Pe. belbahrii* and *Pe. salviae-officinalis* have

proven to be destructive pathogens in the production of the respective crops. When downy mildew disease was first discovered on basil and common sage, it was mostly considered to belong to *Pe. lamii* (McMillan, 1993, Gamliel & Yarden, 1998, Plenk, 2002b, Hill et al., 2004, Belbahri et al., 2005a, Liberato et al., 2006, Humphreys-Jones et al., 2008, Choi et al., 2009a), according to the broad species concept advocated by (Yerkes & Shaw, 1959) for some downy mildew groups. However, this species concept is generally not appropriate for downy mildews as demonstrated by several phylogenetic studies over the past 20 years or so (for a review see (Thines & Choi, 2016). Specifically, it had been shown that the taxon *Pe. lamii* should be restricted to the downy mildew parasitizing *Lamium* spp. or *L. purpureum* only (Choi et al., 2009a, Thines et al., 2009a).

Apart from the sage pathogens reported so far, several other *Salvia* species were reported as hosts for *Peronospora*, such as *S. lanceolata*, *S. pratensis*, *S. reflexa* and *S. sclarea* (Rabenhorst, 1857, Ellis & Kellerman, 1887, Gäumann, 1923, USDA, 1960, Osipjan, 1967, Kochman, 1970, Stanjavičenie, 1984). In checklists of *Peronosporaceae* in Europe and the British Isles downy mildews on *S. sclarea* (clary sage) and *S. pratensis* (meadow sage), have been noted in addition to downy mildew on common sage (Gaponenko, 1972, Dudka et al., 2004, Mulenko et al., 2008, Müller & Kokes, 2008). The downy mildews on clary and meadow sage were usually attributed to *Pe. swinglei* (Gaponenko, 1972, Mulenko et al., 2008), or *Pe. lamii* (Preece, 2002, Dudka et al., 2004, Müller & Kokes, 2008), respectively. While *Pe. lamii* is clearly not an appropriate species name for downy mildews on sage (Choi *et al.*, 2009, Thines *et al.*, 2009), the application of the name *Pe. swinglei* to downy mildew pathogens of various species of sage seemed to be more plausible, because this taxon was originally described from *S. reflexa* (Ellis & Kellerman, 1887, Constantinescu, 1991). However, phylogenetic investigations revealed a very high degree of specialisation in *Peronospora* on *Lamiaceae* in general and on *Salvia* in particular (Choi et al., 2009a, Thines et al., 2009a). These studies demonstrated that *Pe. swinglei* was not only distinct from *Pe. belbahrii* but also from the two downy mildews infecting *S. officinalis* and *S. plebeia,* resulting in the description of *Pe. salviae-plebeiae* and *Peronospora salviae-officinalis* (Choi et al., 2009a). Thus, three individual *Peronospora* taxa are currently reported from sages.

So far common sage is the only known host of *Pe. salviae-officinalis*. From a phytopathological perspective it is important to clarify whether other potential hosts do

exist that could serve as reservoirs of inoculum for the disease caused by *Pe. salviae-officinalis*. Phylogenetic studies of *Lamiaceae* with a special focus on *Salvia* showed that *S. officinalis* and *S. sclarea* are closely related. Together with *S. pratensis*, they belong to the “Clade I” within the mint family (Walker & Sytsma, 2007, Will & Classen-Bockhoff, 2014). Because of their close phylogenetic relationship, it seemed possible that these two sage species could be alternative hosts and could play a role in the infection of sage fields. At the same time, the downy mildew pathogen of coleus that also belongs to the *Pe. belbahrii* species complex seems to be still having a restricted distribution (Gorayeb et al., 2019) suggesting that it is not conspecific with *Pe. belbahrii* and thus representing another downy mildew pathogen posing a potential economic risk.

It was the aim of the current study to better define species boundaries in the *Pe. belbahrii* species complex by detailed morphological and molecular phylogenetic investigations.

Materials and methods

Fungal specimens

The downy mildew specimens analysed in this study are given in Table 1.

Table 3: Specimens analysed

Pathogen	Host	Location	Year	Accession	Collector	ITS	*EF1a*	*ß-tub*	*Hsp90*	*cox1*	*cox2*
Pe. glechomae	*Glechoma hederacea*	Germany, ST, Östliches Harzvorland	2001	GLM-F73803	H. Jage	MN450323	MN546892	MN546919	MN546995	MN546943	KJ654217*
Pe. glechomae	*Glechoma hederacea*	Romania, Suceava, Clit	1992	BUCM 125.616	G. Negreen	MN450332	MN546901	MN546926	MN547002	MN546952	MN546978
Pe. lamii	*Lamium purpureum*	Germany, BW, Ladenburg	2018	# WM 3830	M. Hoffmeister	MN450324	MN546893	–	–	MN546944	MN546970
Pe. lamii	*Lamium purpureum*	Germany, NI, Evessen	2018	# WM 3831	M. Hoffmeister	MN450325	MN546894	–	–	MN546945	MN546971
Pe. salviae-plebeiae	*Salvia plebeia*	Korea, Hongcheon	2008	KUS-F23371		–	–	–	–	–	KJ654299*
Pe. salviae-officinalis	*Salvia officinalis*	Germany, HE, Bad Hersfeld	2017	GLM-F117791	H. Blum	MN308035	MN546878	MN546904	MN546981	MN546929	MN546955
Pe. salviae-officinalis	*Salvia officinalis*	Germany, SN, Dresden	2017	GLM-F117792	C. Grunert	MN308036	MN546879	MN546905	MN546982	MN546930	MN546956
Pe. salviae-officinalis	*Salvia officinalis*	Switzerland, TG, Kesswil	2017	GLM-F117793	H. Blum	MN308034	MN546880	MN546906	MN546983	MN546931	MN546957
Pe. salviae-officinalis	*Salvia officinalis*	Germany, NI, Rittmarshausen	2017	GLM-F117794	M. Hoffmeister	MN450312	MN546881	MN546907	MN546984	MN546932	MN546958
Pe. salviae-officinalis	*Salvia officinalis*	Germany, RP, Worms	2016	GLM-F117795	M. Hoffmeister	MN450318	MN546889	MN546916	MN546992	MN547005	MN546967
Pe. saturejae-hortensis	*Satureja hortensis*	Germany	1996	GLM-F67681	H. Jage	–	–	–	–	KJ654094*	KJ654243*
Pe. choii	*Plectranthus scutellarioides*	USA, Michigan	2007	PsC3	C. Ehrhard	MN450320	MN546891	MN546918	MN546994	MN546942	MN546969
Pe. choii (Holotype)	*Plectranthus scutellarioides*	USA, Tenessee	2015	BPI 893223	A. Windham	MN450333	MN546902	MN546927	MN547003	MN546953	MN546979
Pe. choii (Paratype)	*Plectranthus scutellarioides*	USA, Tenessee	2015	BPI 893222	A. Windham	MN450334	MN546903	MN546928	MN547004	MN546954	MN546980
Pe. choii	*Plectranthus scutellarioides*	USA	2007	HOH HUH945	anonymous	–	–	–	–	–	FJ394343*
Pe. choii	*Plectranthus scutellarioides*	USA	2007	HOH HUH946	anonymous	–	–	–	–	–	FJ394342*
Pe. choii	*Plectranthus scutellarioides*	USA	2007	HOH HUH947	anonymous	–	–	–	–	–	FJ394339*
Pe. choii	*Plectranthus scutellarioides*	USA	2007	HOH HUH948	anonymous	–	–	–	–	–	FJ394340*
Pe. choii	*Plectranthus scutellarioides*	USA, Tenessee	2015	BPI 893223	A. Windham	–	–	–	–	–	KT828759*
Pe. salviae-pratensis (Holotype)	*Salvia pratensis*	Germany, BW, Ladenburg	2016	GLM-F117783	M. Hoffmeister	MN450313	MN546885	MN546911	MN546988	MN546936	MN546962
Pe. salviae-pratensis (Paratype)	*Salvia pratensis*	Germany, NI, Braunschweig	2017	GLM-F117784	M. Hoffmeister	MN450314	MN546886	MN546912	MN546989	MN546937	MN546963
Pe. salviae-pratensis	*Salvia pratensis*	Germany, NI, Evessen	2017	GLM-F117785	M. Hoffmeister & W. Maier	MN450319	MN546890	MN546917	MN546993	MN546941	MN546968
Pe. salviae-pratensis	*Salvia pratensis*	Germany, RP, Mainz	2018	GLM-F117786	M. Hoffmeister	MN450326	MN546895	MN546920	MN546996	MN546946	MN546972
Pe. salviae-pratensis	*Salvia pratensis*	Germany, BW, Dossenwald	2018	GLM-F117787	M. Hoffmeister	MN450327	MN546896	MN546921	MN546997	MN546947	MN546973
Pe. salviae-pratensis	*Salvia pratensis*	Germany, BW, Nussloch	2018	GLM-F117788	M. Hoffmeister	MN450328	MN546897	MN546922	MN546998	MN546948	MN546974
Pe. salviae-officinalis	*Salvia sclarea*	Germany, BY, Schwebheim	2017	# WM 3832	M. Hoffmeister	MN450316	MN546887	MN546914	MN546990	MN546939	MN546965
Pe. salviae-officinalis	*Salvia sclarea*	Germany, NI, Braunschweig	2017	GLM-F117789	M. Hoffmeister	MN450317	MN546888	MN546915	MN546991	MN546940	MN546966
Pe. salviae-officinalis	*Salvia sclarea*	Germany, BB, Glindwo	2017	P1809/1	V. Kummer	MN450331	MN546900	MN546925	MN547001	MN546951	MN546977
Pe. salviae-officinalis	*Salvia sclarea*	Germany, ST, Quedlinburg	2018	GLM-F117790	M. Hoffmeister	MN450329	MN546898	MN546923	MN546999	MN546949	MN546975
Pe. swinglei (Type)	*Salvia reflexa*	USA	1887	FH 00079723		–	–	–	–	–	FJ394338*
Pe. teucrii	*Teucrium botrys*	Germany	2004	GLM-F62880	V. Kummer	MN450322	–	–	–	KJ654108*	KJ654257*
Pe. viciae	*Vicia faba*	Germany, SN, Aschersleben	2017	# WM 3833	T. Kühne	MN450315	–	MN546913	–	MN546938	MN546964

* Sequences taken from Genbank

Morphological analysis

The morphology of the investigated specimens was studied using a Zeiss Axioskop 2 plus compound microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) equipped with a Jenoptik ProgRes® digital camera. Nomarski Differential Interference Contrast (DIC) was used for observations, measurements and pictures. Images were taken using CapturePro 2.8 software (Jenoptik, Jena, Germany). Before measuring, herbarium specimens were moistened with 70 % alcohol and then transferred to 60 % lactic acid on a microscope slide. For all samples 100 conidia and conidiophores and 20 conidiophore stems were measured. All measurements are given in the form (minimum –) border of 30 %– mean – border of 30 % (– maximum) as suggested by (Thines et al., 2009a).

DNA extraction, PCR amplification, and sequencing

For DNA extraction about 1 mm² of infected plant tissue was excised using a sterile razor blade, transferred to a 2.0 mL reaction tube with three metal beads (3 mm diameter, Qiagen), cooled down in liquid nitrogen and disrupted using a mixer mill (TissueLyser LT, Qiagen, Hilden, Germany) by shaking the tubes twice at 50 Hz for 1.30 min with an intervening cooling step. Genomic DNA was extracted using the innuPREP Plant DNA Kit (Analytik Jena, Jena, Germany). Four nuclear and two mitochondrial gene regions were amplified by PCR using newly designed or published primer pairs listed in Table 2. Initially amplification success was low for *ef1a*, *β-tubulin* and *hsp*90. Therefore, new primers were designed based on a draft genome of *Peronospora salviae-officinalis* (data not published). Amplification reactions were carried out in 25 µL including genomic DNA, 10 x Mango PCR Buffer, 1.5 U Mango Taq Polymerase (Bioline GmbH, Luckenwalde, Germany), 0.2 mM dNTPs, 2 mM $MgCl_2$, 0.4 µM forward and reverse primers. In cases where only weak PCR amplification was obtained, PCR was repeated using an ALLin Hot Start Taq Mastermix (HighQu GmbH, Kraichtal, Germany). PCR conditions were as follows: an initial denaturation step of 95 °C for 3 min, 40 cycles of 95 °C for 30 s, primer-specific annealing temperatures for 30 s (see Table 2), 72 °C extension for 90 s and final extension of 72°C for 10 min. PCR products were purified using a DNA Clean & Concentrator TM-5 Kit (Zymo Research Europe GmbH, Germany) and amplicons were

sequenced at Eurofins Genomics (Eurofins Genomics GmbH, Ebersberg, Germany) using the primers that were used for PCR.

Table 4: Primers used in this study

Locus	Primer	Sequence (5´ -> 3´)	T[b]	Reference
Nuclear				
ITS	DC6	GAGGGACTTTTGGGTAATCA	57	(Cooke, 2000)
	LR-0	GCTTAAGTTCAGCGGGT		(Moncalvo, 1995)
EF 1*α*	EF1a Pso fd	ACATTGCCCTGTGGAAGTTCGA	61	This study
	EF1a Pso rv	AGTCTCAAGAATCTTACCCGAACGA		This study
β-tubulin	bTub Pso fd	AATGAGGCTACAGGTGGACGTTA	58	This study
	bTub Pso rv	CACGCTTGAACATTTCTTGAATAGC		This study
Hsp 90	HSP90 Pso fd	GGTACTCATCGCTCACTGATG	54	This study
	HSP90 Pso rv	CAACGCCCTTTACAAATGACA		This study
Mitochondrial				
cox 1	OomCox1-levup	TCAWCWMGATGGCTTTTTTCAAC	42	(Robideau et al. 2011)
	OomCox1-levlo	CYTCHGGRTGWCCRAAAAACCAAA		(Robideau et al. 2011)
cox 2	cox2 forward	GGCAAATGGGTTTTCAAGATCC	42,5	(Hudspeth et al., 2000)
	cox2 reverse	CCATGATTAATACCACAAATTTCACTAC		(Hudspeth et al., 2000)

Phylogenetic analysis

In the phylogenetic analyses newly generated and already published sequences were used (see Table 1). The newly generated sequences were edited using the DNA Sequence Analysis Software Sequencher 5.4.1 (Gene Codes Corporation, Ann Arbor, Michigan, USA). DNA sequences were aligned with the online version of Mafft v.7 (Katoh et al., 2017) using the iterative refinement algorithms Q-INS-i for the ITS rDNA and L-INS_i for all other gene regions. The start and end of the alignments were cut manually in Se-Al v2.0 (Rambaut, 1996) to remove leading and trailing gaps. The final alignments obtained were deposited (www.treebase.org) and are available under accession no S25694 (http://purl.org/phylo/treebase/phylows/study/TB2:S25694).

Phylogenetic trees were inferred based on the alignments using maximum parsimony (MP), Bayesian Metropolis coupled Markov chain Monte Carlo analyses (MC^3), maximum likelihood (ML), and minimum evolution (ME). The MP and ME analysis were carried out in MEGA7 (Kumar et al., 2016) using default settings. Support for internal nodes was estimated by 500 and 1000 bootstrap replicates, respectively (Felsenstein, 1985). The MC^3 analysis was performed using MrBayes version 3.2 (Ronquist &

Huelsenbeck, 2003) applying GTR+I+G as the substitution model. For Bayesian analyses 1.000.000 generations were run for the multi-locus tree and 2.000.000 generations for the *cox*2-based tree, respectively, and trees were sampled every 500 generations. The 50% majority rule consensus trees were computed and *a posteriori* probabilities (pp) estimated from trees of the plateau using a 20 % burnin. Maximum likelihood analyses were performed using RAxML 7.2.8 (Stamatakis, 2014) as implemented in Geneious 8.1.2 (Biomatters Limited, Auckland, New Zealand) applying the general time-reversible (GTR) substitution model with gamma model of rate heterogeneity and 1000 replicates of rapid bootstrapping. The phylogenetic trees were visualised within MEGA7 or using FigTree v.1.4.2 (http://tree.bio.ed.ac.uk/software/figtree).

Results

Morphological analysis

The *Peronospora* species on *Pl. scutellarioides* (coleus) differs from *Pe. belbahrii* on *O. basilicum* (basil) in various aspects (Table 3 and Fig.1). Conidia on *Pl. scutellarioides* were ellipsoid to rounded and with a light brown coloration, whereas conidia of *Pe. belbahrii* were ovoid to long ellipsoid and with a dark brown to olive coloration. *Peronospora* on coleus further differed from *Pe. belbahrii* by a smaller conidial size: 19.9 x 18.7 µm in the former vs. 30.8 x 24.0 µm in the latter. Additionally, the mean length/width ratio from 1.13 to 1.16 of the former was smaller than that of the latter (mean = 1.29). The downy mildew on coleus differs from *Pe. belbahrii* also in the shape of the ultimate branchlets. The shape of ultimate branchlets in *Peronospora* on coleus was curved to almost straight, especially the shorter branch was often straight, while in *Pe. belbahrii* both were curved. In addition, the length of the ultimate branchlets and the ratio of the length of the longer to the shorter ultimate branchlet differed. The mean values of the longer branchlets of *Peronospora* on coleus were shorter (15.6 µm) than those of *Pe. belbahrii* (20.6 µm). With 9.2 and 9.8 µm, respectively (type and paratype) in the mean the shorter branchlets of *Peronospora* on coleus were having a similar length as *Pe. belbahrii* (measuring 9.8 µm). As a consequence, the ratio of the length of the longer to the shorter ultimate branchlet was smaller for *Peronospora* on coleus (1.72) than that of *Pe. belbahrii* (2.29).

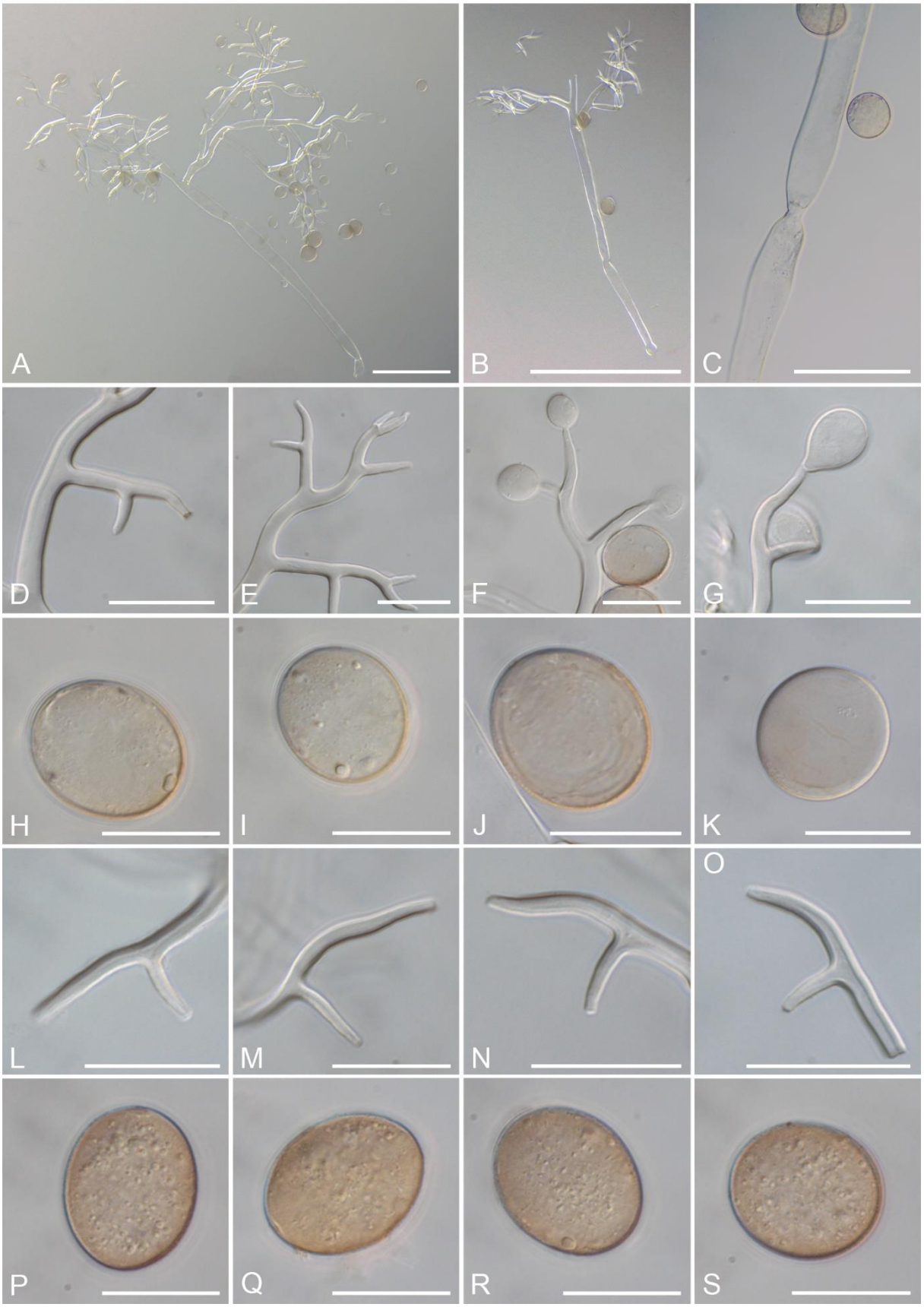

Fig. 1: A-K: *Peronospora* sp. on *Plectranthus scutellarioides* (BPI 893223). L-S: *Pe. belbahrii* on *Ocimum basilicum*. A-C: conidiophores; D, E, L-O: ultimate branchlets of conidophores; F, G: ultimate branchlets of conidophores with developing conidia; H-K, P-S: mature conidia. Scale: A: 100 µm; B and C: 50 µm, D-S: 20 µm

Table 5: Comparison of morphological features of *Peronospora* spp. parasitic to coleus, basil and red deadnettle

Fungus	*Pe* . sp. 1	*Pe* . sp. 1	*Pe* . sp. 1
Host species	*Plectranthus scutellarioides*	*Plectranthus scutellarioides*	*Plectranthus scutellarioides*
Acc. no.	BPI 893223 (Type)	BPI 893222 (Paratype)	HOH, HUH 946
Ultimate branchlets			
Shape	Curved to substrait	Curved to substrait	Curved
Length (longer)	(8.2–)12.7–15.6–17.2(–27.4) µm	(8.0–)14.3–17.6–18.8(–36.5) µm	(6.4–)10.0–13.4–17.0(–26.0) µm
Length (shorter)	(4.2–)7.9–9.2–10.3(–14.9) µm	(4.2–)7.7–9.8–10.9(–25.7) µm	(5.1–)5.4–7.7–8.9(–15.0) µm
Longer/shorter ratio	(1.01–)1.53–1.72–1.83(–3.12)	(0.96–)1.69–1.84–1.96(–2.75)	(1.3–)1.6–1.88–2.2(–3.5)
Shape of tips of ultimate branchlets	Acute to subacute, sometimes rounded	Acute to subacute, sometimes rounded	Acute to subacute
Conidia			
Shape	Ellipsoid to rounded	Ellipsoid to rounded	Ellipsoid
Colour	Light brown	Light Brown	Brown
Base	Rounded	Rounded	Often rounded, sometimes narrowed
Length	(14.6–)17.9–19.9–21.4(–27.3) µm	(20.6–)24.0–25.3–26.5(–31.7) µm	(22.0–)24.0–26.3–28.0(–32.0) µm
Width	(12.8–)15.9–17.8–18.9(–24.9) µm	(18.5–)20.9–22.0–22.8(–27.3) µm	(18.0–)20.0–21.3–23.0(–24.0) µm
Length/width ratio	(1.02–)1.08–1.13–1.16(–1.33)	(1.06–)1.12–1.16–1.18(–1.33)	(1.1–)1.2–1.24–1.3(–1.5)
Pedicel	Absent	Absent	Absent
Wall ornamentation	Obscure	Obscure	
Haustoria			
Shape	Not seen	Not seen	Not seen
			(Thines et al. 2009)

All measurements given in the form (minimum –) border of 30 %– mean – border of 30 %(– maximum).

Fungus	*Pe. belbahrii*	*Pe. belbahrii*	*Pe. lamii*
Host species	*Ocimum basilicum*	*Ocimum basilicum*	*Lamium purpureum*
Acc. no.	HOH, HUH 770 (Type)		
Ultimate branchlets			
Shape	Curved	Curved	Substraight
Length (longer)	(13.0–)18.0–20.6–26.0(–31.0) µm	(8.9–)16.2–18.0–19.9(–28.2) µm	(6.1–)10.6–12.3–14.3(–18.6) µm
Length (shorter)	(3.8–)7.7–9.8–10.0(–15.0) µm	(5.2–)7.5–9.1–10.5(–14.7) µm	(5.0–)6.9–8.3–9.5(–13.6) µm
Longer/shorter ratio	(1.30–)1.80–2.29–2.70(–4.00)	(1.28–)1.88–2.00–2.12(–3.18)	(1.05–)1.36–1.50–1.59(–2.44)
Shape of tips of ultimate branchlets	Acute to subacute	Acute to subacute	Obtuse to subacute
Conidia			
Shape	Ovoid	Ovoid	Ovoid to broadly ellipsoid
Colour	Dark brown to olive	Dark brown to olive	Light brownish to grayish
Base	Rounded	Rounded	Rounded
Length	(24.0–)29.0–30.8–33.0(–36.0) µm	(22.6–)25.9–26.9–27.9(–30.5) µm	(20.3–)22.3–23.4–24.0(–27.3) µm
Width	(20.0–)23.0–24.0–26.0(–29.0) µm	(18.4–)21.9–22.5–23.2(–26.1) µm	(17.0–)19.1–19.7–20.3(–22.6) µm
Length/width ratio	(1.10–)1.20–1.29–1.40(–1.50)	(1.05–)1.16–1.19–1.23(–1.35)	(1.06–)1.16–1.19–1.21(–1.33)
Pedicel	Absent	Absent	Mostly absent, rarely with a scar
Wall ornamentation		Obscure	Obscure
Haustoria			
Shape	Not seen	Pyriform to globose	Pyriform to globose
	(Thines et al. 2009)		

All measurements given in the form (minimum –) border of 30 %– mean – border of 30 %(– maximum).

Conidial size and shape and conidiophore size and shape of the *Peronospora* species on *S. pratensis* were similar in all six sampling sites (measurements are only shown for two specimens, Table 4 and Fig.2). The pathogen on *S. pratensis* differs from *Pe. swinglei* on *S. reflexa* and from *Pe. lamii* on *L. purpureum*. Conidia on *S. pratensis* were ovoid and showed a rounded base, whereas conidia of *Pe. swinglei* were often tear-shaped and narrowing/tapering at the base. Conidia of *Pe. lamii* were ovoidal to broadly ellipsoidal and often slightly narrowing at the base with a short pedicel. *Peronospora* on *S. pratensis* differed from *Pe. swinglei* and *Pe. lamii* by smaller conidial size: 21.0 x 18.3 µm in the former vs. 23.6 x 20.6 µm and 23.4 x 19.7 µm in the latter, respectively. Additionally, the mean length/width ratio from 1.15 of *P*. sp. 3 was smaller than that of *Pe. lamii* (mean = 1.19). Furthermore, conidia of *Peronospora* on *S. pratensis* differed from those of *Pe. glechomae* on *Glechoma hederacea*. With 22.6 x 17.2 µm and a mean length/width ratio of 1.31, conidia of *Pe. glechomae* were longer but less wide than those of *Peronospora* on *S. pratensis*. The conidial colour of *Peronospora* on *S. pratensis* was light greyish with a pale brownish hue whereas conidia from *Pe. glechomae* were vibrant brown. The ovoidal to ellipsoidal conidia of the *Peronospora* species on *S. pratensis* differed in their shape from the conidia of *Pe. salviae-officinalis,* which were ellipsoidal to broadly ellipsoidal. No differences were observed in mean conidial length, width and the mean length/width between these two species. The pathogen on *Salvia pratensis* differs from *Pe. swinglei* and *Pe. lamii* also in the shape of the ultimate branchlets. The shape of the ultimate branchlets in *Peronospora* on *S. pratensis* was slightly curved to almost straight, while in the latter two species it was straight or almost so. Also, the length of the ultimate branchlets and the ratio of the longer to the shorter ultimate branchlet differed. The longer branchlets of *Peronospora* on *S. pratensis* were longer (13.3 µm) than those of *Pe. swinglei* (11.6 µm) and *Pe. lamii* (12.3 µm), respectively. With 7.5 µm in the mean the shorter branchlets of *Peronospora* on *S. pratensis* were longer than the shorter branchlets of *Pe. swinglei* (measuring 7.1 µm) but shorter than the shorter branchlets of *Pe. lamii* (8.3 µm). Although the ultimate branchlets of the *Peronospora* species on *S. pratensis* and those of *Pe. salviae-officinalis* did not differ significantly in morphometric measurements, they tended to be rather rounded in *Peronospora* on *S. pratensis* in contrast to subacute in *Peronospora salviae-officinalis*.

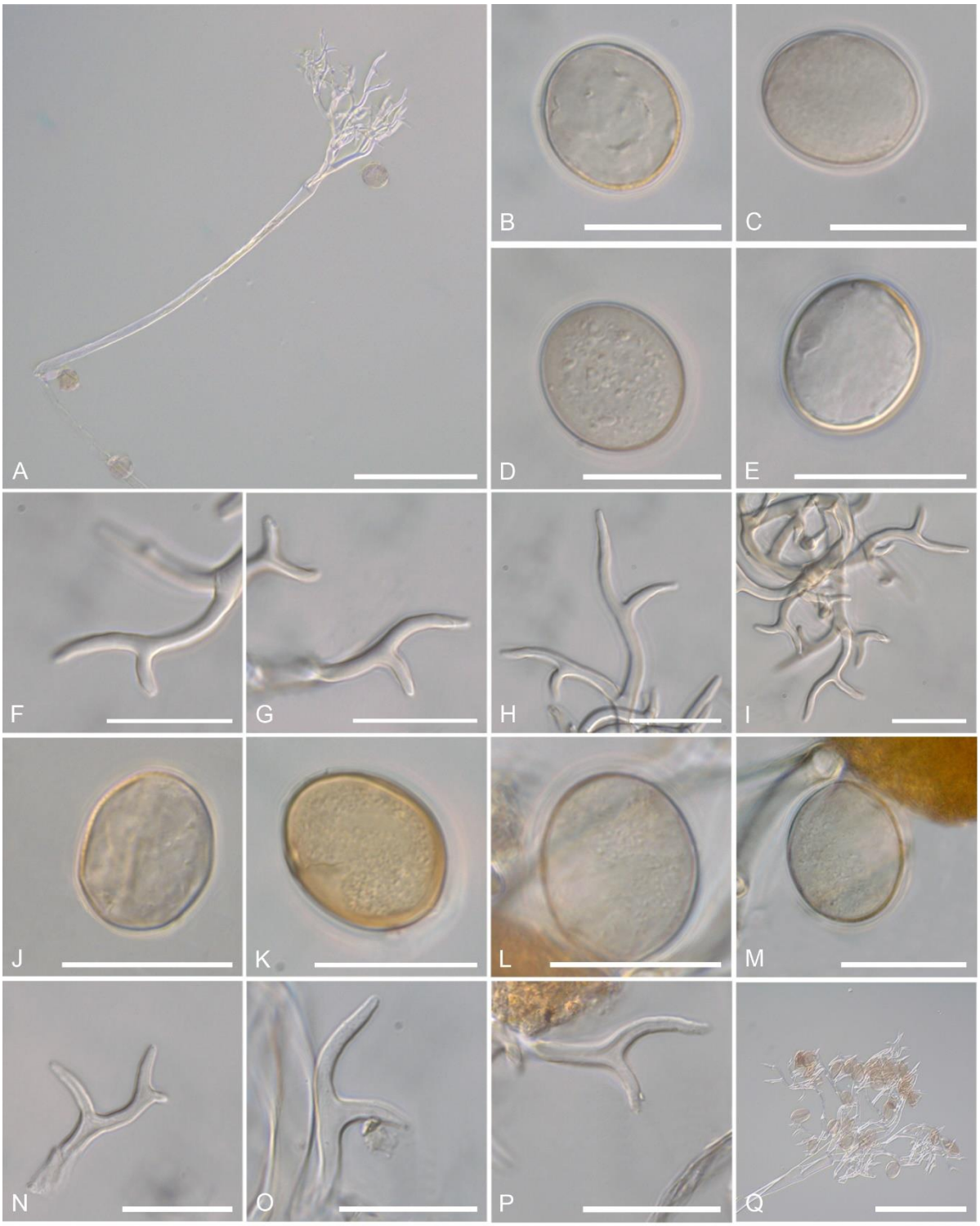

Fig. 2: A-I: *Peronospora* sp. on *Salvia pratensis*. J-Q: *Peronospora glechomae* on *Glechoma hederacea*. A and Q: conidiophore, B - E, J - M: conidia; F - I, N-P: ultimate branchlets. Scale bars: A: 200 µm; B-P: 20 µm; Q: 100 µm

Table 6: Comparison of morphological features of *Peronospora* spp. parasitic to *Salvia* spp. and *Glechoma hederacea*

Fungus	*Pe* . sp. 2	*Pe* . sp. 3	*Pe. swinglei*
Host species	*Salvia sclarea*	*Salvia pratensis*	*Salvia reflexa*
Acc. no.	GLM-F117789	GLM-F117783	FH 00079723 (Type)
Ultimate branchlets			
Shape	Slightly curved to substraight	Slightly curved to substraight	Substraight to straight
Length (longer)	(8.8–)12.1–14.0–15.8(–21.4) µm	(7.2–)11.9–14.0–15.3(–25.2) µm	(8.8–)9.9–11.6–13.4(–15.5) µm
Length (shorter)	(4.9–)6.8–7.9–8.8(–12.8) µm	(4.9–)7.3–8.3–8.9(–14.8) µm	(4.5–)5.7–7.1–8.6(–10.0) µm
Longer/shorter ratio	(1.22–)1.67–1.81–1.91(–2.53)	(1.11–)1.57–1.71–1.81(–2.83)	(1.18–)1.4–1.67–1.93(–2.22)
Shape of tips of ultimate branchlets	Obtuse to subacute	Rounded to subacute	Obtuse to subacute
Conidia			
Shape	Ellipsoid to broadly ellipsoid	Ovoid to ellipsoid	Broadly ellipsoid to tear-shaped
Colour	Light brownish to grayish	Light grayish to pale brownish	Brownish
Base	Rounded	Rounded	Often narrowing
Length	(17.8–)20.6–21.5–22.4(–26.6) µm	(18.0–)21.6–22.3–23.6(–25.7) µm	(20.0–)22.0–23.6–25.3(–27.5) µm
Width	(16.0–)17.6–18.4–19.2(–21.6) µm	(15.1–)18.5–19.2–20.0(–22.2) µm	(17.5–)19.2–20.6–21.9(–22.5) µm
Length/width ratio	(1.04–)1.13–1.16–1.19(–1.28)	(1.05–)1.14–1.16–1.19(–1.25)	(1.02–)1.10–1.15–1.20(–1.25)
Pedicel	Absent	Absent	Absent, rarely with a scar
Wall ornamentation	Obscure	Obscure	Prominent
Haustoria			
Shape	Not seen	Ellipsoid-pyriform	Not seen
			(Thines et al. 2009)

All measurements given in the form (minimum –) border of 30 %– mean – border of 30 %(– maximum).

Fungus	*Pe. salviae-officinalis*	*Pe. salviae-officinalis*	*Pe. glechomae*
Host species	*Salvia officinalis*	*Salvia officinalis*	*Glechoma hederacea*
Acc. no.	GLM-F117795	HOH: HUH 961 (Type)	GLM 73803
Ultimate branchlets			
Shape	Slightly curved to substraight	Slightly curved to substraight	Slightly curved
Length (longer)	(7.3–)12.2–14.2–15.6(–22.2) µm	(8.0–)10.3–12.7–15.0(–17.5) µm	(7.9–)12.1–13.8–15.2(–22.6) µm
Length (shorter)	(4.1–)7.0–8.0–8.8(–13.0) µm	(5.0–)6.2–7.8–9.4(–10.0) µm	(4.9–)6.7–7.6–8.3(–11.5) µm
Longer/shorter ratio	(1.25–)1.63–1.80–1.92(–2.69)	(1.25–)1.33–1.66–1.99(–2.50)	(1.32–)1.64–1.83–1.97(–2.67)
Shape of tips of ultimate branchlets	Subacute	Subacute	Pointed and rounded, somtimes acute
Conidia			
Shape	Ellipsoid to broadly ellipsoid	Ellipsoid to broadly ellipsoid	Broadly ellipsoid
Colour	Light brownish to grayish	Light brownish to grayish	Brownish
Base	Rounded	Rounded	Rounded
Length	(17.5–)19.4–19.9–20.4(–23.1) µm	(18.0–)19.5–21.1–22.7(–25.0) µm	(19,0–)21.8–22.6–23.4(–26.0) µm
Width	(15.3–)16.9–17.4–17.7(–19.7) µm	(16.3–)16.8–18.0–19.1(–22.3) µm	(13.9–)16.5–17.2–18.0(–20.4) µm
Length/width ratio	(1.06–)1.12–1.15–1.17(–1.25)	(1.04–)1.10–1.17–1.25(–1.37)	(1.24–)1.26–1.31–1.34(–1.46)
Pedicel	Absent	Absent	Absent
Wall ornamentation	Obscure	Obscure	Obscure
Haustoria			
Shape	Ellipsoid-pyriform to globose	Globose to lobate	Not seen
		(Thines et al. 2009)	

All measurements given in the form (minimum –) border of 30 %– mean – border of 30 %(– maximum).

Conidia of *Peronospora* on *S. sclarea* are highly similar to those of *Pe. salviae-officinalis* on *S. officinalis*: They measure 21.5 x 18.4 µm in the former and 21.1 x 18.0 µm in the latter (type), and the 1.16 length/width ratio of the conidia was nearly the same as compared to the type of *Pe. salviae-officinalis* (mean = 1.17). The shape and length of the ultimate branchlets and the ratio of the longer to the shorter ultimate branchlet of *Peronospora* on *S. sclarea*, were similar to those of *Pe. salviae-officinalis* (Table 4 and Fig.3).

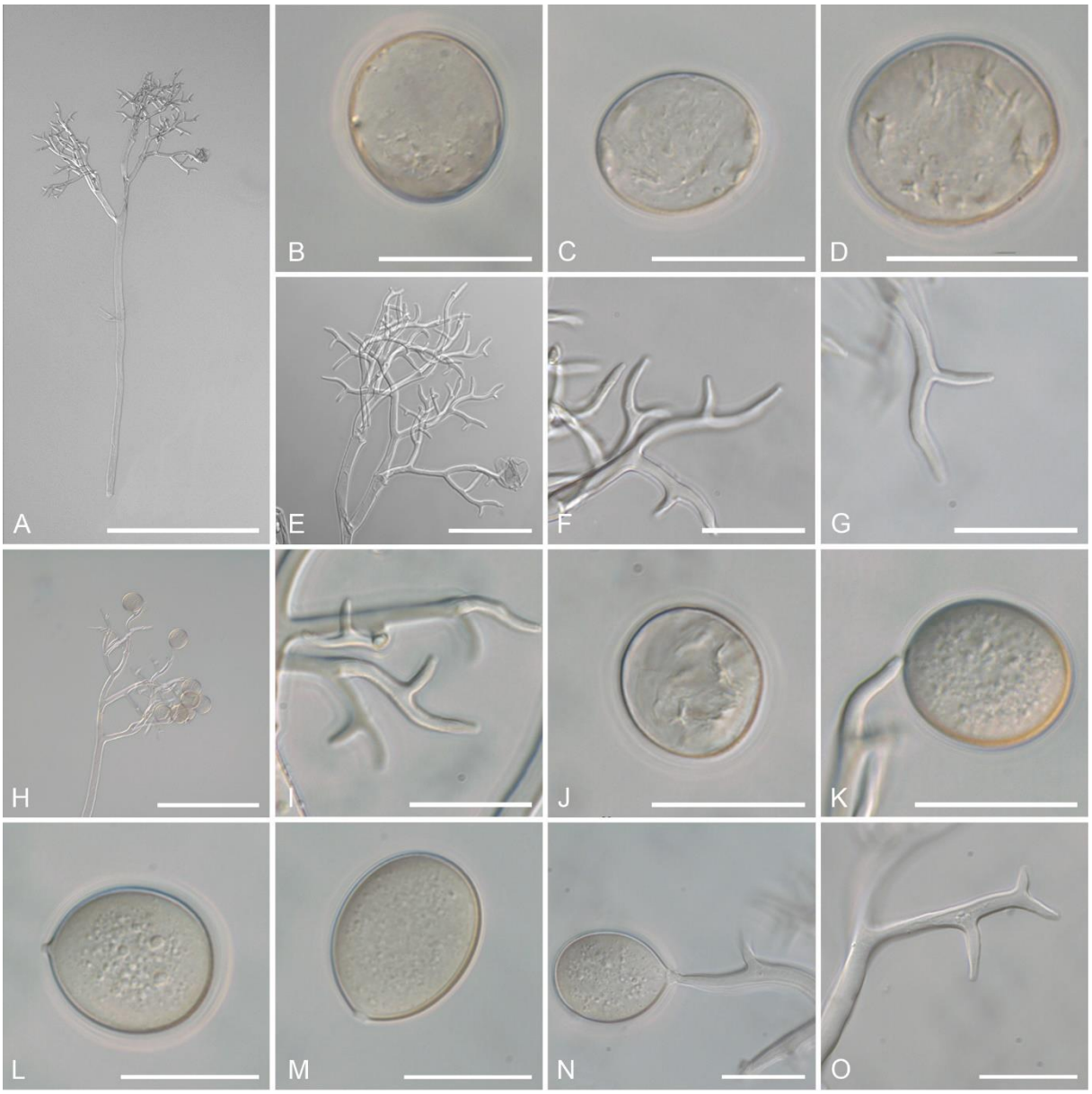

Fig. 3: A-G: *Peronospora salviae-officinalis* on *Salvia sclarea*. H-K: *P. salviae-officinalis* on *S. officinalis*. L-O: *P. lamii* on *Lamium purpureum*. A, E and H: conidiophores; B-D, J-N: conidia; F, G, I and O: ultimate branchlets. Scale bars: A: 200 µm; B-D, E, G, I-O: 20 µm; E: 50 µm; H: 100 µm

Phylogenetic analysis

Phylogenetic relationships inferred using MP, ME, ML and MC³ analyses based on the alignment of *cox*2 only are presented in Fig.4, and the phylogenetic relationships calculated from the concatenated alignment of four nuclear (ITS, *ef*1a*, hsp*90*, β-tubulin*) and two mitochondrial (*cox*1*, cox*2) loci are presented in Fig.5. The *cox*2-only alignment had 419 characters. The concatenated alignment comprised 4049 characters: *i.e. cox*1 (527), *cox*2 (487), ITS (927), *ef*1*a* (677), *hsp*90 (804) and *β-tubulin* (627). Since no conflicts in supported groupings were found between the tree topologies of the MP, ME, ML and MC³ analyses, only the topology of the MP tree is shown for the *cox*2 analysis in Fig.4 and for the multi loci analysis in Fig.5, with addition of the support values of the other analyses. Two most parsimonious trees were found in the MP analysis of the *cox*2-data set and six in the combined data set, respectively with minor differences in the topology of unsupported groupings. One of these trees each was selected for presentation.

The single gene analysis based on *cox*2 sequences showed sufficient resolution to distinguish between *Peronospora* from coleus, basil, and the different sage species (Fig.4), respectively. It also again showed that *Pe. lamii* s.str. on *Lamium purpureum* and *Pe. swinglei* s.str. on *Salvia reflexa* are only distantly related to each other (compare Choi *et al.* 2009; Thines *et al.* 2009) and to the here newly sampled downy mildews on clary and meadow sage. The combined six-gene analysis showed a more resolved and better supported tree topology (Fig.5). The monophyly of lineages parasitic to specific host species received mostly high to maximum support values in the multi gene analyses, except for the two specimens of *Pe. glechomae*, which did not receive any significant support in the analyses. The downy mildew pathogens of meadow sage formed two distinct and each well-supported clades that grouped together with moderate to strong support. The monophyly of *Pe. belbahrii* and coleus downy mildew pathogens, respectively, received maximum support in all analyses in the phylogenetic tree based on six loci. In contrast to *Pe. belbahrii* on basil, which showed intraspecific variability, the downy mildews on coleus were identical in all six gene regions studied. In both trees the downy mildew pathogens on common and clary sage grouped together with mostly strong support in both the *cox*2 and in the reconstruction based on six loci.

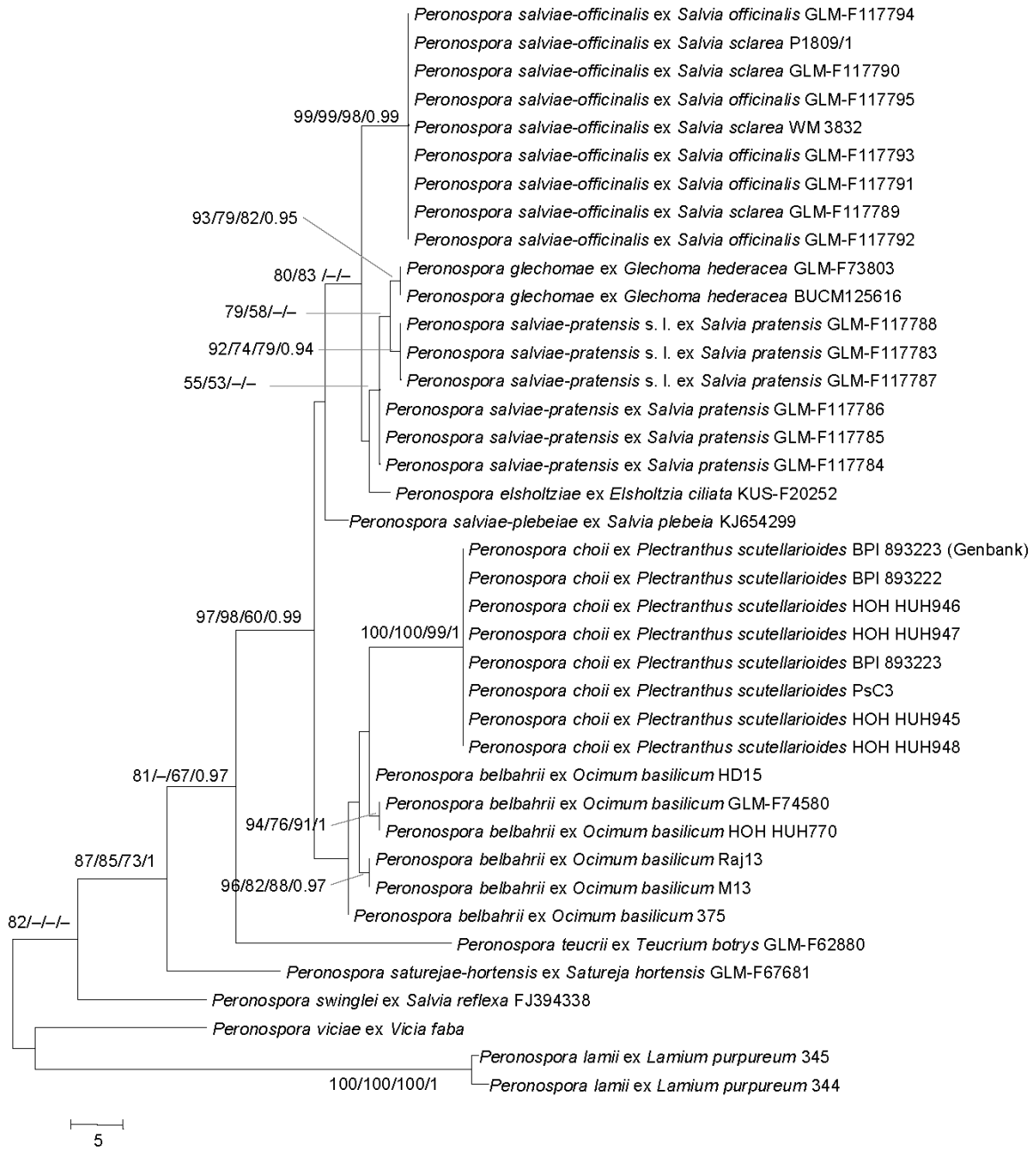

Fig. 4: Phylogentic reconstruction in MP inferred from a *cox*2 alignment. MP, ME and ML bootstrap support values above 50 %, and *a posteriori* probabilities above 0.9 are given at first, second, third, and fourth positions at the branches, respectively. A minus sign denotes lacking support for the present or an alternate topology. No conflicting support was observed

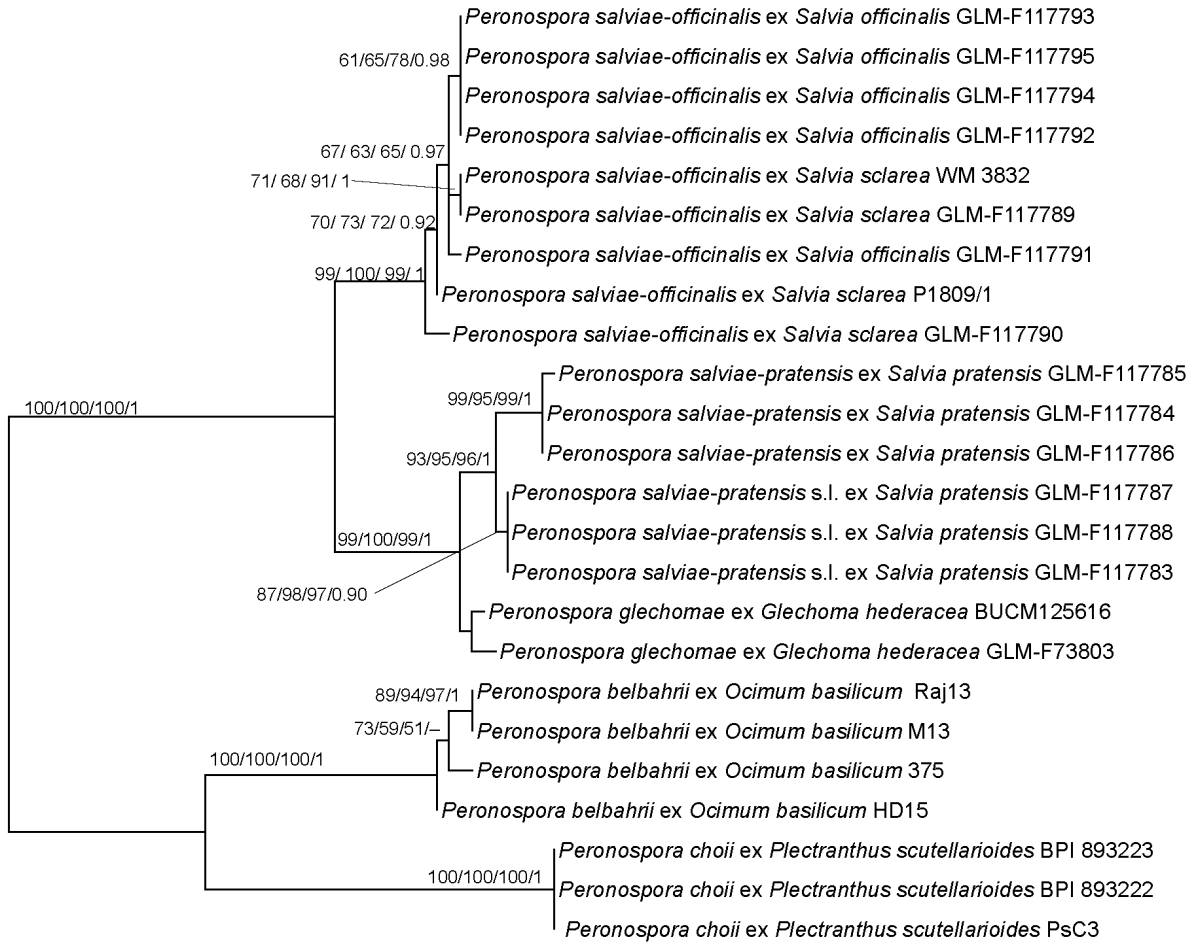

Fig. 5: Phylogentic reconstruction in MP inferred from the concatenated alignment of six genes (ITS, EF1a, HSP90, btub, *cox*1, *cox*2). MP, ME and ML bootstrap support values above 50 %, and *a posteriori* probabilities above 0.9 are given at first, second, third, and fourth positions at the branches, respectively. A minus sign denotes lacking support for the present or an alternate topology

Taxonomy

Due to differences in morphology and on the basis of molecular phylogenetic reconstructions, it is concluded that the *Peronospora* specimens studied from *Pl. scutellarioides* and *S. pratensis* are sufficiently distinct from other *Peronospora* species on *Mentheae* to propose them as new species.

Peronsopora choii Hoffmeister, W. Maier & Thines **sp. nov.,** MycoBank MB 834424

Etymology: The species is dedicated to Young-Joon Choi for his significant contributions to the phylogeny and taxonomy of downy mildews.

Typus: **USA**, Tennessee, on living leaves of *Plectranthus scutellarioides*, August 2015, A. Windham (**holotype** BPI 893223).

Habitat: On living leaves of *Plectranthus scutellarioides* (syn.: *Solenostemon scutellarioides*, *Coleus scutellarioides*; *Lamiaceae*).

Straminipila, Peronosporomycetes, Peronosporales, Peronosporaceae. *Hyphae* intercellular, *haustoria* intracellular. *Down* dainty floccose, greyish to brownish. *Conidiophores* emerging from stomata, hyaline, slender, length 351–831 µm; trunk erect, straight or slightly curved, 222–533 µm long, 10–21 µm broad below the first branch, basal end often slightly swollen, 8–14 µm broad, sometimes constricted at middle height, callose plugs not observed; branching submonopodial, branched 4–6(–7) times, branches slightly curved, arborescent. *Ultimate branchlets* slightly curved to almost straight, obtuse, in pairs with different lengths, the longer being usually (8.2–)12.7–15.6–17.2(–27.4) µm long, the shorter (4.2–)7.9–9.2–10.3(–14.9) µm, longer/shorter branch ratio (1.01–)1.53–1.72–1.83(–3.12). *Conidia* light greyish to pale brownish, ovoidal to ellipsoidal, (14.6–)17.9–19.9–21.4(–27.3) µm lengths, (12.8–)15.9–17.8–18.9(–24.9) µm wide, length/width ratio (1.02–)1.08–1.13–1.16(–1.33), tip and base rounded; wall ornamentation obscure; pedicel absent. *Oospores* not seen.

Infected leaves show discoloured, chlorotic to necrotic spots as seen from the upper surface. On the lower surface of the leaves a grey to brown down of conidiophores with conidia is formed in the lesions.

Additional specimens examined: **USA**, Tennessee, on living leaves of *Plectranthus scutellarioides*, August 2015, A. Windham (paratype BPI 893222).

Peronsopora salviae-pratensis Hoffmeister, W. Maier & Thines ***sp. nov.***, MB 834425

Etymology: *'salviae-pratensis'* refers to the Latin species name of the host plant.

Typus: **Germany**, Baden-Württemberg, Ladenburg, Waldpark (49°28'15.2"N 8°37'04.2E), on living leaves of *Salvia pratensis*, 30 April 2016, M. Hoffmeister (**holotype** GLM-F117783).

Habitat: On living leaves of *Salvia pratensis* (*Lamiaceae*).

Straminipila, Peronosporomycetes, Peronosporales, Peronosporaceae. *Hyphae* intercellular, *haustoria* intracellular, mostly limited to one haustoria per host cell, lobate to globose. *Down* dainty floccose, whitish to cream. *Conidiophores* emerging from stomata, hyaline, slender, length overall 185–541 µm; trunk erect, straight or slightly curved, 85–380 µm long, 8–14 µm wide below the first branch, basal end not differentiated to slightly swollen, 7–12 µm wide at the base, callose plugs absent; branching monopodial to subdichotomous, branched 4–6(–7) times, branches slightly curved, arborescent. *Ultimate branchlets* slightly curved to almost straight, obtuse, in pairs with different lengths, the longer being usually (7.5–)11.8–13.3–14.7(–22.1) µm long, the shorter (4.4–)6.5–7.5–8.4(–11.7) µm, longer/shorter branch ratio (1.18–)1.63–1.78–1.91(–2.66). *Conidia* light greyish to pale brownish, ovoidal to ellipsoidal, (18.3–)20.2–21.0–21.5(–25.3) µm lengths, (15.7–)17.8–18.3–18.6(–21.8) µm wide, length/width ratio (1.04–)1.11–1.15–1.17(–1.25), tip and base rounded; wall ornamentation obscure; pedicel absent. *Oospores* not seen.

Infected leaves show discoloured, yellowed and chlorotic to necrotic, polyangular, clearly vein-limited spots, as seen from the upper surface. On the lower surface of the leaves a light brown down of conidiophores with conidia is formed in the lesions, which darkens with age.

Additional specimens examined: Germany, Lower Saxony, Evessen, quarry (52°11'53.9"N 10°43'19.8"E), 21 July 2017, M. Hoffmeister & W. Maier (GLM-F117785); Germany, Rhineland-Palatinate, Mainz, Botanical Garden (49°59'28.6"N 8°14'27.8"), 27 April 2018 (DE-O-MJG-200809901/1), M. Hoffmeister (GLM-F117786); Germany, Lower Saxony, Braunschweig (52°16'32.2"N; 10°34'04.1"E), 2 May 2018, M. Hoffmeister (GLM-F117784); Germany, Baden-Wuerttemberg, Mannheim, Dossenwald (49°26'34.8"N 8°32'29.9"E), 10 May 2018, M. Hoffmeister (GLM-F117787); Germany, Baden-Württemberg, Nussloch, near quarry (29°19'01.2"N 8°43'01.5"), 13 Mai 2018, M. Hoffmeister (GLM-F117788).

Discussion

Even though more than 400 species have already been described in *Peronosporaceae*, the vast majority of species in this genus remains to be discovered (Thines & Choi, 2016). Especially the downy mildews of *Fabaceae* (Garcia-Blazquez et al., 2008) and *Amaranthaceae* (Choi et al., 2015a) seem to be highly diverse, but also for the *Lamiaceae*, several dozens of hosts have been reported (Constantinescu, 1991, Dick, 2001). Considering the high degree of host specialisation of members of the genus *Peronospora* (Thines & Choi, 2016), it seems likely that this family harbours several undescribed downy mildew agents. Within *Lamiaceae*, the tribe *Mentheae* contains several *Peronospora* species occurring on culinary herbs and medicinal plants (Dick, 2001). Two species belonging to the *Peronospora belbahrii* species complex, *Pe. belbahrii* and *Pe. salviae-officinalis*, have proven to be particularly destructive as emerging pathogens in basil and common sage production, respectively. In this study phylogenetic analyses of downy mildews on *Lamiaceae* were performed using six loci. The combined use of nuclear and mitochondrial gene regions resulted in generally highly-resolved clades and no supported discordance between mitochondrial and nuclear loci was observed, which is in line with previous studies (Choi & Thines, 2015, Choi et al., 2015a), and in contrast to the findings of a recent study on *Peronosporaceae* (Bourret et al., 2018). As previously shown, ITS data were highly similar for closely related species of *Peronospora* (Thines et al., 2009a, Choi et al., 2015b). In contrast, *cox*2 resolved most of the lineages that were found by the six-gene phylogeny and thus qualified as a suitable barcoding marker for *Peronospora* species (Choi et al., 2015b). In addition, DNA extracted from older herbarium samples can be successfully used for amplification of the *cox*2 gene (Telle & Thines, 2008, Choi et al., 2015b). The *cox*1, *ef*1a, *hsp*90 and *β-tubulin* genes also performed well in terms of phylogenetic resolution, and, after primer optimisation (Table 2), could also be amplified reliably.

In the present study, it was shown that the *Peronospora* species on *Pl. scutellarioides* and *Pe. belbahrii* can be reliably distinguished by differences in conidial shape, size and coloration, as well as by the shape of the ultimate branchlets of the conidiophores. In addition, phylogenetic analyses using four nuclear and two mitochondrial gene regions clearly resolved the downy mildew affecting *Pl. scutellarioides* as a highly supported monophyletic group, and, thus, it is described as *Pe. choii* in this study. The downy mildew disease of coleus had initially been lumped within *Pe. lamii* (Daughtrey

et al., 2006, Palmateer et al., 2008), but was then relegated to *Peronospora belbahrii* s.l. (Thines et al., 2009a). In that study it was already suggested that it might represent a species of its own, which is confirmed by the present study. It can therefore be assumed that in nature coleus downy mildew does not serve as inoculum source for basil downy mildew and vice versa although limited artificial infection of basil by *Pe. choii* had been demonstrated (Palmateer *et al.*, 2008). This is in line with infection studies of other downy mildews in which broader potential host ranges than commonly present in nature could be observed under laboratory conditions (e.g. (Runge & Thines, 2008, Runge et al., 2012). Considering this and the fact that *Pe. choii* was so far only reported for Japan (Ito et al., 2015), UK (Denton et al., 2015), the USA (Daughtrey et al., 2006, Palmateer et al., 2008), and recently from Brazil (Gorayeb et al., 2019), but it is not yet as widely distributed as *Pe. belbahrii*, quarantine measures might still be useful to prevent the further spread of this disease throughout the world.

Based on phylogenetic inferences the downy mildews parasitizing *S. sclarea* and *S. pratensis*, respectively, were clearly distinct from *Pe. lamii,* but also from *Pe. swinglei.* Phylogenetic as well as the morphological investigations strongly support that the downy mildew on *S. sclarea* is conspecific with *Pe. salviae-officinalis*, thus this host has to be added to the host range of this species.

Salvia sclarea and *S. officinalis* are closely related and also have an overlapping natural geographical distribution. Whether one of the two host species was initially colonised by a host jump from the other host can only be speculated at this stage.

From a phytopathological point of view, the results from this study showed that the wild sage species *S. pratensis* most likely does not play any role as primary inoculum for downy mildew epidemics in cultivated common sage as it only seems to host a specific downy mildew species. In contrast, clary sage, which is closely related to common sage and is also cultivated as a medicinal plant, likely acts as alternative host for *Pe. salviae-officinalis* and is a potential inoculum source for the dissemination of this disease.

The *Peronospora* accessions from *S. pratensis* are very closely related to *Pe. glechomae* and together they form a sister group to *Peronospora salviae-officinalis*. The morphological and molecular phylogenetic differences between the samples of *Peronospora* found on meadow sage and those from *Pe. glechomae* are subtle. Nevertheless, is seems justified to consider the downy mildew on *S. pratensis* as a

species of its own, *Pe. salviae-pratensis*, and not as conspecific with *Pe. glechomae*, described from *Glechoma hederacea* (Oescu & Radulescu 1939). Interestingly, *Pe. glechomae* was reported only a few times since it was first described as a new species from Romania (Oescu & Radulescu, 1939, Müller & Kokes, 2008). Despite significant efforts we could not find *Pe. glechomae* over a period of three years, whereas downy mildew on *S. pratensis* was easily found at different locations in Germany where *S. pratensis* populations were screened. This is in line with the numerous reports of downy mildew on meadow sage by other authors (Gaponenko, 1972, Preece, 2002, Dudka et al., 2004, Brandenburger & Hagedorn, 2006b, Mulenko et al., 2008, Müller & Kokes, 2008). In contrast to the rare observations of downy mildew on ground ivy, the host plant itself is a very frequent perennial *Lamiaceae*, naturally distributed over large parts of Europe and west-northern Asia, and has also been introduced into North America (Meusel, 1994). Considering that the sister group-relationship of the other sage-downy mildew accessions included in the multilocus analyses received maximum support in all analyses, it could be speculated that *Pe. glechomae* in fact is an incidental host and the few collections resulted from accidental observations of rare host jumps of a downy mildew species originating from meadow sage, for which the original host has not been included in molecular phylogenies, so far. It is also noteworthy that *Pe. salviae-pratensis* accessions from *S. pratensis* formed two distinct clades. It will be interesting to see, if with the addition of more specimens from *S. pratensis* this separation would still be found, suggesting independently evolved populations now both being present in Europe, similar to the situation in *Pseudoperonospora cubensis* (Runge et al., 2011a), or if intermediate lineages will be observed, which would be suggestive of a diversified gene pool, similar to the situation observed in *Albugo candida* (Ploch et al., 2010). In any case, it seems that the *Pe. belbahrii* species complex is still in the phase of active radiation, rendering the discovery of new hosts for some of the species likely, especially if outside their native ranges (Thines, 2019).

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