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Insights into *Papaver somniferum* interactions with the phytopathogenic fungus *Helminthosporium papaveris* and the phytopathogenic bacterium *Xanthomonas papaveri-icola*

Master´s thesis

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Annotation

Papaver somniferum, commonly known as poppy, is an important medicinal crop. In the Czech Republic, poppy seeds are a popular food item and the second biggest exported agricultural commodity. Poppy in the fields can be attacked by plentiful harmful pathogens or pests which severely decrease the poppy yield . Despite its importance, the molecular interactions between poppy and pathogens/pests has not received much attention so far. In this study, we aim to establis three pathosystems of two poppy cultivars with two important pathogens, *Alternaria* sp. (formerly *Helminthosporium papaveris*) and *Xanthomonas papavericola*, and one broad-spectrum plant pathogen, *Botrytis cinerea*. We studied the effect of pretreatment with a common plant immunity elicitor, flg22, on the resistance of poppy towards the *X. papavericola* pathogen. Additionally, we investigated the effect of humidity on the two selected cultivars and the progress of the infection. The pathosystems established in this study can serve as a basis for future research into the molecular basis of *P. somniferum*-pathogen interactions.

Prohlášení

Prohlašuji, že jsem autorem této kvalifikační práce a že jsem ji vypracoval(a) pouze s použitím pramenů a literatury uvedených v seznamu použitých zdrojů.

V Českých Budějovicích dne 13. 4. 2023 Podpis studenta

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CONTENTS

1 INTRODUCTION

Poppy, *Papaver somniferum,* is an important medicinal plant from the family *Papaveraceae.* Its healing and pain-relieving properties have been used for several thousand years, possibly dating back to the Sumerians in Mesopotamia over 7000 years ago. Due to its high alkaloid production, it is the primary ingredient in manufacturing of analgesic opiates and pain relieving substances such as morphine or codeine. Today, poppy is also used in food production for its seeds. The Czech Republic is the world's largest consumer (per capita) of poppy seeds and the leader in culinary poppy seeds production. With cultivars bread for flavour and low amounts of alkaloids, poppy seeds represent one of the leading agriculture export products of the Czech Republic [(1)].

A plethora of microbial diseases threaten yearly poppy harvests. However, only a limited attention has been paid to research of poppy immunity and resistance towards pathogens. Knowledge of the interactions of poppy with harmful pathogens can help reduce the losses and develop modern, sustainable, environmentally friendly cultivation methods.

In this study, we aim to establish pathosystems of opium poppy with some of the most important pathogens causing harmful diseases in the field, which would be suitable for further research on poppy-pathogen interactions. To develop the pathosystems, we test two opium poppy cultivars, “Turec” and “R2” and optimize a protocol for infection of these cultivars with three plant pathogenic microbes, *Alternaria* sp., *Xanthomonas papavericola,* and *Botrytis cinerea*, and develop a system for disease severity evaluation. Additionally, we test the effect of pretreatment of poppy plants with a common plant immunity bacterial elicitor, flg22 (a flagellin epitope), on the plant’s ability to resist the selected pathogen. The pathosystems and tools for their evaluation will be used for further research of the molecular basis of poppy-pathogen interactions in MPMI laboratory.

2 CURRENT STAGE OF KNOWLEDGE

2.1. OPIUM POPPY (PAPAVER SOMNIFERUM)

Opium poppy, or oilseed poppy, is a dicot plant belonging to the order *Ranunculales*, family *Papaveraceae*, subfamily *Papaveroidae*, and order *Papaver*, which comprises about 100 species [(1)]. The name "opium poppy" generally refers to two subspecies, with *Papaver somniferum* subsp. *somniferum* as the domesticated variety of the wild *P. somniferum* subsp. *setigerum* [(1) (2)]. Throughout this thesis, the term poppy will be used as a synonym for *P. somniferum* (Fig. 1).

Figure 1: *Papaver somniferum*, photo by L. Hoskovec 2007 [(24)].

Poppy is a diploid organism with 11 chromosomes [(3) (4) (5)] and a genome length of approximately 2.62 Gb [(6)]. Poppy is native to the Mediterranean and Middle East, spreading to temperate and subtropical regions of Europe, Asia, North and South Africa, North America, and Australia. The centre of its distribution and cultural source is considered to be the Anatolia region in Turkey, which is also the gene centre for this plant. However, poppy cultivation also has long history in Western and Central Europe and was later introduced to India and China around the 17th century [(7)].

2.1.1 POPPY DEVELOPMENT

A Poppy is an annual herbaceous long-day plant which can stand up to 175 cm tall [(7) (8)]. It grows in any soil, but coarse and pressed soils hinder the development of a root system mainly consisting of a tap root with weak lateral roots [(7)]. Stalks have 1-2 cm thick bases, are hollow, and can branch into 2-3 or more than ten branches, depending on the cultivar, in the upper part of the stem. Leaves are covered with a waxy cuticle, giving them greyish-green to glaucus or green colour, with prominent central vein and toothed edges. Upper leaves contribute the most to photosynthesis and are sessile, and more leaves form a protective layer around buds [(7)]. Leaves and stems can have noticeable hairs and trichomes depending on the cultivar [(8)].

P. somniferum is generally regarded as a self-pollinating plants due its bisexual flowers in which ovaries and pollen mature before flowering, with 10-30 % cross-breeding mediated

by pollinators and/or wind [9][10]. Poppy produces a large amount of pollen and therefore attracts pollen beetles (*Meligethes aeneus*) and bees [8]. The fruit of poppy is a capsule crowned with a stigmatic plate of stigmatic rays [11]. Capsules contain carpels with placentas bearing ovules, and generally, cultivars with higher number of stigmatic rays and placentas have higher amount of seeds. Mature capsules contain one to five thousand seeds [7]. The development of the plant is highly dependent on the photoperiod and temperature, with longer days being preferable for earlier flowering [12].

The general developmental cycle of a poppy (Fig. 2) starts with a seed germinating into a seedling with two cotyledons. After the seedling sprouts its first true leaves, the plant grows into a rosette stage, extending up to two months after germination before bolting (change from vegetative to reproductive phase). With bolting, branches start to appear and form buds at the end of the branches. Once buds develop, self-pollination occurs inside the buds before bloom. After flowering, the capsules start maturing and drying and eventually, dry capsules open and release seeds [13].

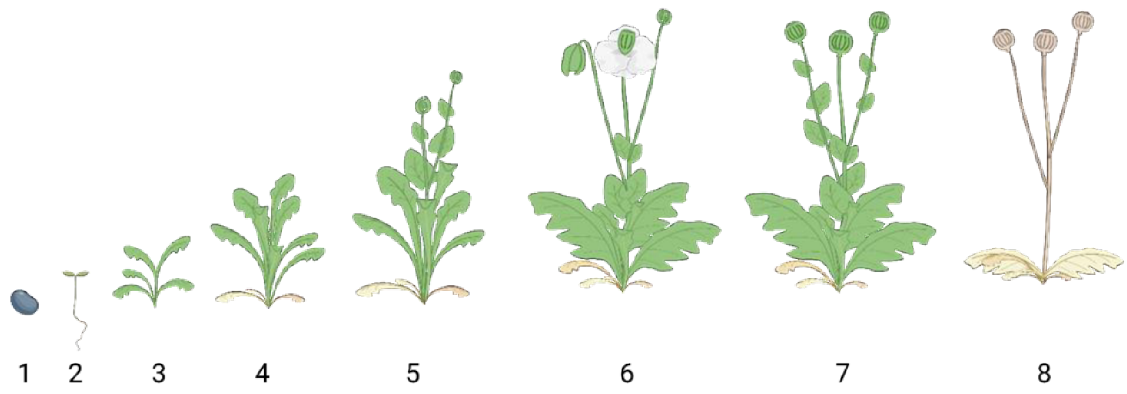

Figure 2: Developmental phases of poppy: 1 – Seed, 2 – Germination and cotyledon appearance, 3 – Seedling, 4 – Rosette stage, 5 – Bolting and branching, 6 – Flowering stage, 7 – Green capsules, 8 – Ripe dry capsules.

2.1.2 POPPY IN THE CZECH REPUBLIC

P. somniferum is cultivated for three main purposes: breadseed, poppy seed used for culinary purposes; pharmaceutical industry, poppy straw used to extract alkaloids used in medicine; and illicit uses, which use unripe poppy capsules for latex extraction and preparation of opium, heroin, and other addictive substances [7]. Most countries cultivating poppy legally do so for the extraction of pharmaceuticals. Turkey and India are the largest producers of poppy straw with comparatively high content of morphine and other alkaloids – poppy cultivars used for medical purposes contain up to 3 %. In comparison, varieties cultivated for food have less than 0.8 %. In the Czech Republic, a poppy is grown specifically for culinary

purposes. The Czech Republic is the world's largest breadseed poppy producer (Table I). Poppy seed constitutes one of the main agricultural export products of the Czech Republic, with ±85% of the total yield being exported [14]. With an average consumption of almost 400 g per capita per year, the Czech Republic is also the largest consumer of breadseed poppy in the world. Since Czech poppy cultivars have been bred specifically for food purposes, the Czech blue poppy is unique in its flavour, smell, and meagre amount of alkaloids (less than 20 mg/kg [14].

Czech blue poppy / Český modrý mák

In 2018, the Czech blue poppy (Český modrý mák) guild norm was established under the number 2019-01-14-0415[1], and in 2021, it was recognized as Protected Geographical Indication by the European Union under the file name PGI-CZ-02236[2]. This trademark covers a total of 14 poppy cultivars , 11 varieties of spring and 3 varieties of winter poppy, although other cultivars can be grown as long as they fulfill the quality requirements set by the EU (Decree No. 399)[3].

1 https://www.cechovninormy.cz/index.php/cechovni-normy/270-modry-mak
2 https://ec.europa.eu/info/food-farming-fisheries/food-safety-and-quality/certification/quality-labels/geographical-indications-register/details/EUGI00000015163
3 https://www.zakonyprolidi.cz/cs/2013-399

Because of the content of alkaloids, the use and monitoring by authorities such as United Nations Office on Drugs and Crime (UNODC) due to its illicit use for heroin and other addictive substances [15]. As of 2017, the Statistics website of the Food and Agriculture Office of the United Nations (FAOSTAT) listed 18 countries in which opium poppy is cultivated legally (Table I) [16].

Table I: Poppy seed cultivation in selected countries [16].

Area	Area harvested (ha)		Yield (hg/ha)		Production (t)	
	Value	Description	Value	Description	Value	Description
India	M	M	M	M	M	M
Italy	M	M	M	M	M	M
Czechia	**32586**	**A**	**6152**	**A**	**20047,64**	**A**
Türkiye	23731	A	6424	A	15244	A
Spain	11000	E	10818	E	11900	E
France	8483	I	5961	E	5056,87	I
Germany	5227	I	5558	E	2905,19	E
Hungary	5168	A	7477	A	3864	A
Romania	3213	I	4091	E	1314,45	I
Slovakia	3144	A	6228	A	1958	A
Austria	3012	A	5973	A	1799	A
Croatia	2752	E	10071	E	2771,47	I
Serbia	948	I	10956	E	1039,09	I
Bulgaria	872	I	6969	E	607,84	I
Netherlands	449	I	7928	E	355,7	E
Palestine	223	E	70697	E	1577,25	E
North Macedonia	75	A	4267	A	32	A
Denmark						

A – Official value; I – Imputed value; E – Estimated value; M – Missing value.

2.1.3 ALKALOIDS IN POPPY

The Latin name of poppy, *P. somniferum*, is derived from the Latin words *somni* (sleep) and *ferrum* (to bring), referring to the relaxing properties of the plant due to the content of alkaloids, many of which have muscle-relaxing properties [17]. To date, opium poppy remains the only commercially viable source of alkaloids used in medicine, especially for treating severe pain [18]. Most research on poppy is connected with alkaloid production. However, the biosynthesis and metabolism of alkaloids in poppies are still not completely understood [18].

Poppy alkaloids belong to the class of benzylisoquinoline alkaloids (BIAs). With over 2500 known structures, BIAs are a group of natural compounds with unprecedented pharmaceutical potential [19] [18].

The biosynthesis of opium poppy alkaloids starts with condensing two L-Tyrosine derivatives: dopamine and 4-hydroxyphenylacetaldehyde, which form the first BIA intermediate (*S*)-Norcoclaurine. By modification of functional groups, (*S*)-Norcoclaurine can be modified into all the final alakaloids including papaverine, codeine, morphine, noscapine or sanguinarine [18].

The individual steps of the pathway are catalyzed by at least 14 enzymes which are frequently promiscuous, catalyzing modification on differing substrates [18]. This substrate promiscuity allows for such a wide structural diversity in BIAs since the biosynthetic pathways can be deeply intertwined, forming a web rather than individual branches [19] [20].

Opium poppy produces up to 80 different alkaloids (EFSA), from which morphine, codeine, thebaine, sanguinarine, papaverin, and noscapin, are the major ones and underwent most frequently monitoring in different poppy cultivars [5] [18] [20] [21] [22].

One of the signature traits of a poppy is a well-developed specialized system of latex-producing cells called the laticiferous system [23] [24]. This system consists of anatomically connected cells with distinct large irregular vesicles in the cytosol [18]. Laticifers are tightly associated with the vascular bundle, forming a complex system with sieve elements and their companion cells[18] [25] [26]. All three types of cells are connected and provide a place for BIAs biosynthesis, with different steps taking place in specific cells [18]. Alkaloids, the end products of BIAs biosynthesis, accumulate in vesicles in the cytoplasm of laticifers. It is commonly referred to as latex and is the source of raw opium [18]. The major part of latex are hydrophobic alkaloids. Besides them, laticifers contain specialized proteins, major latex proteins (MLPs), forming up to 35 % of the laticifer proteome. MLPs are a single family of proteins and belong

the Pathogenesis-Related Proteins 10 (PR-10) family. Ozber et al. discovered that MLP/PR-10 proteins in poppy have the ability to bind to the alkaloids in latex, forming dense protein-alkaloid aggregates and thus allowing the storage of large amounts of BIAs in opium poppy latex [27].

2.1.4 ALKALOIDS IN PLANT DEFENSE

Such an intricate, complicated, and tightly-regulated cell system points towards its useful function for the organism's evolutionary fitness. Generally, plant latex is considered to be part of the first line of defence against herbivores, insects, and pathogens. Its role in defence is supported by latex production upregulation in response to jasmonic acid, a well-known phytohormone in plant defence signalling [28]. The presence of laticifers throughout the entire body of the plant ensures a quick and targeted response to plant wounding and damage, mainly effective against damage caused by herbivores and insects [28]. As for microbial infection, rather than being directly involved in combatting the microorganisms, the laticiferous system poses an ultrastructural barrier confining the microbe to the immediate area of invasion, with the secondary metabolite composition of the latex taking effect later compared to herbivory[28].

In poppy, it has been suggested that the laticiferous system was developed as a form of self-defence from toxic alkaloids, such as the cytotoxic sanguinarine [18]. Additionally, a multitude of BIAs produced by poppy possess a wide array of bioactivities, including anti-herbivorous activity (for example berberin), insecticidal, antifungal, antibacterial (sanguinarine), or antiviral properties [29]. Notable is also the feeding-deterring property of papaverine [29]. Morimoto et al. (2001) discovered a curious physiological function of bismorphins. Compounds synthesized via conversion of two morphine units. Bismorphins accumulate in the cell wall and bind to pectins, promoting cross-linking of cell wall polysaccharides. It happens through bismorphine bridges leading to the strengthening of the cell wall and its resistance to hydrolysis by pectinases [30] [31]. The importance of alkaloids in the defence response of poppy to attack by microbial pathogens is also supported by several studies reporting an increase in alkaloid production upon pathogen attack [32] [33] [34] and the general higher susceptibility of low-latex cultivars towards diseases compared with high-alkaloid varieties.

2.2. DISEASES OF POPPY

Poppy can be affected by many environmental influences, be it abiotic (humidity, water and nutrient availability, weather, light, etc.) or biotic in the form of viral and microbial diseases and pests.

Among abiotic factors, waterlogging is one of the most dangerous for the poppy. It can lead to plant undergrowth, lodging, an overall reduction in plant health, and reduce the viability and germination ability of pollen, causing capsule seedlessness [35].

Severe disease can be caused by boron deficiency in the soil, a common occurrence in the Czech Republic. Symptoms are withering of vegetation apex, small and underdeveloped blooms, and dark deformed capsules without seeds. This disease can be prevented with the addition of boron to the soil before starting the cultivation [35].

General rules for protection of poppy against diseases include careful selection of proper location with soil pH no lower than 6.2. Dense, compacted soil is undesirable. The seeds should be sown with adequate spacing as dense growth can facilitate disease transmission throughout the field. Since several important pathogens of poppy are seedborne, it is strongly advised that only healthy, non-infected seed is used for sowing and that a gap of at least four years is maintained between two consecutive poppy cultivations [35].

As a crop, the poppy is very sensitive and difficult to cultivate as it requires particular conditions to thrive. Damage caused by one agent can easily trigger further disease development, leading to a significant decrease in seed yield [35]. The main pathogens of poppy, are discussed in detail below.

2.2.1 VIRAL DISEASES

Poppy can be affected by a variety of viral diseases, although their occurrence is sporadic [35]. Viruses of the family *Potyviridae* seem to be naturally infecting poppies. For example, the **Turnip mosaic virus** (TuMV) has been reported on opium poppy in the Czech Republic [36] [37], Hungary [38], Australia [39], and South Africa [40]. TuMV causes plant growth to stunt and chlorotic lesions leading to leaf necrosis in the advanced infection stage. Infection can lead to plant death within four weeks of infection. Like other potyviruses, TuMV is transmitted by aphids, especially the green peach aphid *Myzus persicae.* However, it stays viable only for a short period of time [37] [40].

Other poppy-infecting potyviruses include the **Bean yellow mosaic virus** (BYMV) and Poppy mottle virus (PoMV). BYMV was reported from Bulgaria and Poland[40] to cause chlorotic lesions and malformations in young leaves followed by growth stunt and sunken yellow spots on capsules [37]. **Poppy mosaic virus** (PMV-P), a name synonymous with PoMV, is a virus specific to *P. somniferum* and *Argemone mexicana* L. [41]. It has been recorded in Turkey[40] and India [42] [43] to cause mottle and severe green mosaic on capsules and leaves in advanced infection stage[42], stunted growth, and malformed capsules [40] [44]. Zaim et al. (2014)

tested several poppy characteristics, including seed yield and alkaloid content. They found plants infected with PoMV to have a higher content of alkaloids (excluding papaverine), but a lower seed yield. They concluded that while PoMV can be undesirable in poppy cultivars grown for seeds, it may positively affect pharmaceutial poppy after a longer-lasting infection[43]. Other members of the *Potyviridae* family have also been recorded to infect opium poppy or related *Papaver* species, e.g. Beet western yellows virus in *P. rhoeas*[40].

Recently, two new poppy-specific diseases have been reported. **Tomato leaf curl New Delhi Virus** (ToLCNDV), a Begomovirus of the family *Geminiviridae*, was first reported by Srivastava et al. (2016) from India. ToLCNDV, transmitted by the whitefly *Bemisia tabaci*, causes severe curling of *P. somniferum* leaves and was confirmed on 100 % of tested opium poppy plants, suggesting a possible risk to other crops [45]. Another recently idntified virus was described by[46], although the isolate was collected in New Zealand already in 2006. The authors named this new virus from the genus *Umbravirus* **Opium poppy mosaic virus** (OPMV), which was isolated from *P. somniferum* plants displaying leaf mosaic and mottling. They studied OPMV extensively using a range of molecular techniques and determined the virus to be accompanied by a helper virus related to the family *Luteoviridae*, designated "opium poppy mosaic-associated virus" (OPMaV) [46].

Other viruses, such as Tomato spotted wilt virus [47], Plum pox virus[48], or Cucumber mosaic virus [49] have been reported to occur in *P. somniferum* or related *Papaver* species. An overview of poppy viral diseases is given below in Table II.

Notably, viruses have also been used for genetic modifications of poppy using a method called Virus-Induced Gene Silencing (VIGS). This strategy has been used, for example, to study poppy gene function [50] or alkaloid biosynthesis pathway [51] [52]. Achs et al. (2022) were also able to infect the poppy via Plum pox virus-based agroinfiltration, managing to establish the poppy as a candidate host for the production of edible vaccines[48].

Table II. List o poppy infecting viruses

Virus name	Classification	Disease name	Czech disease name	Ref.
Ageratum enation virus	*Geminiviridae*	Ageratum leaf curl	None	(53)
Potyvirus	*Potyviridae*	Mosaic disease	None	(54)
Beet yellow virus	*Closteroviridae*	Yellowing disease	Virus žloutenky řepy	(40)
Beet mosaic virus	*Potyviridae*	Mosaic disease	Virus mozaiky řepy	(37)
Bean yellow mosaic virus	*Potyviridae*	Bean top necrosis	Virus žluté mozaiky fazolu	(37)
Turnip mosaic virus	*Potyviridae*	Brussels sprout mosaic	Virus mozaiky vodnice	(40)
Opium poppy mosaic virus	*Tombusviridae*		None	(46)
Tomato leaf curl New Delhi virus	*Geminiviridae*	Tomato leaf curl	None	(45)
Plum pox virus	*Potyviridae*	Peach sharka	Virus šarky švestky	(37)
Tomato spotted wilt virus	*Tospoviridae*	Tomato spotted wilt	Virus bronzovitosti rajčete	(37)
Beet curly top virus	*Geminiviridae*	Beet curly top	Virus vrcholové kadeřavosti řepy	(37)
Cucumber mosaic virus	*Bromoviridae*	Cucumber mosaic	Virus mozaiky okurky	(37)

2.2.2 MICROBIAL PATHOGENS OF POPPY

Microbial pathogens are among the most severe causing agents of *P. somniferum* diseases and can lead to huge losses in crop yield [(35)(40)]. The most serious microbial pathogens of poppy are oomycetes such as *Peronospora* spp. and fungi such as *Alternaria* spp.. Bacteria, while still decreasing yields, tend to cause less serious damage[(40)]. Below are the most serious diseases of poppy discussed in order of their importance.

2.2.2.1 Downy Mildew: *Peronospora spp*.

One of the most detrimental diseases of oilseed poppy is downy mildew caused by pathogen from the family *Peronosporaceae* (downy mildews), genus *Peronospora* (taxonomic information from NCBI, 2023). These obligate biotrophs belong to the phylum *Oomycota* and have been reported to cause damage on oilseed poppy fields in Tasmania[(40)], India [(55)(56)], Spain [(57)], Austria[(58)], Czechia[(35)], and other poppy-growing countries. In 2022, *Peronospora* was reported on 21 localities throughout the Czech Republic (Fig. 3), out of which 13 location reached a presence over the threshold of harm, totalling to over 190 ha of infected area[(59)].

The main causing agent of downy mildews was considered to be *Peronospora arborescens* (Berk.) du Bary. However, the taxonomy was later refined by Volgmayr et al. (2014), who found that *P. arborescens* could not infect *P. somniferum* L itself but was restricted in its host range to *Papaver rhoeas* L. Instead, Volgmayr et al. (2014) newly defined the pathogen on oilseed poppy to be *Peronospora somniferi* Volgmayr and *Peronospora meconopsidis* Mayor, which were found to be two distinct species and both of which differ from *P. arborescens* on a genetic level [(58)]. While *P. meconopsidis* appears to be widespread and can be

found in *P. somniferum*, it causes minor, only localized symptoms in comparison with highly virulent *P. somniferi* causing systemic infection in oilseed poppy [58].

Downy mildew in poppy is known to cause several symptoms. Typically, irregular chlorotic dark-brown lesions on leaves are limited by leaf veins and white conidial growth on the abaxial side [60], angular necrotic lesion on lowermost leaves[57], curled leaves with dark conidiospore masses on the abaxial side[55], and halting of growth when infected at seedling stage[61] (Fig. 3). Plants growing from infected seeds are stunted with thickened leaves. Plants infected at the rosette stage die or do not produce capsules. Capsules of infected plants are small, deformed, and violet in color. Seeds do not ripe properly[35]. The symptoms are further specified by Voglmayr (2014), who describes the symptoms of *P. somniferi* infection to be systemic, stunting the leaves or the plant growth, causing distorted, curved stems, and *P. mecanopsidis* to cause milder, localized disease mainly manifesting as polyangular leaf lesions [58].

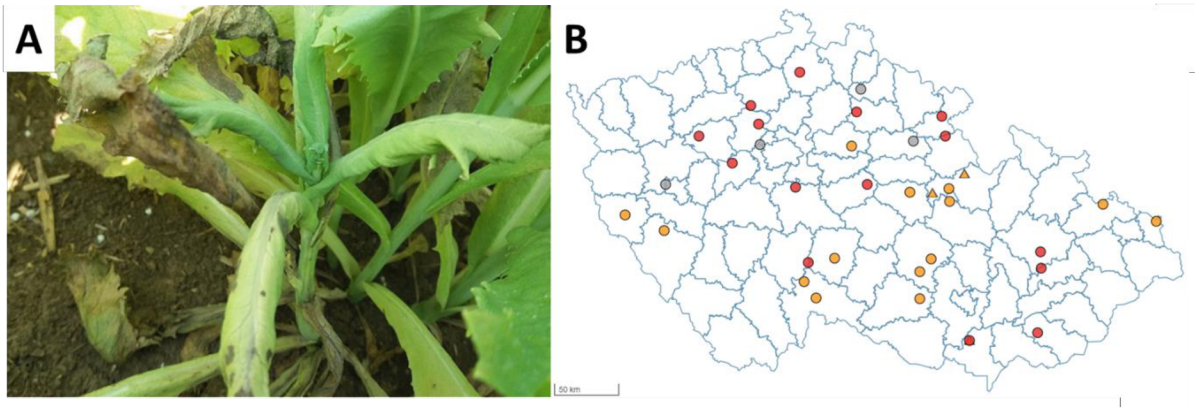

Figure 3: Symptoms and spreading of *P. arborescens*. A) *P. somniferum* infected with *Peronospora* pathogen. ÚKZÚZ phytopathology portal, photo by M. Čadová. **B)** Map of documented ocurrence of *P. arborescenc* on poppy in the Czech Republic in 2020-2021. Red circle – harmful occurrence, orange circle – non-harmful occurrence, orange triangle – low occurrence detected in random monitoring, grey circle – confirmed absence [59].

Peronospora spp. is primarily transmitted via infected seeds[57]. Optimal conditions for its growth are high humidity, lower temperatures, and dense growth, in which the disease spreads quickly via secondary infection from sporangia. Minor source of primary infection can be oospores surviving in soil, which have been demonstrated to infect plants as well. However, plants infected via soil oospores can be asymptomatic[61]. For this reason, it is strongly advised that only healthy seeds are used for sowing. To eliminate the oospores surviving in soil, a proper time distance of at least four years is maintained between two consecutive poppy sowings on the same field [35]. In order to aid in preventing the onset of infection, specific PCR

primers have been developed by Landa et al. (2007) for the detection of *Peronospora* infection in seeds [57].

Given its importance in the agricultural yields of poppy, this pathogen has received significant attention in research. However, most of this effort has been focused on controlling the spred of the infection in fields and breeding resistant types of *P. somniferum*. For example, recently, a physico-chemical treatment of poppy seeds with hypochlorite and electrolytic solution reduced transmission of the pathogen by up to 88%, and additionally, it also reduced the incidence of other *P. somniferum* pathogens such as poppy fire (mentioned in detail below)[62]. Several resistant cultivars of poppy have been identified, such as the 1014 and N3 landraces produced by[55], or the I-14 and Pps-1 genotypes described by Dubey (2009) [56]. Dubey et al. (2010) also developed genotype-specific Amplified Fragment Lenght Polymorphism (AFLP) markers for DM-resistance in poppy based on over ten years of poppy cultivar breeding, and a year later [63], Montes-Borrego (2011) added optimized protocol for RT-PCR quantification of the pathogen load in stem samples as well as commercial seed stocks[64]. Notably, in 2008, a downy mildew epidemics-forecasting model DOWNCAST was adapted for poppy downy mildew in Australia using POPCAST1 and POPCAST2 prediction models with up to 86% accuracy in predicting sporulation of the pathogen [65].

2.2.2.2 Poppy fire: Alternaria spp., formerly Pleospora spp.

Another destructive microbial disease of oilseed poppy is poppy fire[40], also known as helminthosporiosis or poppy blight, caused by members of the *Pleosporaceae* family. As with *Peronospora*, the nomenclature of these pathogens has changed several times in the past. Originally, the disease was thought to be caused by a fungal pathogen *Pleospora papaveracea* (synonymous to *Pleospora calvescens*[66], which had a sexual state named *Dendryphion penicillatum*[67] with synanamorph *Helminthosporium papaveris*[66] [68], hence the name of the disease, Helminthosporiosis. However, in 1950, it was found that *Dendryphion* was a taxon distinct from *Pleospora* [69] and was named *Dendryphion penicillatum* var. *Sclerotiale* M-E Meffert, with the anamorph of *Pleospora papaveraceaea* referred to as *Brachycladium penicillatium* [70] and *Helminthosporium papaveris*[67]. Successively, several studies were undertaken to investigate the relationships between these two taxa. In 2006, Inderbitzin et al. revised the taxonomy with the use of morphological and molecular methods and placed the species in the *Alternaria* group, in which they established a new genus *Crivellia*. Within this new genus, they presented *Crivellia papaveracea* (De Not.) Shoemaker and Inderbitzin as a sexual state formerly known as *Pleospora papaveracea* with *Brachycladium penicillatum* as the asexual state similar to the former *Dendryphion penicillatum* state, and a second, homothallic species

Brachycladium papaveris (K. Sawada) Shoemaker and Inderbitzin synonymous with *Helminthosporium papaveris* and *Dendryphion papaveris* [71]. Later in 2013, the species were renamed again in a paper by Woudenberg et al. based on nucletodie sequences, merging the *Crivellia* group into *Alternaria*. The most recent names of the two pathogens, to the best of my knowledge, are thus *Alternaria penicillata* (formerly *Crivellia papaveracea* and *Pleospora papaveracea*) and *Alternaria papavericola* (formerly *Brachycladium papaveris*) [72].

Typical symptoms of poppy fire include seedling damping-off and strangulation of root collar causing decreased transport of water and nutrients from roots to plant, leading to leaf yellowing, wilting, and eventually drying. Secondary infection symptoms usually appear at the beginning of flowering as angular brown spots easily misidentified as symptoms of downy mildew or bacteriosis. Brown to black stripes can appear on the stem. If capsules are produced, they are small and violet-brown in color. The pathogen can grow inside of the capsules and wrap around unripe seeds, causing them to clump together [35].

Both *A. penicillata* and *A. papavericola* can over-winter in infected poppy straw, which can be the primary source of infection together with infected seed [35] [68]. The pathogens spread

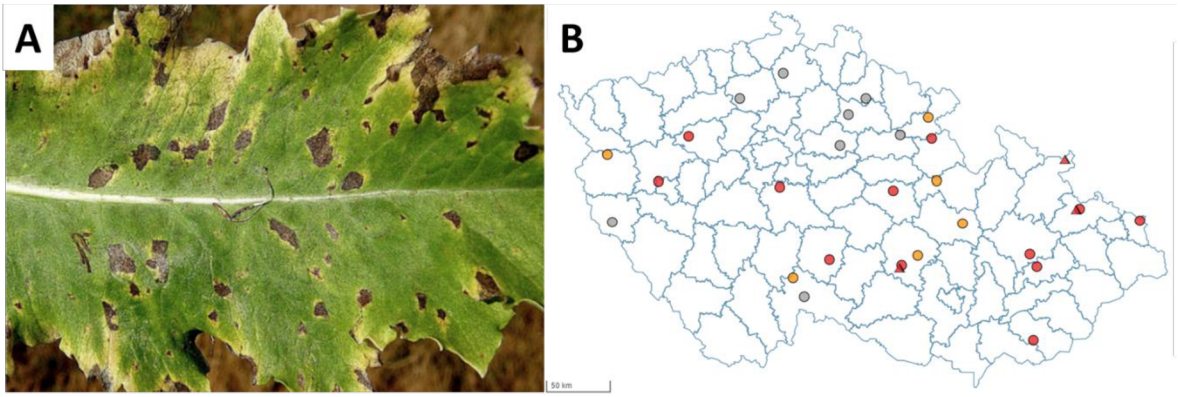

Figure 4. Symptoms and spreading of *Alternaria* spp. A) *P. somniferum* infected with *Alternaria* pathogen. ÚKZÚZ phytopathology portal, photo by J. Rod. **B)** Map of documented ocurrence of *Alternaria* spp. on poppy in the Czech Republic in 2022. Red circle – harmful occurrence, red triangle – harmful occurrence detected in random monitoring, orange circle – non-harmful occurrence, orange triangle – low occurrence detected in random monitoring, grey circle – confirmed absence[76].

very well under lower temperatures in rainy periods with high humidity, in fields with dense poppy growth or fields overgrown with weeds. Similarly to *Peronospora* spp., helminthosporiosis has been reported in almost all countries growing poppy, including Australia (Tasmania [40]), Hungary [70], Austria, USA, Turkey, Colombia, Venezuela, Sweden [71], Russia, Ukraine [40], and Slovakia [73]. According to the czech phytopathology portal, poppy fire was present on 12 locations throughout the Czech Republic over the harm threshold in 2022 (Fig. 4).

As soon as the distinction between the two pathogens was discovered, differences in symptoms and infection severity started to be observed. Although morphologically very similar, *A. penicillata* forms microsclerotia while *A. papavericola* forms chlamydospores. Notably, there seems to be a difference in the virulence of the two species, with *A. papavericola* forming more appresoria than *A. penicillata*, which is also less virulent and competitive [71]. Although this observation was supported by data from O'Neill (2000) [66] and Bailey (2000) [68], Inderbitzin et al. (2006) [71] suggest more work should be carried out on the topic before this conclusion is considered diagnostic.

Although some relatively helminthosporiosis-resistant poppy cultivars are available (e.g. varieties Orel, Racek, or Orfeus registered in the Czech Republic – ÚKZÚZ portal [74]), considerably less work has been done on poppy resistance to this diesase compared to downy mildew. However, poppy fire has been considered for use as biological mycoherbicide for control of illicit poppy cultivation [40]. For example, Bailey et al. (2000b) studied the effect of the Nep1 protein isolated from *Fusarium oxysporum* on the effectivity of infection of opium poppy by *A. penicillata* (*Pleospora papavericola)* [75].

2.2.2.3 Bacterial leaf spot of poppy: Xanthomonas campestris pv. papavericola

Xanthomonas papavericola, newly classified as *X. campestris* pv. *papavericola,* has been reported to cause bacterial blight of poppy, a less serious disease of poppy than previously mentioned pathogens. It was first described by Bryan and Mcwhobter in 1930 as *Bacterium papavericola* sp. nov., who reported the pathogen infecting *Papaver rhoeas* in Franklin, Virginia, USA. In 2023, Harrison et al. published a new phylogenetic analysis study revising the *Xanthomonas campestris* genus and its many pathovars and confirmed the existing position of *X. campestris* pv. *papavericola* in the *X. campestris* strain type [76].

X. papavericola causes irregular chlorotic lesions surrounded by leaf veins (Fig. 5) and is usually only present on plant leaves. At first, the lesions are water-soaked and then turn yellow as the bacteria consume the cellular contents, making the leaves translucent. When the infection is intense, the whole leaves turn necrotic and wither. Compared to the fungal/oomycete pathogen-caused lesion, bacterial blight lesions can be recognised by their transparency [35]. According to Bryan and Mcwhobter, *X. papavericola* enters the leaves through stomata or hydatodes and can cause systemic infection if it penetrates the veins [77].

The available information on the localisation of *X. papavericola* is very limited. In the Czech Republic, the incidence of this pathogen is evidenced by its inclusion in the cultivation methodology published by the Czech Ministry of Agriculture [35] and reports from the Central

Institute for Supervising and Testing in Agriculture [78]. Other than Czechia, it has been observed in Bulgaria [44]and the USA [77].

Curiously, unlike the previous *Peronospora* and *Alternaria* pathogen species, the genome of *X. campestris* pv. *papavericola* has been sequenced and is available on the National Center for Biotechnology Information (NCBI) website under the assembly number ASM2081301v1 [79]. Additionally, when the XhoI endonuclease was first isolated by Gingeras et al. in 1978, *X. papavericola* was also found to contain an enzyme with similar specificity [80].

Figure 5 Symptoms of *X. campestris* : *P. somniferum* infected by *X. campestris* pv. *papavericola*. Photo by Bačová [82].

2.2.2.4 Grey mould: Botrytis cinerea

B. cinerea is a necrotrophic plant pathogen with a broad range of over 1400 plant host species [81]. It belongs to the phylum Ascomycota, family *Sclerotiniaceae*, and was formerly also known as *Botryotinia fuckeliana* de Bary [82] or *Sclerotinia fuckeliana* [83]. *B. cinerea* causes grey mould diseases on various agriculturally important plants such as grapevine, kiwifruit, chickpeas, sweet potatoes, strawberries, and other berries, vegetables, and even ornamental plants. The damage brought about by *B. cinerea* is increased by its post-harvest moulds since, as a necrotrophic pathogen, it can infect and destroy the harvested crops and fruits, especially dicot plants [84]. Given its huge economical impact, costs for fungicides to control *Botrytis* are estimated to be around €40/ha [85] and bunch rot caused by *Botrytis* causes losses of up to AUS $52 million/year [86], it has been selected as number two of the Top Ten fungal pathogens in molecular plant pathology, second only to *Magnoporthe oryzae* in a survey conducted by Dean et al. (2012) [87]. As such, it is one of the best described and most studied plant pathogens, with its genome fully sequenced and available [87]. *B. cinerea* has been extensively used in research of fungi-plant interactions, including by my supervisor [88]. Although a necrotrophic pathogen, *B. cinerea* can also be used for production of sweet wine when it forms a slower-developing infection called “noble rot” on grapes, which occurs naturally under very

specific conditions and increases sugar content in the berries as well as accumulatio of specific aromating compounds which improve the flavor and aroma of wine [89].

B. cinerea usually manifests as water-soaking of parenchyma tissues, especially leaves, soft rots, and subsequent development of gey masses of conidia. It commonly appears on senescent tissues as soft rot and then spreads to the rest of the plant. However, given its wide range of host plants, the symptoms vary just as much with regard to the host in question – for example, on tomato plants, the most affected part of the plant is generally the stem. On raspberry, it causes the development of wedge-shaped brown lesions with yellow margins spreading into the node, from where it infects the stem [84].

B. cinerea has been reported as a seed-borne disease in over 50 hosts (e.g. chickpeas) and can survive without causing symptoms up to several months after sowing [90]. In dying tissues, the pathogens form sclerotia which in the spring produce conidia, serving as the primary source of infection. The production and spreading of conidia is regulated by changes in temperature and humidity and exposition to specific wavelengths of light [84].

Given the attention that has been given to this pathogen in research, many aspects of its mode of action during infection have been studied, with ongoing research producing new information every year. For example, the signalling pathways for the perception of the environment and signalling during pathogenesis are under investigation [91].*B. cinerea* played an indispensable role in the research field focused on RNA communication between plants and pathogens [92]. *B. cinerea* produces a variety of enzymes and effectors which subvert plant immunity responses, allowing the pathogen to enter and feast on the necrotic tissues of its host. Notable, among these are cutinases, lipases, pectinases, superoxide dismutase BcSOD1, phytotoxic metabolites such as botrydial and NEP1-like proteins, or, famously, oxalic acid [84]. A large array of secondary metabolites has been reported from *B. cinerea*, with over 40 biosynthetic gene clusters and 110 secondary metabolites identified to date [93]. *B. cinerea* produces germ tubes and unicellular appresoria (specialized cells which apply turgor pressure on the plant cell wall) as well as multicellular infection cushions [94].

In poppy, *B. cinerea* infects several members of the *Papaveraceae* family, including *P. somniferum* and other *Papaver* spp.„ *Corydalis* spp., and *Eschscholzia* spp., on which it causes grey mould [81]. It has been reported on poppies from Slovakia [73], Portugal, Spain, India (44), and Czechia (Fig. 6). It can affect every part of the plant shoot, causing soggy brown lesions with dense growths of myelium with conidiophores during humid conditions, followed by spread to other leaves and stem, which are deformed and snap. The infected leaves

are yellow and wither. When maturing or ripe capsules are infected, they can display irregularly shaped patterns and, during humid weather, are covered in grey-brown mycelium with conidiophores [59].

B. cinerea has been used in several studies focused on *P. somniferum* and other poppy species such as the California poppy (*Eschscholtzia californica* CHAM.), mainly as a source of fungal elicitors applied in the form of a homogenate or hydrolysate or isolated compounds (34). For example, it has been found by Bobák et al. (1995) that elicitation of callus cells of *P. somniferum* with *B. cinerea* homogenate increased production of sanguinarine alkaloid by up to 30 times [95].

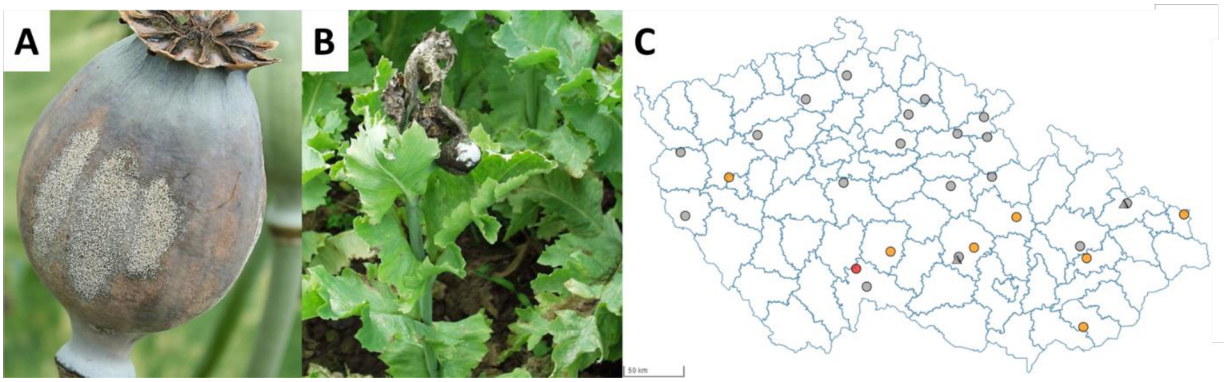

Figure 6. Symptoms and spreading of *Botrytis cinerea* in poppy A) *P. somniferum* capsule and **B)** stem infected by *Botrytis cinerea*. Photos by P. Kraus [99] **C)** Map of occurrence of *B. cinerea* on poppy in the Czech Republic in 2022. Red circle – harmful occurrence, orange circle – non-harmful occurrence, grey circle – confirmed absence, grey triangle – confirmed absence detected in random monitoring [99].

2.2.2.5 Other microbial pathogens

Apart from the above-mentioned pathogens, *P. somniferum* can be affected by a number of other microbial pathogens, although their economic impact is minor. Among the important microbe-caused diseases is capsule black mold, which can be caused by a variety of pathogens (including *Alternaria* species other than poppy fire-associated *Alternaria* spp., *Stemphylium* spp., and *Cladosporium* spp.,) manifesting as black coating and brown-green to black blemishes on capsules, decreasing seed quality in breadseed poppy, and reducing alkaloid content in pharmaceutical poppy [35] [40]. Other fungal pathogens include *Fusarium* spp. (*F. oxysporum* in Hungary, *F. semitectum* in India), which causes fusarium wilt, stem wilting and rotting of roots and stems [35] [40]. White mould, cause by another fungal pathogen *Sclerotinia sclerotiorum*, causes water-soakes lesions and bleaching of stems and forms black sclerotia inside infected tissues [35] [40]. *Rhizoctonia solani*, another fungal pathogen with a wide range of hosts [96], causes collar rot which can be especially severe in tropic conditions and is considered one of the most severe diseases of poppy in India [44]. The pathogen attacks roots and collar, leading to chlorosis and lodging of plants [40] [44]. *Erysiphe* spp., the causal agent of

powdery mildew of poppy (*E. cruciferarum, E. macleayae*)[97], forms cushions of white mycelium on mature leaves, followed by development of necrotic lesions. Powdery mildew of poppy is considered a disease of major importance in India [35] [40]. Other than *Peronospora*, the oomycete *Pythium* spp. (*P. dissotocum, P. ultimum*) can cause the damping-off of poppy seedlings, which develop chlorosis and wilt. *Pythium* has not been recorded in Europe so far (40). However, rather curiously, *P. oligandrum*-based product was used by Satranský et al. to study the influence of poppy seed pre-treatment on field emergence [98]. From bacterial pathogens, *Pectobacterium carotovorum* (syn. *Erwinia carotovora*) causes soft rot disease, causing young rosette-stage plants to turn violet, soften, wither and rot. Older infected plants quickly wilt in two to three days, leaves wilt and turn grey due to the rapid decay of inner tissues. Stems of infected plants is violet-coloured, lower parts are softened to oozing mash, and root tissues turn brown and decay, causing the plant to break and lodge. Soft rot disease was recorded in Spain in 2008 [44] and caused considerable damage in the Czech Republic in 2009 [35].

An overview of microbial disease of *Papaver* sp. is given below in **Table III**.

2.2.3 PESTS OF POPPY

Poppy is damaged mainly by insects commonly from the orders Coleoptera, Hemiptera, and Hymenoptera. While aphids, such as *Aphis fabae* or the green peach aphid *Myzus persicae*, cause mostly indirect damage by acting as vectors for the transmission of viral diseases, many other insects feed and reproduce on a poppy.

One of the most significant insect pests is root weevil ***Stenocarus ruficornis***, a 30-35 mm long beetle with brown wing cases. Root weevils overwinter in the soil and attack young poppy plants early in the spring when they are the most vulnerable, causing a decreae in crop yield or even plant death. *S. ruficornis* feeds on the leaves, leaving behind skeletonized leaves leading to slower and reduced plant growth. Females lay eggs into lower leaf tissues. Young larvae feed on leaves and roots, causing extensive root system damage [40]. Larvae are the primary cause of damage. However, adults can completely destroy the crop in years of high abundance [99]. Until 2013, insecticides for seed treatment were available as protection against root weevil; from then on, foliar protection must be used instead [99]. *S. ruficornis* has been reported from the Czech Republic, Romania, Sweden [99], Slovakia [100], United Kingdom [40], Iran [101], North Macedonia, Turkey [40], and was mentioned in a catalogue of Curculinoidea in Spain, although without specification of the host plant [102].

Another weevil affecting poppy is the capsule weevil ***Neoglacianus macula-alba*** from the order Coleoptera. Females of the capsule weevil chew holes in the capsules in which they

lay eggs. The damaged parts of the capsules produce white latex/milk, which later dries and produces dark spots called boreholes [103]. Larvae feed on developing seeds and can destroy the whole capsule. After four to five weeks, the larvae eat out of the capsule and bury themselves in cocoons in soil, where they wait for the following spring.

Wounds caused to the plants by capsule weevils are frequently points of entrance for fungal pathogens, which can bring destruction to completeness (35) (40) (103).Given its specific requirements for larvae development inside capsules, *N. maculaalba* was considered for opium poppy biocontrol program [40].

The capsule midget ***Dasineura papaveris*** is a secondary pest of poppy, as females lay eggs in the holes left behind by larvae of *N. macula-alba*. However, it seems to be capable of completing its life cycle even without the capsule weevil [104]. Its tiny yellow to orange-red larvae sucks on inner capsule tissues, which swell and fail to develop seeds [35] correctly. Capsule midgets overwinter in soil or inside of the galls [104].

Another harmful pest is the stem gall wasp ***Iraella luteipes*** (formerly *Timaspis papaveris*). Females of this species lie eggs inside of the stems, which can be recognized by violet spots and tissue necrosis. Up to several dozen larvae can be developing inside a single stem, damaging vascular tissue and causing yellowing and drying up of capsules or withering of the whole plant. The larvae themselves can be subject to parasitism by two species of the order Hymenoptera, *Trichomalus bracteatus* and *Pseudotorymus papaveris* [35].

Damage to oilseed poppy can also be done by the aphid ***Aphis fabae***, a pest with a wide range of hosts. This aphid usually overwinter on winter hosts commonly european spindle tree *Euonymus europaeus* [105] and in summer, the parthenogenetic females produce offspring which reproduce asexually on secondary hosts such as *P. rhoeas* or *P. somniferum*. They feed on phloem sap of secondary host, excreting carbohydrate-rich honeydew [105]. Common symptoms of aphid infestation on poppy include visible deformities and inward curling of leaves as well as colonies of black aphids on the abaxial side of leaves, stems, and sometimes green unripe capsules [35].

Other pests such as the potato bug *Calocoris norvegicus* or root weevil *Ceutorhynchus parmassicus* are listed in Table IV.

Table III: Microbial pathogens of poppy.

Pathogen	Alternative name	Classification	Disease name	Czech disease name	Reference
Alternaria penicillata	*Crivellia papaveracea* *Dendryphion penicillatum* (anamorph) *Helminthosporium papaveris* (holomorph) *Pleospora papaveracea* (teleomorph)	Ascomycota, Pleosporaceae, Alternaria sec. Crivellia	Helminthosporiosis, Blight of poppy, Poppy fire	Hnědá skvrnitost máku Helmintosporióza máku	(71) (72)
Alternaria papavericola	*Brachycladium papaveris*	Ascomycota, Pleosporaceae, Alternaria sec. Crivellia	Helminthosporiosis, Blight of poppy, Poppy fire	Helmintosporióza máku, spála máku	(72)
Peronospora spp.		Oomycota, Peronosporaceae	Downy mildew	Plíseň maková	(57)
Fusarium spp.		Ascomycota, Nectriaceae	Fusarium wilt, Root rot	Srpovnička špičatovýtrusá	(44)
*Pectobacterium carotovorum*subsp. *carotovorum*	*Erwinia carotovora* subsp. *carotovora*	Gammaproteobacteria, Enterobacterales	Soft rot disease	Bakteriální černání stonku	(44)
Pythium spp.		Oomycete, Pythiaceae	Damping-off	Pythiové padání máku	(40)
Alternaria spp.		Ascomycota, Pleosporaceae	Black spot, fruit rot	Čerň střídavá	(73)
Botrytis cinerea (anamorph)	*Botryotinia fuckeliana*(teleomorph), *Botrytis fabae, Sclerotinia fuckeliana, Peziza fuckeliana*	Ascomycota, Sclerotiniaceae	Grey mould	Plíseň šedá, šedá plísňovitost máku	(73)
Xanthomonas campestris pv. *papavericola*		Gammaproteobacteria, Xanthomonadaceae	Bacterial leaf spot of poppy	Bakteriální listová skvrnitost	(40)
Rhizoctonia solani	*Thanatephorus cucumeris*	Basidiomycota, Ceratobasidiaceae	Damping-off, collar rot	Kořenomorka, Rohoplodník bramborový, Rhizoktoniová kořenová hniloba máku	(40)
Sclerotinia sclerotiorum	*Sclerotium varium* (anamorph)	Ascomycota, Sclerotiniaceae	White mould of poppy	Hlízenka hlíznatá	(40)
Stemphylium vesicarum	*Stemphylium herbarum, Pleospora herbarum, Helminthosporium vesicarium*	Ascomycota, Pleosporaceae	Black spots of poppy	Zďovka bylinná, čerň máku	(40)
Pseudomonas viridiflavia		Gammaproteobacteria, Pseudomonadaceae	Baterial blight		(40)
Erysiphe cichoracearum	*Golovinomyces cichoracearum*	Ascomycota, Erysiphaceae		Padlí máku	(40)
Cladosporium herbarum	*Mycosphaerella tassiana* (teleomorph), *Dematium herbarum, Byssus herbarum*	Ascomycota, Cladosporiaceae	Black spots of poppy	Čerň obilná	(97)
Verticillium spp.		Ascomycota, Plectosphaerellaceae		Přeslenatka, Verticiliové vadnutí	(44)
Entyloma fuscum	*Entyloma bicolor*	Basidiomycota, Entylomataceae	Leaf smut, leaf spot	Sněť maková	(40)

Table IV: Pests of poppy

Organism	Alternative name	Classification	Common name	Czech name	Ref.
Neoglocianus maculaalba	*Ceutorhynchus macula-alba*	Insecta, Coleoptera	Capsule weevil, poppy weevil	Krytonosec makovicový	(40)
Dasineura papaveris	-	Insecta, Diptera	Capsule midges	Bejlomorka maková	(104)
Aphis fabae Scopoli	-	Insecta, Hemiptera	-	Mšice maková	(105)
Calocoris norvegicus (Gmelin)	*Closterotomus norwegicus*	Insecta, Hemiptera	Potato bug	Klopuška dvoutečná	(40)
Aylax minor Hartig	*Aulax minor*	Insecta, Hymenoptera	Gallwasp	Žlabatka maková	(106)
Aylax papaveris (Perris)	-	Insecta, Hymenoptera	Herb gallwasp	Žlabatka makovicová	(106)
Iraella luteipes (Thompson)	*Timaspis papaveris Kieffer*	Insecta, Hymenoptera	Stem gall wasp	Žlabatka stonková	(107)
Stenocarus ruficorni, Stenocarus fuliginosus	-	Insecta, Coleoptera	Root weevil	Krytonosec kořenový	(40)
Tettigometra sp.	-	Insecta, Hemiptera	-	Plochulka	(40)
Ceutorhynchus parnassicus	*Ethelcus denticulatus*	Insecta, Coleoptera	Root weevil	Krytonosec	(40)
Clinodiplosis cilicrus	*Carpodiplosis papaveris*	Insecta, Diptera	-	Plodomorka makovicová	(40)
Agrotis spp	-	Insecta, Lepidoptera	Cutworm	Osenice	(108)
Myzus persicae	-	Insecta, Hemiptera	Green peach aphid	Mšice broskvoňová	(108)
Meligethes spp.	-	Insecta, Coleoptera	-	Blýskáček	(109)

2.2.4 BENEFICIAL ORGANISMS AND POPPY

Not all interactions of poppies with microbes and other organisms are harmful. A number of bacterial and fungal species are used in commercial fertilizers or additives to improve soil quality with regard to poppy cultivation, such as *Clonostachys* spp. (CLONOPLUS additive), Trichoderma spp. (GLIOREX), and other species of fungi (POLYMIX) which decompose sclerotia, spores, and inactive stages of poppy pathogens (*Botrytis*, *Rhizoctonia*, *Sclerotinia*) in soil. Similarly, *Pseudomonas veronii* is added for vitality improvement (PROMETHEUS CZ), and addition of *Bacillus subtilis* QST 713 (Serenade ASO) or *Pythium oligandrum* M1 (Polyversum) has positive effect on resistance against pathogens such as *Alternaria* spp. [(35)].

A few endophytic microbes of poppy have been studied. For example, Pandey et al. (2016) isolated 22 endophytic microbes from roots, leaves, capsules, and poppy seeds. They found them to modulate specific traits according to the plant part from which they were isolated. For example, endophytes isolated from leaves could modulate photosynthesis and endophytes isolated from capsules increased morphine content [(110)]. Later, Ray et al. (2019) [(32)] followed up on Pandey's work, studying a consortium of two root endophytes isolated from poppy roots, *Acinetobacter* sp. and *Marmoricola* sp., and their effect on alkaloid production and the influence of 19 different endophytic root isolates on resistance of poppy towards

Peronospora-caused downy mildew. They identified one isolate that increased resistance and triggered a boost in salicylic acid content, suggesting resistance might be improved via systemic-acquired resistance induced by the endophyte (111).

Some research has also been conducted on an endophytic strain of *Beauveria bassiana* (04/01-Tip), an Ascomycete fungus belonging to the order Hypocreales. *B. bassiana* is an entomopathogen infecting *Iraella luteipes*, poppy stem gall wasp, and can be found as endophyte of poppy. For this reason, Quesada-Moraga et al. investigated the ability of *B. bassiana* to impart systemic protection against the stem gall wasp on poppy plants treated with conidial suspensions [112]. Subsequently, they also developed a method for PCR-based *B. bassiana* detection and quantification *in planta* [113].

2.3. PLANT IMMUNITY

In nature, plants have to share their environment with other organisms, which makes interactions with life forms from viruses and microbes to fungi, insects, animals, and even other plants inevitable [114]). And as was described in chapter 2, poppy is no exception. Being sessile organisms, running away or changing environments is not an option for plants, and a functional immune system is therefore essential for their survival [115].

2.3.1 BASAL DEFENCE MECHANISMS AND STRUCTURES

Before an interaction is initiated, plants employ a variety of mechanisms and features to protect themselves from harm. On the surface of a plant, a layer of waxes forms a cuticle, which protects the plant from desiccation, and UV radiation, contains antimicrobial compounds, and represents the first defence layer against pathogens [116]. Beneath the cuticle and forming a continuous layer, the cell wall represents a second physical barrier pathogens must penetrate to get to the live cells [116]. Both the cuticle and cell wall are dynamic systems which participate in signalling and can change their composition in case of pathogen attack [116] [117]. For example, the inner cell wall of plant cells is reinforced with lignin, a phenolic polymer forming a network of unpredictably joined monomers, a challenging obstacle to trespass for pathogens [117]. In response to pathogens, cells can react by deposition of callose and phenolics on certain places to enhance the mechanical strength of the cell wall and release toxic secondary metabolites [117].

In contrast, pieces of broken-down cell walls digested by pathogen lytic enzymes serve as DAMPs (Damage-Associated Molecular Patterns), elicitors of plant immunity, recognized by receptors localized on cell surface. Other structures such as hairs and trichomes also play a role in plant defence against organisms, from microbes to large herbivores [117]. Inside the

cells, a cytoskeleton comprised of actin microfilaments and microtubules provide structural framework allowing for controlled movement of organelles, cell growth, and intercelullar communication. This system is highly dynamic and tightly regulated. The role of cytoskeleton in plant-microbe interactions is evidenced by its participation in pathogen recognition by forming functional complexes with immune receptors, trafficking and delivery of immune-connected cargoes (callose deposition, lignin accumulation, signalling molecules, vesicles, or entire organelles) to the site of infection [118]. Additional protection mechanisms can be employed by plants, such as latex production in opium poppy [18], see chapter 2.1.3.

2.3.2 DANGER SIGNAL RECOGNITION

The first essential step in plants' defence against harmful organisms is recognition of the presence of a potential source of harm [119]. This may include a whole spectrum of signals, from the plants' cell components (such as extracellular ATP) released during tissue damage by both biotic and abiotic factors (the so-called "damage-associated molecular patterns", DAMPs) to molecules typical for pathogens, such as the bacterial flagellum component flagellin ("pathogen-associated molecular patterns", PAMPs) [120]. To perceive these signals, plants employ a wide array of receptors referred to as "pattern-regonition receptors" (PRRs) located on the plasma membrane, in the cytoplasm, or on inner organelle membranes ((121)).

Receptors on the cell surface are designated "receptor-like proteins" (RLPs) and "receptor-like kinases" (RLKs) and are functional analogues of metazoan Toll-like receptors [122]. These PRRs are generally considered to recognize more conserved or general molecular structures such as oligogalacturonides (OGs), fragments of pectin released from the plant cell wall upon the damage. At the same time, intracellular receptors perceive more "specialized" and varied molecules [120]. RLPs and RLKs belong to a superfamily of proteins featuring a leucine-rich repeat extracellular domain and represent an enormous array of proteins, with over 600 identified in *Arabidopsis*. An example of RLKs is the FLAGELLIN-SENSITIVE 2 (FLS2), a receptor for flagellin epitope flg22 derived from bacterial flagellum [123]. Since flagella are essential for bacterial motility, flg22 is a good indicator of the presence of bacteria, and plants deploy specialized lytic enzymes to release the epitope from the flagellum [122].

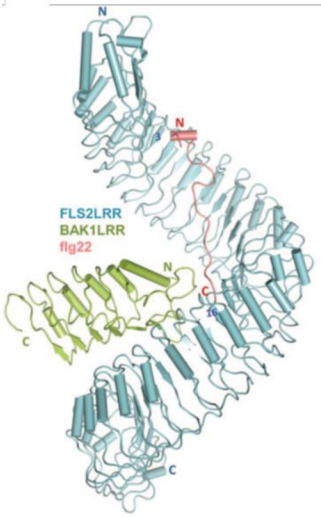

Figure 7. Interaction of flg22 with the FLS2 receptor [128].

Nucleotide-binding leucine-rich repeat receptors (NLRs) are located in the cell cytoplasm and recognize microbial effectors, specialized molecules produced by pathogenic microbes to subvert plant immunity and facilitate infection [122]. The recognition by NLRs leads to a potent immune response that can result in programmed cell death, restricting the pathogen's spread, and making it especially effective towards biotrophic pathogens feeding on life tissues [124]. For example, RESISTANCE-RELATED KINASE 1 (RKS1 or ZRK1) directly recognizes the AvrAC effector of the plant pathogenic bacterium *Xanthomonas campestris* [125].

2.3.3 SIGNAL TRANSDUCTION DURING PLANT IMMUNE RESPONSE

Plants' perception of molecular markers of "danger" is not as simple as binding a molecule. Instead, the receptors located either on the plasma membrane or inside the cytoplasm tend to cooperate with co-enzymes, co-receptors, or helper proteins, which help fine-tune the assessment of the danger posed by the molecules. Upon ligand recognition, PRRs send a signal to a receptor-like cytoplasmic kinases (RLCKs), which propagate the signal through a signalling pathway(s) leading to an immune response(s) by the cell [125]. Such activation of extracellular receptors triggers the production of reactive oxygen species, spikes in Ca^{2+} levels and activation of cognate protein kinases, activation of mitogen-activated protein kinase (MAPKs) cascades, and ultimately transcriptional changes [126]. For example, the FLS2 receptor for flagellin epitope flg22 requires a co-receptor BAK 1 (BRASSINOSTEROID RECEPTOR-ASSOCIATED KINASE 1) and, interestingly, these co-receptors are highly conserved in land plants (114). The cytoplasmic NLRs are usually present in an inactive state and require activation, for example, by dimerization of a sensor NLR and a helper NLR to trigger a downstream signalling cascade which initiates immunity response, frequently culminating in cell death, and event termed "hypersensitive response" HR) [122] [125]. Many co-receptors or general receptors are highly conserved within plants, and, curiously, the signalling pathways from different PRRs and NLRs seem to converge, i.e., although many receptors are present on the plasma membrane and the cytosol, once ligands are bound, they elicit similar types of responses. The complicated network of receptors and signalling molecules may result from an evolutionary arms race between plants and microbes, who try to evade the plant immune system by employing more specialized molecules that interject with plant signalling. In contrast, plants evolve ever more specialized receptors to spot the microbes' presence without compromising their signalling [114].

Apart from receptors, phytohormones, especially salicylic (SA) and jasmonic acid, play an important role in plant defence signalling. SA is essential for plant defence against biotrophic and hemibiotrophic pathogens [127] by amplifying the signals mediated by PRR and NLR proteins. SA biosynthesis is significantly increased upon pathogen detection by the plant [128], and is tightly regulated by multiple complicated levels of regulators, which include Ca^{2+} signalling. SA then binds to regulators of transcription of pathogenesis-related genes, ultimately increasing their transcription to facilitate plant defense and induces expression of some NLRs [122].

2.3.4 IMMUNE RESPONSE EXECUTION

Once pathogens are sensed, and the signal is delivered to the appropriate place, it has to be translated to take effect against the attacker. One of the most prominent response is, when signal reachs the cell nucleus and modulate gene expression. The transcriptome changes are quite intensive upon pathogen attack [122] and initiate various defence responses, such as modifications in cell wall composition, cytoskeleton rearrangement, callose deposition, ROS burst, secondary metabolite and toxin deposition in the apoplast, potentiation of PRR and NLR gene expression, expression off pathogenesis-related proteins, production of enzymes such as chitinases and glucanases, and a variety of other physiological changes are several examples of defense mechanisms of plants [116] [117] [114].Two additional physiological responses are potent in plants' defence arsenal: hypersensitive response (HR) and systemic acquired resistance (SAR) (114).

Hypersensitive response is predominantly a response induced by NLR-mediated immunity. Nowadays, the view on the interactions of NLRs and PRRs is changing, as new evidence suggests that NLRs cannot induce HR without PRRs, and more complicated regulation is at play [114]. HR results in the rapid local death of host cells at the site of infection to stop the spreading of a biotrophic or hemibiotrophic pathogen to living tissue. During HR, plant cells generate large quantities of ROS, reactive nitrogen species such as NO, or antimicrobial metabolites [129]. However, since this response is quite costly to the plant in terms of resources and can be advantageous to necrotrophic pathogens, HR is under strenuous regulation in plants to avoid contra-productive outcomes [129].

Unlike animals, plants lack specialized immune cells, and most immune responses happen in all affected cells. However, that does not mean plants do not have an organized system comprising the whole plant. While directly attacked tissues initiate an immune response to the pathogens, distant parts of the plant enter an enhanced immune state, preparing

the cells for a possible attack and allowing for a more robust and faster response. Therefore, the information about the attack must be communicated throughout the whole plant, and is mainly mediated and regulated by SA (or/and MeSA), N-hydroxypipecolic acid (NHP), glycerol-3-phosphate or azelaic acid and some other molecules[130]. Cells and tissues warned in advance in such a way react by transcriptional changes (for example, by induction of SA-biosynthetic genes in an auto-induction loop), decreasing photosynthetic capacity and halting growth-related processes, accumulation of defence metabolites such as camalexin in *A. thaliana*, and even facilitating HR [131].

2.4. STUDIES ON POPPY IMMUNITY

Despite its agricultural importance, little attention has been paid to the research of *P. somniferum* immunity towards pathogens. Several studies which dealt with resistance to diseases focused on the genetic variations and markers towards the breeding of resistant pathogens [132]. However, the molecular background of the interactions has not been comprehensively studied to date. From the available information on the topic, most research deals with alkaloids and induction of their production by microbial elicitors, or identification of cultivars resistant to some major diseases and sometimes finding the genomic locus conferring resistance. Unfortunately, most studies did not continue further to identify the mechanisms underlying the increased resistance. The following section attempts to summarize the information available on poppy immunity research using microbial elicitors (Section 4.1) and on the interactions of *P. somniferum* with numerous microbes (sections 2.4.2 – 2.4.4; but also rpartly reviewed in chapter 2.2) with special attention on the mechanisms of immunity and resistance in poppy. Finally, section 2.4.5 briefly deals with interactions of poppy and non-pathogenic, i.e. endophytic, microbes.

2.4.1 MICROBIAL ELICITORS

Several studies have been conducted on the effect of microbial elicitors on opium poppy as far back as 1985 when preparations from fungal pathogens were used to treat poppy cell cultures. This elicitation induced increased accumulation of sanguinarine, reaching up to 2600 μg/g of dry weight, in the cells and medium while no morphinan production was elicited. Interestingly, cells (and media) cultured with preparation from *Botrytis cinerea* or *Rhodotorula* sp. turned reddish brown [133]. After elicitation with *B. cinerea* elicitor preparation, Facchini et al. (1998) observed increased content of dopamine and tyramin (precursors for BIAs biosynthesis), BIA-synthetic gene mRNA, and accumulation of sanguinarine,

providing further evidence of the metabolite's integral role in response to pathogens. Additionally, incorporation of hydroxycinnamic acid amides into cell walls was observed in cell cultures treated with fungal elicitors [134]. They, however, mentioned the inability of dedifferentiated cells to produce morphinans, likely because specific cell structures are needed.

Sanguinarine has been consistently linked to elicitation by microbial elicitors in numerous studies. To better observe the effect of elicitors in differentiated cells, Huang and Kutchan studied transcription of the genes leading to morphinan and sanguinarin in cell cultures, seedlings, and mature plants. Their results showed that sanguinarine, but not morphine, is induced by fungal elicitor [135]. However, Khan et al. (2011) treated poppy plants with alginate oligosaccharides, compounds inducing cell signalling in plants. They observed enhanced growth and a considerable increase in content of morphine and codeine [136]. Very interesting for Czech poppy research is the study of Verma et al. (2014), who used both biotic (four endophytic fungi) and abiotic (salicylic acid, hydrogen peroxide, and carbon dioxide) elicitors to stimulate embryonic cell suspensions of latex-less variety of poppy. Notably, sanguinarine production was also enhanced in this latex-less variety by application of *T. harzianum* preparation and treatment with 250 μM salicylic acid, one of the main biotic stress-related phytohormones, possibly linking sanguinarine regulation to salicylic acid defense signalling [137].

Elicitors were also used in multiple studies of BIAs biosynthesis [138]. For example, Yu (2000) studied the role of detoxifying glutathione *S*-transferases in poppies and found them to be inducible by fungal elicitors in seedlings and cell cultures [139]. A substantial amount of work on the biosynthesis of alkaloids using elicitors was carried out by the group of P. Facchini, who recently published an updated review on the topic in 2019[18]. In 2005, they were able to link sanguinarine biosynthesis to the endoplasmic reticulum undergoing ultrastructural changes upon elicitor treatment [140]. In 2008, the group showed massive metabolic reprogramming in poppy cell cultures with elicitors using ^{1}H NMR metabolomics and analyzed proteome by LCMS/MS and DNA microarrays, detecting a large amount of alkaloid biosynthetic genes/enzymes and pathogenesis-related proteins in elicitor-treated cells [141].

Holková et al. (2010) used the elicitor-inducible cell cultures producing sanguinarine platform to investigate lipoxygenases, enzymes producing oxylipins. Oxylipins play a significant role as signal molecules that activate defence-related genes. They observed an increase in lipoxygenase activity directly preceding sanguinarine accumulation[34].

A comprehensive genome-wide transcriptome profiling in methyl jasmonate-elicited capsules shows increased morphine and noscapine content. They also observed a significant increase in stress-related transcripts in treated capsules [142].

Apart from elicitors, Mishra et al. (2013) studied poppy response to wounding, identifying a wound-inducible transcription factor termed PsWRKY [108]. Two years later, they inserted the previously identified transcription factors, PsAP2, into a tobacco plant, in which they observed increased antioxidant levels and enhanced biotic and abiotic stress tolerance through antioxidant regulation [143].

2.4.2 RESISTANCE TO P. ARBORESCENS-CAUSED DOWNY MILDEW

As the most destructive disease of poppy, downy mildew (caused by *P. arborescens*) has been the target of various researchers to limit its adverse effects on opium yield. Downy mildew is noted as soon as in 1920 by Harold Annett, who obtained seeds for pure poppy lines from H. M. Leake, an economic botanist breeding poppy towards resistance to the abovementioned pathogen[144]. This endeavour was followed by many other researchers [145] [146] studying the genetic aspects of resistant cultivars, resulting in identification of partly downy mildew-resistant cultivar Pps-1, which later became a "model" cultivar for studying downy mildew resistance in this research group [147]. In 1999, was observed a correlation of higher alkaloid yield with higher resistance to downy mildew in Indian poppy landraces. However, none of the cultivars studied was simultaneously resistant to *P. arborescens* and damping-off disease caused by *P. dissotocum*, pointing towards separate resistance mechanisms specific for each pathogen rather than a single trait being responsible for increased resistance. High alkaloid content, although possibly a contributing factor, did not seem to cause resistance (55). However, it was later observed that selection for downy mildew resistance is possible together with high alkaloid seed, straw yield, and other traits (capsule diameter, number of stigmatic rays, number of capsules per plant), a highly advantageous fact for pharmaceutical poppy improvement [148].

In 2009, Dubey et al. identified the resistance traits in cultivar Pps-1 to be maternally transmitted indicating the involvement of cytoplasmic genes in addition to nuclear regulation, polygenic, and recessive [56]. Further, they confirmed these results in another paper published the same year, in which they compared chloroplast DNA from the Pps-1 resistant mutant with a susceptible cultivar H-9. Using random primers, they identified a unique fragment in Pps-1 encoding plastid RNA polymerase β' subunit (*rpoC1*), which transcribes most plastid genes and pinpointed four single nucleotide substitutions. The hypothesis that these changes were responsible to DM resistance were supported by a report of microcin J25-resistant *E. coli* strain with mutations in *rpoC* gene. However, the influence of other genes could not be ruled out [56]. Based on these results, the group developed a method for identification of downy mildew

resistance using Amplified Fragment Length Polymorphism (AFLP) markers, which identified more maternally-inherited cytoplasmic and nuclear polymorphic regions in the resistant mutant, further supporting the polygenic nature of resistance traits [63]. Resistance was also linked to antioxidant content in poppy, with Pps-1 mutant exhibiting the increased need for enhanced ROS for immune response leading to a hypersensitive response. As *P. arborescens* is an obligate biotrophic pathogen, cell death is an essential feature of plant defence against this pathogen [63].

An interesting finding was made by Montes-Borrego et al. (2017), who found that even resistant, asymptomatic plants could be systemically infected with the pathogen and produce infected seeds[149].

2.4.3 Resistance to *Alternaria* (*Pleospora*)-caused Helminthosporiosis

Although helminthosporiosis is one of the most damaging diseases of poppy (besides downy mildew) no information is available on the mechanisms of resistance towards *Alternaria* pathogens in poppy. Most research of these pathogens focused on a completely opposite goal, as *Alternaria* (*Pleospora*) was considered a mycoherbicide of poppy for biological control of weeds and illicit poppy cultivation. This idea was postulated in 1989, when Del Serrone investigated its potential for controlling various weeds in crop fields, with corn poppy (*Papaver rhoeas*) frequently found in cereal crops in Italy found to be susceptible. While cereals were immune to the disease, other *Papaver* species could be affected. Interestingly, a long dew period of up to 24 h was required for successful infection resulting in 100% plant mortality, presumably due to the pathogen entering through stomata [150].

The use of *Alternaria/Pleospora* as a biological control agent was reevaluated in the year 2000, when *Pleospora* was found to be able to overwinter in the field [66][68]. The pathogen was later deemed to have a good potential as mycoherbicide when used with Tween 20 (1% v/v) as an adjuvant to help adhesion to the waxy surface of poppy leaves to reduce extractable morphine from capsules [151]. The addition Nep1, a phytotoxic protein isolated from *Fusarium oxysporum*, was found to further enhance the infectivity by acting synergistically with *P. papaveracea* [75].

2.4.4 Resistance to Rhizoctonia solani -cause collar rot

Collar rot is another damaging disease of poppy. Unlike poppy fire caused by *Alternaria* spp., collar rot-resistant cultivars such as Ib-38 and IS-22 have been. The resistance was shown to be recessive with no influence of cytoplasmic genes and a possibility of only nuclear

genes being involved. Further, evidence for one single gene governing collar rot resistance in poppies was observed in cultivar crossing and back-crossing experiments [152].

2.4.5 INTERACTIONS WITH OTHER MICROBES

Some other *P. somniferum* interactions with microbes have been studied. Bonilla et al. investigated the influence of ten endophytic strains and their culture media on seed germination and identified three strains, *Stenotrophomonas maltophilia, Chryseobacterium balustinum,* and *Pseudomonas fluorescens*, which were able to increase the alkaloid content, height, and straw dry weight in poppy plants. An increase in morphine and decrease in thebaine was observed after treatment with *S. maltophilia*, which was identified as a good candidate to increase productivity of the crop[153]. Altered metabolite accumulation was also observed upon infection with Ageratum enation virus together with increased ROS production in infected tissues [53].

An entomopathogenic fungal strain *Beauveria bassiana* was found to be able to colonize poppy and possibly provide systemic protection against the larvae of *Timaspis papaveris*, and important poppy pest in Spain [154]. The effect of endophytic bacteria on poppy was further explored by Pandey et al, who studied the differences in bacterial community from different parts of the plant and their tissue-specific functions. Culturable leaf-associated bacteria exhibited the ability to modulate photosynthesis, plant growth, and productivity, and increased BIAs content event in latex-less poppy varieties. The most pronounced effect on alkaloid accumulation had the capsule-associated bacteria, which upregulated specific BIA-synthetic genes in isolate-specific fashion - e.g., one particulate isolate caused the increase in morphine, papaverine, and noscapine production by upregulation of almost all key biosynthetic genes [110]. One of the root isolates, *Pseudomonas putida*, was shown to increase tolerance against downy mildew while promoting growth [155]. Similarly, Mishra et al. (2020) observed antagonistic effects of *P. fluorescence* treatment as a foliar spray on *P. arborescens*, and was found to be effective as a means of management of the disease when used with metalaxyl, and plants treated with *P. fluorescence* showed increased antifungal phenol content, possibly by enhancement of systemic resistance [156].

One endophytic bacteria isolated by Pandey et al., *Microbacterium* sp., decreased downy mildew biochemical markers in treated poppy plants. The researchers conducted a comparative transcriptome analysis in treated and untreated plants and found differential ex-

pression of genes involved in signal transduction, protein modification, defence proteins, transcription factors, and phytohormones. Concomitantly, they observed increased salicylic acid content in treated plants, pointing towards SA-regulated endophyte-induced resistance [111].

3 WORK OBJECTIVES

- Establish P. *somniferum* - *H. papaveris* and *P. somniferum* - *X. papavericola* pathosystems in the MPMI laboratory
- Monitor the resistance of two *P. somniferum* cultivars (Turec and R2) to *H. papaveris* and *X. papavericola*
- Observe the effect of flg22 treatment on *P. somniferum* and its resistance to *X. papavericola*

4 MATERIALS AND METHODS

4.1. PLANT MATERIAL

4.1.1 POPPY CULTIVARS

The seeds of the two cultivars used in this project, "Turec" (designation 15O08) and "R2" (designation 15O0800119), were kindly provided by Dr. Rychlá from Oseva PRO s.r.o. The communication with OSEVA was carried out by Ing. Ondřej Hejna, Ph.D. from the Laboratory led by Prof. Vladislav Čurn at the Faculty of Agriculture and Technology at the University of South Bohemia in České Budějovice (FZT JU), who also kindly provided space, and equipment necessary for the growth monitoring. The cultivars were evaluated by the provider for their yield, alkaloid content, fatty acids content, growth and flowering stages and resistance to common diseases. The diseases monitored in the fields were caused by *Peronospora* sp., "Helminthosporiosis" of poppy caused by *A. penicillata* or *A. papavericola,* and "Bacteriosis" without specification of the causal bacterial agent.

4.1.2 POPPY GROWTH CONDITIONS

Poppy seeds without pretreatment were sown in 6-section pots (4.5×4.5×5 cm) in non-sterile perlite and watered with a solution of G2 fertilizer and Jungle garden base (both purchased from Numazon s.r.o.) prepared according to the manufacturer's instructions (for 10 L of the solution, 25 mL of G1 fertilizer and 10 mL of Jungle garden base were thoroughly mixed in non-sterile tap water and immediately used for watering the plants). The plants were kept in a growing chamber with a photoperiod of 16 h day/8 h night at 8000 lux illumination. Constant temperature of 20±1°C and 80% humidity at night/60% humidity during the day were mantained. The plants were kept under these conditions for 4 weeks and were watered every 3 days (or according to need). The growth was carried out in collaboration with Prof. Čurn laboratory at FZT JU.

4.2. PATHOGEN CULTIVATION

4.2.1 *XANTHOMONAS PAPAVERICOLA*

X. papavericola was purchased from Czech Collection of Microorganisms (CCM) at Masaryk University, Brno, listed in the cataloge under the code CCM 452. The culture was originally isolated from *Argemone* sp. (a species of prickly poppy) on Mauritius island. The pathogen was mantained on potato dextrose agar (PDA) plates (1,5% agar) at 25°C in darkness and re-inoculated on fresh plates every two weeks.

4.2.2 *BOTRYTIS CINEREA*

Botrytis cinerea BMM isolate was obtained from the Laboratory of Pathological Plant Physiology, Institute of Experimental Botany, Czech Academy of Sciences [88]. It was mantained on PDA (1,5% agar) at 25°C in darkness. Every two weeks, a slice of agar from the center of the agar plate was places on a fresh plate to propagate.

4.2.3 *ALTERNARIA* SPP. ISOLATION AND CULTURING

Poppy leaves from the field infected with a supposed *Alternaria* spp. pathogen were kindly provided by Dr. Ondřej Hejna. The leaves were pressed onto agar plates containing brewer's yeast extract (10 g/L) and glucose (20 g/L) (following Bailey 2014 [151]) and the antibiotics tetracyclin (50 μg/mL) and kanamycin (50 μg/mL) to prevent growth of bacteria. After 1 week at 25°C in darkness, once different colonies formed on the plates, each colony was inoculated separately onto fresh plates with antibiotics and cultivated for another 1 week to obtain individual colonies and/or homogenous cultures of mycelia. Individual cultures were used to inoculate liquid media (brewer's yeast extract and glucose in the amounts given above, without agar) with antibiotics and cultivated for another 3 days at 25°C in darkness with 180 rpm shaking. These cultures were then used to prepare 25 % glycerol stocks, flash-frozen in liquid nitrogen, and stored at -80°C. Live cultures were mantained on the same agar plates with antibiotics.

4.3. FLG22 TREATMENT AND PRE-TREATMENT

To observe the reaction of the two poppy cultivars to elicitation using bacterial MAMP (microbe-associated molecular pattern), here represented by flg22 peptide, 40-days-old plants (~ 6 weeks) were watered with 1 L of standard aquaponic medium (Jungle garden fertilizer as mentioned above) supplied with 100 nM flg22 (QRLSTGSRINSAKDDAAGLQIA; EZBiolab). Control (non-treated) plants were watered with 1 L of medium without flg22. The plants were observed and samples collected after 6, 12, 24, 48, 72, and 168 hours. The assay was performed in 12 replicates per cultivar per treatment.

For pre-treatment of plants for infection assays, the plants were treated with flg22 in the same way as described above 1 day before infection with pathogens.

4.4. GROWTH PARAMETERS MEASUREMENTS

4.4.1 CHLOROPHYLL FLUORESCENCE MEASUREMENT

The rate of photosynthesis was measured using a Pulse Amplitude Measurement (PAM) PAM-2500 portable chlorophyll fluorometer (Walz) with leaf clip attachment. Fv/Fm

ratio was measured in mature leaves on the upper part of leaves. The measurement was always performed between 7-8 AM after the plants have been in darkness during the night. Plants that were not measured at the moment were covered with dark cloth to prevent exposure to measuring lights. During measurement, the leaf clip with inserted leaf was covered with dark cloth to prevent light affecting the neighboring plants.

4.4.2 GROWTH AND WEIGHT DETERMINATION

At the end of the experiment, the plants were taken out of the pots, and divided into leaves and stems. The leaves were picked off at the base attaching to the stem and dried at 65°C overnight. Stems were cut from roots at the hypocotyl site, length was measured, and stems were dried at 65°C overnight. The dried weights of all plant parts were determined using analytical scale.

4.5. INFECTION OF POPPY PLANTS WITH PATHOGENS

4.5.1 *XANTHOMONAS PAPAVERICOLA*

For infection assay, we followed a modified protocol from Cerutti et al. 2017 (157). *X. papavericola* was cultured in liquid PDB medium for 2-3 days at 25°C in darkness. Before infection, the OD_{600} was measured and the culture diluted either to $OD_{600} = 0.01$, $OD_{600} = 0.05$, or $OD_{600} = 0.2$ using sterile distilled water and supplied with 0.001% Tween. The solution was then thoroughly mixed to homogenize and sprayed onto all parts of the plants (both ad- and abaxial sides of leaves) using a spray bottle until runoff. Control plants were sprayed with distilled water containing 0.001% Tween. The assays were performed in replicates of 3 or 6 (plants) per cultivar per treatment.

One day before infection, all plants were placed in clear plastic boxes to increase the humidity. After infection, the plants were (1) kept inside the boxes at high humidity during the entire course of the experiment (7 days) or (2) removed from boxes and kept at normal humidity after 1 day post infection.

The course of infection was allowed to developed for 7 days after infection and assessed every day. The infection development was rated on a scale of 1-10 (Fig. 8) of increasing percentage of leaves infected by the pathogen on each plant (1 – 10% or less leaves showing symptoms, 10 – 100% of leaves showing symptoms) and documented on photos. On the last day of the experiment, the plants were removed from the boxes, leaves carefully picked from the plants so as not to damage them mechanically, arranged from oldest (senescent) to youngest, and photographed. Leaves with visible infection were collected, wrapped in aluminium foil, flash-frozen in liquid nitrogen, and stored at -80°C.

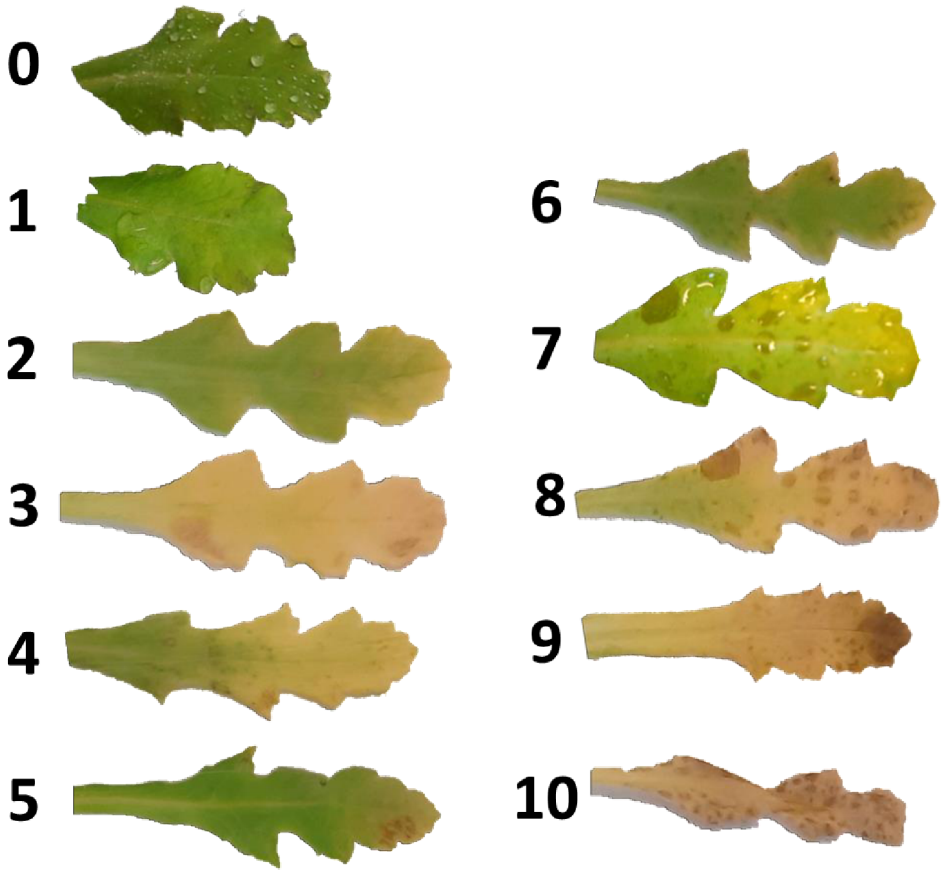

Fig. 8: Evaluation scale of *X. papavericola* symptoms on poppy leaves.

0	Non-infected plants; completely green asymptomatic leaves with water droplets from spraying with inoculum.
1	Visible on or two small brown dots.
2	Light-brown lesion, yellowing near the margins of the leaves or on the site of inoculum droplets.
3	Light-brown lesions occupying less than 30% of the leaf area.
4	Brown lesions accompanied by yellowing of he surrounding area on multiple places of the leaf.
5	Pronounced brown lesions accompanied by yellowing of the surrounding leaf, lesions spread from original site of inoculation. Infection appears not only on senescent leaves but spreads on mature green leaves.
6	Small brown lesions on cca 50% of the leaf accompanied with yellowing of surrounding areas.
7	Brown lesions on over 50% of the leaf grow in size and number, almost whole leaf is yellow.
8	Lesion continue to spread over more than 60% of leaf area, brown lesion sites start to dry up in the centers.
9	Infection in to most affected regions do not conform to shape and start spreading to the surrounding area creating dark brown spots on the leaf, leaf starts to dry up.
10	Whole leaf is yellow and covered in dark brown spots, leaf is completely dry.

4.5.2 *BOTRYTIS CINEREA*

B. cinerea infection was performed using a drop of conidial suspension according to modified protocol from Kalachová et al. 2020 [88]. For production of conidia, a slice of *B. cinerea*-containing agar was placed on a fresh agar and cultivated at 25°C in darkness for 4 days, then under daylight at room temperature (RT) for 7 days.

On the day of infection, the conidia were collected by washing the agar plate with ±5 mL of PDB medium and gently released from the mycelium using ethanol- and flame-sterilized glass spatula. The concentration of the conidia in the obtained suspension was counted using a Bürker counting chamber and Olympus BX61 microscope. The suspension was diluted using sterile distilled water to 1×10^5 or 1×10^6 conidia/mL and supplied with 0,001% Tween. 5 µL of the suspension was then transferred using a micropipette onto three leaves of the same age labeled with a marker on each plant. The infection development was rated on a scale of 1-10 (Fig. 9). The assays were performed in 3 or 6 replicates per cultivar per treatment and infection was allowed to develop for 7 days.

One day before infection, all plants were placed in clear plastic boxes to increase the humidity. After infection, the plants were (1) kept inside the boxes at high humidity during the entire course of the experiment (7 days) or (2) removed from boxes and kept at normal humidity after 1 day post infection

Size and morphology of lesions were monitored daily for the duration of the experiment. On the last day of experiment, the leaves of each plant were picked and arranged oldest to youngest and photographed. Leaves with lesions were collected, wrapped in aluminium foil, flash-frozen in liquid nitrogen, and stored at -80°C.

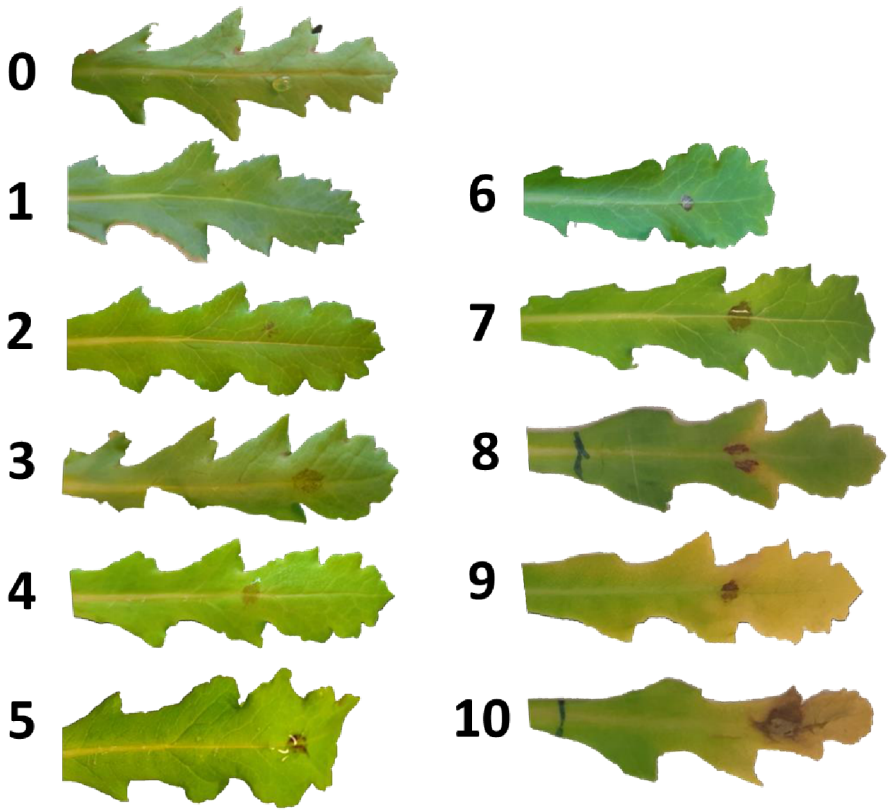

Fig. 9: Evaluation scale of *B. cinerea* symptoms on poppy leaves.

0 Non-infected plants; completely green asymptomatic leaves with drop of water.

1 Visible discoloration of leaves, light-brown misshapen lesions in the place of drop with inoculum.

2 Light-brown lesions scattered on area the size of the original drop or slightly bigger.

3 Light brown lesions scatter on an area the size of the original drop or slightly bigger occupying almost all the space but not a continual lesion yet.

4 One continual light brown leasion the size of the original drop or slightly bigger.

5 Brown lesion on the leaf the size of the original drop of inoculum or slightly bigger, pronounced brown color, visible on the abaxial side of the leaf.

6 Dark brown lesion outgrowing the original drop with inoculum, lesion reaches abaxial side of the leaf and is as prominent as on the adaxial side, white mycelium may be visible.

7 Brown lesion on both sides of the leaf, yellow discoloration hinted on the sides of the lesions.

8 Dark brown lesion on both sides of the leaf not limited by veins, growing larger than original drop with inoculum, starts affecting the surrounding area causing yellow spots to appear in a characteristic "V" shape.

9 Light or brown spots affecting the surrounding area and causing yellowing of a large part of the leaf behind growing lesion.

10 Large, dark brown lesion considerably bigger than original drop with inoculum, growing intensively on both sides of the leaf, expanding into yellow region behind the lesion, whole part of leaf yellow following the lesion and outreaching in front of the lesion.

4.5.3 PUTATIVE *ALTERNARIA* SP.

For production of conidia, *Alternaria* sp. was cultivated on brewer's yeast and glucose medium for 10 days according to Bailey et al. 2004[151]. On the day of infection, the conidia were collected as described above and concentration calculated using Bürker counting chamber. The conidial suspension was diluted with sterile distilled water to 1×10^5 or 1×10^6 conidia/mL and supplied with 0,001% Tween. 5 µL of the suspension was then transferred onto the leaves using a micropipette onto three leaves of the same age labeled with a marker on each plant. The assays were performed in 3 or 6 replicates per cultivar per treatment and infection was allowed to develop for 7 days. Size and morphology of lesions were monitored daily for the duration of the experiment. On the last day of experiment, the leaves of each plant were picked and arranged oldest to youngest and photographed. Leaves with lesions were collected, wrapped in aluminium foil, flash-frozen in liquid nitrogen, and stored at -80°C.

To assess the progress of infection, a scale of 0-10 was developed based on the symptoms exhibited by the plants (Fig. 10).

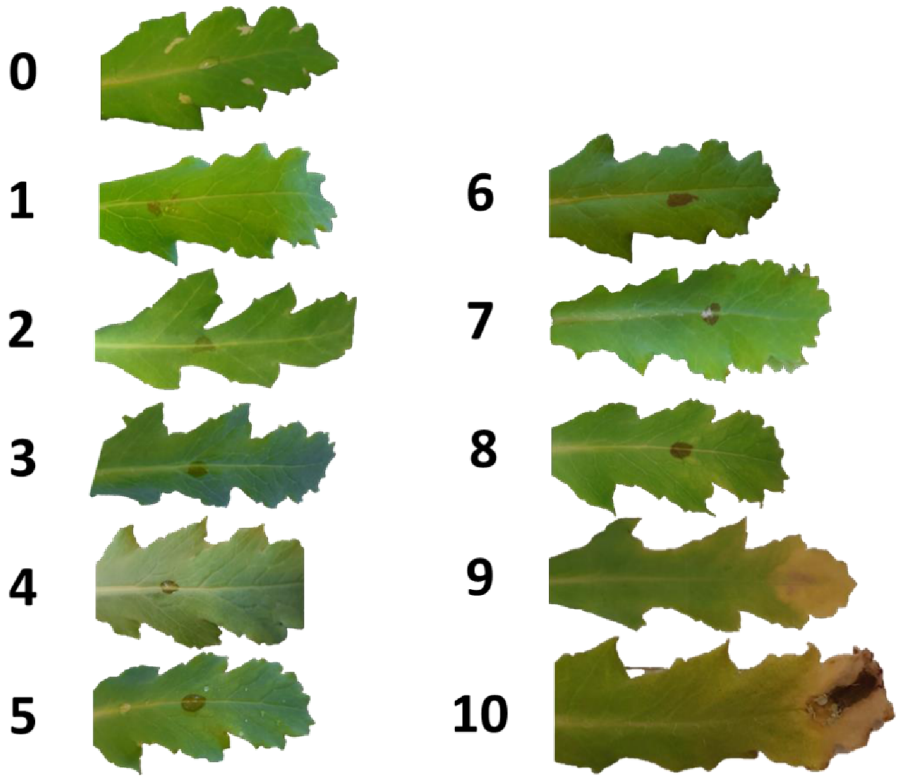

Fig. 10: Evaluation scale of putative *Alternaria* sp. symptoms on poppy leaves

0 Non-infected plants; completely green asymptomatic leaves with drop of water.

1 Visible discoloration of leaves, light-brown misshapen lesions in the place of drop with inoculum.

2 Light-brown lesion the size of the original drop or slightly bigger.

3 Brown lesion on the leaf limited by central vein; central vein unaffected.

4 Brown lesion on the leaf limited by central vein; central vein unaffected, lesion reaches the abaxial side of the leaf.

5 Brown lesion on the leaf the size of the original drop of inoculum or slightly bigger, not limited by veins, lesion reaches abaxial side of the leaf.

6 Brown lesion outgrowing the original drop with inoculum, not limited by veins, lesion reaches abaxial side of the leaf and is as prominent as on the adaxial side.

7 Brown lesion on both sides of the leaf not limited by veins, white fluffy mycelium may start growing on the leaf.

8 Dark brown lesion on both sides of the leaf not limited by veins, growing larger than original drop with inoculum, starts affecting the surrounding area causing yellow spots to appear following side veins directly behind lesion.

9 Light or brown spots affecting the surrounding area and causing yellowing of a large part of the leaf behind lesion.

10 Large, dark brown lesion considerably bigger than original drop with inoculum, growing intensively on both sides of the leaf, expanding into yellow region behind the lesion, whole part of leaf yellow following the lesion and outreaching in front of the lesion.

4.5.4 STATISTICAL ANALYSIS

Statistical analysis was performed in GraphPad Prism 5 software.

5. RESULTS

5.1. DESCRIPTION OF POPPY CULTIVAR PHENOTYPES

Our colleague, Natálie Hradecká, obtained four poppy cultivars thanks to a collaboration with Dr. Hejna (Faculty of Agriculture and Technology, South Bohemia Unversity) a Dr. Rychlá (OSEVA PRO s.r.o.). According to field screening experiments by OSEVA PRO s.r.o., these four cultivars are distinct in their resistance/susceptibility to fungal pathogens *Alternaria penicillata/Alternaria papavericola* (formerly known as *Pleospora papveris*, a pathogen causing the so-called helminthosporiosis of poppy) and *Peronospora arborescens*. Out of these four cultivars, we selected two cultivars "Turec" and "R2" based on experiments with flagellin treatment of these cultivars in our lab conditions previously performed by Natálie Hradecká. She observed that Turec was more sensitive to flg22 than R2.

In this thesis, we compared the growth phenotype and basic photosynthetic parameters of the selected cultivars in our lab growth conditions. Additionally, we analyzed the reaction of the selected cultivars to treatment with flagellin epitope flg22 and high humidity (a growth condition used for later pathogen infection studies).

5.1.1 GROWTH PHENOTYPE OF TUREC AND R2 CULTIVARS

Both cultivars were grown in stable conditions as described in section XX without interference to assess the standard growth phenotype of the cultivars and observe the differences between them. Several notable distinctions were observed.

At the beginning of cultivation, there is little difference between the germination and growth rate of the two cultivars (Fig. 11A), and they reach similar heights once they grow into the rosette stage (Fig. 11 B). However, once in the rosette stage, the stem of the cultivar Turec begins bolting much sooner than cultivar R2. There is a delay of a whole three weeks: while Turec starts bolting 6 weeks after sowing and forms buds and flowers in the 9th week, R2 does not start bolting until 9 weeks after sowing (Fig. 11-C). However, once bolting does start in R2, the stem is much thicker than that of cultivar Turec (Fig. 11 D).

To quantify the difference, we measured leaf weight, stem weight, and stem length in the 9th weeks old poppy plants (Fig. 12). The results showed that Turec cultivar has higher biomass production compared to R2, as was shown by the higher dry leaf and stem weight (Fig. 12). The higher stem weight of Turec cultivar correlates with the longer stems (Fig. 12).

Fig. 11: Comparison of gowth phenotype of poppy cultivars Turec and R2. **A)** 5 days old seedlings of Turec (left) and R2 (right). **B)** 5 weeks old plants of Turec and R2 cultivar in rosette stage. **C)** 9 weeks old plants of cultivars Turec (bolting stage) and R2 (rosette stage). **D)** 4 months old plants of R2 (bolting stage) and Turec (capsule ripening stage).

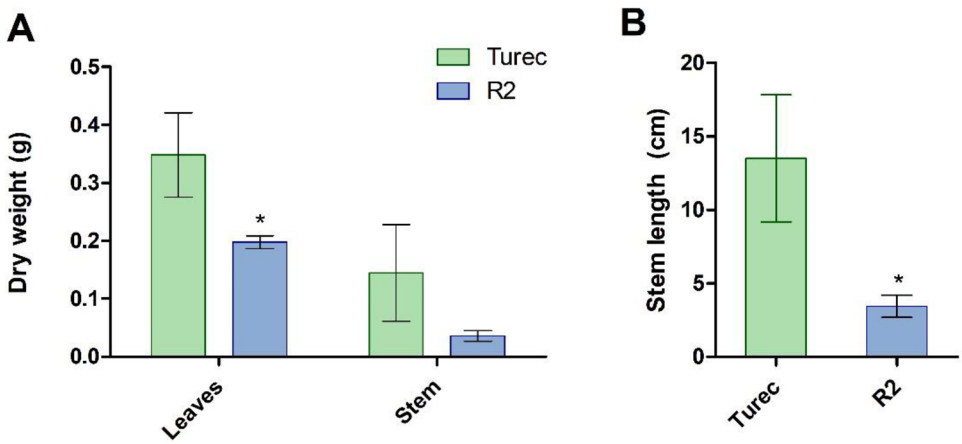

Fig. 12: Comparison of biomass production of the two cultivars. **A)** Dry weights of leaves and stems of the two cultivars. **B)** Stem length of the two cultivars. Statistical analysis (t-test) was performed in GraphPad Prism 5 using data from 3 replicates of each category. Asterisk (*) indicates statistically significant difference ($p<0,05$).

The leaves of the two cultivars also differ in some aspects. While cultivar Turec has bright green leaves with many crevices, the leaves of R2 are more round (Fig.13 A and B), darker, and frequently colored a deep reddish brown color on the abaxial side (Fig. 13 C). A frequent anomaly observed in R2 are plants with white spots in leaf crevices on all leaves (Fig. 13 -D), which constitute about 10% of the population.

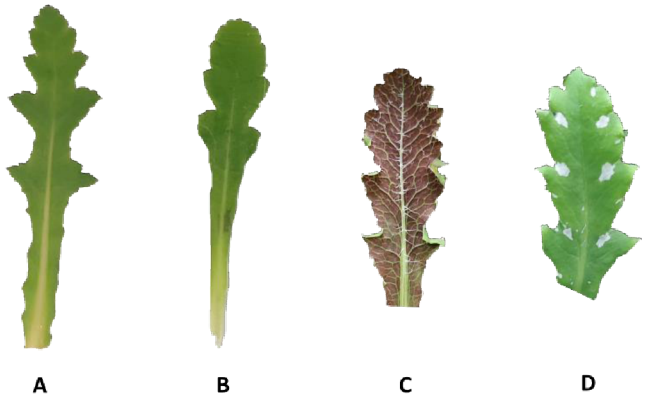

Fig. 13: Differences in leaf phenotype of 6-weeks-old plants between the two cultivars. **A)** Leaf of cultivar Turec. **B)** Leaf of cultivar R2. **C)** Abaxial side of a leaf of cultivar R2 showing reddish coloring. **D)** Anomalous leaf of cultivar R2 showing white spots on the adaxial side of the leaf.

Additionally, the two selected cultivars differs in flower and seed colour. The Turec seeds are milky white and seeds of R2 cultivar has blue colour (Fig. 14 A). The petals of Turec are completely white, while petals of R2 sport a purple spot near the center of the flower (Fig. 14 B and C).

Fig. 14: Differences in seed and flower phenotype of the two cultivars. **A)** White seeds of cultivar Turec (up) and blue seeds of R2 (down). Photo provided by J. Kubásek. **B)** White petals of cultivar Turec. **C)** White petals with purple spots of cultivar R2.

5.1.2 PHOTOSYNTHESIS PARAMETERS OF TUREC AND R2 CULTIVARS

Chlorophyll fluorescence was measured as an indicator of the photosynthetic ability of the plant and the influence of environmental conditions. First, the Fv/Fm value of leaves in different state of maturity were measured to determine the variability of values within a plant and its dependence on the age of the leaf (Fig. 15). It was found that the highest photosynthetic ability was in the youngest leaves at the top of the rosette, where Fv/Fm values consistently reached over 0,80. Mature leaves had slightly lower values usually between 0,70-0,79. As expect, the oldest, senescent leaves, had the lowest values dropping to 0,4 and lower. No significant differences were found between the two cultivars.

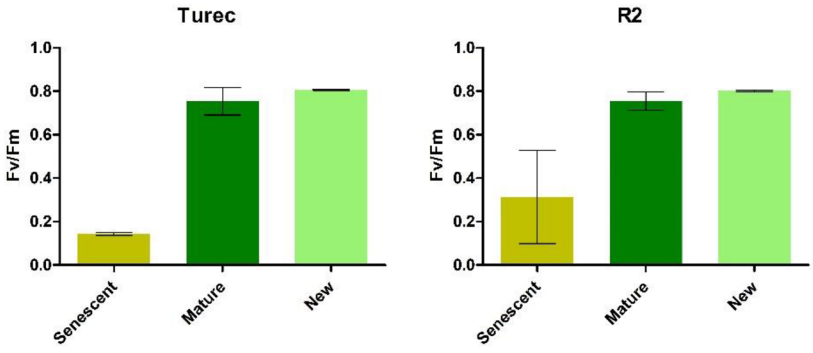

Fig. 15: Chlorophyll fluorescence in leaves of different ages in 8-weeks-old plants. **A)** Fv/Fm measurements in differently aged leaves of the cultivar Turec. **B)** Fv/Fm measurements in differently aged leaves of cultivar R2. Bars represent means and error bars represent SD from 3 replicates.

5.1.3 EFFECT OF FLAGELLIN ON GROWTH AND PHOTOSYNTHESIS

In our work, we focus on plant-microbe interactions. One of the easiest way how to simulate bacterial attack is to treat the plants with flg22 (Zipfel et al. 2004). Based on the result obtained by Natálie Hradecká (not yet published but will be part of her bachelor thesis submitted in 2023), we knew that poppy reacts to flg22. We did basic analysis of the effect of flg22 on growth and photosynthetic parameters. Plants were treated with flg22 in the 6th week after sowing and the growth phenotype and photosynthetic parameters were monitored over the course of two weeks. We observed the effect of flg22 treatment on the growth of Turec cultivar, while the R2 seemed to be not affected (Fig. XY A and B). No effect of flagellin on the chlorophyll fluorescence was observed (Fig. XY C).

Additionally to plant growth, we measured the basic photosynthetic parameter Fv/Fm in mature poppy leaves after flg22 treatment. The results of controls were in accordance with our previous measurements (Fig. 14). The data show that flg22 treatment with flagellin did not have any effect on the chlorophyll fluorescence of mature leaves (Fig. 16 C).

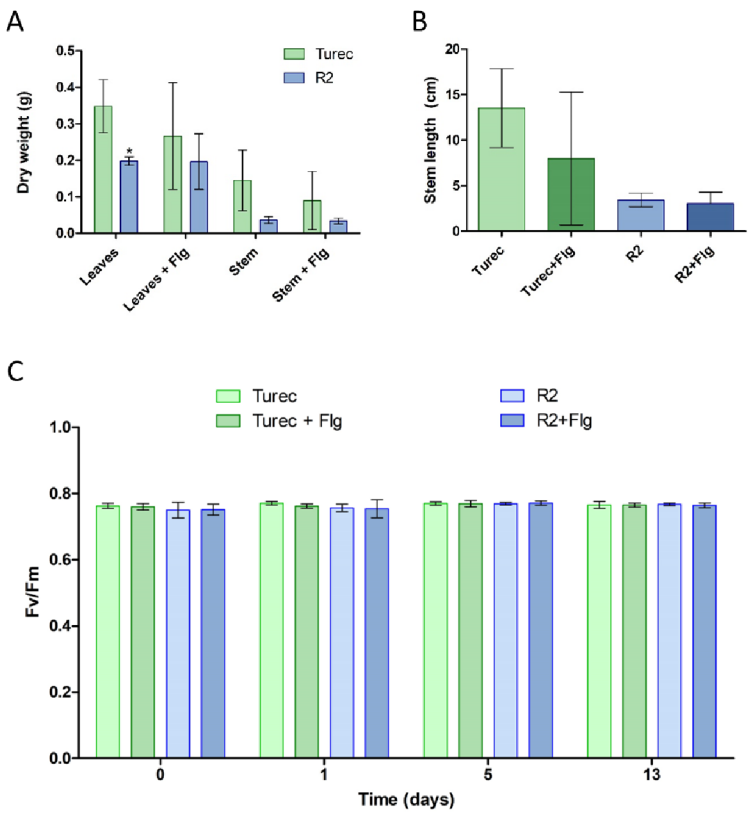

Fig. 16: Effect of flg22 treatment on the growth of Turec and R2. Leaves and stems were collected from 8-weeks-old plants. **A)** Dry weight of leaves and stems of the two cultivars. **B)** Lenght of freshly collected stems of the two cultivars. **C)** Chlorophyll fluorescence of mature leaves over the course of two weeks after flg22 treatment. Bars represent means and error bars represent SD from 6 replicates. Flg = Treatment with flg22. Statistical analysis was performed in GraphPad Prism 5 using t-test. Asterisks (*) indicate statistically significant difference.

5.1.4 EFFECT OF HUMIDITY

Humidity is an important factor for microbial disease development [158]. Because the plant infection with pathogens occurs under high humidity and reduced CO_2 availability (plants are enclosed in plastic boxes), we wanted to know the effects of high humidity on poppy growth phenotype and photosynthetic parameters.

Fig. 17: High huidity-caused granulation in the leaf.

First day after enclosing the plants in the plastic boxes, gutation of water from hydatodes was observed in the morning. Occassional water droplets on the leaves were observed due to reduced ability of the water to evaporate. After several days, senescent leaves of the plants seemingly filled with water, causing small dark granulation to appear all over the leaves

(Fig. 17). After 7 days in high humidity, leaves of the plants started to curl inwards along the axis of the main leaf vein and plants started to wilt (Fig. 18). Since no pathogen was introduced to the plants, the growth rate was not generally affected, however, the space-constrained conditions of the box caused the growing plants (especially the cultivar Turec) to curve their stems to fit inside in the second week of the experiment (Fig. 18).

Fig. 18: Wilting, leaf curling, and stem curving of cultivar Turec cultivated for 10 days in high humidity conditions.

Additionally, we compared the fluorescence values of plants in normal vs. high humidity (Fig. 19). We observed slight decrease in the Fv/Fm ratio of plants after three days in high humidity and significant decrease after 6 days (Fig. 19). We also analyzed the effect of flg22 treatment in low vs. high humidity (Fig 19), however, no significant difference in the effect was observed.

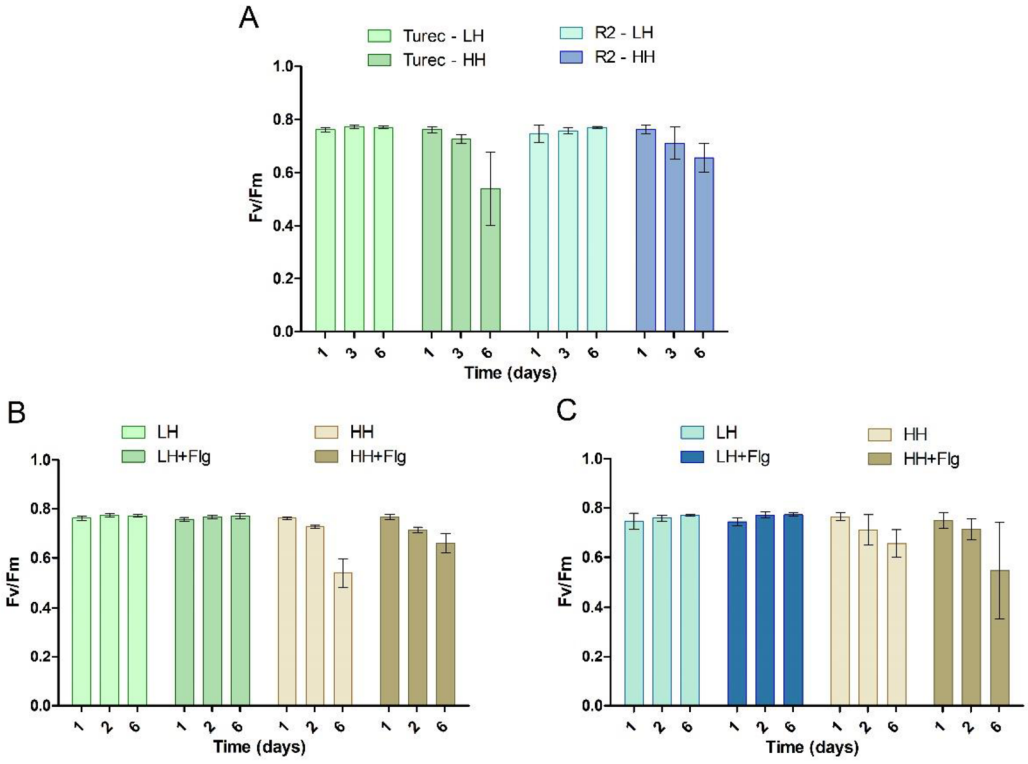

Fig. 19: Chlorophyll fluorescence measurements of mature leaves of 6-weeks-old plants taken over the period of one week. **A)** Comparison of Fv/Fm measurements of the two cultivars in low vs. high humidity conditions. **B)** Comparison of low vs high humidity with and without flagellin treatment effect on Fv/Fm measurement of Turec. **C)** Comparison of low vs high humidity with and without flagellin treatment effect on Fv/Fm measurement of R2. The bars represent means and error bars represent SD from 6 replicates.

5.2. INTERACTION OF POPPY CULTIVARS WITH PATHOGENS

Seeds of different poppy cultivars were obtained from OSEVA PRO s.r.o.. Our colleagues from OSEVA kindly provided us with data from field screening tests of resistance of these cultivars to three poppy diseases: downy mildew, helminthosporiosis, and bacteriosis. Based on this data, the cultivar "R2" seemed to be more resistant to pathogens than cultivar "Turec" (Fig. 20).

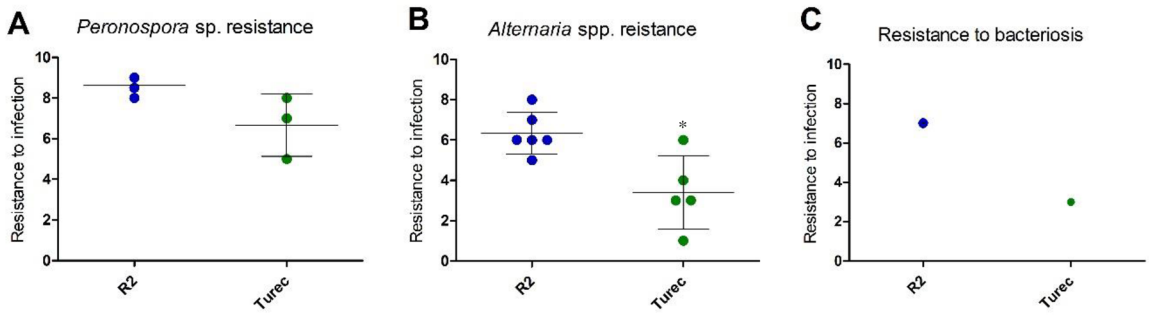

Fig. 20: Comparison of resistance of R2 and Turec cultivar based on available data from OSEVA. Resistance was measured on a scale of 1 (severely affected plants) to 9 (completely asymptomatic plants). **A)** Resistance of cultivars to *Peronospora* sp. Analysis of difference by t-test did not show statistical significance. **B)** Resistance of cultivars to Helminthosporiosis (*A. penicillata/A. papavericola*). Analysis by t-test showed statistically significant difference ($P<0.05$). **C)** Resistance of cultivars to Bacteriosis (only one datapoint available).

The work carried out in this project has been the first time we worked with poppy pathogens in our lab, and therefore pathosystems of the selected cultivars with pathogens needed to be established first. For this purpose, we tested three distinct pathogens in our growing conditions, one bacterial (*Xanthomonas papavericola,* the causal agent of bacterial leaf spot of poppy) and two fungal (*Alternaria* sp., the causal agent of helminthosporiosis, and *Botrytis cinerea*, the causal agent of grey mould).

5.2.1 ESTABLISHMENT OF THE PATHOSYSTEM WITH *XANTHOMONAS PAPAVERICOLA*

For establishment of *P. somniferum* - *X. papavericola* patosystem, we modified a protocol published previously by Van Hulten et al. 2019 [159]. From our previous experiences, we knew that poppy leaves are not easy to infiltrate with needleless syringae, a typical approach for tests of *Arabidopsis* resistance to bacteria [160]. We thus decided to employ a spraying method for poppy leaf infection instead. A suspension of bacterial culture in water supplied with a detergent (0.001% Tween) was sprayed on the entire aboveground part of the plant to facilitate movement of bacteria towards entry points (e.g. stomata, hydatodes). To establish an

infection protocol in our lab, we tested the infection progress under three inoculum concentrations (OD_{600} of 0.01, 0.05, and 0.2) and two distinct growing conditions after infection (a period in high or normal humidity). The plants were rated on the scale of 0 to 10, where 0 meant no signs of infection and 10 meant 100% of leaves infected (see section 4.5.1).

5.2.1.1 Infection of poppy cultivars with *X. papavericola*

First, we started with low inoculum of OD_{600} = 0.01 to monitor the progress of the infection in high humidity. We found this concentration to be insufficient as first symptoms of disease only occurred after two to three days (Fig. 21), and we therefore decided to try a higher concentration.

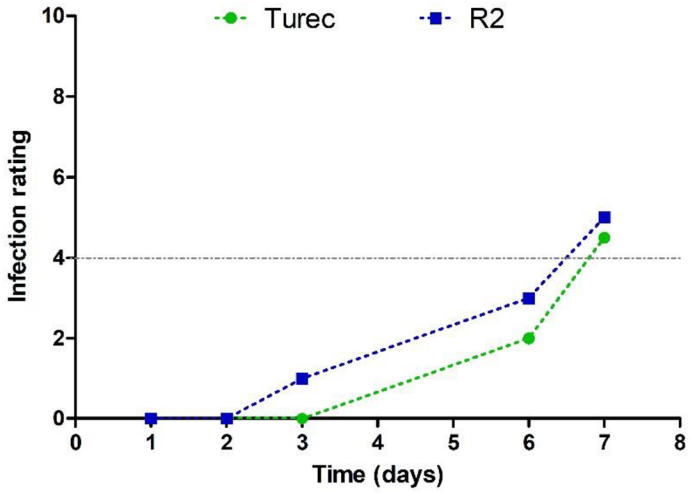

Fig. 21: Progress of infection of poppy cultivars with low inoculum (OD600 = 0.01) of *X. papavericola* bacterial suspension. Grey dashed line indicates infection rating of 4 out of 10 Data points represent means and error bars represent SD from 6 replicates.

In order to facilitate the infection of the plants by *X. papavericola*, we also investigated the effect of low vs. high humidity on the progress of infection. Plants were inoculated with *X. papavericola* inoculum of OD_{600} = 0.05 and placed in normal (LH) conditions and inside of plastic boxes (HH). We found the higher inoculum to cause faster infection as symptoms were already visible 1 day after inoculation (Fig. 22 A). We observed a favorable effect of low humidity on the infection of Turec, but cultivar R2 did not exhibit any differences between the two humidity conditions (Fig. 22 B). When comparing the two cultivars, the infection progressing slightly ($P<0,05$ on day 3) faster in Turec than R2 in low humidity (Fig. 22 C), while we observed no difference in infection rating in plants cultured in high humidity (Fig. 22 D).

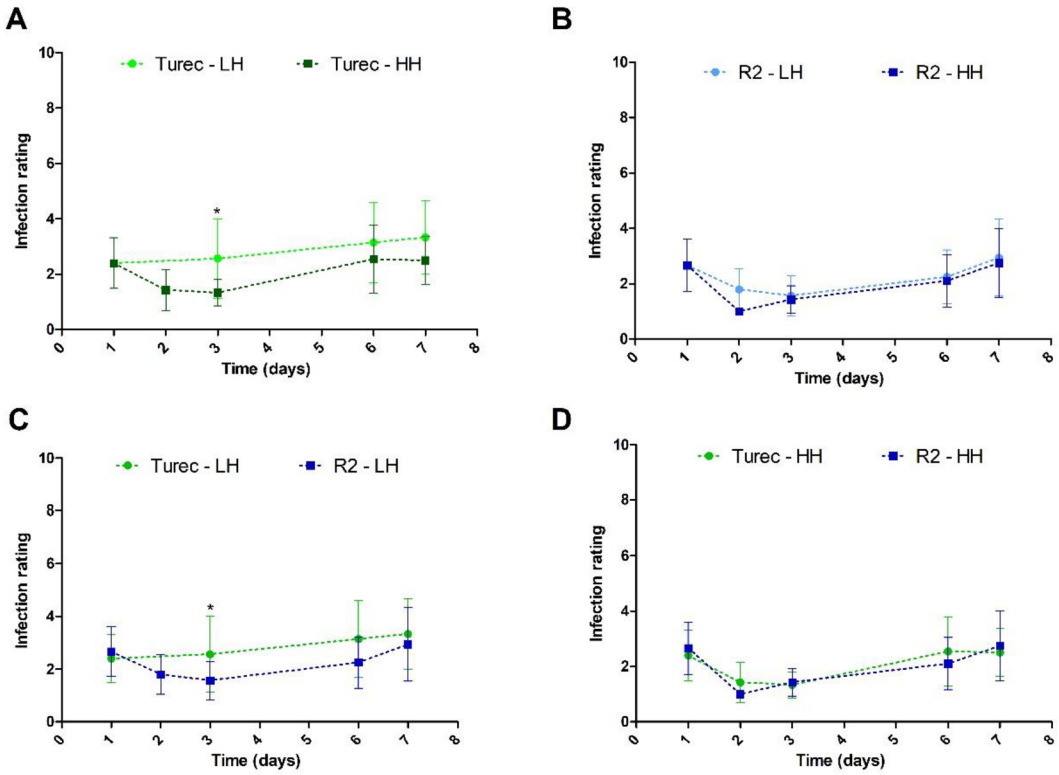

Fig. 22: Progress of infection of poppy cultivars with *X. papavericola* inoculum concentration of OD_{600} = 0.05. The infection was monitored in 6-weeks-old plant over the course of 1 week in low (LH) and high (HH) humidity conditions. **A)** The effect of humidity on the *X. papavericola* infection of Turec cultivar. **B)** The effect of humidity on the *X. papavericola* infection of R2 cultivar. **C)** Comparison of the infection progress in the two cultivars at low humidity. **D)** Comparison of the infection progress in the two cultivars at high humidity Data points represent means and error bars represent SD from 3 replicates. Statistical analysis was performed in GraphPad Prism 5. Asterisks (*) indicate statistically significant difference ($P<0.05$).

Finally, the highest inoculum concentration tested was OD_{600} = 0.2. Following the previous example, we tested the progress of infection in two humidity conditions. We did not observe any significant increase in disease severity after 7 days, although an increasing trend was observed. However, a significant difference was observed between low and high humidity in both cultivars (Fig. 23 A and B), with low humidity favoring higher infection ratings. Nevertheless, no difference was observed between the cultivars in the two conditions (Fig. 23 C and D).

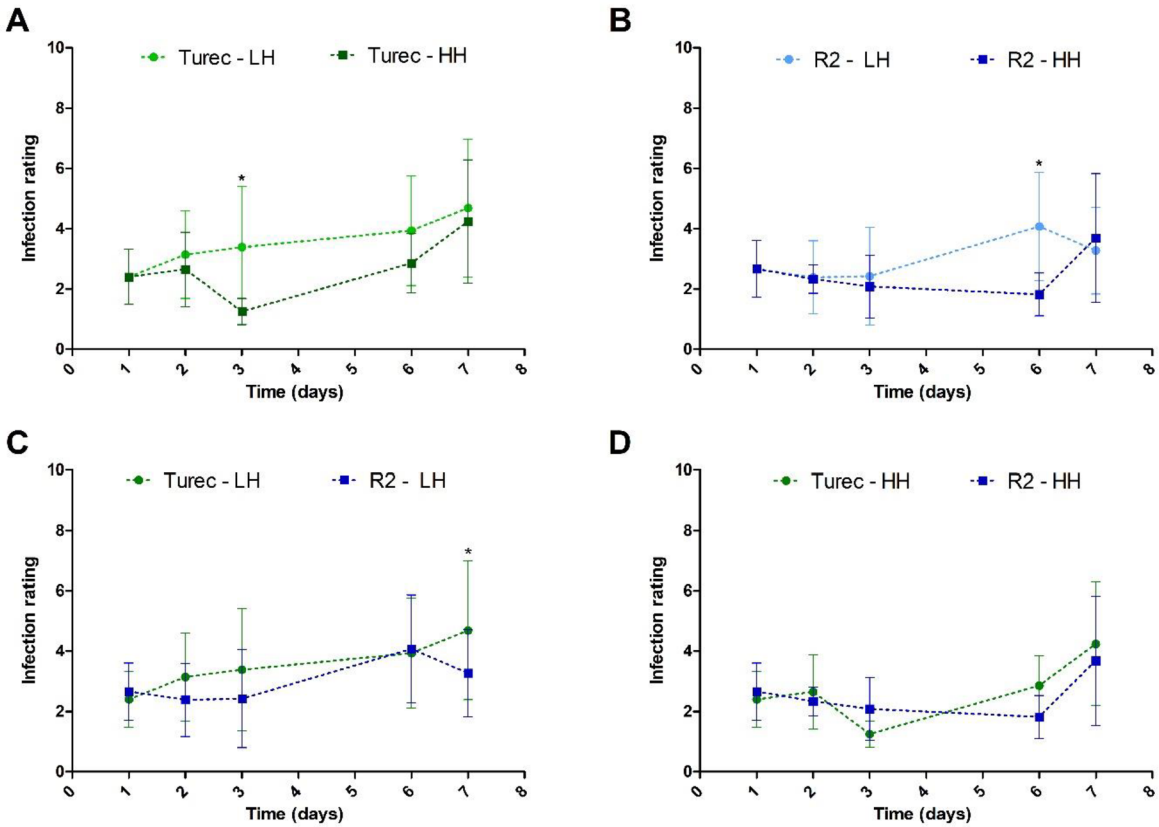

Fig. 23: Progress of infection of poppy cultivars with *X. papavericola* inoculum concentration of OD_{600} = 0.2. The infection was monitored in 6-weeks-old plant over the course of 1 week in low (LH) and high (HH) humidity conditions. **A)** The effect of humidity on the *X. papavericola* infection of Turec cultivar. **B)** The effect of humidity on the *X. papavericola* infection of R2 cultivar. **C)** Comparison of the infection progress in the two cultivars at low humidity. **D)** Comparison of the infection progress in the two cultivars at high humidity. Data points represent means and error bars represent SD from three replicates. Statistical analysis was performed in GraphPad Prism 5. Asterisks (*) indicate statistically significant difference ($P<0.05$).

5.2.1.2 Effect of flg22 on cultivar resistance to X. papavericola

Next, we investigated the effect of flg22 treatment on the resistance of the two cultivars towards *X. papavericola* infection. 6-weeks-old plants were pre-treated with flg22 and infected with low inoculum (OD_{600} = 0.01) of *X. papavericola* and cultivated in high humidity. No significant effect on the infection rate was observed in either cultivar (Fig. 24).

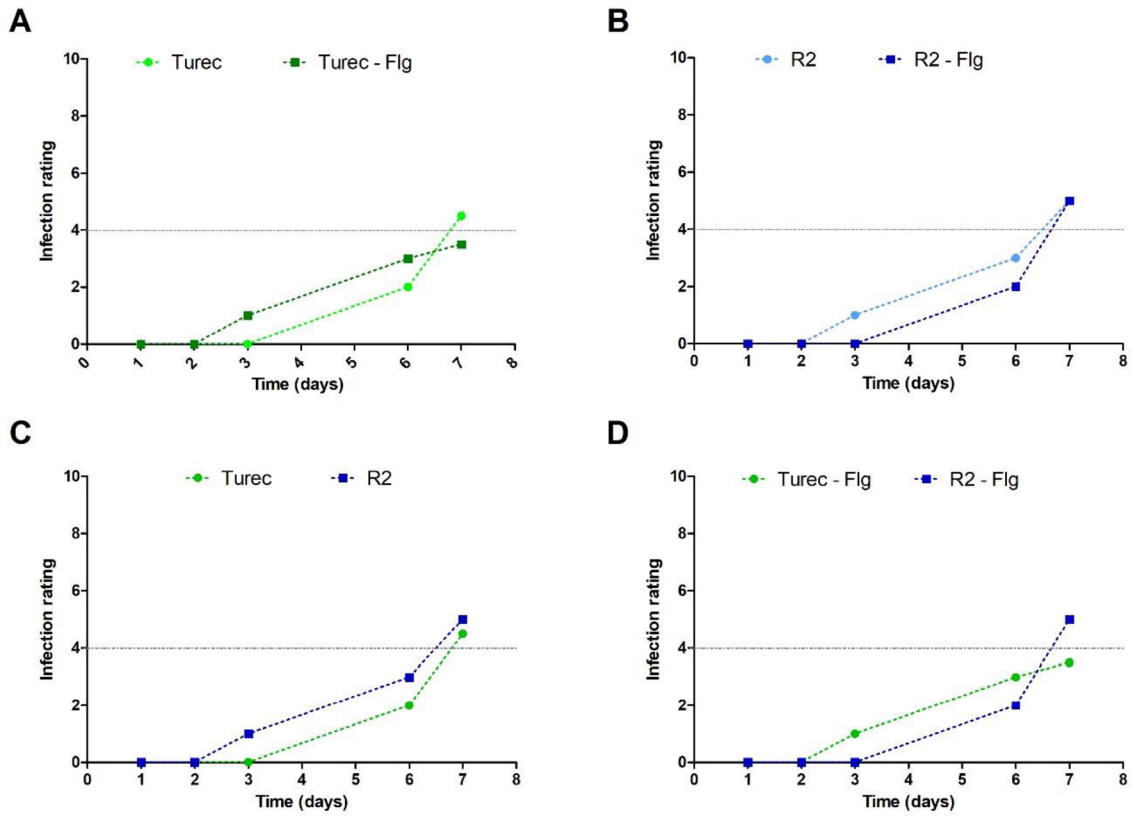

Fig. 24: Effect of flagellin (Flg) on the progress of infection of poppy cultivars with *X. papavericola* inoculum concentration of OD_{600} = 0.01. The infection was monitored in 6-weeks-old plant over the course of 1 week in high humidity conditions. **A)** The effect of flg22 on the *X. papavericola* infection of Turec cultivar. **B)** The effect of flg22 on the *X. papavericola* infection of R2 cultivar. **C)** Comparison of the infection progress in untreated cultivars at high humidity. **D)** Comparison of the infection progress in flg22-treated cultivars at high humidity. Data points represent means and error bars represent SD from Each treatment was performed in six replicates.

5.2.1.3 Effect of infection on photosynthetic capability of poppy

To monitor the plant's physiological state, we monitored chlorophyll fluorescence throughout the experiment in high humidity over the period of one week. We did not observe any effect of flagellin on the chlorophyll fluorescence of the plants (Fig. 25).

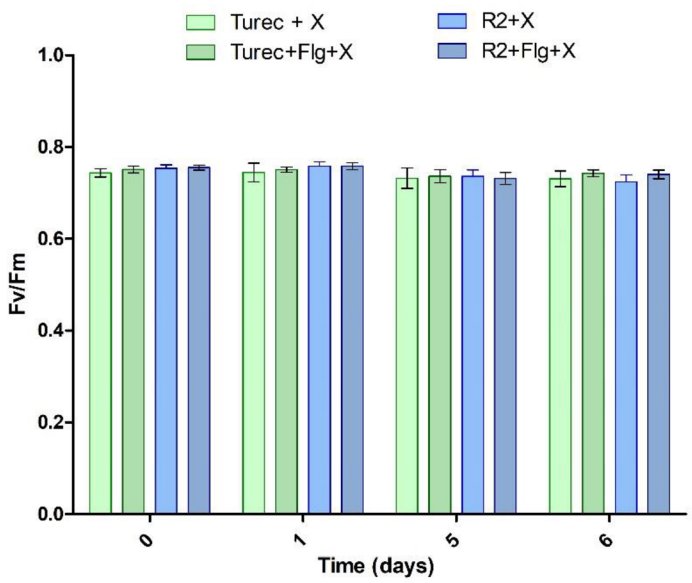

Fig. 25: Effect of flagellin (Flg) on the Fv/Fm measurement throughout the progress of infection of poppy cultivars with *X. papavericola* (X) inoculum concentration of OD_{600} = 0.01. The infection was monitored in 6-weeks-old plant over the course of 1 week in high humidity conditions. Bars represent means and error bars represent SD from. Statistical analysis was performed in GraphPad Prism 5 and showed no statistical difference among the measured values.

5.2.2 BOTRYTIS CINEREA

For the establishment of infection of the plants with *B. cinerea*, we used a modified method published by Valeri et al (2020) [161]. Plants were inoculated using two different concentrations of *B. cinerea* conidial suspensions, 10^5 conidia/mL and 10^6 conidia/mL, and were kept in low and high humidity for one week. The development of lesions on the infection sites was monitored over the course of 7 days.

As shown on Fig. 26, infection using 10^5 conidia/mL was unsuccessful in both high and low humidity conditions.

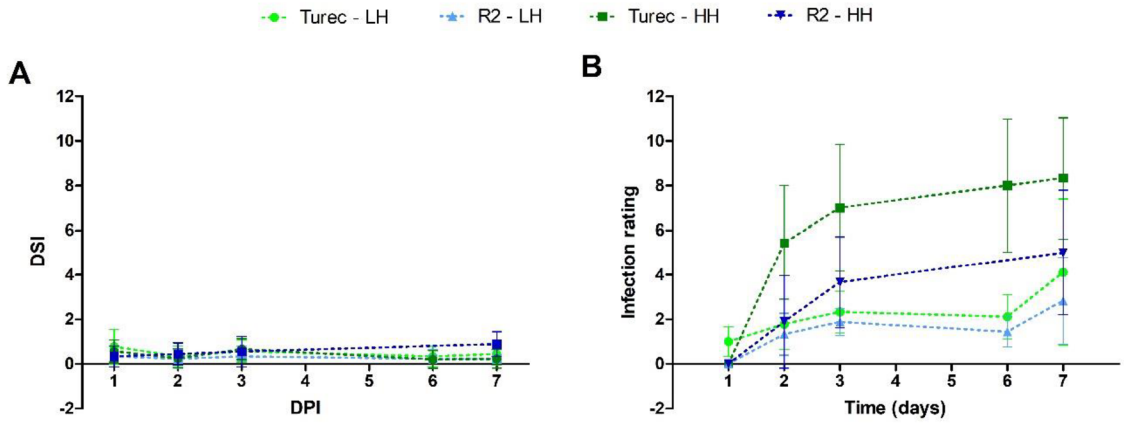

Fig. 26: Infection of 6-weeks-old poppy plants with *B. cinerea* conidial suspension. **A)** Infection with inoculum concentration of 10^5 conidia/mL. **B)** Infection with inoculum concentration of 10^6 conidia/mL. Each treatment was performed in triplicates.

However, we observed great results with the higher inoculum of 10^6 conidia/mL. The infection developed rapidly in both cultivars with both showing significantly higher rating in high humidity compared to low humidity conditions (Fig. 27 A and B). Interestingly, both cultivars showed almost exactly the same infection progress in low humidity (Fig. 27 C), but in high humidity, cultivar R2 was significantly more resistant than Turec (Fig. 27 D).

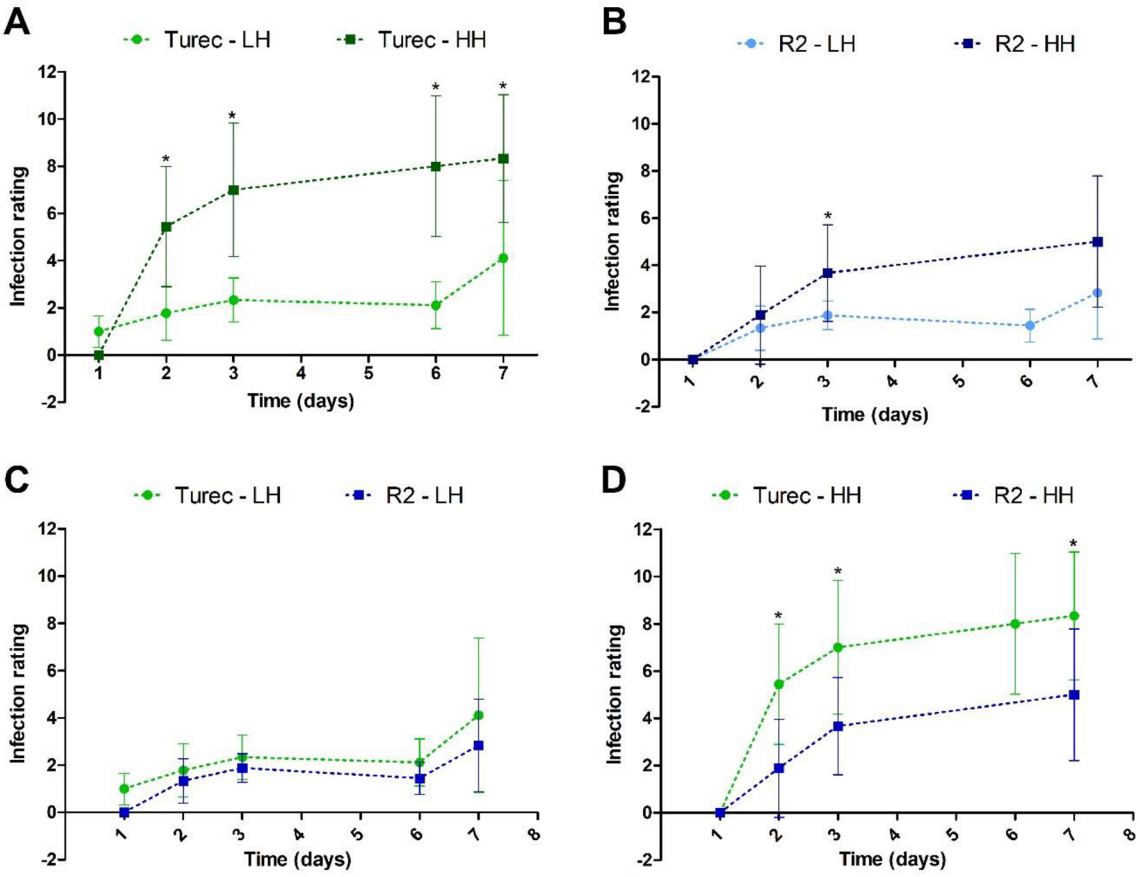

Fig. 27: Effect of low humidity (LH) and high (HL) humidity on the progress of infection of poppy cultivars with *B. cinera* inoculum concentration of 10^6 conidia/mL. The infection was monitored in 6-weeks-old plants over the course of 1 week. **A)** The effect of humidity on the *B. cinera* infection of Turec cultivar. **B)** The effect of humidity on the *B. cinera* infection of R2 cultivar. **C)** Comparison of the infection progress in *B. cinerea*-infected cultivars at low humidity. **D)** Comparison of the infection progress in *B. cinerea*-treated cultivars at high humidity. Data points represent means and error bars represent SD from three replicates. Statistical analysis was performed in GraphPad Prism 5. Asterisks (*) indicate statistiaclly significant differences.

5.2.3 *ALTERNARIA SP.*

5.2.3.1 Isolation of *Alternaria* sp. pathogen

Unlike *X. papavericola* and *B. cinerea* which were obtained from our colleagues or commercially purchased, *Alternaria* sp. had to be isolated from infected poppy leaf sample. The supposed *Alternaria* pathogen was collected by Dr. Hejna (as described in chapter 4),

grown on agar plate and selected based on morphological appearance when compared to photos from similar studies [162]. However, genetic sequencing is needed to ascertain the identity of the isolates.

Infection with *Alternaria* sp. pathogen was performed following the method established for *B. cinerea*. Plants were inoculated with 10^6 conidia/mL suspension and placed in low and high humidity conditions. Strong symptoms were observed immediately one day after inoculation. Interestingly, the effect of humidity seems to be opposite to that observed in *B. cinerea* infection. While Turec shows no statistically significant difference between the two humidity conditions with implied increasing trend in high humidity (Fig. 28 A), cultivar R2 exhibits clear, statistically significant increase of disease severity compared in low humidity compared to high humidity conditions (Fig. 28 B). In low humidity, cultivar R2 is significantly more susceptible to *Alternaria* pathogen than Turec (Fig. 28 C), while in high humidity, no statistically significant difference was observed (Fig. 28 D).

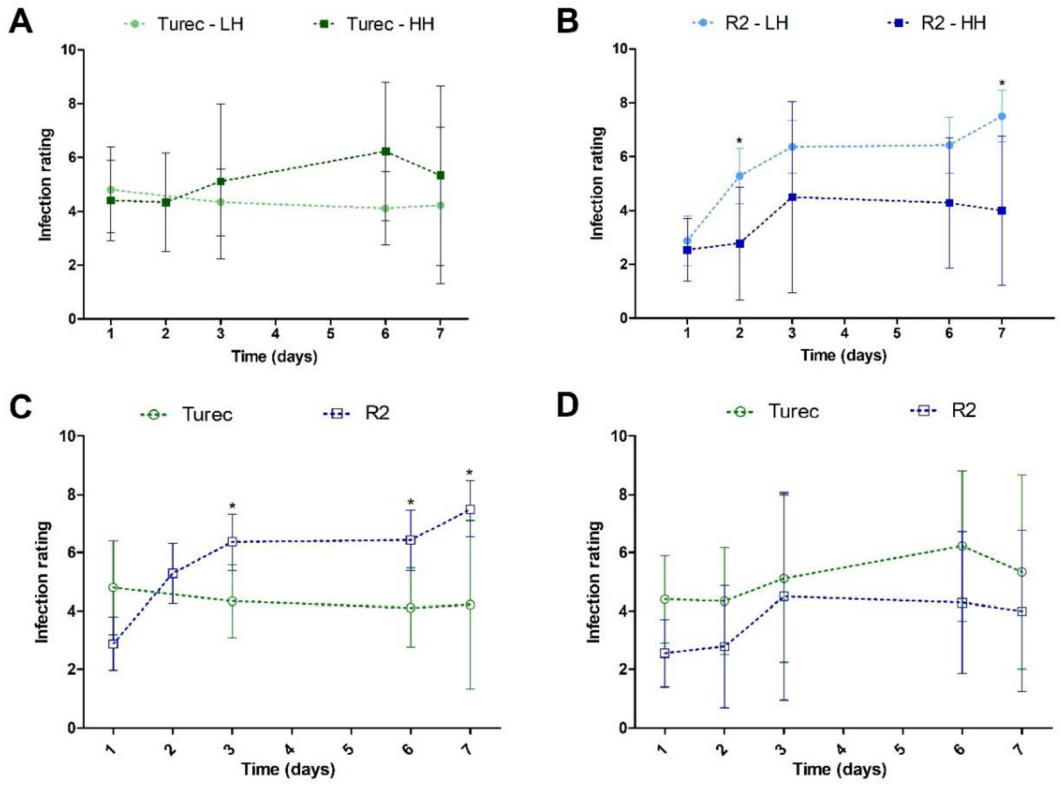

Fig. 28: Effect of low humidity (LH) and high (HL) humidity on the progress of infection of poppy cultivars with *Alternaria* sp. inoculum concentration of 10^6 conidia/mL. The infection was monitored in 6-weeks-old plants over the course of 1 week. **A)** The effect of humidity on the *Alternaria* sp. infection of Turec cultivar. **B)** The effect of humidity on the *Alternaria* sp. infection of R2 cultivar. **C)** Comparison of the infection progress in *Alternaria*-infected cultivars at low humidity. **D)** Comparison of the infection progress in *Alternaria*-treated cultivars at high humidity. Data points represent means and error bars represent SD from three replicates Statistical analysis was performed in GraphPad Prism 5. Asterisks (*) indicate statistiaclly significant differences.

6 DISCUSSION

Our laboratory is a relative new research group focused on the research of plant-microbial interactions. Until now, the predominantly used model plant in our research was *Arabidopsis thaliana.* However, my supervisor, decided to expand his work into the study of immunity of *Papaver somniferum* in order to identify possible better ways of its protection in the field. This thesis is part of the effort to establish the experimental procedures needed for the study of interactions between poppy and its pathogens in our laboratory.

Before this project, the study of poppy was initiated by a bachelor student, Natálie Hradecká. During her bachelor's thesis, a collaboration was established with Prof. Čurn and Dr. Hejna from the Faculty of Agriculture and Technology, University of South Bohemia, which enabled the cultivation of poppy plants for our experiments. This collaboration allowed us to start a cooperation with OSEVA PRO s.r.o., who are able to provide us with interesting poppy cultivars along with their data.

For this research, we obtained four different cultivars. Natálie Hradecká performed a series of initial experiments with flagellin treatment of the four cultivars (data in her bachelor thesis), which were used as a basis for selecting of the two cultivars used in this study, Turec and R2. Once the pathosystems and research tools developed in this study are properly established in our lab, the other two cultivars will be tested as well. The cultivars were provided to us based on the results of field experiments performed by OSEVA PRO s.r.o., in which they monitored the resistance of these cultivars towards microbial pathogen *Peronospora arborescens* and two microbial diseases, helminthosporiosis and bacteriosis.

We hope that our research can help to find the molecular mechanisms underlying the interactions of poppy with its most harmful pathogens and identify new, safe, environmentally friendly ways of poppy protection in the fields.

6.1. COMPARISON OF GROWTH, DEVELOPMENT, AND PHYSIOLOGICAL PARAMETERS BETWEEN CULTIVARS

We observed clear difference in the **growth rate differences** between the two selected poppy cultivars. Turec grew much faster than R2 (Fig. 11C), although the field data available from Dr. Rychlá and Dr Plachká from OSEVA PRO s.r.o. suggest that R2 tends to grow taller than Turec. The field data also suggest Turec develops in the early spring while R2 grows more in summer, and the difference in growth observed during the experiment could be therefore attributed to the time of year in which the experiments were performed regardless of the fact that the plants were grown in controlled conditions without access to outside light and

temperatures. Another possible explanation for the difference in growth speed, however, is a different content of phytohormones. Specifically, salicylic acid (SA) is the main phytohormone in plant defense signalling, and high SA-containing *A. thaliana* plants are dwarfed compared to wild type plants. Other than stunting the growth of the plant, SA confers higher resistance to pathogens to *A. thaliana* mutants [163]. Higher SA content in R2 could therefore be a possible explanation for slower development and higher disease resistance in R2. To explore this possibility, we established a collaboration with Dr. Kahoun and Dr. Fojtíková from the Department of Chemistry, Faculty of Sciences, South Bohemia University with whom we started developing an UHPLC-based method for salicylic acid detection in poppy leaves. Although we do not have any results yet, the method has been optimized in *A. thaliana* and is now in the process of implementation in poppy.

Interestingly we observed during the cultivation, even without any stress conditions, the **higher pigmentation** in R2 cultivar compared to Turec. Especially regarding the red coloration on abaxial side of the leaves. Red or purple color is generally associated with anthocyanin pigmets such as cyanidin (red), peonidin (purple), or malvidin (lilac) [164]. Anthocyanins are important plant flavonoids with potent antioxidant activity with role in protection against oxidative stress [164]. In 2017, Krošlák et al. studied the antioxidant activity of seed extracts of selected poppy genotypes and found increased content of antioxidants in blue-colored poppy seed extracts compared to poppies with white seeds, and proposed this fact could be related to higher susceptibility to staling of the seeds during storage [165]. Additionally, *A. thaliana* has been observed to accumalte anthocyanins in response to infection with pathogenic fungi [166], on the other hand anthocyanin production is inhibited by activated plant immunity in *A. thaliana* [167]. It seems reasonable to expect that higher production of flavonoids/antioxidants might be linked to higher resistance of blue-seed cultivar R2 towards pathogens. We have tried to establish a link between the red coloration and elicitation by flagellin, however, these efforts have been unsuccessful so far. However, flavonoids are generally produced in plants as protection against high-intensity and UV light, with their biosynthesis being activated upon exposure to UV light [168]. It is more probable that cultivar R2 produces these compounds due to light spectrum composition of the lighting used during the experiment, and experiments with different light spectra are needed to confirm this link. Likewise, an analytical approach is needed to link the red color to a certain phenolic compound. However, research of flavonoid content and their role in poppy immunity seems worthwile as higher antioxidant content can improve the nutritional and pharmaceutical values of plants [168].

Photosynthetic measurements using PAM is very easy and useful method analyzing essential plant physiological parameter. Chlorophyll *a* fluorescence, which is analysed by PAM, is important as an indicator of plant health under different experimental conditions and were crucial for interpretation of symptoms under high humidity conditions. To facilitate infection of the plants with pathogens, plants were enclosed inside plastic boxes, which restricted access to air and light availability for the plants. The comparison between plants grown under normal conditions and plants grown in boxes showed a sligt decrease in chlorophyll fluorescence in plants grown in boxes (roughly 0.78 dropped to 0.7, i.e., 10 % decrease) (Fig. 19). This rate was mantained for approximately 5 days during which the conditions were stable and plants did not show any major signs of damage by high humidity apart from dew drops forming at the hydatodes. This decrease in photosynthetic rate is likely to be caused by blocking of some light by the plastic lid rather than unsuitability of the conditions inside the boxes. Nevertheless, this should be further verified by measuring the light intensity and spectrum inside and outside the box to compare.

It is important to note that PAM only measures the fluorescence of chlorophyll *a* reflecting the capability of the photosynthetic apparatus to functions, not the entire rate of photosynthesis including input and output of gases etc. The ratio used in this study, Fv/Fm, is a ratio of fluorescence measured at a given timepoint compared to maximum fluorescence value, and it provides information about the photochemical efficiency of PSII [(169)]. It can be used indirectly to assess the overall health of a plant attacked by a pathogen, however, it does not provide enough information to be a diagnostic tool of infection. For example, in the case of *X. papavericola* infection of poppy leaves, the symptoms first start to develop on old yellow leaves and considerable symptoms can be seen without the photosynthetic ability of green mature leaves being affected.

The plant developmental stage affects its defense system activation [(170)], all plants should therefore be in the same stage of growth in order for the results of the experiments to be comparable. This has to be accounted for in future experiments as the two cultivars grow at different rates. We generally observed bolting in Turec cultivar around the 6th week after sowing and 8th week after sowing in R2. In future experiments, maximum 4 weeks old plants should be used. It could also be advantageous to test even younger plants for the similarity of responses to pathogen infection as using smaller and younger plants would help streamline the experiments.

As our group is focused on the study of plant immune reactions, once the growth conditions for poppy were established, we tested the effect of flg22 on poppy. Flg22 is a well-

known plant immunity elicitor which is frequently used for stimulation of bacteria-induced plant immune responses [114]. The initial data obtained by Ms. Hradecká showed that flg22 treatment of poppy plants leads to decreased biomass weight and elicitation of reactive oxygen species, an indication of plant immune response. The data obtained in this study further indicate that flagellin does not have an effect on the chlorophyll fluorescence of the plants (Fig.19). By the time of writing this thesis, we have tested the effect of flagellin pretreatment on the infection progress of *X. papavericola*, which is discussed in further detail in the next section. In the future, we plan to investigate its effects further as knowing the reactions of poppy to this bacterial elicitor is an important tool for the research of poppy immunity.

Another direction of plant immunity research employed in our laboratory by a Ph.D. student, Tereza Kalistová, is the study of plant cuticle and its role in plant immunity. This fact prompted us to examine the cuticle of our poppy cultivars. An apparent qualitative difference between the leaves of Turec and R2 cultivars was observed, and we have thus collected samples in collaboration with Tereza Kalistová who will perform analysis of these samples in the near future. To the best of our knowledge, cuticle and its role in immunity has not been extensively studied in poppy, and constitutes a point of interest for future work

6.2. POPPY-PATHOGEN INTERACTIONS

Since this project is constituted as the beginning of poppy immunity research in our lab, the most important goal of this study was the establishment of poppy pathosystems with some of the most important pathogens as tools for future research. Pathogens for these pathosystems were selected based on their importance in field cultivation in poppy, availability, and possibility of culturing in our lab. The chosen pathogens were *X. papavericola*, *Botrytis cinerea,* and a putative *Alternaria* sp. isolate. The most harmful pathogen, *Peronospora* sp., was not selected for this study due to its nature as a biotroph, which means it cannot be cultivated in a medium and needs live plants for propagation. As the cultivation conditions for poppy plants were not established yet, mantainance of biotrophic pathogens on live plants was not yet possible. However, upon further advancement of poppy research in our lab, we plan to investigate the interaction of poppy with *Peronospora* sp. as well.

The first pathogen, *X. campestris* pv. *papavericola,* was chosen as a representative bacterial species infecting opium poppy. Furthermore, *X. campestris* is a well-known bacterial plant pathogen infecting, for example, many *Brassica oleracea* species, and its modes of virulence and development of infection have been studied [171]. However, little information is available on the disease development and resistance in opium poppy. The *X. papavericola*

bacterial culture was obtained from microorganism culture collection of Masaryk University, Brno, as a clean culture.

In this study, poppy plants were inoculated with different concentrations of inoculum using spray bottle. Originally, infiltration method was tried by Ms. Hradecká, however, this method did not work and an alternative approach was chosen based on literature [171].Over the course of two weeks, plants were rated based on the percentage of leaves showing signs of symptoms. *X. campestris* has two stages, biotrophic and necrotrophic, and during the biotrophic stage, the leaves are generally symptomless [172]. Photosynthesis measurements were obtained in paralell with disease severity ratings, and both infection indicators showed the same trend of infection development. Since the initial stages of infection can be without symptoms and therefore cannot be seen and adequately rated, photosynthesis measurements seem like a good alternative approach. Unfortunately, in this experiment, PAM measurements did not show any significant advantage as a disease indicator over direct symptom rating, and differences in values could be largely attributed to differences in light availability and humidity. In advanced stage of the infection, fluorescence values did start to drop as the health of the plants deteriorated, however, symptom ratings stand superior as a disease detection method since it can more accurately detect small symptoms that might not necessarilly affect the photosynthetic activity yet. Additionally, plant grown in high humidity inside the plastic boxes can be affected by the stressful environment, leading to leaf yellowing and consequently lower PAM values unrelated to pathogen infection.

This study did not identify any significant differences in resistance to the pathogen between the two studied cultivars. Now that better scoing system is developed, more experiments with larger amount of replicates is needed to corroborate the minor differences observed and determine their statistical significance. Although the difference is small, low humidity seems to be a better or at least equally suitable condition for the development of the disease than high humidity. Since low humidity removes a significant amount of stress posed on the plants, performing future experiments in low humidity conditions seems to be the better option. There is a clear effect of the amount of starting inoculum on the development of the disease and the speed of its progression.

Additionally, we investigated the effect of flg22 pretreatment on the progress of infection of *X. papavericola*. We did not find any significant effect, however, we plan to repeat the experiment with higher inoculum and larger amount of replicates. The results were affected by imperfect disease scoring system, which was not fully developed at the time of the experiment, making it impossible for us to perform any statistics on the obtained data. But we are

aware of it and we will work on improvement on the evaluation of bacterial infection in next experiments.

The next pathosystem was *P. somniferum – Botrytis cinerea*. Fungal culture of *B. cinera* was provided by our collaborators from the group of prof. Čurn. In the case of *B. cinera* infection, high humidity had a clear effect on the resistance of the two cultivars and showed much larger difference (Fig. 27). The available data on the resistance of the cultivars towards pathogens suggested a slight difference between the two cultivars, with cultivar Turec being more sensitive towards diseases than cultivar R2 (data from OSEVA). Although increased resistance of R2 towards helminthosporiosis and bacteriosis was not confirmed in this study, infection of the two cultivars with *B. cinerea* did show a significant difference in their response. Cultivar R2 was indeed more resistant and showed less severe symptoms than cultivar Turec, especially under high humidity conditions (Fig. 27). Nevertheless, more data is necessary to support this observation.

B. cinerea is the only pathogen used in this study which is not a specialized pathogen of poppy. Both *X. papavericola* and the putative *Alternaria* sp. are isolates collected from diseased poppy plants, whereas *B. cinerea* is a general pathogen with broad host spectrum. It is possible that *B. cinerea* infection is met with more general response and revealing a more "fundamental" difference between the cultivars, while the two other pathogens are able to subvert poppy immunity with more specialized systems, effecting more complicated response with less obvious differences. Resistance to *B. cinerea* has been linked to higher SA content in tomato and SA is crucial for flg22-triggered immunity response in *Arabidopsis*. On the other hand, SA-insensitive and -deficient *A. thaliana* mutants did not show higher susceptibility to *B. cinerea* (173). The possible link between higher resistance to *B. cinerea* and higher SA content in poppy cultivar R2 is an exciting direction to undertake in future experiments.

The last pathosystem established focused on a devastating poppy pathogen formerly known as *Pleospora papaveracea,* which was later renamed *Crivellia papaveracea* and most recently reclassified into two closely related fungal species, *Alternaria penicillata* and *Alternaria papavericola*, both of which are capable of infecting poppy. These strains were not available on the market, and thus we used infected leaves from Dr. Hejna and attempted isolation of environmental *Alternaria* sample. The putative *Alternatia* strain was isolated from poppy leaf infected with unidentified disease collected in the field. Antibacterial antibiotics have been used to select for fungi and eliminate bacterial contamination. Since *Alternaria* sp. is the most prevalent fungal disease of poppy in the fields [(40)], an isolate that repeatedly ap-

peared on multiple plates infected with different infected leaves exhibiting the same morphology each time (a white foam-like mycelium) was selected as a good candidate for a pathogen infecting poppy (Fig. 10). Nevertheless, we cannot prove for a 100 % the identity of the isolate with certainty at this point. In order to identify the pathogen, ITS primers for fungal genotyping as described by Martin et al. (2005) [174] have been purchased and sequencing of the pathogen is planned as the next step. Since there is a chance the selected isolate is not the desired *Alternaria* sp., all other fungal pathogens observed on the plates were isolated and preserved as glycerol stocks to act as a backup.

Infection with *Alternaria* isolate was performed in teh same way as *B. cinera* and displayed largely similar symptoms. Unlike *B. cinerea* however, white mycelium could be seen on more severe lesions on adaxial side of the leaves. A significant difference in resistance towards this pathogen was observed under low humidity conditions, in which cultivar R2 proved much more susceptible to the disease than Turec (Fig. 27). This is a rather surprising discovery, as field data obtained from OSEVA PRO s.r.o. suggested an opposite trend. Further research is needed to corroborate this observation with more data.

The most surprising observation in *Alternaria* infection was the completely opposite effect of high humidity on the disease development in cultivar R2 compared to Turec. This effect is completely opposed to the results obtained in *B. cinera* infection, in which high humidity had a positive effect on the development of the disease. Since this finding goes against our expectations, we want to repeat the experiment with larger sample size to eliminate the possibility of data misinterpretation. Nevertheless, an effective method of pathogen cultivation as well as infection procedure and assessment has been established.

7. CONCLUSION

- Cultivation, growth, and physiological parameters of two poppy cultivars Turec and R2 were established.
- Three pathosystems of *P. somniferum* with *X. papavericola, B. cinerea*, and a putative *Alternaria sp*. isolate were established and tools for infection severity evaluation were developed.
- Cultivar Turec has more rapid growth than cultivar R2 under our growth conditions.
- No differences were observed in resistance to *X. campestris* between Turec and R2 cultivars.
- Flg22 treatment did not have any effect on *X. campestris* infection rate and on chlorophyll fluorescens.
- Turec is more susceptible to *B. cinerea* under high humidity conditions.
- R2 is more susceptible to *Alternaria sp.* under low humidity conditions

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