Germination and differentiation of *Blumeria graminis* ascospores and effects of UV-C and white light irradiation on *B. graminis* conidial prepenetration

Keimung und Differenzierung von *Blumeria graminis* Ascosporen und Effekte von UV-C und Weißlichtbestrahlung auf die Konidienpräpenetration von *B. graminis*

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Summary

Blumeria graminis, the obligate biotrophic grass powdery mildew, is a highly pathogenic fungus capable of inflicting foliar diseases and of causing severe yield losses. There is asexual and sexual propagation in the life cycle of *B. graminis*. In the epidemiological processes of this pathogen, both types of spores - asexual conidia and sexual ascospores – are crucial.

Conidia of *B. graminis* are demonstrated to perceive cuticular very-long-chain aldehydes as molecular signal substances notably promoting germination and differentiation of the infection structure (the appressorium) – the prepenetration processes – in a concentration- and chain-length-dependent manner. Conidial germination and appressorium formation are known to be dramatically impeded by the presence of free water on the host surface. However, sexually formed ascospores are reported to easily germinate immersed in water. There are abundant assays on conidial prepenetration processes. However, with respect to the stimulating effects of very-long-chain aldehydes and to the influence of the presence of free water, ascosporic prepenetration processes are still obscure.

In order to study the effects of very-long-chain aldehydes on the ascosporic prepenetration processes of wheat powdery mildew fungus *B. graminis* f. sp. *tritici*, Formvar®-based *in vitro* systems were applied to exclude the secondary host effects (such as host resistance) and to reproducibly provide homogeneous hydrophobic substratum surfaces. By the presence of even-numbered very-long-chain aldehydes (C_{22} - C_{30}), the appressorium formation of the ascospores was notably triggered in a chain-length dependent manner. *N*-octacosanal (C_{28}) was the most inducing aldehyde tested. Unlike conidia, ascospores could easily differentiate immersed in water and showed a more variable differentiation pattern even with a single germ tube differentiating an appressorium.

To evaluate the alternative management against barley powdery mildew fungus *Blumeria graminis* f. sp. *hordei*, the suppressing effects of UV-C irradiation on the developmental processes of conidia on artificial surfaces (*in vitro*) and on host leaf surfaces (*in vivo*) were assayed. *In vitro* and *in vivo*, a single dose of 100 J m^{-2} UV-C was adequate to decrease conidial germination to < 20 % and to reduce appressorium formation to values < 5 %. UV-C irradiation negatively affected colony pustule size and vegetative propagation. Under photoperiodic conditions of 2h light/16h dark, 6h dark/12h light or 6h dark/18h light, UV-C-treated conidia showed photoreactivation (photo-recovery). White light-mediated photoreactivation was most effective immediately after UV-C irradiation, suggesting that a prolonged phase of darkness after UV-C application increased the efficacy of management against *B. graminis*. UV-C irradiation increased transcript levels of three putative photolyase genes in *B. graminis*, indicating those were probably involved in photoreactivation processes.

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However, mere white light or blue light (wavelength peak, 475 nm) could not induce the up-regulation of these genes.

To determine whether visible light directly impacted the prepenetration and penetration processes of this powdery mildew pathogen, conidia of *Blumeria graminis* f. sp. *hordei* and *Blumeria graminis* f. sp. *tritici* were inoculated onto artificial surfaces and on host leaf surfaces. Samples were analyzed after incubation periods under light conditions (white light intensity and spectral quality). Increasing white light intensities directly impaired conidial prepenetration processes *in vitro* but not *in vivo*. Applying an agar layer under the wax membrane compensated for conidial water loss as a consequence of high white light irradiation. Light stimulated *in vitro* and *in vivo* the appressorium elongation of *B. graminis* in a wavelength-dependent manner. Red light was more effective to trigger the elongation of appressorium than blue light or green light assayed.

Taken together, the findings of this study demonstrate that 1) a host surface recognition principle based on cuticular very-long-chain aldehydes is a common feature of *B. graminis* f. sp. *tritici* ascospores and conidia; 2) the transcriptional changes of three putative photolyase genes in *B. graminis* are mediated in a UV-C-dependent manner; 3) light directly affected the (pre)penetration processes of *B. graminis.*

Zusammenfassung

Blumeria graminis, der obligate biotrophe Grasmehltau, ist ein hochpathogener Pilz, der in der Lage ist, Blätter zu schädigen und in Getreidekulturen schwere Ertragsverluste zu verursachen. Im Lebenszyklus von *B. graminis* kommen sowohl sexuelle als auch asexuelle Reproduktion vor. In den epidemiologischen Prozessen dieses Erregers sind beide Arten von Sporen - asexuelle Konidien und sexuelle Ascosporen - von entscheidender Bedeutung.

Conidien von *B. graminis* reagieren nachweislich auf kutikuläre, sehr langkettige Aldehyde als molekulare Signalstoffe, die insbesondere die Keimung und Differenzierung der Infektionsstruktur (das Appressorium) - die Präpenetrationsprozesse - konzentrations- und kettenlängenabhängig fördern. Es ist bekannt, dass die Konidienkeimung und Appressoriumbildung durch die Anwesenheit von freiem Wasser auf der Wirtsoberfläche dramatisch behindert wird, während sexuell gebildete Ascosporen unter diesen Bedingungen keimen. Es gibt zahlreiche Untersuchungen zu Konidien-Präpenetrationsprozessen. Im Hinblick auf die stimulierende Wirkung von langkettigen Aldehyden und den Einfluss von freiem Wasser sind die askosporischen Präpenetrationsprozesse jedoch noch unklar.

Um die Effekte langkettiger Aldehydmoleküle auf die ascosporischen Präpenetrationsprozesse von Weizenmehltaupilz zu untersuchen, wurde *B. graminis* f. sp. *tritici* auf Formvar®-basierten *In-vitro*-Systemen angewendet. Dadurch konnten sekundäre Wirtseffekte (wie Wirtsresistenz) ausgeschlossen und homogene hydrophobe Substratoberflächen reproduzierbar bereitgestellt werden. Die Anwesenheit geradzahliger sehr langkettiger Aldehyde (C_{22} – C_{30}) erhöhte deutlich die Ausbilung des Appressoriums von Ascosporen. Von den getesteten Aldehyden zeigte *n*-Octacosanal (C_{28}) die größte Induktionskraft. Im Gegensatz zu Konidien konnten bei Ascosporen auch unteri Anwesenheit von freiem Wasser variable Differenzierungsmuster festgestellt werden, bei denen auch einzelne Keimröhren zur Differenzierung des Appressoriums führten.

Zur Bewertung der alternativen Bekämpfung gegen Gerstenmehltaupilz *Blumeria graminis* f. sp. *hordei* wurde die Unterdrückungswirkung von UV-C-Strahlung auf die Entwicklungsprozesse von Konidien auf künstlichen Oberflächen (*in vitro*) und auf Wirtsblattoberflächen (*in vivo*) untersucht. *In vitro* und *in vivo* war eine Einzeldosis von 100 J m^{-2} UV-C ausreichend, um die Konidienkeimung auf < 20 % zu verringern und die Appressoriumsbildung auf Werte < 5 % zu reduzieren. Die UV-C-Bestrahlung beeinflusste die Größe der Kolonepusteln und die vegetative Vermehrung negativ. UV-C behandelte Konidien wurden unterschiedlichen Lichtverhältnissen ausgesetzt: 2 h hell / 16 h dunkel, 6 h dunkel / 12 h hell und 6 h dunkel / 18 h hell. Dabei zeigten sie unterschiedliche Photoreaktivierungsaktivitäten. Die Weißlicht-vermittelte Photoreaktivierung war unmittelbar

Zusammenfassung

nach der UV-C-Behandlung am effektivsten, was darauf hindeutet, dass eine verlängerte Phase der Dunkelheit nach der UV-C-Anwendung die Wirksamkeit des Managements gegen *B. graminis* erhöhte. UV-C-Bestrahlung erhöhte die Transkriptmengen von drei mutmaßlichen Photolyase-Genen in *B. graminis*, was darauf hindeutet, dass sie wahrscheinlich an Photoreaktivierungsprozessen beteiligt waren. Weißes oder blaues Licht (Wellenlängen-Peak, 475 nm) konnte die Hochregulation dieser Gene nicht induzieren.

Um zu bestimmen, ob sichtbares Licht direkt auf die Präpenetrations- und Penetrationsvorgänge dieses Mehltau-Erregers einwirkt, wurden Konidien von *Blumeria graminis* f. sp. *hordei* und *Blumeria graminis* f. sp. *tritici* auf künstliche Oberflächen und auf Wirtsblattoberflächen inokuliert. Die Proben wurden nach Inkubationunter verschiedenen Lichtbedingungen (Weißlichtintensität und Spektralqualität) analysiert. Das Auftragen einer Agarschicht unter der Wachsmembran kompensierte den Wasserverlust der Konidien als Folge der Bestrahlung mit hoher Lichtintensität. Zunehmende Weißlichtintensitäten beeinträchtigten Konidienpräpenetrationsprozesse direkt *in vitro*, aber nicht *in vivo*. Eine wellenlängenabhängige Stimulation der Verlängerung des Appressoriums konnte sowohl *in vitro* als auch *in vivo* beobachtet werden. Dabei war rotes Licht wirksamer, um die Verlängerung des Appressoriums auszulösen, als blaues oder grünes Licht.

Zusammengefasst zeigen die Ergebnisse dieser Studie, dass 1) ein Prinzip der Wirtsoberflächenerkennung basierend auf kutikulären, sehr langkettigen Aldehyden ein gemeinsames Merkmal von *B. graminis* f sp. *tritici* Ascosporen und Konidien ist; 2) die Transkriptionsänderungen von drei putativen Photolyase-Genen in *B. graminis* in einer UV-C-abhängigen Weise vermittelt werden und 3) Licht direkt die (Vor-) Penetrationsvorgänge von *B. graminis* beeinflusste.

1 Introduction

1.1 The plant cuticle

Plants are exposed to a variety of biotic and abiotic stressful elements. The interface between plant and its environmental stress factors is cuticle, a continuous membrane covers outer walls of the epidermal cell of petals, green stems, fruits and leaves (Schuster *et al.*, 2017). The plant cuticle is a multi-functionary layer, which constitutes of barriers to protect against water loss, plant herbivores, ultra-violet radiation and surrounding microorganisms (Riederer & Schreiber, 2001; Müller & Riederer, 2005; Dominguez *et al.*, 2011). The most crucial eco-physiological function of the plant cuticle is to establish an efficacious barrier against uncontrolled nonstomatal water loss from inner tissues under varying environmental conditions (Schuster *et al.*, 2017). This pivotal function is considered to be an indispensable requirement for the colonization of land by terrestrial plants (Kerstiens, 1996; Burghardt *et al.*, 2006; Buda *et al.*, 2013; Renault *et al.*, 2017).

As a barrier, the cuticle efficiently supports the structural integrity of plant organs and directly protects plants against biotic invasions. The physical and chemical properties of this initial contact surface can be sensed and recognized by phyllosphere microorganisms, including non-pathogenic and pathogenic fungi (Zabka *et al.*, 2008; Ringelmann *et al.*, 2009; Hansjakob *et al.*, 2010; Weidenbach *et al.*, 2014; Kou & Naqvi, 2016; Zeilinger *et al.*, 2016; Aragón *et al.*, 2017). In many cases, pathogenic fungi need to complete pre-invasion stages in order to infect hosts. The surface sensing process of fungal pathogens is considered to be one of the most crucial phases for fungal development and for plant-fungus interactions (Aragón *et al.*, 2017).

1.2 Surface sensing of plant pathogenic fungi

The interaction between plants and pathogenic fungi begins when fungal pathogens initially contact with the plant surface – the cuticle (Kou & Naqvi, 2016). In most cases, fungal spores can sense and respond to the appropriate physio-chemical cues of plant cuticle to germinate, develop and differentiate infection structures. The plant cuticle structurally composes of a polymer matrix (cutin) and cuticular waxes, which are classified into intracuticular waxes that are embedded in the cutin matrix and epicuticular waxes (Jetter *et al.*, 2000). The epicuticular waxes, which coat the plant surface and interact with pathogens, predominantly consist of fatty acids, alkanes, alcohols, esters and very-long-chain aldehydes ($> C_{18}$) (Jetter *et al.*, 2000). As common and ubiquitous wax components, so far very-long-chain aldehydes can be found in most of the cuticular plant waxes analyzed (Jetter *et al.*, 2007). Very-long-chain aldehydes are

crucial signals for the development of spores of plant pathogenic fungi (Kou & Naqvi, 2016; Aragón *et al.*, 2017). It was shown that C_{28} aldehyde *n*-octacosanal was involved in the appressorium formation of uredospores of the wheat stem rust fungus - *Puccinia graminis* (Reisige *et al.*, 2006). It was reported that very-long-chain aldehydes were capable of notably promoting the conidial development of the crop powdery mildew fungi - *Blumeria graminis* (Tsuba *et al.*, 2002; Zabka *et al.*, 2008; Ringelmann *et al.*, 2009; Hansjakob *et al.*, 2010; Hansjakob *et al.*, 2011) and - *Erysiphe cruciferarum* (Weis *et al.*, 2014).

In addition, the development (such as germination and the formation of infection structure) of fungal pathogens is stimulated by surface hydrophobicity (Kou & Naqvi, 2016). It was shown that the processes of filament formation and infection structure growth of the corn smut fungus - *Ustilago maydis* - were promoted by plant surface hydrophobicity (Mendoza-Mendoza *et al.*, 2009). Surface hydrophobicity induced the germination of spores of the anthracnose fungus – *Colletotrichum graminicola* (Chaky *et al.*, 2001). In a very recent study, it was illustrated that surface hydrophobicity plays an essential role in regulating prepenetration phases of the pear black spot fungus - *Alternaria alternata* (Tang *et al.*, 2017). In previous studies, it was demonstrated that conidial germination and appressorium differentiation of *B. graminis* were related to surface hydrophobicity (Zabka *et al.*, 2008; Ringelmann *et al.*, 2009; Hansjakob *et al.*, 2010). However, it was suggested that chemical signals combined with the surface hydrophobicity were comparatively more effective to stimulate the development of fungal pathogens (Francis *et al.*, 1996; Zabka *et al.*, 2008; Mendoza-Mendoza *et al.*, 2009; Hansjakob *et al.*, 2010; Lanver *et al.*, 2014).

1.3 The fungal pathogen - *Blumeria graminis*

The powdery mildew fungus – *Blumeria graminis* – is an obligate biotroph, one of the most frequently encountered plant pathogens in the worldwide (Dean *et al.*, 2012). *B. graminis* belongs to Ascomycota of the order the Erysiphales, and it is a widespread causative foliar disease (Kuhn *et al.*, 2016). This destructive phyllosphere plant disease invades hosts and produces propagation structures (i.e. conidiophores and conidia), which show easily visual and recognizable aspects as white powdery pustules (**Figure 1.1**). Based on strict host specialization (host genus), *B. graminis* was classified into eight formae speciales (ff. spp.): *hordei, tritici, avenae, secalis* and four ff.spp. on wild grasses (Marchal, 1902; Oku *et al.*, 1985; Wyand & Brown, 2003). *B. graminis* f. sp. *hordei* (Bgh) and *B. graminis* f. sp. *tritici* (Bgt) respectively infest the host plants - barley (*Hordeum vulgare*) and wheat (*Triticum aestivum*), two of the most essential food crops in the world. The infection of *B. graminis* directly causes serious qualitative and quantitative yield losses (Murray & Brennan, 2010).

Figure 1.1 *Blumeria graminis* on host leaf surface. (A) "White powdery" pustules of *B. graminis.* (B) A colony of *B. graminis.* (C) Conidia chains of *B. graminis.* Scale bars, 100 μm.

Like many biotrophic parasites, *B. graminis* requires an appropriate living plant tissue to finish sexual and asexual propagation processes by establishing colonies without causing host cell death. Therefore, it cannot be preserved on the pure artificial culture/media under sterile conditions. Although in many studies, *B. graminis* has been inoculated and grown on detached host leaves, there is still no method of routinely cultivating it on artificial surfaces nowadays. Unlike other common model fungi of Ascomycota (i.e. *Neurospora crassa* (Raju, 2009) and *Aspergillus nidulans* (Etxebeste *et al.*, 2010)), there is lack of stable transformation techniques/protocols of *B. graminis.* There is no mutant strain, which is a prerequisite of analyzing the impacts and functions of single genes in *B. graminis*. There is the restriction of studying comprehensively biological questions of this powdery mildew fungus.

Nevertheless, because of the biological characters, *B. graminis* has been regarded as one of the most common and popular models to study the cellular- and molecular-level interactions between plants and obligate biotrophic pathogens (Glawe, 2008; Hückelhoven & Panstruga, 2011). Several years ago, the genomes of Bgh (Spanu *et al.*, 2010) and Bgt (Brunner *et al.*, 2012; Wicker *et al.*, 2013) were sequenced, annotated and published. The approximate genome sizes of *B. graminis* are about 120 Mb, which are slightly smaller than the genome sizes of the pea (*Pisum sativum*) powdery mildew fungus - *Erysiphe pisi* (ca. 151 Mb) and the *Arabidopsis thaliana* powdery mildew fungus - *Golovinomyces orontii* (ca. 160 Mb) (Spanu *et al.*, 2010). Interestingly, the average genome size of Ascomycota is only 37 Mb (Mohanta & Bae, 2015). Therefore, the approximate genome sizes of *B. graminis* are around three times larger than the average size of Ascomycota. The number of coding genes in *B. graminis* and the average number of coding genes in Ascomycota are about 5900 and 11129, respectively (Mohanta & Bae, 2015). These indicate that, in comparison to the sizes of

genomes, *B. graminis* has a relatively lower number of coding genes among ascomycetes. It was hypothesized that approximate 64% of the Bgh genome (Spanu *et al.*, 2010) and about 85 % of the Bgt genome (Parlange *et al.*, 2011) were mainly consisted of repetitive DNA and transposable elements. The repeat contents of *B. graminis* are more prominent than in the genomes of *Botrytis cinerea* (~ 4% transposable elements) (Amselem *et al.*, 2011), *Puccinia graminis* (~ 45 %) (Duplessis *et al.*, 2011) and *Tuber melanosporum* (~ 58 %) (Martin *et al.*, 2010).

1.4 Life cycle of *Blumeria graminis*

There are two types of propagation in the life cycle of *B. graminis* (**Figure 1.2**): sexual propagation (teleomorphic sexual state) and asexual propagation (anamorphic asexual state) (Glawe, 2008). In these two propagation, the pleomorphic *B. graminis* forms two morphologically typical spores: asexually produced conidia and sexually formed ascospores.

1.4.1 Asexual propagation of *Blumeria graminis*

Under natural conditions, conidia are produced in conidia chains and they are mostly dispersed by wind, animals, insects or human activities (Kuhn *et al.*, 2016). Conidia are considered to be released under the conditions of high temperatures and low relative humidity (RH) (Hammett & Manners, 1971). However, conidial dispersal is hypothesized to be inhibited when host surfaces are wet or under the conditions of low temperatures and high RH (Hammett & Manners, 1971).

After landing on a suitable host surface, the air-borne conidia start to release a liquid extracellular matrix (ECM), which assists the attachment of spores to the host plant surface (Carver *et al.*, 1999). This ECM release process rapidly begins within twenty seconds when a conidium initially contacts with a hydrophobic substratum (Carver *et al.*, 1999; Wright *et al.*, 2002). Subsequently, conidia perceive surface signals and further initiate the germination and appressorium (APP) differentiation processes – the so-called prepenetration processes.
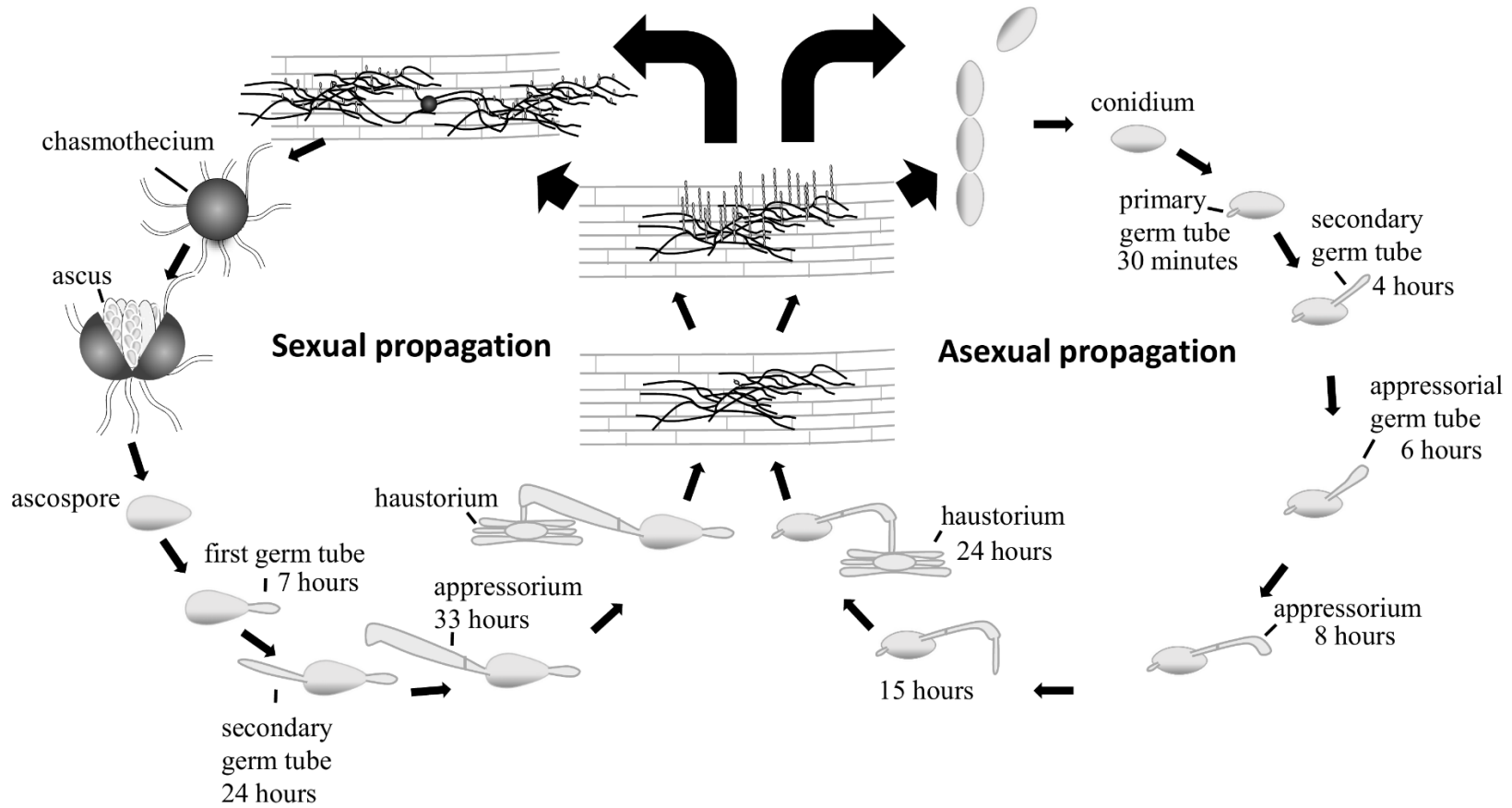
Figure 1.2 Schematic life cycle of *Blumeria graminis*. The black arrows indicate propagation in the life cycle. Asexual propagation stages consist of the prepenetration processes (conidial germination and formation of appressorium, up to ca. 15 hpi) and the postpenetration processes (establishment of haustorium, development of hyphae and conidiation). Sexual propagation stages mainly compose of chasmothecium formation, ascospore production, ascosporic infection, development of epiphytic mycelium and conidiation. (According to Both, *et al.* (2005) and Zhu, *et al.* (2017))

Introduction

In these prepenetration processes, conidia interpret a highly ordered consecutive morphogenesis. After initial contact (ca. 30 min) with a host surface, the non-germinated (NG) conidia germinate and form a short (ca. 6.5 µm) unique primary germ tube (PGT). The PGT was hypothesized to be involved in water uptake (Carver & Bushnell, 1983), spore attachment (Edwards, 2002), host-derived signals perception (Zhang *et al.*, 2005) and host-resistance suppression processes (Yamaoka *et al.*, 2007). By the presence of free water on a host surface, the conidial germination and differentiation processes are significantly impaired (Yamaoka & Takeuchi, 1999). However, under arid conditions, the conidial development dependents on water obtained from hosts through the PGT (Carver & Bushnell, 1983). After the germination process, conidia powerfully attach to their host surface via the PGT, which penetrates plant cuticle and contacts with the epidermal cell wall of their host but does not breach any further (Edwards, 2002). Conidia, as well as the PGT, are able to recognize and comprehend contacting surface features (Nielsen *et al.*, 2000). By comparing suspended (on spider threads) and unsuspended (on host surface) germlings, Carver and Ingerson (1987) demonstrated that the suspended conidia germinated by forming several short germ tubes (multiple germ tubes) but did not elongate or form an appressorial germ tube (AGT). However, by transferring the suspended germlings, which formed only one germ tube, to a host surface, the following germ tube of conidia elongated, stretched and produced an AGT(Carver & Ingerson, 1987). These findings suggested that the conidial PGT was able to perceive surface properties/signals. Moreover, it was reported that when the PGT and the APP of conidia penetrated the same host cell, the ratio of the feeding structure (the haustorium) formation was dramatically higher than when the PGT and the APP penetrated different cells (Yamaoka *et al.*, 2007). In line with this, the PGT of *B. graminis* conidia was hypothesized to be capable of functionally suppressing the resistance of a host epidermal cell and of effectively stimulating accessibility of host cells (Yamaoka *et al.*, 2007).

Following the formation of a PGT, conidia subsequently produce a secondary germ tube (SGT) (2 to 4 hours post-inoculation, hpi). The SGT elongates (ca. 30 µm), stretches, swells and forms an AGT (4 to 6 hpi). The emergence of SGT and AGT was considered to be necessarily promoted by stopping the PGT elongation, due to the fact that when a PGT continuously elongated without termination, the outgrowth of an AGT was relatively delayed (Yamaoka *et al.*, 2006). Then the AGT differentiates into a mature and hooked infection structure – an APP, which attempts to pierce both plant cuticle and the host epidermal cell wall to invade the host cell. The prepenetration processes of *B. graminis* are completed within 15 h with the establishment of a functional APP and play an essential role in the invasion processes of this fungal pathogen. *B. graminis* is a biotrophic pathogen, which needs to obtain nutrient and water from the host epidermal cell via a feeding structure – a haustorium (HAU) – at the beginning of colony formation. Because the HAU is produced by the penetration hyphal

peg of a functional APP, the appressorium formation and penetration successes of the APP are extremely crucial to the following pathogenesis and the fungal life cycle. It was demonstrated that both enzymatic activities and high mechanical forces (turgor pressures up to ca. 2 - 4 MPa) were involved in the piercing processes of penetration peg of *Blumeria graminis* conidia (Pryce *et al.*, 1999). However, it seems that conidia mainly depend on the physical force to invade host cells, as *B. graminis* has a comparatively much lower number of carbohydrate-active enzyme coding genes than many other phytopathogenic fungi (Spanu *et al.*, 2010; Kuhn *et al.*, 2016).

In postpenetration processes, if a penetration hyphal peg of functional APP successfully pierces the host cell wall and enters an epidermal cell without interrupting the host plasma membrane, it differentiates a bubble-like initial HAU (ca. 18 to 24 hpi). Then the initial HAU develops and grows into a perihaustorial membrane surrounded digitate HAU (Spanu, 2012), which was hypothesized to enhance the potential of nutrient absorption. As the essential feeding structure of *B. graminis*, the HAU is the only cell resides within a host cell to obtain water and nutrients. Once the first functional HAU is established, nutrients (such as glucose) are gained and transported from the HAU to epiphytic hyphae (Sutton *et al.*, 1999). The epiphytic hyphae then develop and produce new appressoria, which can penetrate nearby host cells and subsequently form secondary haustoria. The haustoria feed a young colony to grow, develop and produce new conidia. The main immune interaction site between host plants and *B. graminis* is the HAU, which produces the perihaustorial membrane (the extra-haustorial membrane) to separate the plant cell and fungal structures (Kuhn *et al.*, 2016). Like the PGT, the HAU can control and suppress host immunities by secreting effector molecules during infection processes (Panstruga & Dodds, 2009). In a recent study, it was speculated that the vesicle traffic of infected host epidermal cell was controlled by effectors introduced by the HAU of *B. graminis* (Kwaaitaal *et al.*, 2017).

The young colony of *B. graminis* results in the development (elongation and branch) of hyphae (epiphytic mycelia) on the host surface and eventually production of abundant conidia in chains. The production of conidia starts 3 – 4 days post-inoculation. This production process is supported by secondary haustoria that actively take up sources to feed the superficial structures (Both *et al.*, 2005). Newly produced conidia (up to 20000 conidia per colony (Zhang *et al.*, 2005)) may be then distributed or transferred onto plant leaf surfaces and subsequently infect the hosts. It was shown that the liberation of Bgt conidia was positively associated with wind, low RH, high temperatures, and surface dryness of wheat leaves (Hammett & Manners, 1971).

1.4.2 Sexual propagation of *Blumeria graminis*

Under natural field conditions, the sexual propagation of *B. graminis* begins when hyphae of opposite mating strains fuse/mate at the end of summer period. It was hypothesized that this mating process was stimulated by dry weather and it results in typically forming fruiting bodies (**Figure 1.3** A) – chasmothecia (formerly called cleistothecia) (Wicker *et al.*, 2013). Under laboratory/greenhouse and field conditions, the artificial mating-cross processes of *B. graminis* were achieved, which allowed to perform the recombination/hybridization of target mating isolates/strains to produce chasmothecia (Brown *et al.*, 1992; Bousset & de Vallavieille-Pope, 2003; Wicker *et al.*, 2013; Menardo *et al.*, 2016). The newly formed chasmothecia are generally light-coloured, then turning yellowish and finally black when mature (Glawe, 2008). At the beginning of senescence of host plant, chasmothecia are generally embedded in mycelia of *B. graminis* colonies on the lower leaf surfaces (Gotz *et al.*, 1996). Chasmothecia are known to remain dormant during laboratory storage or adverse weather conditions for an extended period. This dormant phase allows *B. graminis* to survive a long period of drought and then to sexually produce spores when a condition is optimum for this fungus.

The sexually produced spores – ascospores – mature in asci (**Figure 1.3** B and C) when chasmothecia are wetted by rainfall or artificially supplied moisture. The wetting processes were hypothesized and applied to promote ascosporic maturation (Menzies & MacNeill, 1989). In general, each single ascus (**Figure 1.3** D) contains eight matured ascospores. Then the ascospores are forcibly ejected from asci and they are regarded as the initial inoculum of the volunteer host seedlings according to the coordination of the propagation-cycles of hosts and pathogens (Koltin & Kenneth, 1970; Liu *et al.*, 2012).

After reaching a suitable host leaf surface, ascospores can germinate and produce (a) germ tube(s). It was reported that, unlike conidia, ascospores generally formed 1 – 4 germ tube(s) on glass slides or on the host surface (Jankovics *et al.*, 2015). After the germination process, ascospores on host surface produce an infection structure – an appressorium, which may subsequently penetrate both cuticle and host epidermal cell wall to develop into a haustorium (**Figure 1.3** E). It was shown that, in some cases, all germ tubes of one single ascospore differentiated into appressoria and then contributed to the young colony formation (Jankovics *et al.*, 2015).

Upon establishing a parasite relationship with a host, a young colony of ascospore (**Figure 1.3** F) develops and initiates conidiophores (conidial chains) after 3 to 5 days following an ascosporic inoculation. The newly produced conidia may be mainly distributed and infect the hosts.
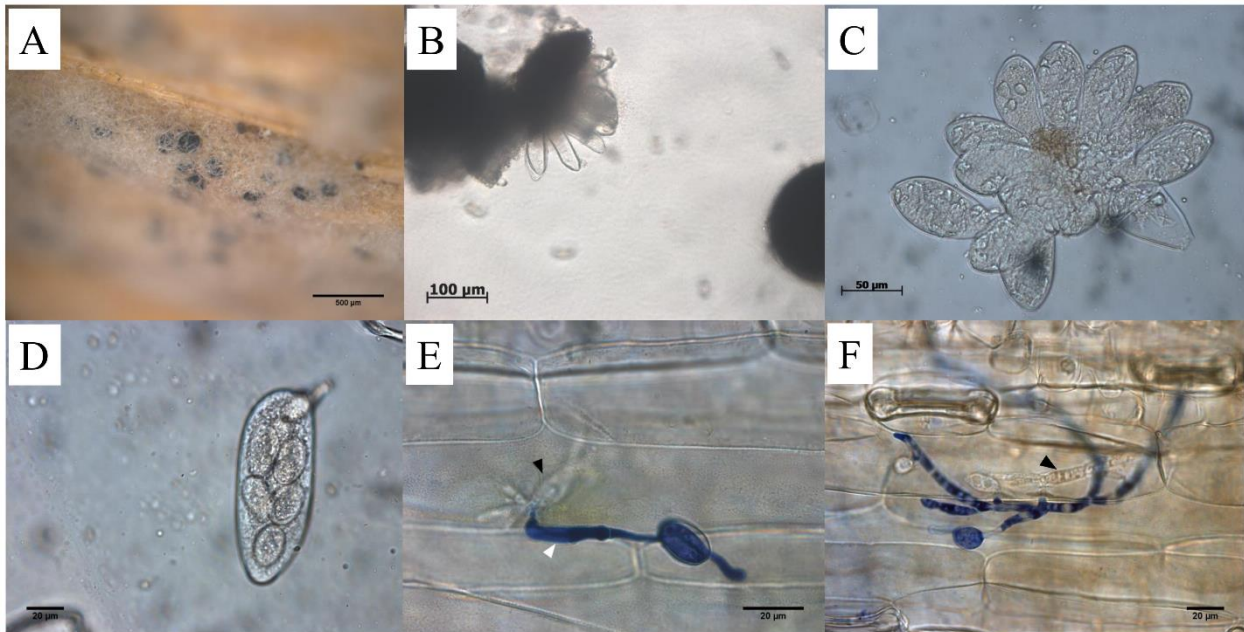
Figure 1.3 chasmothecia, asci, ascospores and a young colony of *Blumeria graminis*. (A) *B. graminis* chasmothecia and mycelium on leaf debris. Bar = 500 µm. (B) Dehisced chasmothecium with several asci. Bar = 100 µm. (C) Isolated asci with protoplasm. Bar = 50 µm. (D) Formed ascospores surrounded by protoplasm in a single ascus. (E) Differentiated ascospore with haustorium. White arrowhead indicates appressorium on host leaf surface. Black arrowhead shows haustorium inside host epidermal cell. (F) A young ascosporic colony with haustorium (black arrowhead). (D) to (F), bar = 20 µm.

1.5 *In vivo* and *in vitro* conidial prepenetration processes of *Blumeria graminis*

As the prerequisite for postpenetration processes, the prepenetration processes (**Figure 1.4**) of *B. graminis* are the essential stages of fungal propagation. Because *B. graminis* needs to penetrate the host cuticle and subsequently the epidermal cell wall by an appressorium to form a haustorium, in most cases, conidial prepenetration stages occur on the epicuticular wax layer before the initial interactions of a fungal haustorium and a host cell take place.

The influences of physio-chemical cues of cuticular wax on the conidial prepenetration processes of *B. graminis* were first demonstrated by the researchers who applied the *eceriferum* (*cer*) barley mutants to show the importance of properties of host surface to spore development (Yang & Ellingboe, 1972). When *B. graminis* was inoculated onto the host barley leaves, conidia generally germinated and regularly formed a mature appressorium. However, on the host leaf without wax or on artificial surfaces (such as water agar, glass slide, and cellulose paper) conidia could germinate but rarely differentiate, indicating the development of

B. graminis in prepenetration stages might be in a substratum-dependent manner (Yang & Ellingboe, 1972; Carver & Ingerson, 1987). In other studies, it was shown that conidia of *B. graminis* could germinate and differentiate on two types of cellulose membranes (semi-inductive and inductive) at relatively high levels (Kobayashi *et al.*, 1991; Hall *et al.*, 1999; Kinane *et al.*, 2000). Conidia on other types of films, such as Formvar® membranes, were stimulated to differentiate at moderate levels (Hansjakob *et al.*, 2010). However, on hydrophilic glass slides, the germination of conidia was notably reduced and differentiated conidia were rarely monitored.

It was found that *B. graminis* developed and infected at different levels on the abaxial and adaxial leaves of *Lolium* spp. grasses, suggesting the properties (forms of epicuticular waxes) of leaf surface affected prepenetration processes of conidia (Carver *et al.*, 1990). The following experiments were conducted to re-assess the hypothesis that components of the abaxial epicuticular waxes impaired the prepenetration processes of *B. graminis* (Ringelmann *et al.*, 2009). It found that there were differences of chemical composition, physical microstructure and surface hydrophobicity between the adaxial and abaxial surfaces of leaves of *Lolium perenne*, demonstrating the development of *B. graminis* depended on the physio-chemical properties of cuticular waxes (Ringelmann *et al.*, 2009). It was shown that single chemical factors of leaf cuticular waxes was involved in the developmental morphogenesis of *B. graminis* and that the C_{26} aldehyde *n*-hexacosanal was capable of significantly stimulating APP differentiation of conidia *in vitro* (Tsuba *et al.*, 2002). This triggering effect was confirmed by the following study, which showed C_{26} aldehyde *n*-hexacosanal notably triggered the differentiation (about 50 %) of *B. graminis* conidia when the surface contact angles were above 80° (Zabka *et al.*, 2008). In addition, a comprehensive study illustrated that very-long-chain aldehydes (C_{22}-C_{30}) were able to stimulate prepenetration processes of *B. graminis* conidia in a chain length- and dose-dependent manner by employing an easily reproductive Formvar®-based *in vitro* system (Hansjakob *et al.*, 2010).

The Formvar®-based *in vitro* system is a platform, which provides highly homogeneous hydrophobic (contact angle > 90 °) surfaces to determine the promoting effects of single constituents in cuticular waxes on *B. graminis* conidia. Because the Formvar®-based system is an *in vitro* tool, there is the absence of living host tissue. Therefore, the secondary effects from plants in the studies were absolutely excluded (Hansjakob *et al.*, 2010). Then this system was improved by applying an agar layer as a water/source reservoir between a glass slide and a wax membrane. The improved platform - the so-called Formvar®/wax/agar-based *in vitro* system – allowed the application of versatile substances (such as cell cycle inhibitors, staining substance), which might be obtained by conidia on wax membrane (Hansjakob *et al.*, 2012).
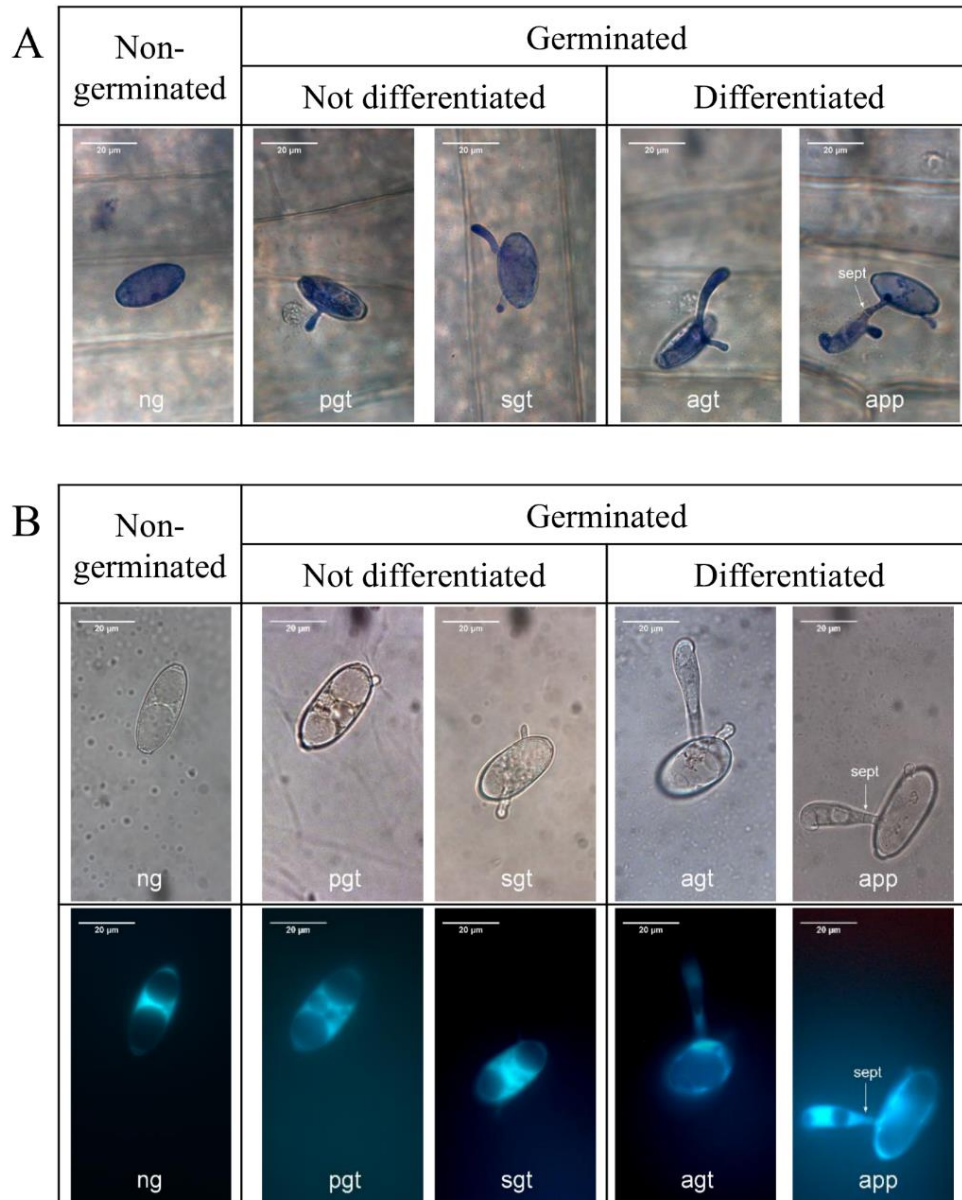
Figure 1.4 Development of *Blumeria graminis* conidia on (A) leaf surfaces and (B) on glass slides coated with Formvar®/ barley wax/agar. (A) *In vivo* conidia were stained with trypan blue. (B) Representative bright field (upper) and fluorescent (lower) microscopy images of conidia *in vitro.* Conidia were inoculated onto glass slides coated with Formvar®/ barley wax/agar. The agar was supplemented with fluorescence diacetate (500 µg/ml) in order to stain conidia. Prepenetration processes of *B. graminis in vitro* and *in vivo*: ng, nongerminated conidium; pgt, germinated conidium with a primary germ tube only; sgt, conidium with a secondary germ tube; agt, conidium with a swollen appressorial germ tube; app, conidium with fully differentiated, lobed and septate appressorium. Scale bars, 20 µm.

1.6 UV-mediated suppression of powdery mildews

Ultraviolet (UV) light, consisting of UV-A (wavelength, 315 - 400 nm), UV-B (wavelength, 280 - 315 nm) and UV-C (wavelength, 100 - 280 nm), is regarded as a natural mutation-causing agent. In most cases, UV irradiation was considered as one of the harmful and stressful factors to phyllosphere fungi, including powdery mildews (Janisiewicz *et al.*, 2016; Suthaparan *et al.*, 2018). During managements against fungal pathogens, the intensive application of fungicides against powdery mildews*,* whose propensity of selectively developing resistance to various anti-fungal agents, trends to be less efficient and very costly to control the phytopathogens (McGrath, 2001; Suthaparan *et al.*, 2016a). The biological control of powdery mildews has been attempted and achieved under practical conditions (Kiss, 2003; Laur *et al.*, 2018). However, the biological control alone is hypothesized to be not as effective as conventionally chemical fungicide treatments against fungal pathogens (Janisiewicz *et al.*, 2016a). The application of UV irradiation was regarded as an alternative method to manage against powdery mildews.

It was known that *in vitro* UV-mediated suppression of conidia of powdery mildew fungi was in a wavelength- and dose-dependent manner. It was illustrated that there was a potential in controlling of powdery mildews via the application of UV-B and/or UV-C irradiation in several pathosystems (hosts/pathogens), including Barley (*Hordeum vulgare*)/*Blumeria graminis* (Buxton *et al.*, 1957), grapevine (*Vitis vinifera*)/*Uncinula necator* (Willocquet *et al.*, 1996), pot rose (*Rosa × hybrid* 'Toril')/*Podosphaera pannosa* (Suthaparan *et al.*, 2012a), cucumber (*Cucumis sativus*)/ *Podosphaera xanthii* (Suthaparan *et al.*, 2012b; Suthaparan *et al.*, 2014), strawberry (*Fragaria × ananassa*)/*Podosphaera aphanis* (Janisiewicz *et al.*, 2016a; Suthaparan *et al.,* 2016a), rosemary (*Rosmarinus officinalis* L.)/ *Golovinomyces biocellatus* (Suthaparan *et al.*, 2016a) and tomato (*solanum lycopersicum*)/*Oidium neolycopersici* (Suthaparan *et al.*, 2016b). Moreover, the management of grape powdery mildew has been investigated by employing a mobile device to treat plants and pathogen with UV-C light in a vineyard, indicating the application was valid under field conditions (Michaloski, 1991).

1.7 Visible light-regulated development of powdery mildews

Visible light (wavelength ranging from ca. 380 nm (violet) – ca. 700 nm (red)) is an environmental source of information and energy that is capable of modulating many physiological aspects of plants and fungi (Kangasjärvi *et al.*, 2012; Fuller *et al.*, 2014). Plant defense responses, photosynthesis, and metabolism processes are affected by light (Idnurm & Heitman, 2005; Canessa *et al.*, 2013; Lu *et al.*, 2017). In the entire fungal kingdom, photo-responsive proteins are highly conserved (Fuller *et al.*, 2014). It is hypothesized that plant

pathogenic fungi are able to perceive light source/information to deal with various degrees of plant defense (Tisch & Schmoll, 2010; Fuller *et al.*, 2014). Moreover, the development and behaviors of phytopathogenic fungi are regulated by light (Corrochano, 2007; Fuller *et al.*, 2014). For powdery mildew fungi, light is shown to influence the germination, differentiation, penetration, colonization and conidiation processes of these ascomycetes in a dose- and quality-depend manner (Edwards, 1993; Carver *et al.*, 1994; Kenyon *et al.*, 2002; Jacob *et al.*, 2008; Mieslerová & Lebeda, 2010; Wang *et al.*, 2010; Suzuki *et al.*, 2018).

It was shown that the colony formation of *rhododendron* powdery mildew fungus – *Erysiphe* sp. – was affected by both light intensity and photoperiod (Kenyon *et al.*, 2002). It was reported that light intensity affected development (such as conidial germination, germ tube length, appressorial formation and disease severity) of the tomato powdery mildew fungus – *O. neoplycopersici* (Jacob *et al.*, 2008). Conidial germination and conidiation of *O. neoplycopersici* were influenced by conditions of light spectral quality, intensity and photoperiod (Mieslerová & Lebeda, 2010). When the cucumber powdery mildew fungus – *Sphaerotheca fuliginea* – was incubated under a red light condition, the disease severity of conidia was decreased (Wang *et al.*, 2010). In addition, it was reported that the lengths of conidiophore of the melon powdery mildew fungus – *Podosphaera xanthii* – were affected in a light wavelength (light quality) - dependent manner (Suzuki *et al.*, 2018).

1.8 Aims of this study

1.8.1 Influence of very-long-chain aldehydes on the ascosporic prepenetration processes of *B. graminis*

Plant cuticular waxes are demonstrated to be important chemical cues that triggering the prepenetration phases of *B. graminis* conidia (Carver *et al.*, 1990; Tsuba *et al.*, 2002; Zabka *et al.*, 2008; Hansjakob *et al.*, 2010; Hansjakob *et al.*, 2011; Weis *et al.*, 2014). Very-long-chain aldehydes – the specific constituents of many plant cuticular waxes – are pertinent to the conidial germination and differentiation of *B. graminis*. Even numbered very-long-chain aldehydes (C_{22} - C_{30}) are demonstrated to play crucial roles in chemically stimulating the prepenetration processes of *B. graminis* conidia in a dose- and chain-length dependent manner (Tsuba *et al.*, 2002; Zabka *et al.*, 2008; Hansjakob *et al.*, 2010; Hansjakob *et al.*, 2011). In addition, many environmental factors (such as atmospheric humidity and surface hydrophobicity) can notably influence conidial prepenetration processes (Manners & Hossain, 1963; Carver *et al.*, 1990; Zabka *et al.*, 2008; Ringelmann *et al.*, 2009).

B. graminis conidial prepenetration processes are dramatically impeded by the presence of free water (Manners & Hossain, 1963; Sivapalan, 1994; Iwamoto *et al.*, 2002). Interestingly, *B. graminis* ascospores are illustrated to easily germinate even when being submerged in water (Wolff, 1874; Jankovics *et al.*, 2015). This substantial difference with regard to environmental conditions might involve (a) variable mode(s) of surface recognition in ascospores of *B. graminis*. There are extensive studies with respect to conidial prepenetration processes of *B. graminis*. In contrast, to date there is only scarce information available on the perception of potential host surface signals, the timing of events and preferential conditions of the ascosporic prepenetration processes.

Therefore, the central aims of **Chapter 1** are mainly to address the following two questions:

1. Does the presence of very-long-chain aldehydes promote the ascosporic prepenetration processes of *B. graminis*?
2. Does the presence of free water affect the ascosporic prepenetration processes?

In order to enhance the knowledge of the (pre)penetration biology of the agriculturally and economically relevant phytopathogen *B. graminis*, *in vivo* experiments are combined with an appropriate and versatile experimental platform - Formvar®-based *in vitro* system to study the ascosporic prepenetration.

1.8.2 UV-C-mediated suppression of development of Barley powdery mildew (*B. graminis* f. sp. *hordei*)

It was demonstrated that UV-B and UV-C irradiation were efficiently to control powdery mildews in several pathosystems (hosts/powdery mildews) (Willocquet *et al.*, 1996; Suthaparan *et al.*, 2012a; Suthaparan *et al.*, 2012b; Suthaparan *et al.*, 2014; Janisiewicz *et al.*, 2016b; Suthaparan *et al.*, 2016a; Suthaparan *et al.*, 2016b; Suthaparan *et al.*, 2018). The suppressing effect of UV irradiation on powdery mildews is in a wavelength- and dose-dependent manner (Suthaparan *et al.*, 2016b; Suthaparan *et al.*, 2018). UV-C radiation was shown to be the best in controlling of the tomato powdery mildew – *O. neoplycopersici*, comparing to the same doses of UV-B and UV-A (Suthaparan *et al.*, 2016b; Suthaparan *et al.*, 2018). It was reported that UV-C irradiation negatively affected *B. graminis* conidial germination and infectivity (Buxton *et al.*, 1957). However, the impact of UV-C on appressorium formation of *B. graminis* remains scarce.

In many fungi, there are many repair mechanisms, which efficiently repair UV-induced damages (Mei & Dvornyk, 2015; Cohrs & Schumacher, 2017; Hu & Adar, 2017). Besides the dark repair (i.e. nucleotide excision repair), the UV-induced damage is recognized and fixed by photolyases, the enzymes utilize blue light/near UV light energy to repair damages during photoreactivation processes (Mei & Dvornyk, 2015). In *B. graminis* genome, there are three putative genes encoding photolyases, which might be involved in the photo-repair processes. To date, however, there is no information available with respect to the transcriptional regulation of these photolyase genes in *B. graminis* and other powdery mildew pathogenic fungi.

Therefore, the study of **Chapter 2** mainly focused on the following three questions:

1. What is the influence of doses of UV-C irradiation on the conidial prepenetration processes of *B. graminis*?
2. Are there the photoreactivation processes in *B. graminis* under varied photoperiod conditions?
3. How does the transcription of photolyase genes change under UV-C and visible light irradiation conditions?

It was shown that UV-C irradiation could notably enhance the resistance of host tissues against pathogens (Miyagawa *et al.*, 1994; Christensen *et al.*, 1998; Nigro *et al.*, 1998; Mercier *et al.*, 2000; Pombo *et al.*, 2011; Mintoff *et al.*, 2015; Urban *et al.*, 2018). In order to exclude secondary effects caused by host tissue, besides *in vivo* studies, the Formvar®-based *in vitro* system was applied to analyze the influence of UV-C irradiation on the conidial prepenetration processes of *B. graminis*.

1.8.3 Impact of light irradiation on conidial germination, differentiation and penetration of *B. graminis*

In previous studies, it was illustrated that light influenced germination, differentiation, penetration and conidiation processes of powdery mildews in the dose- and quality-depend manners (Edwards, 1993; Carver *et al.*, 1994; Kenyon *et al.*, 2002; Jacob *et al.*, 2008; Mieslerová & Lebeda, 2010; Wang *et al.*, 2010). To date, with respect to the influence of light conditions on conidial prepenetration and penetration processes of *B. graminis*, only a few of studies have been identified.

The haustorial development of *B. graminis* f. sp. *hordei* (Bgh) was reported to be relatively delayed in the experiments that used various lighting regimes (Edwards, 1993). It was speculated that there was no effect of light on the fungus (Edwards, 1993). However, in contrast, Carver *et al*. (1994) demonstrated that white light and a wide variety of light qualities influenced the appressorium maturation and haustorium formation of *Blumeria graminis* f. sp. *avenae* (Bga) and that there is no significant difference between the development of conidia inoculated onto detached leaves and intact leaves. Carver et al. (1994) hypothesized that there was a direct effect of white light on the fungus. Despite the differences of pathosystems (barley/Bgh and oat/bga systems), due to biotrophic characters of *B. graminis*, both of the studies applied a pathogen/host system, which might include the secondary effect of host tissue when illuminated plants with lights. To date, the scientific evidence with respect to the direct influence of light on *B. graminis* conidial prepenetration processes is not clear.

The Formvar® resin-based *in vitro* system, which provides reproducible homogeneous hydrophobic surfaces, is shown to be a novel platform to analysis conidial prepenetration processes of *B. graminis* (Hansjakob *et al*., 2010; Hansjakob *et al*., 2012; Zhu *et al*., 2017). Moreover, this *in vitro* system allows the study of *B. graminis* excluding secondary effects caused by host tissues (Zabka *et al.*, 2008; Hansjakob *et al.*, 2010).

In line with this, the work of **Chapter 3** focused on the questions:

1. Are there direct effects of white light on *B. graminis* conidial germination and appressorium differentiation processes?
2. Do qualities of light affect conidial developments?

2 Materials and methods

2.1 Plant and fungal materials

Dry leaves of the spring wheat cultivar "Tschermaks Blaukörniger Sommerweizen" (*Triticum aestivum* var. *tschermakianum*, Dreschflegel GbR, Witzenhausen, Germany) grown in the Botanical Garden of the University of Würzburg bearing chasmothecia of *Blumeria graminis* f. sp. *tritici* were collected in July 2015. Leaf segments were cut (approximately 5 cm long) and then soaked in sterile distilled water for ca. 4 h, subsequently fixed horizontally on sterile wooden sticks and finally got physically contact with primary leaves of 7 d old wheat germlings (*Triticum aestivum* cv. Winnetou, Saatzucht Firlbeck, Atting-Rinkam, Germany) (Jankovics *et al.*, 2015). Wheat seedlings were sprayed with distilled water twice a day (once in the morning (10:00 a.m.) and once in the afternoon (3:00 p.m.)) to maintain humid conditions. The first visible symptoms of *B. graminis* infection on the seedlings were observed after 7 days. Ascospores from the collected chasmothecia and asexually produced conidia from these initial ascosporic infections were applied for experimentation. This isolate was named as *B. graminis* f. sp. *tritici* Wue1 (BgtWue1). *Blumeria graminis* (DC.) E.O. Speer f. sp. *hordei* Em. Marchal [isolate A6] (Bgh) was propagated on its host (barley, *Hordeum vulgare* L. cv. Stendal) under the same conditions.

Wheat (cv. Winnetou) or barley (cv. Stendal) was, respectively, sown in plastic pots (diameter 9 cm) filled with standard potting soil (Typ ED73; SteuderComp, Schermbeck, Germany) and kept in growth chambers in a 16 h : 8 h (light/dark) photoperiod (white light intensity, ~ 300 µmol photons $m^{-2} s^{-1}$) at 20°C : 18°C and 70 % relative humidity. The white light in growth chamber was provided by the fluorescent bulbs (F17T8/TL741, Philips, USA). This light was used as the white light source in the whole study.

Bgh and BgtWue1 were, respectively, propagated on its hosts until distinct "white powdery" pustules appeared. In order to obtain freshly emerged conidia available for assays, one day before conidia were required for experimentations, spore-bearing leaves were carefully shaken to remove older conidia.

2.2 Coating of glass slides

Histobond® glass slides (Marienfeld, Lauda-Königshofen, Germany), standard microscopy glass slides or cover glasses (21 x 26 mm) were cautiously cleansed (described in **table 2.1**) and finally dried at room temperature. A 0.5% solution of Formvar® resin (polyvinyl formal, CAS 63450-15-7, Applichem) in chloroform was supplemented with the C_{28} alkane *n*-octacosane

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(Sigma-Aldrich, Munich, Germany) to a final concentration of 7×10^{-4} mol l^{-1} (0.25 mg ml^{-1} n-octacosane) only, or additionally with the respective even-numbered very-long-chain aldehyde to final concentrations of 7×10^{-6} mol l^{-1}.

Table 2.1 glass slides washing

Step		Time
1	Deconex® 11 UNIVERSAL detergent(Applichem, Darmstadt, Germany)/water (2:100, v:v)	10 min
2	Distilled water	5 min
3	Isopropanol	20 s
4	Distilled water	2 min

To obtain total leaf wax extracts of 14d old wheat or barley plants, the entire leaves (apart from cut edges) were dipped into 25 ml chloroform (>99% Roth, Karlsruhe, Germany) for 90s. In order to remove the solvent and residual water, chloroform/wax solution was treated with a gentle stream of nitrogen gas. To remove polar contaminants, wax extracts were re-dissolved in chloroform for at least 1 h and then added to the 0.5 % Formvar solution yielding concentrations of 0.5 – 1 mg ml^{-1} (Hansjakob et al., 2010). The cleansed glass slides or cover glasses were dipped into respective coating solution for ca. 10 s, subsequently dried completely for at least 16 h at room temperature and finally stored in boxes for further experimentation. Very-long-chain aldehydes were synthesized as described in the previous study (Hansjakob et al., 2010).

In order to obtain slides with an agar layer underlying the Formvar®/wax membrane, cleansed glass slides (Elka Sondheim, Germany) were dipped into a solution of 1% (w/v) polyvinylpyrrolidone 40 (PVP40) (Sigma-Aldrich) for 20 s and dried completely for about 24 h at room temperature. The PVP40-coated slides were then dipped into a 0.5% solution of Formvar® resin in chloroform supplemented with total wheat leaf wax extract (500 μg ml^{-1}), total barley leaf wax extract (500 μg ml^{-1}) or n-octacosane (0.25 mg ml^{-1}). Subsequently, slides were dried for 16 h at room temperature. The edges of the coated slides were gently scratched with fine sandpaper. Then the slides were slowly immersed in distilled water in an angle of around 45°. This procedure resulted in the solubilisation of the PVP40 coating and the subsequent release of the Formvar®/wheat wax, Formvar®/barley wax or Formvar®/n-octacosane membranes floating on the water surface. One side of a glass slide was covered with 670 μl of 2 % (w/v) Bacto water agar and then submersed below the floating membrane. The agar-covered glass slide was slowly pulled out again at a 30° angle. This process resulted

in the transfer of a membrane attached to the agar surface. Residual water was dabbed away by gently contacting the edges of the slide with a paper towel (Hansjakob *et al.*, 2012).

2.3 Contact angle measurements

To measure surface hydrophobicity, contact angles of 2 µl droplets of distilled water were determined (contact angle system OCA 15, software system SCA20; Dataphysics Instruments, Filderstadt, Germany). A total of 20 measurements on each of at least five independent surface samples was performed. Data are given as means ± SD.

2.4 Experiments with ascospores and conidia

Adaxial surfaces of Detached 12-d-old coleoptile (Bushnell *et al.*, 1967) or primary leaves of the host were used for *in vivo* study. Glass slides with different surface coatings were applied for *in vitro* assay. Wheat and glass slides coated with Formvar®/wheat wax extracts were employed for the study of BgtWue1. Barley and glass slides coated with Formvar®/barley wax extracts were utilized for the essay of Bgh.

A fine needle was used for scraping off *B. graminis* f. sp. *tritici* chasmothecia. Only mature black pigmented chasmothecia were selected as samples. In order to reduce contaminations with other organisms, chasmothecia were isolated from the appendages/mycelium. Single isolated chasmotecia were then transferred onto uncoated Histobond® glass slides or Histobond® glass slides coated with Formvar®/wheat wax extracts or Formvar®/wax compound mixtures forming several groups of 5 - 10 chasmothecia per slide. Subsequently, 100 µl of sterile distilled water was pipetted onto the slides to immerse chasmothecia. To constantly immerse chasmothecia in water, a cover glass was placed on modelling clay to form a water-filled gap (approximately 1 mm) between the glass slide and cover glass. Then the samples were incubated on moist filter paper for 5 – 7 days in darkness at 20 °C.

To determine ascospore germination and differentiation of *B. graminis,* Chasmothecia that had been submerged in distilled water for 24h were placed onto the adaxial wheat leaf surfaces. Then the leaves were placed onto glass slides. Fifty percent of the isolated chasmothecia were covered by a droplet of distilled water. The slides were then placed on moistened filter paper and incubated under conditions of > 90 % relative atmospheric humidity for 5 - 7 days at 20°C in darkness.

During conidia inoculation processes, to ensure even distribution of conidia on substrata, infected wheat leaves bearing conidial colonies were placed on the top of a settling tower and blown by pressurized air. After blowing process, conidia were allowed to land onto substrata for 1 min. The density of conidia is ca. 2 x 10^3 conidia cm^{-2}. Subsequently, the glass dishes were sealed with transparent film. Artificial surfaces, coleoptiles and primary leaves were kept moist with wet filter paper applied underneath the slides to achieve a relative humidity of at least 90 %. In order to monitor prepenetration processes in water, conidia were initially inoculated onto surfaces and allowed to attach to the surfaces for 30 min. Then a droplet of water was pipetted onto the surface to immerse conidia. Finally, the samples were incubated in darkness at 20°C for 18 h.

To de-stain leaves or coleoptiles, samples were carefully placed with the inoculated surface up onto filter paper moistened with solution 1. When surfaces were completely bleached, they were transferred to filter paper moistened with solution 2 (**Table 2.2**). Finally, staining solution (**Table 2.3**) was carefully pipetted onto inoculated surfaces to stain conidia (Lyngkjaer & Carver, 1999). To obtain clear visible fungal structures, inoculated conidia were stained for at least 30 min.

Table 2.2 Leaf de-staining processes

Step		Time
1	Solution 1 (ethanol : acetic acid (1 : 1, v∕v))	≥ 24 h
2	Solution 2 (lactic acid: glycerol : water (1 : 1 : 1, v∕v∕v))	≥ 3 h

Table 2.3 Staining solution

trypan blue (Merck) (final concertation: 0.1 %, w∕v)
acetic acid : glycerol : water (1 : 1 : 1, v∕v∕v)

2.5 UV-C light irradiation

The source of UV-C (wavelength, 254 nm) was provided by a Vilber Lourmat UV crosslinker (Bio-Link 254, Marne la Vallee, France). Inoculated conidia were treated with different doses of UV-C light for *in vitro* and *in vivo* experiments. Conidia without UV-C radiation were conducted as the control. After an incubation period of 18 - 48 h in darkness, conidia were analyzed.

2.6 Analysis of photoreactivation processes in *B. graminis*

To determine the photoreactivation processes of Bgh, conidia were treated with UV-C light and immediately incubated under the photo-period condition (white light/darkness, 2 h/ 16 h). To avoid the greenhouse effect on the development of conidia under light conditions, the temperature of growth chamber was set at 18 ± 2 °C to keep the inoculated surface temperature as 20 ± 1 °C.

To evaluate the ability of photoreactivation after an incubation period in darkness, conidia were treated with 100 J m^{-2} or 200 J m^{-2} of UV-C irradiation, incubated in darkness for 6 h and subsequently under the white light condition for 12 h or 18 h. Then conidia *in vitro* and *in vivo* were analyzed. To evaluate the severity of conidial infection, irradiated conidia were incubated under the photo-period condition (darkness /white light, 6 h/18 h) for 5 days and then examined.

2.7 RNA isolation of *B. graminis* conidia

After an incubation period, inoculated conidia were harvested by brushing surfaces with 5 % (w/v) cellulose acetate dissolved in acetone. After fast evaporation of acetone, the cellulose stripes were collected, then immediately frozen in liquid nitrogen and finally stored at - 80 °C for further experiments (Both *et al.*, 2005).

By using isolation method (**Table 2.4**), total RNA from conidia was extracted. The concentrations of isolated RNA were determined with a nanodrop spectral photometer (NanoDrop™ One/OneC, Thermo Scientific, USA). The integrity of RNA used for complementary DNA (cDNA) synthesis was confirmed by denaturing agarose gel electrophoresis according to the "Golden Rules of Quantitative RT-PCR" (Udvardi *et al.*, 2008).

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Table 2.4 RNA isolation of *B. graminis* conidia

1	Prepare fresh nucleic acid extraction buffer (5% SDS; 150 mM NaCl; 5mM EDTA pH 9; 50 mM tris/Cl ;).
2	Grind cellulose acetate strips with sea sand in liquid nitrogen.
3	Add 1.8 ml prepared extraction buffer.
4	Grind until the buffer is thawed.
5	Dispense extraction in Eppendorf tube.
6	Centrifuge the solution for 10 min at 14000 g.
7	Transfer 1 ml of the supernatant to a new Eppendorf tube.
8	Add 1ml of Roti phenol/Chloroform/Isoamyl alcohol (24:25:1) in the supernatant.
9	Centrifuge the solution for 5 min at 16000 at 2 °C.
10	Transfer 900 ml of the supernatants to a new Eppendorf tube.
11	Add 900 ml of chloroform/Isoamyl alcohol (24:1) in the supernatant.
12	Centrifuge the solution for 25 min at 16000 at 2 °C.
13	Transfer 400 ml of the supernatant to a new Eppendorf tube.
14	Add 40 µl of 3 M sodium acetate (pH 5.2) and 1 ml of 100 % ethanol to the supernatant.
15	Precipitate over night at -20 °C.
16	Centrifuge the solution for 40 min at 16000 g at 2 °C.
17	Discard the supernatant and then add 500 µl of ice-cold 75% ethanol.
18	Centrifuge the solution for 10 min at 16000 g at 2 °C.
19	Repeat steps 17 – 18.
20	Discard supernatant and dry pellet with the tube for 30 min at room temperature.
21	Re-suspend the pellet in 100 µl DEPC water for 10 min.
22	Treat the solution with DNase I (Qiagen, Hilden) according to the manufacturer's protocol.
23	Add 100 µl of LiCl (5 M LiCl, 34 mM EDTA, pH 8.0).

24   Precipitate over night at -20 °C.

25   Repeat steps 16 – 20.

26   Re-suspend the pellet in 20 µl DEPC water for 10 min.

27   Store at – 80 °C for cDNA synthesis.

2.8 cDNA synthesis

cDNA was synthesized by using the reverse transcription kit (Invitrogen SuperScript III Reverse Transcriptase, Cat. No. 18080-044) with Oligo(dT)$_{20}$ primers (Invitrogen, Karlsruhe), according to the manufacturer's instructions. Then the cDNA was checked to ensure non-contamination of gDNA (an example in **Figure 2.1**). The exon-exon primers – TUBA-G was used in the check processes.

TUBA-G (5' – 3'): TUBA-GF (GTGCCCCACTGGATTCAAGA) and TUBA-GR (TCGTCAAGCAGCAAAAAGCC). The checked cDNA was then stored at -20 °C until further use.
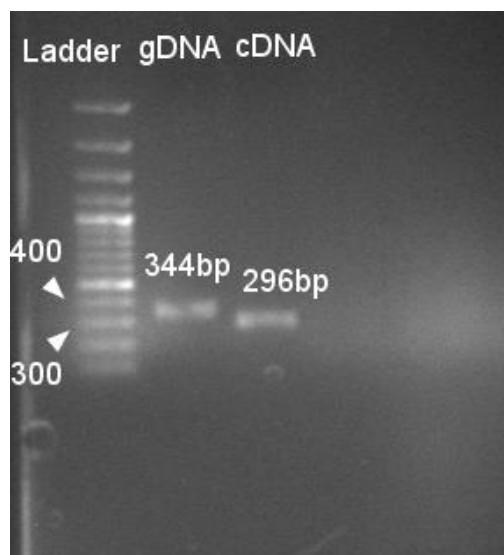
Figure 2.1 PCR Products of gDNA and cDNA using primers - TUBA-G. The product of gDNA is 344bp. The product of cDNA is 296bp. PCR was conducted as described in **2.9** and the cycle of PCR is 32. Then 1 µl of the product was mixed with loading dye and pipetted in a gel.

2.9 PCR

One µl of cDNA was added with PCR reaction master-mix (**Table 2.5**). Then PCR was performed with a mastercycler gradient PCR machine (Eppendorf, Hamburg) with the program described in **table 2.6**.

Table 2.5 PCR reaction master-mix

10x dreamTaq Buffer (Thermo Scientific, Germany)	5 µl
10 mM dNTP (KAPA Biosystems)	1 µl
Forward primers	10 µM
Reverse primer	10 µM
Template DNA	10 pg -1 µg
DreamTaq DNA polymerase (Thermo Scientific, Germany)	1.25 U
water	To 50 µl
Total volume	50 µl

Table 2.6 PCR program

1	95 °C	3 min
2	95 °C	30 s
3	60 °C	30 s
4	72 °C	1 min
5	Repeat 2 - 4	20 – 40 cycles
6	72 °C	2 min
7	4 °C	Store

2.10 RT-qPCR

In order to investigate the transcriptional regulation process of photolyase gene, conidia *in vitro* were treated with or without UV-C light (100 J m^{-2}) and then incubated in darkness, under white light or blue light conditions. The source of blue light was provided by LED (wavelength peak, 475 nm, Philips, USA) as the intensity of 80 µmol photons m^{-2} s^{-1}. The intensity of blue light (wavelength peak, 475 nm) was measured with a light meter (LI-250A, LI-COR®, USA) connected to a quantum sensor (LI-190, LI-COR®).

RNA and cDNA of treated conidia were prepared as described above. RT-qPCR was performed with the Maxima SYBR Green qPCR Master Mix (2X) (Thermo Fisher Scientific, Waltham, MA, USA) in a real-time thermocycler (C1000™ with CFX96™ Real-Time system, BioRad, Muenchen). The RT-qPCR program (**Table 2.7)** was processed according to Pennington *et al.*, (2016). The glyceraldehyde-3-phosphate Dehydrogenase (GAPDH) gene (Pennington *et al.*, 2016) was confirmed to be constitutively expressed in *B. graminis* conidia. Transcript levels of photolyase genes (Bgh isolate DH14, Accession: BGHDH14_bgh00666, BGHDH14_bgh01427 and BGHDH14_bghG001129000001001) were calculated relative to GAPDH gene, according to the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

Primers of photolyase gene (5' – 3'): BGHDH14_bgh00666 (5' – 3'): PHLF (TGATAGGGCGTGTGAGTGTG) and PHLR (TCGTCAAGCAGCAAAAAGCC). BGHDH14_bgh01427 (5' – 3'): CRYF (TGACGATGAGGATTGGGCTG) and CRYR (CCGCCACCTCTACATGGTTT). BGHDH14_bghG001129000001001: DASHF (GAGCATCCCAACACGAATGC) and DASHR (TCAGCGGAGCAAATTGAGGT). Primers of GAPDH (5' – 3'): GAPDHF (GGAGCCGAGTACATAGTAGAGT) and GAPDHR (GGAGGGTGCCGAAATGATAAC).

Table 2.7 RT-qPCR program

1	95 °C	3 min
2	95 °C	10 s
3	60 °C	30 s
4	Repeat 2 - 3	40 cycles
5	60 °C – 95 °C (increment 0.5 °C)	Melt curve analysis

2.11 White light irradiation

To determine the influence of intensity and quality of light on conidial prepenetration and penetration processes, conidia of Bgh and BgtWue1 were inoculated on host leaf surfaces and artificial surfaces. Then the conidia were incubated under various light conditions. Conidia incubated in darkness were conducted as the control. To avoid the greenhouse effect on the development of conidia, the incubation temperature was set at 18 ± 2 °C to keep the inoculated surface temperature as 20 ± 1 °C.

For each of experimentation with white light treatment, the intensity of white light was measured by a light meter (LI-250A, LI-COR®, USA) connected to a quantum sensor (LI-190,

LI-COR®). In order to study the effect of qualities of light on *B. graminis* conidia, LED lights were employed as the light sources. The blue LED (wavelength peak, 475 nm, Philips, USA), green LED (wavelength peak, 525 nm, Philips, USA) and red (wavelength peak, 660 nm, Philips, USA) LED were set at the certain distance in order to achieve same light intensity (80 µmol m^{-2} s^{-1}) at inoculated surfaces. The samples *in vitro* were analyzed after an incubation period of 18 h. *In vivo* samples were determined after an incubation period of 18 h to 48 h.

2.12 Microscopic analysis

Individual ascospores and conidia were monitored on each surface by light microscopy (Leica DMR with Leica IM1000 software package, Wetzlar, Germany). The determined conidia or ascospores were classified as nongerminated (ng), had formed a primary or first germ tube only (pgt/fgt), a secondary germ tube (sgt), a swollen appressorial germ tube (agt) or a septate appressorium (app). In addition, the loss rate of spores that were apparently damaged, burst or desiccated during the inoculation procedure was recorded (aberr). Additionally, conidia *in vivo* were recorded whether they formed haustorium (hau) only or hau with secondary hypha (hyp). Only single, well-separated conidia or ascospores were counted at each observation to eliminate the possibility of inhibition caused by crowding.

2.13 Scanning electron microscopy

BgtWue1 conidia and ascospores were inoculated onto Formvar®/wheat wax coated cover glasses (Ø 10 mm) and air dried at room temperature. The inoculated cover glasses were stuck to aluminum holders. Then the samples were sputtered with gold palladium at 25 mA for 150 s with Bal-Tec SCD 005 (Leica Microsystems, Wetzlar, Germany). Finally, they were monitored under a field emission scanning electron microscope (JEOL GSM-7500F, Nihon Denshi K.K., Akishima-Shi, TKY, Japan)(Zhu *et al.*, 2017).

2.14 Statistics

The statistical analyses were performed with IBM SPSS statistics software (version 23). The basis for statistical analyses was n = 2 - 3 independent experiments. In each independent assay, 250 ascospores or 300 - 600 conidia were examined. Shapiro test was applied to check significant differences (P < 0.05) of normality. Levene's test was used to check significant differences (P < 0.05) of homogeneity. Significant differences (P < 0.05) between multiple datasets were tested by Kruskal-Wallis test followed by a Bonferroni multiple comparison *post*

hoc test, Welch ANOVA followed by a Games-Howell *post hoc* test or one-way ANOVA followed by a Tukey *post hoc* test. Significant differences ($P < 0.05$) between pairwise comparisons were tested by a Student's T test or a Mann–Whitney U test.

3 Chapter 1 Very-long-chain aldehydes induce germination and differentiation of *Blumeria graminis* f. sp. *tritici* ascospores

This chapter is based on the following publication:

Zhu, M., Riederer, M., Hildebrandt, U. (2017). Very-long-chain aldehydes induce appressorium formation in ascospores of the wheat powdery mildew fungus Blumeria graminis. *Fungal Biology*, 121(8), 716-728. (doi: 10.1016/j.funbio.2017.05.003)

Statement of individual author contributions in the manuscripts:

Publication (complete reference): Zhu, M., Riederer, M., Hildebrandt, U. (2017). Very-long-chain aldehydes induce appressorium formation in ascospores of the wheat powdery mildew fungus Blumeria graminis. *Fungal Biology*, 121(8), 716-728. (doi: 10.1016/j.funbio.2017.05.003)				
Participated in	**Author Initials**, Responsibility decreasing from left to right			
Study Design	M.Z.	U.H.	M.R.	
Data Collection	M.Z.	U.H.	M.R.	
Data Analysis and Interpretation	M.Z.	U.H.	M.R.	
Manuscript Writing	U.H.	M.Z.	M.R.	

All figures and tables included in this chapter are also illustrated in the publication.

I confirm that I have obtained permission from both the publishers and the co-authors for legal second publication and the correctness of the above mentioned assessment.

I also confirm my primary supervisor's acceptance.

Mo Zhu	11.06.2018	Würzburg	
Doctoral Researcher's Name	Date	Place	Signature

3.1 Introduction

Blumeria graminis is an obligate biotrophic powdery mildew fungus, which is considered as one of the most oftentimes encountered plant pathogens in the world wild (Dean *et al.*, 2012). In the life cycle, *B. graminis* produced two spore types – conidia and ascospores (**Figure 1.2**). Both types of spores are crucial inoculums in the epidemiology (Koltin & Kenneth, 1970; Glawe, 2008; Liu *et al.*, 2012). However, there is only scarce information on *B. graminis* ascosporic germination and infection structure differentiation. The fruiting bodies – chasmothecia (**Figure 3.1** A), are formed when compatible strains/isolates mate. Chasmothecia are regarded as the structures that allow *B. graminis* to remain dormant and survive during adverse conditions. When the condition is optimum for the fungus, ascospores are sexually formed and matured (**Figure 1.3** D and **Figure 3.1** E) in chasmothecia. Following the dehisce of chasmothecia, ascospores of *B. graminis* are forcibly ejected from asci (**Figure 3.1** F). After reaching an appropriate host surface, ascospores germinate with one or several germ tubes and form an infection structure - appressorium (app) (**Figure 1.3** E and **Figure 3.2** A). Then appressoria may forcibly penetrate the host cuticle and epidermal cell wall forming the feeding structure – the haustorium (**Figure 1.3** E - D) (Reed, 1913; Jankovics *et al.*, 2015).

The likewise asexually produced conidia begin prepenetration processes (**Figure 1.4** A) after landing on a suitable host surface and form a primary germ tube (pgt). Then a secondary germ tube (sgt) elongates, swells and subsequently differentiates into an appressorial germ tube (agt) that finally matures and becomes an appressorium (app) (**Figure 3.2** B). Approximately 12 - 15 hpi, conidial prepenetration processes are completed (**Figure 1.2**) (Zhang *et al.*, 2005).

Plant cuticular waxes are demonstrated to be important chemical cues that triggering the prepenetration phases of *B. graminis* conidia (Carver *et al.*, 1990; Tsuba *et al.*, 2002; Zabka *et al.*, 2008; Hansjakob *et al.*, 2010; Hansjakob *et al.*, 2011; Weis *et al.*, 2014). Very-long-chain aldehydes – the specific constituents of many plant cuticular waxes – are pertinent to the conidial germination and differentiation of *B. graminis*. Even numbered very-long-chain aldehydes (C_{22} - C_{30}) are demonstrated to play crucial roles in chemically stimulating the prepenetration processes of *B. graminis* conidia in a dose- and chain-length dependent manner (Tsuba *et al.*, 2002; Zabka *et al.*, 2008; Hansjakob *et al.*, 2010; Hansjakob *et al.*, 2011). Nevertheless, many environmental factors (such as atmospheric humidity and surface hydrophobicity) can notably influence conidial prepenetration processes (Manners & Hossain, 1963; Carver *et al.*, 1990; Zabka *et al.*, 2008; Ringelmann *et al.*, 2009).

However, *B. graminis* conidial prepenetration processes are dramatically impeded by the presence of free water (Manners & Hossain, 1963; Sivapalan, 1994; Iwamoto *et al.*, 2002). Interestingly, *B. graminis* ascospores are illustrated to easily germinate even when being

submerged in water (Wolff, 1874; Jankovics *et al.*, 2015). This substantial difference with regard to environmental conditions might involve (a) variable mode(s) of surface recognition in ascospores of *B. graminis*. There are extensive studies concerning conidial prepenetration processes of *B. graminis*. In contrast, to date, there is only scarce information available on the perception of potential host surface signals, the timing of events and preferential conditions of the ascosporic prepenetration processes.

Therefore, the central aims of this chapter were to determine (1) whether very-long-chain aldehydes can notably trigger the prepenetration processes of *B. graminis* ascospores and (2) whether the ascosporic prepenetration processes are affected by the presence of free water. In order to enhance the knowledge of the (pre)penetration biology of the agriculturally and economically relevant phytopathogen *B. graminis*, *in vivo* experiments are combined with an appropriate and versatile experimental platform - Formvar®-based *in vitro* system.
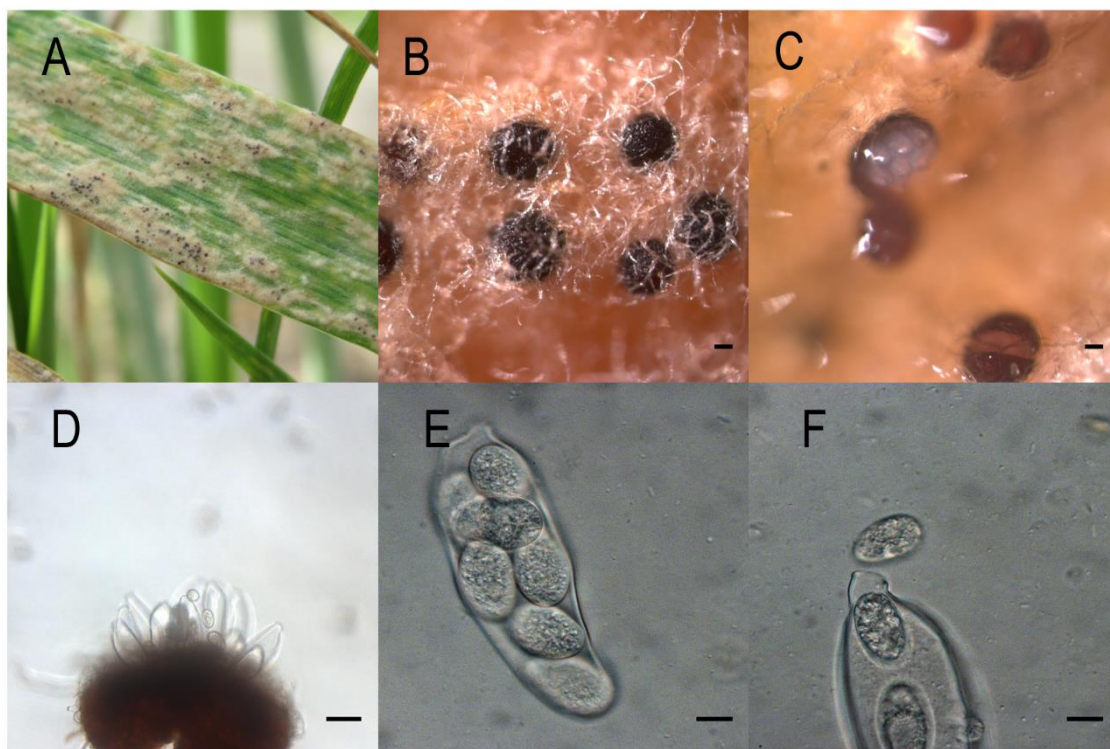
Figure 3.1 Chasmothecia, asci and ascospores of *B. graminis* f. sp. *tritici*. (A) Bgt mycelium and chasmothecia on a host leaf. (B) Imbibed chasmothecia surrounded by Bgt mycelium. Scale bar = 50 µm. (C) Dehisced chasmothecium on a host leaf surface presenting several asci. Bar = 50 µm. (D) Dehisced chasmothecium with several empty asci. Bar = 50 µm. (E) Single ascus containing mature Bgt ascospores. Bar = 10 µm. (F) Release of ascospores from a single ascus. Bar = 10 µm. (Zhu *et al.*, 2017)

A
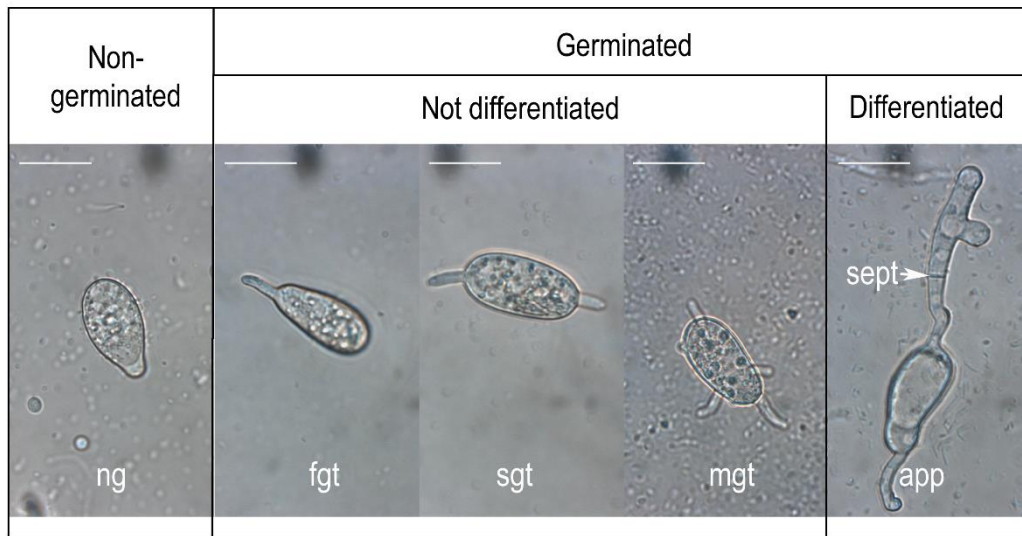
B
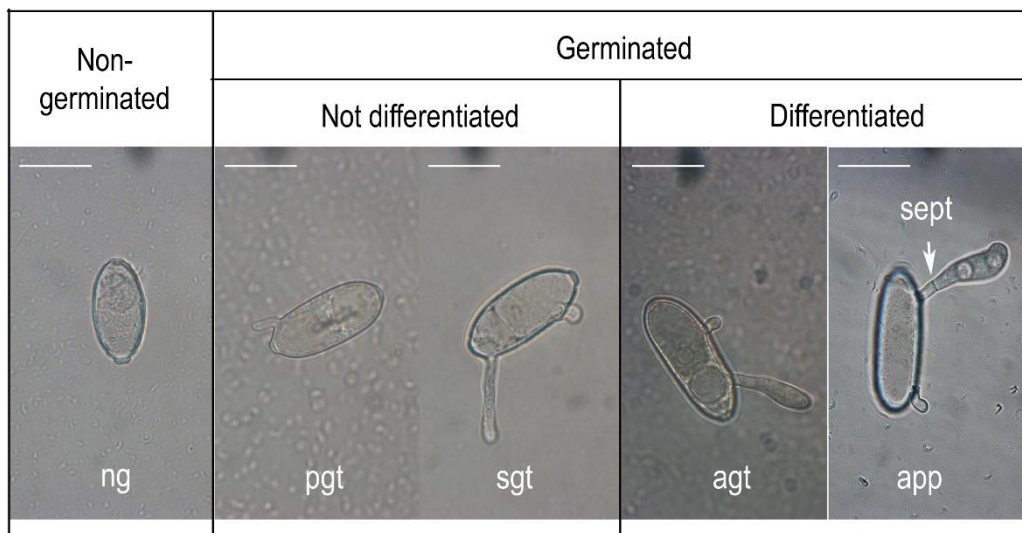
Figure 3.2 *In vitro* development of *B. graminis* f. sp. *tritici* (BgtWue1) ascospores and conidia on glass slides coated with Formvar®/wheat wax. (A) Prepenentration processes of *B. graminis* ascospores: ng, non-germinated; fgt, first germ tube formed; sgt, with an elongated secondary germ tube; mgt, with multiple non-differentiated germ tubes; app, fully differentiated appressorium with a septum (sept). (B) Prepenetration processes of *B. graminis* conidia: ng, nongerminated; fgt, with a first germ tube only; sgt, with an elongated secondary germ tube; agt, with a swollen appressorial germ tube; app, fully differentiated, lobed appressorium with a septum (sept). Scale bars = 20 μm. (Zhu *et al.*, 2017)

3.2 Results

3.2.1 *B. graminis* **ascospore prepenetration** *in vitro*

In initial experiments, pre-wetted *B. graminis* f. sp. *tritici* (BgtWue1) chasmothecia were submersed in a droplet of water. Then they were placed either onto glass slides or onto glass slides coated with a mixture of *n*-octacosane (7 x 10^{-4} mol l^{-1} in the coating solution) and Formvar® resin. The contact angle of the uncoated slides was 43 ± 1°. While Formvar®/*n*-octacosane coated slides were much more hydrophobic with contact angles of 112 ± 1° (**Table 3.1**).

Seven days after initial wetting, germination and differentiation of ascospores were determined (**Figure 3.2**). However, 88 % of the ascospores on the uncoated Histobond slides stayed non-germinated, this proportion was substantially decreased on the Formvar®/*n*-octacosane coated slides to 29 %. The numbers of ascospores with a first germ tube or a secondary germ tube increased to 46 % and 25 %, respectively. The formation of appressoria was neither found on the Formvar®/*n*-octacosane nor uncoated slides. To find out whether extracted host leaf wax was able of triggering ascospore germination and differentiation, chasmothecia were incubated for 7d in water on Formvar®/wheat wax-coated glass slides (contact angles, 105 ± 1°). In contrast to the situation on Formvar®/*n*-octacosane slides and uncoated slides, more than half of ascospores on Formvar®/wheat wax slides formed an appressorium. Although Formvar®/*n*-octacosane slides and Formvar®/wheat wax slides exhibited comparable hydrophobicity. The proportion of non-germinated ascospores on Formvar®/wheat wax slides was 12 %, which distinctly decreased below the numbers seen on Formvar®/n-octacosane slides.

In general, the lengths of BgtWue1 ascospores and the corresponding conidia are similar. However, ascospores were frequently pear shaped and exhibited a 21 % greater width than conidia (**Table 3.2**). Significant differences were noticed for fgt length that was nearly twice the value as for conidia. Likewise, under *in vitro* conditions, when compared with the dimensions of appressoria formed by conidia, the length and width of appressorium of ascospores increased by approximately 50 %.

Table 3.1 Surface contact angle of different substrata

Substratum	Contact angle(deg)
Histobond glass	43 ± 1
Coleoptile	94 ± 5
Formvar®/wheat wax	105 ± 1
Formvar®/*n*-octacosane	112 ± 1
Formvar®/*n*-octacosane /*n*-docasanal	112 ± 2
Formvar®/*n*-octacosane /*n*-tetracosanal	112 ± 2
Formvar®/*n*-octacosane /*n*-hexacosanal	112 ± 3
Formvar®/*n*-octacosane /*n*-octacosanal	112 ± 3
Formvar®/*n*-octacosane /*n*-triacontanal	112 ± 3

A total of 20 measurements on each of at least 5 independent surface samples were performed. Data are given as means ± SD. (Zhu *et al.*, 2017)

Table 3.2 Size parameter (µm) of *Blumeria graminis* f. sp. *tritici* (BgtWue1) conidia and ascospores

	Length	Width	Pgt length	Pgt width	App length	App width
Conidia	30.68[a] ± 3.31	12.52[a] ± 1.83	6.54[a] ± 1.66	3.45[a] ± 0.75	30.65[a] ± 5.26	6.60[a] ± 1.42
Ascospores	30.74[a] ± 2.27	15.16[b] ± 1.72	13.85[b] ± 3.63	4.40[b] ± 0.84	47.12[b] ± 8.94	9.47[b] ± 1.15

Dimensions of *Blumeria graminis* f. sp. *tritici* (BgtWue1) conidia and ascospores were determined after inoculation on glass slides coated with Formvar®/wheat wax. Each value is given as means ± SD of 50 spores. Different letters within a column indicate significant differences ($P < 0.05$) determined in Mann – Whitney U test. (Zhu *et al.*, 2017)
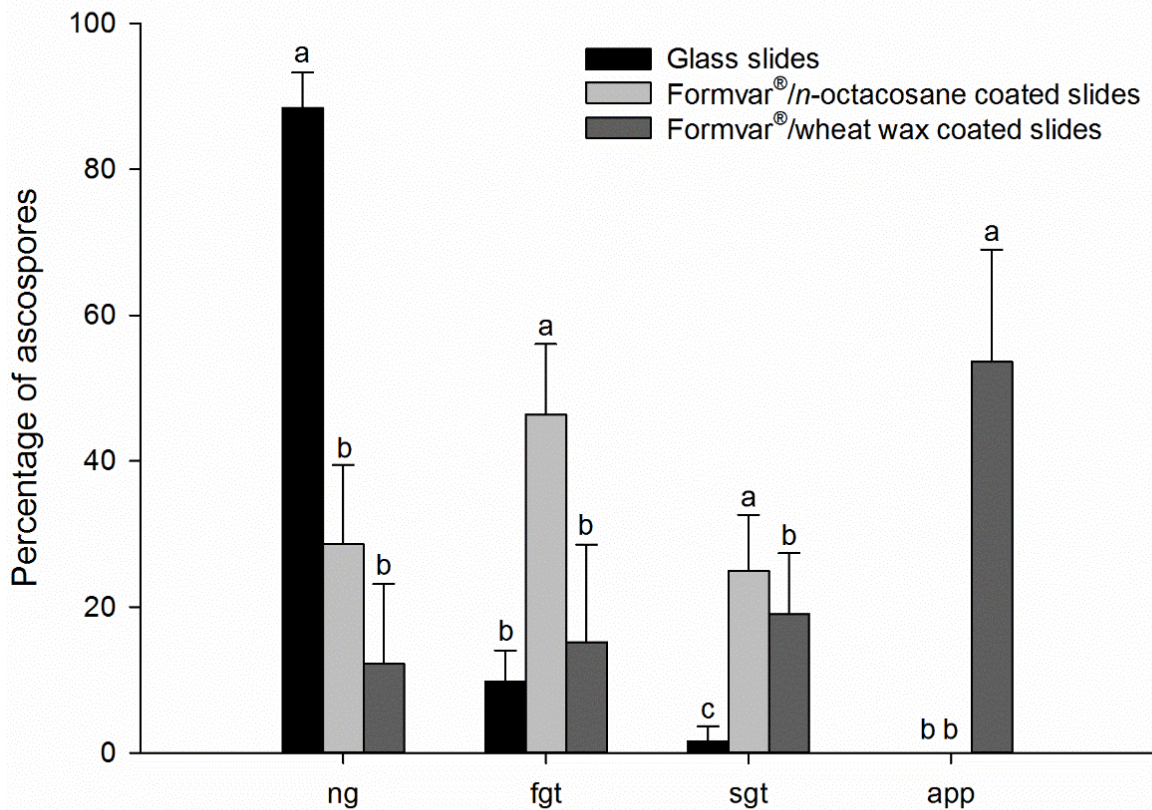
Figure 3.2 *In vitro* development of *Blumeria graminis* f. sp. *tritici* (BgtWue1) ascospores on coated and uncoated glass slides, glass slides coated with Formvar®/*n*-octacosane or Formvar®/wheat wax after an incubation period of 7 d in darkness. Prepenetration developmental stages of *B. graminis* ascospores: ng, non-germinated ascospore without a germ tube; fgt, with first germ tube only; sgt, ascospore with an elongated secondary germ tube; app, with a fully differentiated appressorium. Each value is given as mean ± SD of five replicates (50 spores each) of two independent experiments. Significant differences were determined in a Welch ANOVA followed by a Games-Howell *post hoc* test: different letters indicate significant differences ($P < 0.05$). (Zhu *et al.*, 2017)

3.2.2 Very-long-chain aldehydes trigger ascospore prepenetration

In previous studies, it was shown that very-long-chain aldehydes qualitatively influenced conidial prepenetration of *B. graminis* (Zabka *et al.*, 2008; Hansjakob *et al.*, 2010; Hansjakob *et al.*, 2011). To determine the effect of very-long-chain aldehydes on ascosporic prepenetration processes, chasmothecia of BgtWue1 were isolated and placed on glass slides

coated with Formvar®/*n*-octacosane or Formvar®/*n*-octacosane additionally supplemented with individual very-long-chain aldehydes. Then the chasmothecia were incubated for 7 days in darkness. Prepenetration processes of released ascospores were notably triggered by all of the analyzed aldehydes (**Figure 3.3**). Only 8 % and 11 % of ascospores formed an appressorium by the presence of *n*-docosanal and *n*-triacontanal, respectively. The presence of *n*-tetracosanal resulted in 15 % of differentiated ascospores. The number of ascospores that formed an appressorium significantly increased up to 43 % and 55 %, when they were incubated on the slides coated with *n*-hexacosanal and *n*-octacosanal, respectively. Ascosporic germination and differentiation were dramatically influenced by the aldehydes with different chain length.
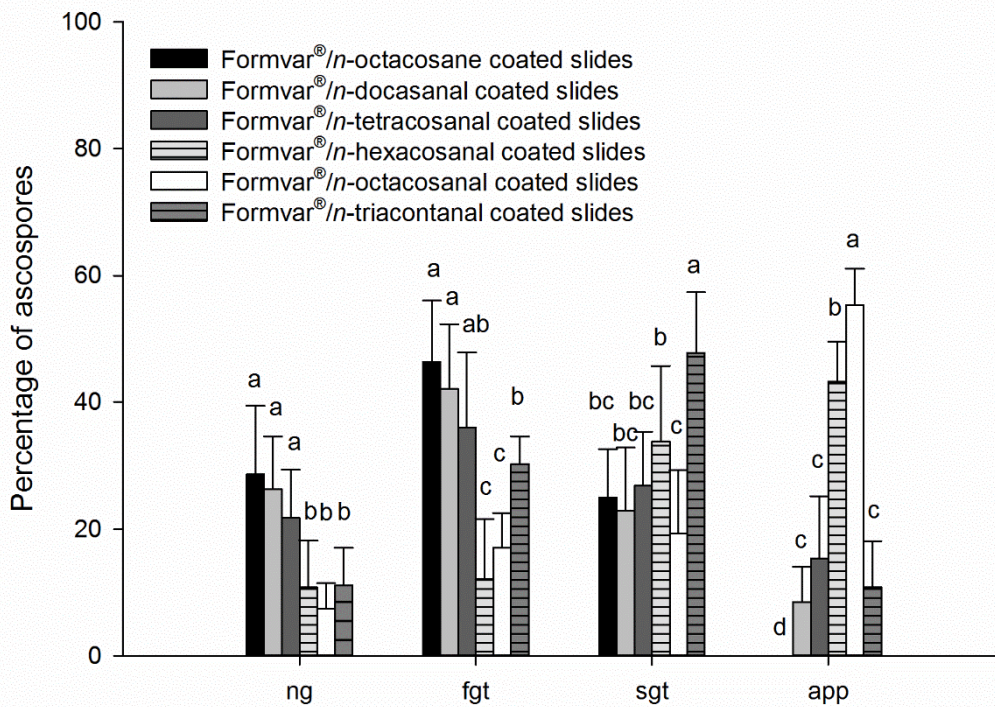
Figure 3.3 *In vitro* development of *Blumeria graminis* f. sp. *tritici* (BgtWue1) ascospores on glass slides coated with different very-long-chain aldehydes. Slides were incubated for 7 d in darkness. Prepenetration developmental stages of *B. graminis* ascospores: ng, non-germinated; fgt, with a first germ tube only; sgt, ascospore with an elongated secondary germ tube; app, fully differentiated appressorium. Each value is given as mean ± SD of five replicates (50 ascospores each) of two independent experiments. Significant differences were determined in a Welch ANOVA followed by a Games-Howell *post hoc* test: different letters indicate significant differences ($P < 0.05$). (Zhu *et al.*, 2017)

By the presence of *n*-docosanal and *n*-tetracosanal, the numbers of non-germinated ascospores were not significant to those on glass slides with *n*-octacosane only. However, the presence of *n*-triacontanal, *n*-hexacosanal and *n*-octacosanal resulted in only around 10 % of ascospores, remaining non-germinated pattern. When the propagated conidia (BgtWue1) were inoculated onto coated slides supplemented with very-long-chain aldehydes, they also showed a notable preference of *n*-octacosanal (**Figure 3.4**).
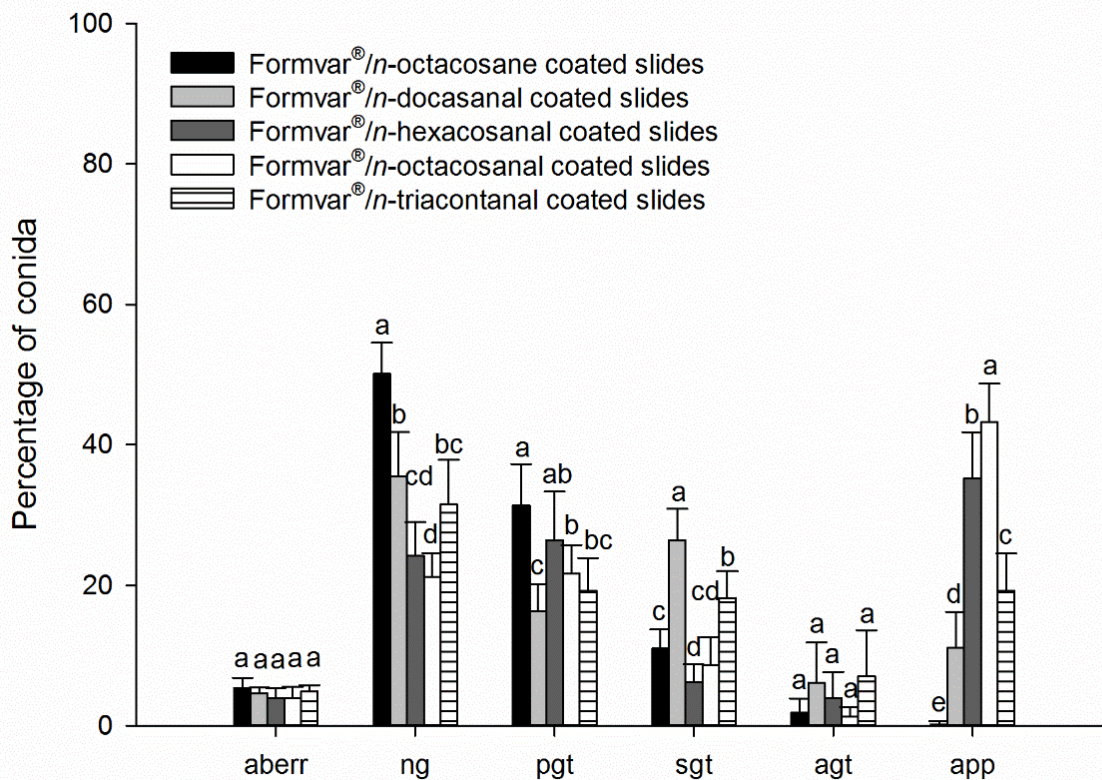
Figure 3.4 *In vitro* development of *Blumeria graminis* f. sp. *tritici* (BgtWue1) conidia on glass slides coated with different very-long-chain aldehydes. Slides were incubated for 18 h in darkness. Prepenetration developmental stages of conidia: aberr, damaged, burst or desiccated conidia; ng, non-germinated; pgt, with a primary germ tube only; sgt, with an elongated secondary germ tube; agt, with a swollen appressorial germ tube; app, fully differentiated appressorium. Each value is given as mean ± SD of five replicates with 100 conidia each of two independent experiments. Significant differences were determined in a Welch ANOVA followed by a Games-Howell *post hoc* test: different letters indicate significant differences ($P < 0.05$). (Zhu *et al.*, 2017)

3.2.3 *In vitro* ascospore prepenetration chronology

In order to characterize the timing of events regarding ascosporic prepenetration, development of single ascospores was monitored over 3 days after the discharge of ascospores from asci (**Figure 3.5**). To frequently check ascospores, chasmothecia were placed on Formvar®/wheat wax coated slides. More than 70 % of ascospores had formed first germ tube within the first 7 hours, while 5 % of them exhibited a secondary germ tube. None of the germinated ascospores showed patterns of appressorium formation at that time point. After 24 h around 80 % of the BgtWue1 ascospores had germinated and the proportion of germination was no further increased for the following 48 h. The maximal number of secondary germ tube formation was monitored at around 48 h after ascospore release. The first differentiated ascospores were seen at 33 h after spore discharge. The number of differentiated ascospores was 58 % within a time frame of 9 h (from 24 h to 33 h post-release). Consecutively, the proportion of differentiated ascospores stayed almost constant.
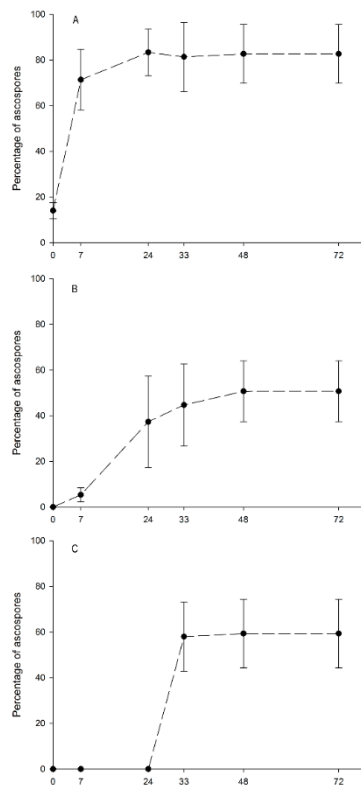
Figure 3.5 *In vitro* germination and differentiation of submerged *Blumeria graminis* f. sp. *tritici* (BgtWue1) ascospores on Formvar®/wheat wax coated slides. Development was followed over 72 h in darkness after ascospore release; (A) germination, formation of a first germ tube (B) secondary germ tube formation, (C) ascospores with appressoria. Each value is given as mean ± SD of three replicates, each monitoring 100 ascospores. (Zhu *et al.*, 2017)

3.2.4 *In vivo* and *in vitro* water relations in ascospore prepenetration

To further study ascosporic prepenetration processes, dry chasmothecia that still attached to dry host leaves were re-wetted on the moist filter paper in a petri dish. The lid of the petri dish was lined with glass slides coated with Formvar®/wheat wax, Formvar®/*n*-octacosane, Formvar®/wheat wax/agar or Formvar®/*n*-octacosane/agar. Seven days after re-wetting, the BgtWue1 chasmothecia had ejected ascospores. The ascospores that landed onto the coated surfaces were allowed to germinate and differentiate under humid conditions (>90% atmospheric humidity) (**Figure 3.6**). Neither Formvar®/*n*-octacosane/agar nor Formvar®/*n*-octacosane coated surfaces resulted in formation of appressoria. Interestingly, on Formvar®/wheat wax coated slides 55 % of the released ascospores had formed appressoria, while 25 % exhibited the formation of multiple germ tubes without distinct signs of differentiation of appressoria (**Figure 3.6** A). However, the percentage of differentiated ascospores statistically significant increased to 77 % and the proportion of ascospores with multiple germ tubes notably decreased to 6 %, when applied an agar layer beneath the membrane.

In addition, the germination and differentiation signs of released ascospores were analyzed. The proportion of germ tubes and appressoria per germinated and differentiated spore was quantified (**Figure 3.6**). Around 25 % of the differentiated ascospores on wheat wax coated slides had produced a single germ tube that had formed into an appressorium. The majority of 73 % exhibited 2 – 5 germ tubes with only single appressorium and a minor proportion (2 %) showed the formation of 2 – 5 germ tube and two of the germ tubes became appressoria. Interestingly, on wheat wax/agar coated slides, 96 % of the differentiated ascospores produced 2 – 5 germ tubes and one appressorium. Only 3 % of ascospores formed one germ tube that differentiated into an appressorium. The proportion of ascospores with 2 – 5 germ tubes and two appressoria was 1 %.

To determine more about the preferential prepenetration condition of ascospores, BgtWue1 chasmothecia were prepared on adaxial surfaces of detached wheat primary leaves or coleoptiles. The discharged ascospores were immersed in distilled water or under conditions of > 90 % relative air humidity (**Figure 3.7**). Intriguingly, in contrast to the experiments with coated glass slides *in vitro*, ascospores landed onto coleoptiles under high humidity conditions showed a notable formation of appressoria (76 %), however, only 53 % of the ascospores discharged in water showed the differentiation of an appressorium (**Figure 3.7** A). The numbers of ascospores that had formed 2 – 5 germ tubes but no appressorium were almost identical for both conditions (7%). Pretty similar results were found using primary leaf surfaces (**Figure 3.7** B). Consequently, under conditions of high air humidity, 87 % of the ascospores differentiated an appressorium. While a decreased number (67 %) of ascospores formed an appressorium when submerged in distilled water. Only 9 % of the ascospores showed the

differentiation of an appressorium from a single germ tube. While 89 % of ascospores had formed multiple germ tubes with one differentiating an appressorium. The proportion of ascospores that differentiated with two appressoria was 1 % (**Figure 3.7** C).
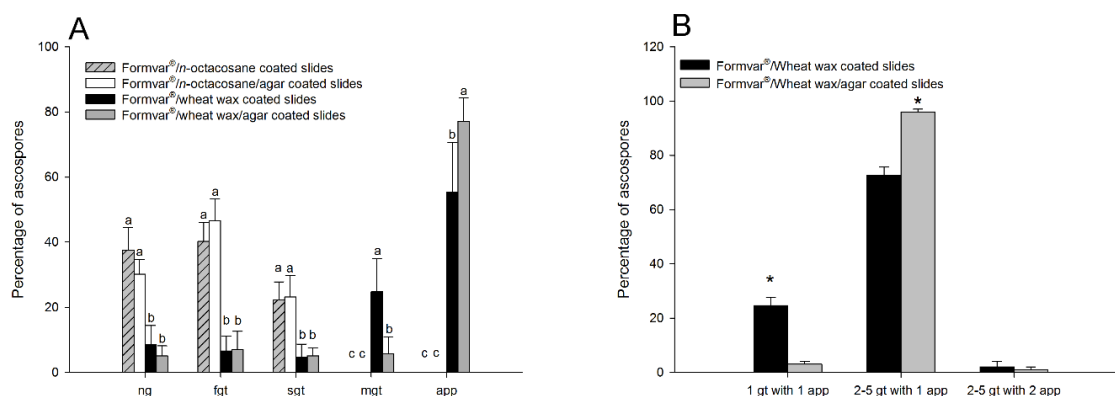
Figure 3.6 *In vitro* development of air-borne *Blumeria graminis* f. sp. *tritici* (BgtWue1) ascospores. Development on glass slides coated with Formvar®/*n*-octacosane, Formvar®/*n*-octacosane/agar, Formvar®/wheat wax or Formvar®/wheat wax/agar after incubation for 72 h in darkness with a high relative humidity (>90%). (A) Prepenetration developmental stages of ascospores: ng, non-germinated; fgt, with a first germ tube only; sgt, with an elongated secondary germ tube; mgt, with multiple non-differentiated germ tubes; app, with a fully differentiated appressorium (app). Each value is given as mean ± SD of five replicates with 100 conidia each of two independent experiments. Significant differences were determined in a Welch ANOVA followed by a Games-Howell *post hoc* test: different letters indicate significant differences ($P < 0.05$) (B) Proportions of developmental types of ascospores with fully differentiated appressoria: 1 germ tube (gt) with 1 appressorium (app), 2-5 gt with 1 app, 2-5 gt with 2 app. Each value is given as mean ± SD of three independent experiments with 100 ascospores each. Significant differences were determined in a Mann-Whitney U test: * significant differences ($P < 0.05$). (Zhu *et al.*, 2017)
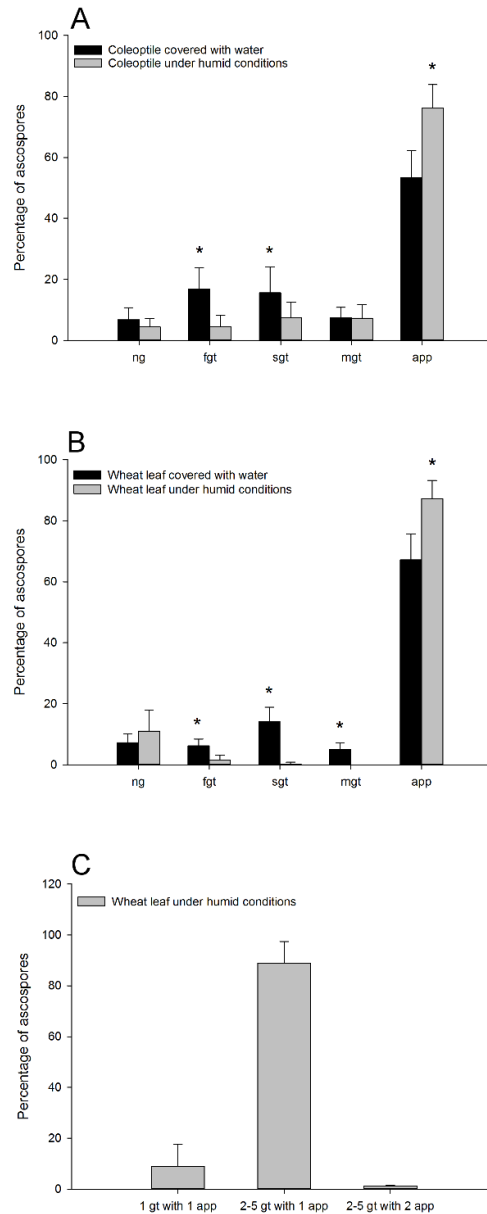
Figure 3.7 *In vivo* development of *Blumeria graminis* f. sp. *tritici* (BgtWue1) ascospores. Development on wheat coleoptile (A) and wheat primary leaf (B) surfaces immersed in water and under conditions of >90% relative humidity after 7d of incubation in darkness. Prepenetration developmental stages of *B. graminis* ascospores: ng, non-germinated; fgt, first germ tube formed; sgt, with an elongated secondary germ tube; mgt, with multiple non-differentiated germ tubes; app, with fully differentiated appressorium. (C) Proportions of ascospores with fully differentiated appressoria on wheat primary leaves under conditions of >90% relative humidity after 7d of incubation in darkness: 1 germ tube (gt) with 1 appressorium (app), 2-5 gt with 1 app, 2-5 gt with 2 app. Each value is given as mean ± SD of five replicates (50 ascospores each) of two independent experiments. Significant differences were determined in a Mann-Whitney U test: * significant differences ($P < 0.05$). (Zhu *et al.*, 2017)

Immersed conidia of BgtWue1, however, showed a significant decrease of germination and differentiation on coleoptile surfaces and glass slides coated with Formvar®/wheat wax (**Figure 3.8**). In contrast to conditions of high air humidity, the majority (ca. 60 %) of submerged conidia remained non-germinated phase and less than 10 % formed an appressorium.
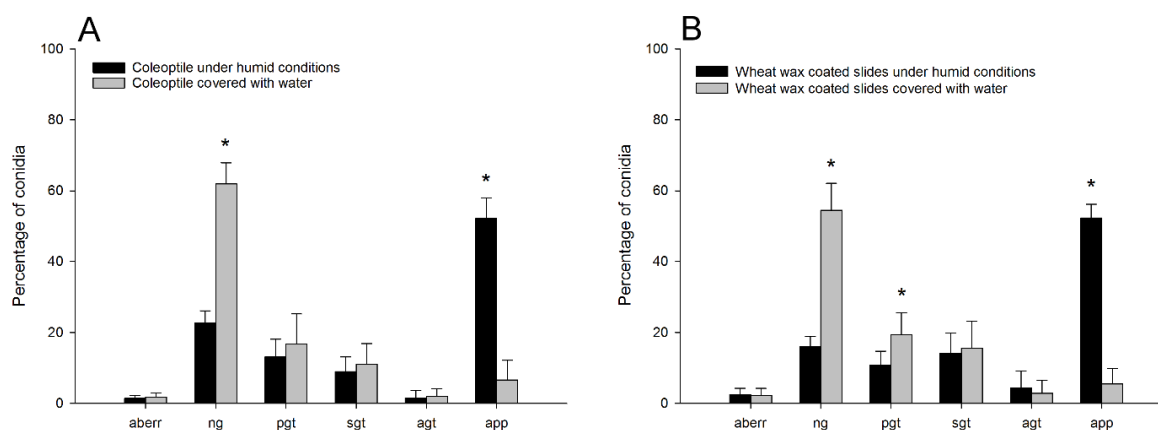
Figure 3.8 *In vivo* and *in vitro* development of *Blumeria graminis* f. sp. *tritici* (BgtWue1) conidia. (A) inoculated onto wheat coleoptiles and (B) onto Formvar®/wheat wax coated glass slides under submerse or high humidity conditions 18h after inoculation. For submerse conditions conidia were immersed in a 500 µl droplet of distilled water 30 min after inoculation. Prepenetration developmental stages of *B. graminis* conidia: aberr, damaged, burst or desiccated conidia; ng, non-germinated; pgt, with a primary germ tube only; sgt, with an elongated secondary germ tube; agt, with a swollen appressorial germ tube; app, fully differentiated appressorium. Each value is given as mean ± SD of five replicates with 100 conidia each of two independent experiments. Significant differences were determined in a Mann-Whitney U test: * significant differences ($P < 0.05$). (Zhu *et al.*, 2017)

3.2.5 Characterization of ascospore and conidium surfaces

To elucidate the characters of ascospore and conidium surfaces, spores were inoculated onto Formvar®/wheat wax coated cover-glasses and then analyzed by SEM (**Figure 3.9**). The ascospores showed the presence of a distinguishable reticulate network on the spore surfaces (**Figure 3.9** A and B). Similar structures were also monitored on ungerminated and germinated BgtWue1 conidia (**Figure 3.9** C and D), however, with a more prominent and much denser appearance. For ascospore and conidium, such irregular ridges were not observed on newly produced germ tubes or appressoria (**Figure 3.9** B and D).
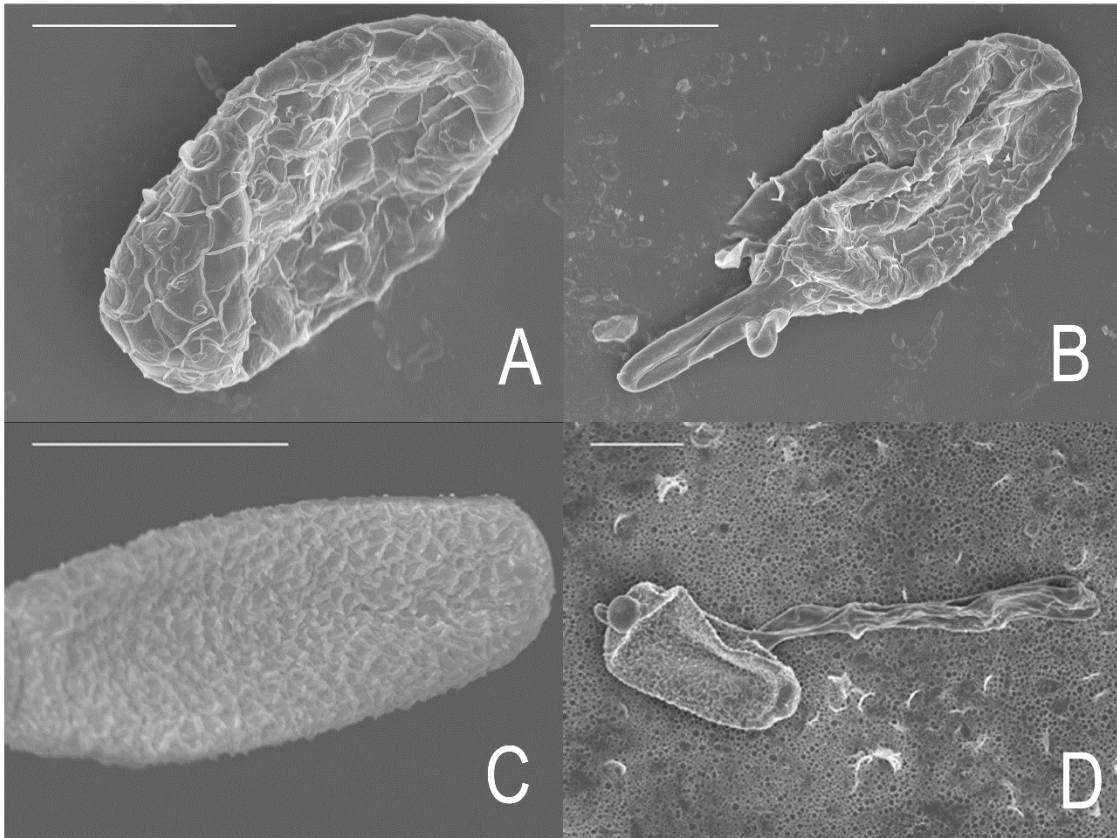
Figure 3.9 SEM pictures of *B. graminis* ascospores (A, B) and conidia (C, D) on glass slides coated with Formvar®/wheat wax. (A) Non-germinated ascospore. (B) Germinated ascospore with first germ tube. (C) Non-germinated conidium. (D) Fully differentiated conidium. Scale bars are 10 µm. (Zhu *et al.*, 2017)

3.3 Discussion

To date, there is only scarce study and information available on ascosporic prepenetration processes (Jankovics *et al.*, 2015). It is probably due to the fact that, in comparison to conidia, the direct observation of the discharge and germination of ascospores of *Blumeria graminis* is pretty difficult. So far, the majority of studies have primarily dealt with chasmothecium and ascospore formation pursuing the role of ascospores in fungal epidemiology (Wheeler *et al.*, 1973; Menzies & MacNeill, 1989; Bousset & de Vallavieille-Pope, 2003). It was reported that ascosporic formation and maturation were more rapid when the chasmothecia were immersed in water than when they were incubated in a moist atmosphere (Moseman & Powers, 1957). When incubated under humidity condition, the chasmothecia collected in July 2015 (at Botanical garden, Wuerzburg, Germany) required at least a period of 96 hours (4 days) until the discharge of the first mature ascospores. This incubation period is in accordance with the ca. 3 – 7 days demonstrated previously (Salmon, 1903; Koltin & Kenneth, 1970; Menzies & MacNeill, 1989; Jankovics *et al.*, 2015).

The earliest report on ascospore germination of *B. graminis* was published more than 140 years ago (Wolff, 1874). It was illustrated that Bgt ascospores were ejected from the asci into the air and needed around 6 h to form the first germ tubes after landing on a glass slide (Wolff, 1874). Wolff (1874) observed that, even in water, ascospores were able to form single or multiple germ tubes, but died soon afterward on the glass slide. The formation of one or two germ tubes producing from terminal or subterminal ascospore positions in water on glass slides was affirmed by a recent study (Jankovics *et al.*, 2015).

However, it was reported that, on host surface, ascospores frequently formed two to four germ tubes of the same type (Jankovics *et al.*, 2015). Some of the germ tubes, in some cases, then elongated and produced septate hyphae that were normally swollen towards their tips, evidently differentiating appressoria (Jankovics *et al.*, 2015). It was also demonstrated that in some cases, when regarding young colonies, all germ tubes of a single ascospore developed septate upon elongation eventually contributing to the infection phase (Jankovics *et al.*, 2015). In this study, the differentiation signs of ascospores *in vitro* and *in vivo* were also elucidated. Indeed, the formation of at best two appressoria originating from one ascospore was found, however, only in rare cases (**Figure 3.6** B and **3.7** C). On a suitable inductive substratum, the vast majority of *B. graminis* ascospores formed a non-differentiating first gem tube. Subsequently, ascospores produced one or several additional gem tubes. Then only one of the germ tubes finally differentiated into a septate appressorium. However, to date, there is no line of evidence that the ascosporic first germ tube functions in a same/similar way as the conidial primary germ tube involving anchorage (Edwards, 2002), uptake of water/solutes (Carver & Bushnell, 1983; Edwards, 2002), communication with the host cells (Yamaoka *et al.*,

2007) and nutrients from the plant apoplast (Edwards, 2002). In contrast to the asexually formed conidia, the more prominent variability in the sequence of ascosporic germination and differentiation might reflect their higher genetic diversity due to the processes of sexual reproduction.

3.3.1 *In vitro* ascospores germinate and differentiate more slowly than conidia

Approximately 70 % of *B. graminis* conidia were demonstrated to produce their primary germ tube within 2 hours after landing on a glass slide coated with Formvar®/host wax (Hansjakob *et al.*, 2010; Hansjakob *et al.*, 2012). Within the following 14 h, more than 80% of the germinated conidia differentiated infection structures (Hansjakob *et al.*, 2010; Hansjakob *et al.*, 2012).

Due to the fact that the prepenetration development of non-immersed *B. graminis* ascospores was dramatically affected by several relative air humidity changes during the microscopic analysis, immersed ascospores were followed to determine the prepenetration processes. After a 4 days period of ascospore maturation in the asci, the exact time/moment of ascospore discharge, however, cannot be predicted. Therefore, after noticing the first ascospores being discharged from the submerged *B. graminis* chasmothecia, the fate of the ascospores was monitored. Consequently, the germination and differentiation processes of ascospores were less synchronous than those of conidia. The ascosporic prepenetration processes hence showed a higher degree of variation. Nevertheless, more than half of ascospores germinated within the first 7 hours after the first observed spore release. This is in accordance with the report made by Wolff (1874). The formation of ascosporic appressorium was seen at 24 h and 33 h after the onset of observation. Hence, the results indicate that ascospore prepenetration processes take at least around twice the time illustrated for conidia. However, the observed ascosporic differentiation took place immersed in distilled water and not under high humidity conditions as for the monitored conidia. Therefore, the different conditions might have affected the time needed for prepenetration development.

3.3.2 Surface hydrophobicity and host wax promote ascospore prepenetration

In this study, the germination process of submerged ascospores was determined applying substrata with different contact angles (**Figure 3.2**). The experiments showed that an elevated surface hydrophobicity notably influences germination but apparently not the formation of appressoria, which is in contrast to the observation with conidia (Zabka *et al.*, 2008; Hansjakob *et al.*, 2010; Hansjakob *et al.*, 2011). About 70 % of the released ascospores had germinated in response to Formvar®/*n*-octacosane-coated surfaces (contact angles, 112 °), which is

dramatically exceeding the ca. 40 % of germinated BgtWue1 conidia on equivalent surfaces. Hence, these results suggest that surface hydrophobicity might play a more notable role in the initiation of germination of *B. graminis* ascospore. Despite slightly decreased surface contact angles (105 °), glass slides coated with Formvar®/wheat wax resulted not only in increased germination rates but also in a substantial percentage with differentiated appressoria (54 %). These results unequivocally indicate that - just as with conidia – constituents of the cuticular host leaf wax can stimulate prepenetration processes of *B. graminis* ascospore.

3.3.3 Very-long chain aldehydes and ascosporic prepenetration

The Formvar®-based coating *in vitro* system constitutes an effective platform. This system is a good tool to determine the influences of single wax components on the prepenetration phases of plant pathogenic fungi. In addition, the effect of other plant factors is remoted during analysis. Even-numbered very-long-chain aldehydes, especially the *n*-hexacosanal (C_{26}-aldehyde) and the *n*-octacosanal (C_{28}-aldehyde), were identified to be most effective in promoting germination and differentiation of *B. graminis* f. sp. *tritici* and f. sp. *hordei* conidia (Hansjakob *et al.*, 2010). Likewise, *in vitro n*-octacosanal followed by *n*-hexacosanal was the most effective aldehyde molecule capable of triggering ascospore and conidia differentiation of BgtWue1. As hypothesized for conidia (Hansjakob *et al.*, 2010), the chain-length dependent stimulation of ascosporic germination and differentiation may indicate a protein with a size-selective hydrophobic pocket or patch involved in the perception of host cuticular aldehydes. The results of this study hence strongly illustrate the involvement of the same or (a) very similar molecular mechanism(s) responsible for very-long-chain aldehyde perception in ascospores and conidia during prepenetration. While conidia of another Bgt strain used in a previous study (Hansjakob *et al.*, 2010), however, demonstrated a preference for *n*-hexacosanal (C_{26}-aldehyde) with a somewhat stronger response than C_{28}-aldehyde *n*-octacosanal. This might possibly indicate a specific adaption of Bgt to wheat wax composition, with C_{28}-aldehyde *n*-octacosanal as the most eminent aldehyde constituent (Hansjakob *et al.*, 2010). Therefore, the results of this study on BgtWue1 ascospores and conidia indicate the incidence of isolate-specific traits regarding to wax constituent preference within the same *B. graminis forma specialis.* The detailed molecular analysis of potentially involved candidate receptor proteins from different *B. graminis* strains or *formae speciales* showing a varied aldehyde chain-length preference might help in discovering the currently hidden molecular principles of aldehyde perception of the grass and cereal powdery mildew fungus *B. graminis*.

3.3.4 Water relations during ascospore prepenetration

It is well known that free water negatively affects the conidial germination of *B. graminis* (Manners & Hossain, 1963; Sivapalan, 1994; Iwamoto *et al.*, 2002). In line with this, germination and appressorium formation of submerged BgtWue1 conidia *in vitro* and *in vivo* were considerably reduced (**Figure 3.8**). It was shown that only 10 % of immersed conidia germinated on hydrophilic petri dish surface. However, in this study, 44 % of immersed conidia germinated. This might be attributable to the presence of very-long-chain aldehydes and the higher substratum hydrophobicity of the underlying surface. When ascospores were incubated in water, the germination was also reduced, however, at a much lower level. Submerged ascospores of *Erysiphe cichoracearum* were demonstrated to exhibit decreased germination percentage on clean hydrophilic glass slides (Wheeler *et al.*, 1973). With respect to differentiation, however, *B. graminis* ascospores exhibited a varied behavior than their counterparts – conidia. On coated glass slides, submerged *B. graminis* ascospores and ascospores under conditions of high relative humidity showed almost the same number forming appressoria (**Figure 3.2** and **Figure 3.5**). However, a substantial percentage of germinated ascospores formed 2 – 5 germ tubes without differentiating appressoria under conditions of high relative humidity. An almost fifty percent increase of differentiation values was found with a water agar layer placed underneath the Formvar®/wheat wax membrane. This indicates that an improved water availability under the condition.

On primary leaves and coleoptiles, however, the highest numbers of differentiation were found under the conditions of high relative humidity and not submerged in water (**Figure 3.7**). Since the released ascospores were ejected already in water from the asci on the leaves, this response may be attributable to the phenomenon demonstrated for conidia in a previous study (Yamaoka & Takeuchi, 1999). In such way, ascospores were regarded to be inoculated onto the hydrophilic surface of pre-wetted surfaces. This may have resulted in a weaker adherence of *B. graminis* ascospores and consequently in a decreased percentage of mature appressoria. While substantial differences in the formation of appressorium between submerse and high air humidity conditions were not found *in vitro*. Hence, it is tempting to hypothesize that the varied behavior of immersed ascospores on leaf surfaces might be attributable to a more intense leakage of osmotically active or possibly antifungal solutes from the leaf apoplast impairing ascosporic prepenetration processes. Under high humidity conditions, the percentage of differentiated ascospores on slides coated with Formvar®/wheat wax/agar almost fully matched those observed on natural host surfaces, indicating the functionality of the Formvar® -based *in vitro* system.

Likewise, the high percentage of differentiated ascospores on the primary wheat leaf surface and on slides coated with Formvar®/wheat wax/agar is almost the same as the

numbers obtained with conidia in a previous study (Hansjakob *et al.*, 2010), demonstrating comparably high prepenetration ability of Bgt ascospores and conidia.

It is found that the dramatical occurrence of ascospores forming multiple germ tubes on the coated slide surfaces and the notable reduction of this phenotype on natural host surfaces and on slides coated with Formvar®/wheat wax/agar. These indicate that upon failure to establish nutrient or water uptake from the surface ascospores might try to form multiple germ tubes to escape that situation. To date, it is not known whether at least one of functions of ascospore germ tubes in a similar/same way as the conidial primary germ tube, which exclusively breaches the cuticle and then provides access to nutrient and water reservoirs of the host apoplast in prepenetration processes (Carver & Bushnell, 1983; Edwards, 2002). More research is needed studying the fate of each single germ tube by more closely observing germination and establishment of the colony over time.

To find out whether specific surface cues that might contribute to the differential behavior of spores in the presence of free water, both types of spores – ascospores and conidia – were inoculated on cover glass slides coated with Formvar®/wheat wax and subjected to conventional SEM observation. The results of conidia were coherent with previous images describing a dense network of irregular ridges on the conidia surface (Johnson *et al.*, 1976). By low-temperature scanning electron microscopy observation, it was shown that the presence of such a reticulate network was on conidia surface in particular before germination. Due to the release of an extraconidial matrix, more and more this structure were disappeared and conidial surface was covered with spinelike or globular protrusions after surface contact the ridges (Kunoh *et al.*, 1988; Carver *et al.*, 1995). Nonetheless, the ascospore results show that, when compared with the conidial surface, the mesh aperture produced by this reticulate network is distinctly enhanced. This might suggest that the existence of a special spore wall structure in Bgt ascospores and conidia. By producing a more or less water-tight cuticle, conidia of *B. graminis* are well adapted to wind-distribution and prepenetration even under low atmospheric humidity conditions (Hammett & Manners, 1971; GAY *et al.*, 1985). Nonetheless, it remains to be illustrated whether the distinct features/characters of the *B. graminis* ascospore surface reflect the adaptation to more humid conditions during prepenetration processes.

3.4. Conclusions

Ascospore formation and maturation is an integral component of the ascosporic epidemiology of *Blumeria graminis*. Ascospores are most likely to be discharged following a period of lengthened rain when the fruiting body – chasmothecia – have imbibed water, and the asci are fully turgid (Wheeler *et al.*, 1973; Menzies & MacNeill, 1989). The results confirm that – unlike conidia – *B. graminis* ascospores are capable of effectively germinating, differentiating and penetrating host epidermis even when being submerged in water. The results indicate that released ascospores may be distributed not only by the wind but also by splashes of rainfall. However, in the absence of free water, a high relative atmospheric humidity is conducive and requisite to the prepenetration processes of *B. graminis* ascospores and conidia. Nonetheless, under humid conditions, the capability to additionally germinate and differentiate in water offers the ascospores an obvious biological advantage over conidia by broadening ascosporic ecological amplitude facilitating germination, differentiation and subsequent infection of the host plant even when the foliage is still wet after a rainfall (Wheeler *et al.*, 1973).

In contrast to conidia (Zabka *et al.*, 2008; Hansjakob *et al.*, 2010), the results show that a high surface hydrophobicity is not sufficient for stimulating appressorium differentiation of ascospore. This highlights the significance of the signal of cuticular very-long-chain aldehyde for ascosporic infections of *B. graminis.* Due to the fact that another powdery mildew species - *Erysiphe cruciferarum* – that infects dicotyledonous plants is also responsive towards very-long-chain aldehydes, the cuticular wax signal is debated to have been a powdery mildew pathogenicity determinant for the past 140 – 150 million years since divergence of monocot and dicot host species (Weidenbach *et al.*, 2014; Weis *et al.*, 2014). The results illustrate that this chemical signal is also conserved among asexually and sexually formed spore types and that its perception in ascospores is not influenced by the presence of free water on the host plant cuticle.

4 Chapter 2 Determination of UV-C irradiation affecting conidial prepenetration processes and photolyase gene transcription in barley powdery mildew fungus, *Blumeria graminis* f. sp. *hordei*

4.1 Introduction

Barley powdery mildew - *Blumeria graminis* f. sp. *hordei* (Bgh), is an obligate biotrophic fungus, which causes destructive foliar disease and inflicts severe economic losses in crop production. *B. graminis* is the world's sixth most crucial fungal pathogen (Dean *et al.*, 2012) and it has been regarded as one of the most destructive powdery mildews. The intensive application of fungicides against powdery mildews, trends to be less efficient and very costly to control the phytopathogens (McGrath, 2001; Suthaparan *et al.*, 2016a). Biological control of *B. graminis* has been attempted and achieved under practical conditions (Kiss, 2003; Laur *et al.*, 2018). However, biological control alone is considered to be not as effective as conventional fungicide treatments against fungal pathogens (Janisiewicz *et al.*, 2016a). More research is needed to explore alternative managements against *B. graminis.*

Ultraviolet (UV) light, consisting of UV-A (wavelength, 315 - 400 nm), UV-B (wavelength, 280 - 315 nm) and UV-C (wavelength, 100 - 280 nm), is considered as a potential mutation-causing agent (Suthaparan *et al.*, 2016b; Suthaparan *et al.*, 2018). It was illustrated that there was the potential in controlling of powdery mildews via the application of UV irradiation in several pathosystems (Michaloski, 1991; Suthaparan *et al.*, 2012a; Janisiewicz *et al.*, 2016b; Suthaparan *et al.*, 2016b). UV-B and UV-C light were demonstrated as powerful factors to suppress powdery mildew developments (Buxton *et al.*, 1957; Newsham *et al.*, 2000; Suthaparan *et al.*, 2012a; Suthaparan *et al.*, 2014; Janisiewicz *et al.*, 2016b; Suthaparan *et al.*, 2016a; Suthaparan *et al.*, 2017; Suthaparan *et al.*, 2018). The management of grape powdery mildew has been investigated by employing a mobile device to treat plants and pathogen with UV-C light in vineyards (Michaloski, 1991). It was shown that there was a negative effect of UV-B irradiation on *Erysiphe necator* conidial germination (Willocquet *et al.*, 1996).

B. graminis conidial germination and appressorium formation processes – the so-called prepenetration processes (**Figure 4.1**) – are prerequisites for the fungal infection and propagation. After contacting with a suitable host surface, the asexually produced conidia germinate and typically produce a primary germ tube (pgt), which attaches and then penetrates

the plant cuticle (Edwards, 2002; Yamaoka *et al.*, 2006). Afterward, a secondary germ tube (sgt) forms, stretches, swells and differentiates into an appressorial germ tube (agt). Later the agt develops into a lobed and septate appressorium (app) – an infection structure, which subsequently attempts to forcefully penetrate both host cuticle and the epidermal cell wall to form a feeding structure – a haustorium. The prepenetration processes are completed within about 12 – 15 h post-inoculation (Zhang *et al.*, 2005). The functional haustorium can take up water and nutrient to feed back to the appressorium to subsequently form secondary hyphae, mycelium and conidia chains.

UV-C was demonstrated to suppress conidial germination *in vitro* and to reduce infectivity in *B. graminis* (Buxton *et al.*, 1957). Boxton *et al.*, (1957) reported that a single UV-C dose of around 55 J m^{-2} resulted in a 95% reduction of infectivity in *B. graminis*, while the germination of *B. graminis* conidia that were inoculated onto ordinary glass slides prior to irradiation was reduced by less than 90%. However, there is no information available with the respects to the effects of UV-C irradiation on the differentiation of appressorial germ tubes and formation of functional appressoria in *B. graminis*. It was reported that a dark period followed the UV-C treatment was necessary to avoid photo-recover to control the fungal pathogen (Janisiewicz *et al.*, 2016a). *B. graminis* was demonstrated to process photoreactivation immediately after UV irradiation (Buxton *et al.,* 1957). However, it is not clear whether irradiated *B. graminis* conidia can process photoreactivation to complete the prepenetration processes after a dark incubation period.

In addition, UV-C has been shown to have deleterious impacts on proteins and DNA. Two major products indicative of DNA damage - cyclobutane-pyrimidine dimers (CPD) and pyrimidine(6–4)pyrimidone photoproducts – were induced after absorption of UV-C photons by DNA. Although both photoproducts lead to mutagenesis, the major of UV-C-induced products is the CPD, which ordinarily accounts for ca. 75% of UV-C photoproducts in DNA (Manova *et al.*, 2016). The photoproducts impede transcription, prevent genome replication and hence cell division. As efficient countermeasures, organisms develop a number of highly conserved repair mechanisms/processes, such as base excision repair, mismatch repair, nucleotide excision repair, and photoreactivation that utilizes blue or near UV light energy for repair (Rastogi *et al.*, 2010). Photoreactivation reverses CPD and 6-4 photoproducts into the intact monomer bases, catalyzed by a light-dependent photolyase enzymatic process (Thiagarajan *et al.*, 2011). Photoreactivation has also been illustrated for *B. graminis* and other powdery mildew fungi after UV-C treatment (Buxton *et al.*, 1957; Suthaparan *et al.*, 2018). The annotated *B. graminis* f. sp. *hordei* (DH14) genome (Spanu *et al.*, 2010) contains three members of the cryptochrome/DNA photolyase gene family encoding proteins that might be involved in photoreactivation processes. The first gene - BGHDH14_bgh00666 - codes for a protein (CCU77936.1) orthologous to CPD DNA photolyase CRY1 from the closely related pathogenic

gray mold fungus - *Botrytis cinerea*, in which this protein exhibits the major photolyase activity in photoreactivation (Cohrs & Schumacher, 2017). The product of the second gene - BGHDH14_bgh01427 (CCU75653.1) represents a 58% amino acid identity to the 6-4 DNA photolyase Cry1 of filamentous fungus - *Trichoderma reesei* (Guzmán-Moreno *et al.*, 2014) and thus may be also involved in 6-4 photoproduct repair activity. Both genes constitute the EggNog ortholog group: OG5_127186 in *B. graminis* (Huerta-Cepas *et al.*, 2016). A third gene, BGHDH14_bghG001129000001001, codes a putative Cry-DASH protein (CCU75260.1), whose orthologue Cry2 in *B. cinerea*, as in other fungi, appears to be largely dispensable for photorepair (Cohrs & Schumacher, 2017). Whereas, Cry-DASH proteins were demonstrated to repair CPD lesions in single-stranded DNA and loop structures of double-stranded DNA (Selby & Sancar, 2006; Pokorny *et al.*, 2008). To date, however, there is no information available regarding photolyase gene expression and transcriptional regulation in *B. graminis* and other powdery mildew pathogenic fungi.

It was demonstrated that UV-irradiated host tissue could enhance resistance against pathogens (Nigro *et al.*, 1998; Pombo *et al.*, 2011; Urban *et al.*, 2018). In order to exclude secondary effects caused by host tissue after UV-C application, besides *in vivo* studies, a Formvar® resin-based *in vitro* system was employed. This platform consists of a Formvar membrane/layer containing host leaf wax constituents or host leaf wax that trigger(s) the *B. graminis* prepenetration processes (Hansjakob *et al.*, 2010; Zhu *et al.*, 2017; Hildebrandt *et al.*, 2018). In addition, it provides reproducible homogeneous hydrophobic surfaces to permit analysis of the direct impact of UV-C and visible light irradiation on the prepenetration processes of Bgh conidia (Hansjakob *et al.*, 2012; Zhu *et al.*, 2017). Therefore, the central aims of the present research were: 1) to evaluate the influence of doses of UV-C light on the conidial prepenetration processes *in vitro* without host defense; 2) to study the photoreactivation processes in Bgh under different photoperiod conditions; 3) to determine the transcriptional changes of putative photolyase genes in this powdery mildew under UV-C and visible light irradiating conditions.

Non-germinated	Germinated			
	Not differentiated		Differentiated	
ng	pgt	sgt	agt	app
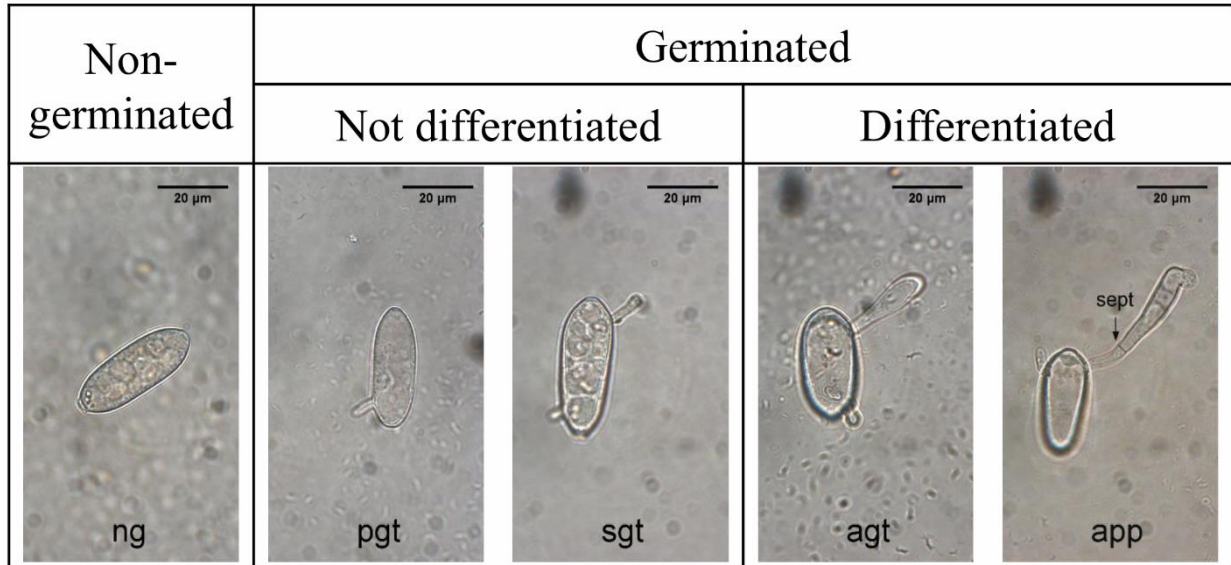
Figure 4.1 *In vitro* development of *B. graminis* f. sp. *hordei* conidia on glass slides coated with Formvar®/barley wax. Prepenetration processes of *B. graminis* conidia: ng, non-germinated conidium; pgt, germinated conidium with a primary germ tube only; sgt, conidium with a secondary germ tube; agt, conidium with a swollen appressorial germ tube; app, fully differentiated appressorium with a septum (sept).

4.2 Results

4.2.1 Effect doses of UV-C irradiation on *B. graminis* conidial prepenetration processes *in vitro* and *in vivo*

In initial experiments, *B. graminis* f. sp. *hordei* (Bgh) conidia were inoculated either on Formvar®/barley wax coated slides or on Formvar®/barley wax/agar coated slides. Subsequently, conidia were treated with different doses of UV-C irradiation and then incubated in darkness. Conidia treated without UV-C irradiance were as the control. Germination and differentiation of conidia was studied 18 h after initial treatment (**Figure 4.2** A and B). When compared with control, all of the UV-C treatments had a significantly negative effect on conidial germination and differentiation *in vitro.* When conidia were treated with 100 J m^{-2} of UV-C irradiance, the proportion of non-germinated conidia significantly increased to 76 ± 6 %. Consequently, the numbers of germinated and differentiated conidia notably decreased. When conidia were treated with 80 J m^{-2} or 100 J m^{-2} of UV-C irradiation, the formation of appressoria was not observed.

To find out whether UV-C irradiation was capable of efficient suppressing conidial germination and differentiation *in vivo*, conidia were inoculated on adaxial barley leaf surfaces. Then inoculated conidia were treated with different doses of UV-C irradiation and incubated in darkness for 18 h (**Figure 4.2** C). The similar results of UV-C-exposed conidia *in vivo* were found when compared with treated conidia *in vitro*. All of UV-C treatments significantly increased the percentages of non-germinated conidia and dramatically reduced the proportions of differentiated conidia when compared with the numbers of control. When conidia *in vivo* were irradiated with 100 J m^{-2} of UV-C, 68 ± 7 % of spores showed nongerminated patterns and none of them differentiated an appressorium.
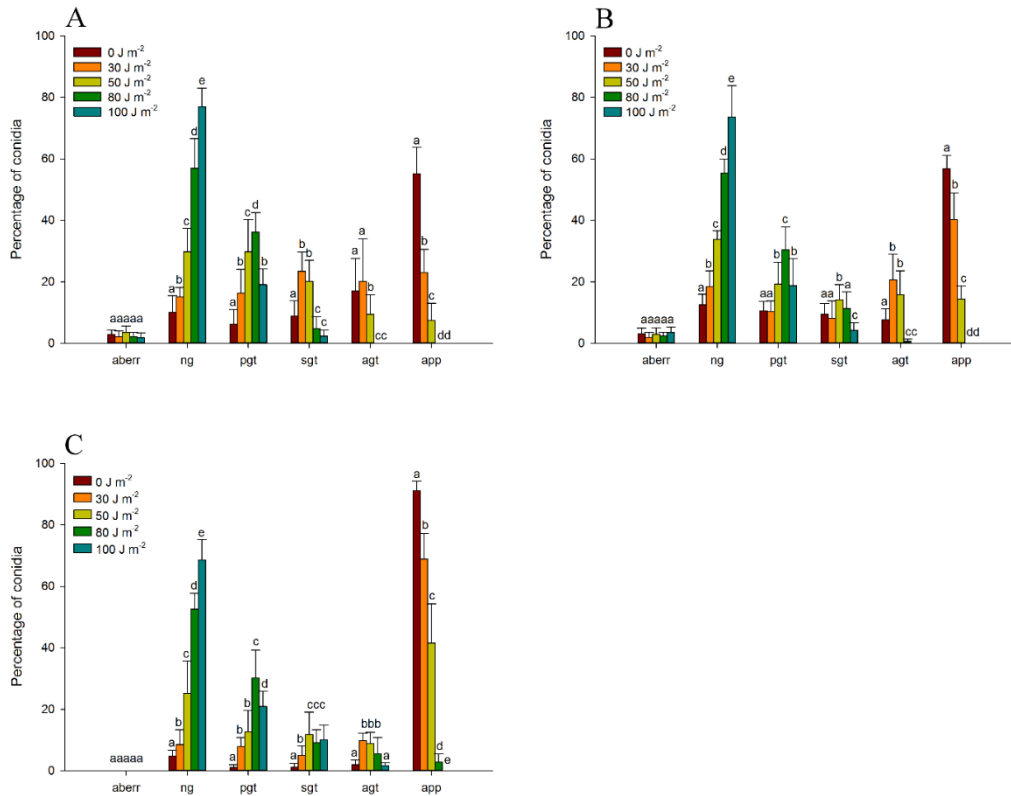
Figure 4.2 Development of *Blumeria graminis* f. sp. *hordei* conidia on (A) Formvar®/barley wax coated glass slides, (B) Formvar®/barley wax/agar coated glass slides and (C) adaxial surface of barley leaf after UV-C treatment and an incubation period of 18 h in darkness. Prepenetration developmental stages of *B. graminis* conidia: aberr, burst or dead conidia; ng, non-germinated conidium; pgt, germinated conidium with a primary germ tube only; sgt, conidium with an elongated secondary germ tube; agt, conidium with a swollen appressorial germ tube; app, conidium with a fully differentiated appressorium. In (A) and (B), each value is given as mean ± SD of six replicates (100 conidia each) of three independent biological experiments. In (C), each value is given as mean ± SD of three replicates (100 conidia each) of three independent biological experiments. Significant differences were determined in a Kruskal-Wallis test, with *post hoc* comparison by Bonferroni multiple comparison test: different letters indicate significant differences ($P < 0.05$).

4.2.2 Dark recovery of *B. graminis* conidia after UV-C treatment

In order to determine whether *B. graminis* could recover the capacity to germinate and differentiate after UV-C exposure and an incubation period in darkness, conidia *in vitro* and *in vivo* were treated with 100 J m^{-2} or 200 J m^{-2} of UV-C light. The germination and differentiation of conidia were studied after an incubation period of 48 h (**Figure 4.3**). When conidia *in vitro* were treated with 100 J m^{-2} or 200 J m^{-2} of UV-C irradiation, the proportion of nongerminated spores was 54 ± 9 % and 79 ± 4 %, respectively. Additionally, none of the irradiated conidia was able to form appressoria.

The numbers of nongerminated conidia *in vivo* were 52 ± 7 % and 78 ± 8 %, respectively, after UV-C treatment and dark incubation. Interestingly, when conidia *in vivo* were treated with 100 J m^{-2} of UV-C irradiance, 7 ± 2 % of spores could form appressoria. To compare the development of conidia in postpenetration processes, the percentages of haustorium only or haustorium with secondary hypha formed by conidia were recorded. In control group, 60 % of conidia formed haustoria or haustoria with secondary hyphae. However, when conidia were irradiated 100 J m^{-2} of UV-C, only 2 ± 2 % of spores could form haustoria or secondary hyphae. Neither haustorium nor secondary hypha was formed when conidia were treated with 200 J m^{-2} of UV-C irradiance.

When Bgh conidia were treated with 100 J m^{-2} of UV-C irradiance and incubated for 18 h in darkness, more than 70 % of spores did not germinate (**Figure 4.2**). However, when the exposed conidia were incubated in darkness for 48 h, the percentages significantly decreased to less than 55 % (**Figure 4.3**). Moreover, 7 % of exposed conidia were capable of forming appressoria when spores *in vivo* were treated with 100 J m^{-2} of UV-C irradiance and incubated for 48 h in darkness. While none of the conidia was able to differentiate when they were exposed with 100 J m^{-2} of UV-C irradiance and incubated for 18 h in darkness.
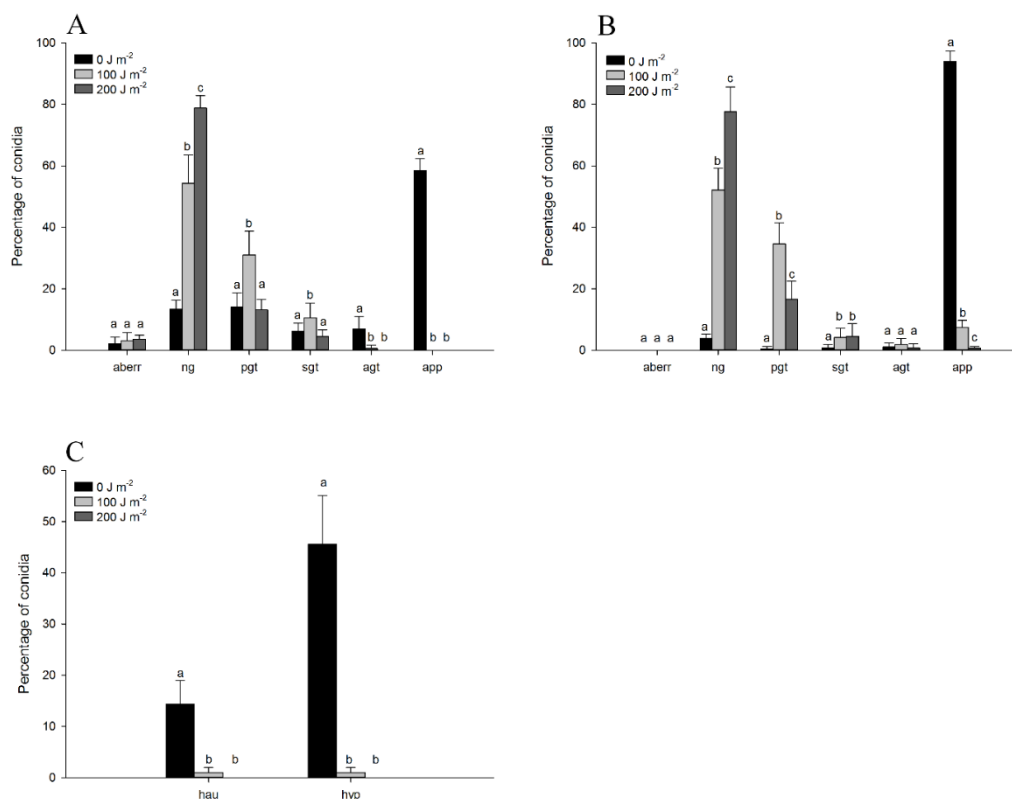
Figure 4.3 Development of *Blumeria graminis* f. sp. *hordei* conidia on (A) Formvar®/barley wax/agar coated glass slides and (B, C) adaxial surface of barley leaf after UV-C treatment and an incubation period of 48 h in darkness. Developmental stages of *B. graminis* conidia: aberr, burst or dead conidium; ng, non-germinated conidium; pgt, germinated conidium with a primary germ tube only; sgt, conidium with an elongated secondary germ tube; agt, conidium with a swollen appressorial germ tube; app, conidium with a fully differentiated appressorium; hau, fully differentiated conidium formed haustorium in the epidermal cell of barley; hyp, conidium formed haustorium and secondary hyphae on barley leaf surface. In (A), each value is given as mean ± SD of six replicates (100 conidia each) of three independent biological experiments. In (B) and (C), each value is given as mean ± SD of three replicates (100 conidia each) of three independent biological experiments. Significant differences were determined in a Kruskal-Wallis test, with *post hoc* comparison by Bonferroni multiple comparison test: different letters indicate significant differences ($P < 0.05$).

4.2.3 White light-mediated recovery of *B. graminis* conidia after UV-C treatment

To determine whether *B. graminis* could process photo-recovery to develop after UV-C exposure, conidia *in vitro* and *in vivo* were treated with different doses of UV-C light. The germination and differentiation of conidia were studied after a photoperiod of 18 h (white light/dark, 2 h/16 h) (**Figure 4.4**). *B. graminis* conidia were capable of efficiently utilizing white

light to recover and then to develop. After photo-repair processes, the proportions of nongerminated conidia *in vitro* and *in vivo* were less than 27 %. Furthermore, irradiated conidia were able to form appressoria. The percentages of UV-C-treated conidia with appressoria were gradient increased by reducing doses of UV-C irradiation and, however, were still significantly lower than the numbers of control (non-treated conidia).
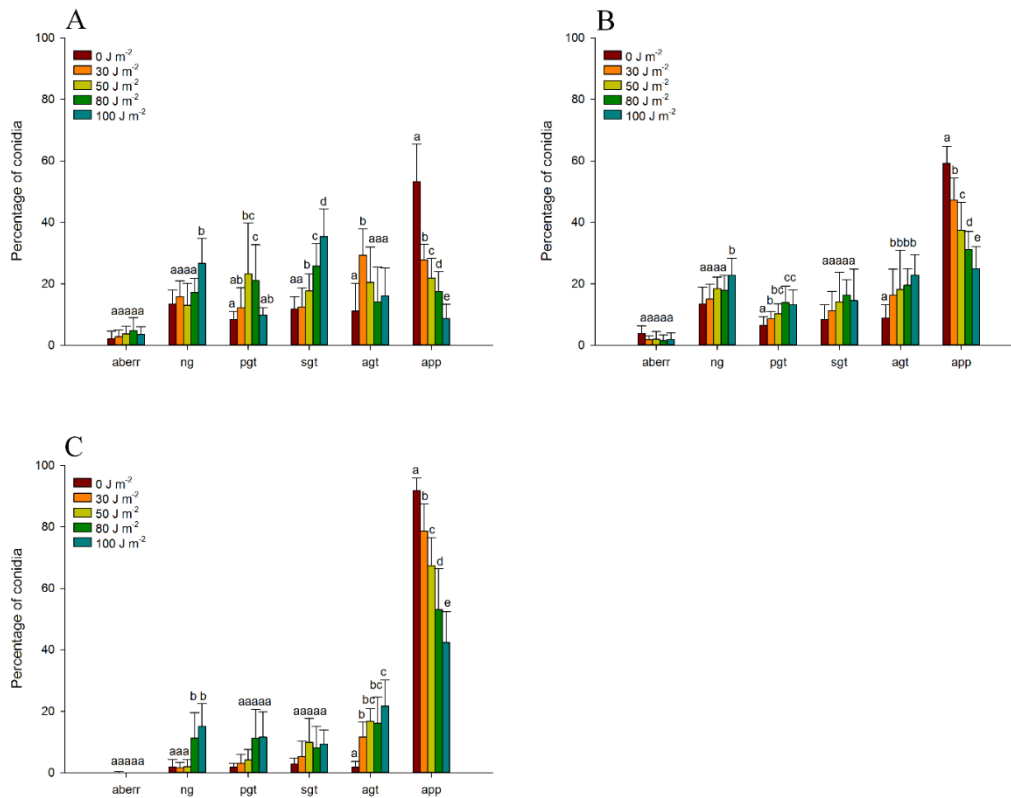

Figure 4.4 Development of *Blumeria graminis* f. sp. *hordei* conidia on (A) Formvar®/barley wax coated glass slides, (B) Formvar®/barley wax/agar coated glass slides and (C) adaxial surface of barley leaf after UV-C treatment and after an incubation period of 18 h (white light/ dark, 2 h/ 16 h). White light intensity, 234 µmol m^{-2} s^{-1}. Prepenetration developmental stages of *B. graminis* conidia: aberr, burst or dead conidia; ng, non-germinated conidium; pgt, germinated conidium with a primary germ tube only; sgt, conidium with an elongated secondary germ tube; agt, conidium with a swollen appressorial germ tube; app, conidium with a fully differentiated appressorium. In (A) and (B), each value is given as mean ± SD of six replicates (100 conidia each) of three independent biological experiments. In (C), each value is given as mean ± SD of three replicates (100 conidia each) of three independent biological experiments. Significant differences were determined in a Kruskal-Wallis test, with *post hoc* comparison by Bonferroni multiple comparison test: different letters indicate significant differences ($P < 0.05$).

4.2.4 Effect of dark incubation on prepenetration processes of *B. graminis* conidia treated with UV-C irradiation

To assay whether irradiated conidia could successfully process white light-mediated recovery to develop after a period of dark incubation, spores *in vitro* and *in vivo* were treated with 100 J m^{-2} or 200 J m^{-2} of UV-C irradiation. Then the exposed conidia were incubated under the photoperiod conditions for 18 h (dark/white light, 6 h/12 h) (**Figure 4.5** A and B). Compared with the numbers of nongerminated conidia in control groups, the proportions of nongermination of irradiated conidia were notably higher. Consequently, the percentages of differentiation of exposed conidia were significantly lower. When conidia *in vitro* and *in vivo* were treated with 100 J m^{-2} of UV-C irradiation, the numbers of nongerminated conidia were 49 ± 12 % and 54 ± 10 %, respectively. Furthermore, 7 % of conidia *in vitro* and 11 % of conidia *in vivo* were capable of differentiating. While with the treatment of 200 J m^{-2} of UV-C irradiation, the proportions of nongerminated conidia *in vitro* and *in vivo* significantly increased to about 70 %. Consequently, almost none of the exposed conidia were able to form appressoria.

To get an idea whether a longer period of white light treatment could improve the photo-recovery of *B. graminis*, irradiated conidia were incubated in a photoperiod of 24 h (dark /white light, 6 h/ 18 h) (**Figure 4.5** C and D). The rather similar results were found when conidia *in vitro* were irradiated with 100 J m^{-2} or 200 J m^{-2} of UV-C. The number of nongerminated conidia was 41 ± 8 % and 60 ± 9 %, respectively. Subsequently, the proportions of differentiated conidia were only 8 ± 4 % and 0 %, respectively. However, when conidia *in vivo* were exposed with 100 J m^{-2} of UV-C light, the proportion of conidia with appressoria was 20 ± 5 %. Nevertheless, none of the conidia *in vitro* and *in vivo* differentiated after treatment of 200 J m^{-2} of UV-C.

To test the infection ability of irradiated conidia, the percentages of haustorial formation or haustoria with secondary hyphae were recorded after a photoperiod of 24 h (dark/ white light, 6 h /18 h). Conidia incubated in darkness were performed as the control (**Figure 4.5** E). Accordingly, 14 ± 7 % of non-irradiated conidia were capable of forming haustoria, while almost none of the irradiated conidia were able to infect the host.
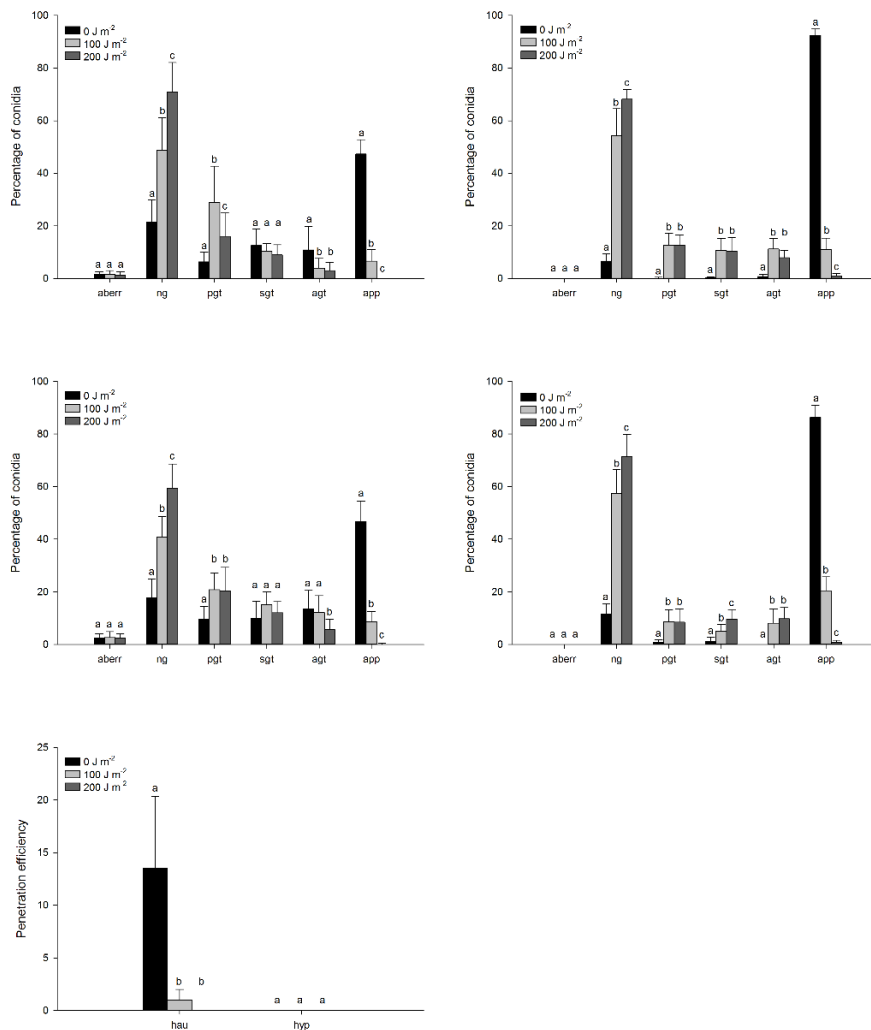
Figure 4.5 Development of *Blumeria graminis* f. sp. *hordei* conidia after UV-C treatment and an incubation period of 18 h (dark/light : 6h / 12h) (A and B) or 24 h (dark/light: 6h/ 18 h) (C to E) on different substrata. (A) and (C) Conidia on Formvar®/barley wax/agar coated glass slides; (B) (D) and (E) Conidia on adaxial surface of barley leaf. White light intensity, 234 µmol m^{-2} s^{-1}. Developmental stages of *B. graminis* conidia: aberr, burst or dead conidia; ng, non-germinated conidium; pgt, germinated conidium with a primary germ tube only; sgt, conidium with an elongated secondary germ tube; agt, conidium with a swollen appressorial germ tube; app, conidium with a fully differentiated appressorium; hau, conidium with haustorium in host epidermal cell; hyp, conidium with haustorium and secondary hypha. In (A) and (C), each value is given as mean ± SD of six replicates (100 conidia each) of three independent biological experiments. In (B), (D) and (E), each value is given as mean ± SD of three replicates (100 conidia each) of three independent biological experiments. Significant differences were determined in a Kruskal-Wallis test, with *post hoc* comparison by Bonferroni multiple comparison test: different letters indicate significant differences ($P < 0.05$).

4.2.5 The suppressing effect of UV-C irradiation on colony formation of *B. graminis*

To find out more about the suppressing effect of UV-C light on *B. graminis* prepenetration, infection and conidiation processes, conidia *in vivo* were treated with 100 J m^{-2} or 200 J m^{-2} of UV-C irradiation and incubated under a photoperiod (6 h dark/18 h light) condition for 5 days (**Figure 4.5**). Conidia treated without UV-C irradiation (control) showed pronounced infection and conidiation (formation of conidia chains - white powder like pustules) patterns (**Figure 4.6 A**). However, irradiated conidia on leaf surfaces rarely produced secondary hypha. The formed mycelium showed limited sizes and non-functional (non-conidiation) signs (**Figure 4.6 B**).

The exposed conidia in different developmental stages were also quantified to analyze the recovery and infection abilities of *B. graminis* after UV-C treatment (**Figure 4.6** C and D). The irradiated conidia were able to recover and finish prepenetration processes in a dosage-dependent manner. Conidia exposed with 100 J m^{-2} of UV-C light were capable of infecting host in a low percentage level (less than 25 %) and, however, were not able to produce conidia. After 200 J m^{-2} of UV-C treatment, conidia were rarely able to infect plant (the number of hypha formation was less than 5 %). The colonies emerging from UV-C exposed conidia three days after inoculation were significantly smaller than those formed from untreated conidia (**Table 4.1**). The dose of UV-light affected the average size of the colonies. Doubling the UV-C dose from 100 J m^{-2} to 200 J m^{-2} reduced colony size by around 50%. While after 4 days the first colonies of non-UV-C irradiated conidia started to produce conidia, the colonies of UV-C treated conidia did not exhibit conidiation even 5 d post-inoculation.

Table 4.1 Colony Size of *Blumeria graminis* f. sp. *hordei* after UV-C treatment and incubation period of 3 days

	Colony size (x 10^{-3} mm^2)
0 J m^{-2}	80 ± 28a
100 J m^{-2}	20 ± 8b
200 J m^{-2}	9 ± 4c

Note, conidia of *B. graminis* were treated with different doses of UV-C irradiation and incubated under photoperiod condition (Dark/light, 6/18 h) for 3 days. Colonies of *B. graminis* were stained by trypan blue and then photographed by light microscopy. The sizes of colonies were measured by imageJ using the freehand-selections function. Each value is given as mean ± SD of 50 individual colonies. Significant differences were determined in a One-way ANOVA with *post hoc* Tukey test: different letters indicate significant differences ($P < 0.05$).
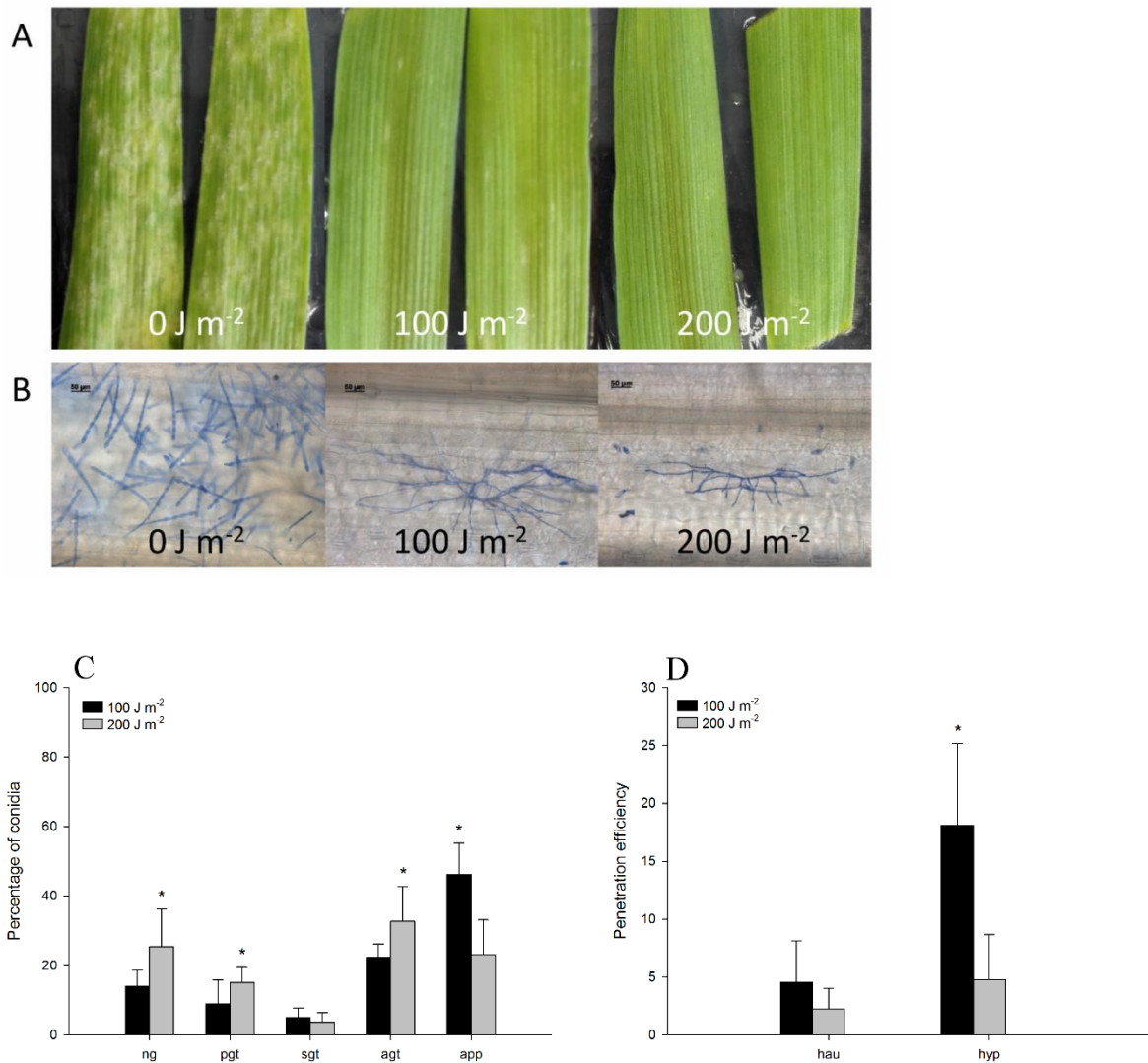
Figure 4.6 Development of *Blumeria graminis* f. sp. *hordei* conidia on adaxial surface of barley leaf after UV-C treatment and an incubation period of 120 h (dark/white light, 6h / 18h). Light intensity, 234 µmol m^{-2} s^{-1}. (A) Macroscopic pictures of pustules on barley leaf after conidia inoculation and UV-C treatments; (B) Microscopic pictures of conidial developments on adaxial surface of barley leaf after UV-C treatments; (C) and (D) Conidial development on adaxial surface of barley leaf. Developmental stages of *B. graminis* conidia: ng, non-germinated conidium; pgt, germinated conidium with a primary germ tube only; sgt, conidium with an elongated secondary germ tube; agt, conidium with a swollen appressorial germ tube; app, conidium with a fully differentiated appressorium; hau, fully differentiated conidium with haustorium in the epidermal cell of barley; hyph, conidium with haustorium and secondary hyphae on barley leaf surface. In (C) and (D) each value is given as mean ± SD of three replicates (100 conidia each) of three independent biological experiments. Significant differences were determined in a student T test: * indicates significant differences ($P < 0.05$).

4.2.6 UV-C irradiation induced upregulation of photolyase genes in *B. graminis*

In *B. graminis*, irradiated conidia could process photo-recovery under white light conditions (**Figure 4.4** to **4.6**). These might indicate that photolyases, the key enzymes utilizing white light/blue light to repair UV-induced damage, were involved in repairing phases. Because there is no stable and reproducible method/protocol of transformation of *B. graminis*, the transcriptional changes of photolyase gene were explored to determine the gene regulation processes in conidia under different irradiation conditions. RT-qPCR analyses were employed to check the transcriptional regulation of three putative photolyase genes (BGHDH14_bgh00666, BGHDH14_bgh01427 and BGHDH14_bghG001129000001001). In the initial experiment, conidia *in vitro* were irradiated with or without UV-C light (100 J m^{-2}). Subsequently, the conidia were incubated in darkness or under constant white light conditions for 30 min, 2 h or 6 h (**Figure 4.7**). Conidia treated without UV-C and incubated in darkness were performed as a control.

In the initial analyses, it was shown that the transcript levels of the putative photolyase gene BGHDH14_bgh00666 were significantly up-regulated about threefold 30 min and 2 h after UV-C irradiation, irrespective of whether the conidia had been incubated in white light or in darkness (**Figure 4.7** A and B). However, after 6 h in light the transcript levels in UV-C exposed conidia decreased to values seen for the conidia without UV-C irradiation, while the transcripts in the UV-C treated conidia incubated in darkness were around eightfold in number and thus remained at a significantly increased level (**Figure 4.7** C). For the putative Cry-DASH gene (BGHDH14_bghG001129000001001), induction patterns were rather similar to BGHDH14_bgh00666 and BGHDH14_bgh01427 30 min after UV-C irradiation (**Figure 4.7** D and G). The six-fold increase after 2 h post UV-C irradiation in darkness (**Figure 4.7** E) was followed by a more than 20-fold up-regulation 6 h after UV-C irradiation (**Figure 4.7** F). White light treatment, however, resulted in significantly decreased transcript numbers at 2 h and 6 h post UV-C irradiation. Transcripts of the putative 6-4 DNA photolyase gene (BGHDH14_bgh01427) showed only a 2-fold increase 2 h after UV-C irradiation (**Figure 4.7** H) and subsequent incubation in darkness while after 6 h the levels were increased by factor 6 (**Figure 4.7** I). Incubation under white light irradiation resulted only at 6 h post UV-C irradiation in a significant decrease of transcript numbers when compared with the conidia in controls.
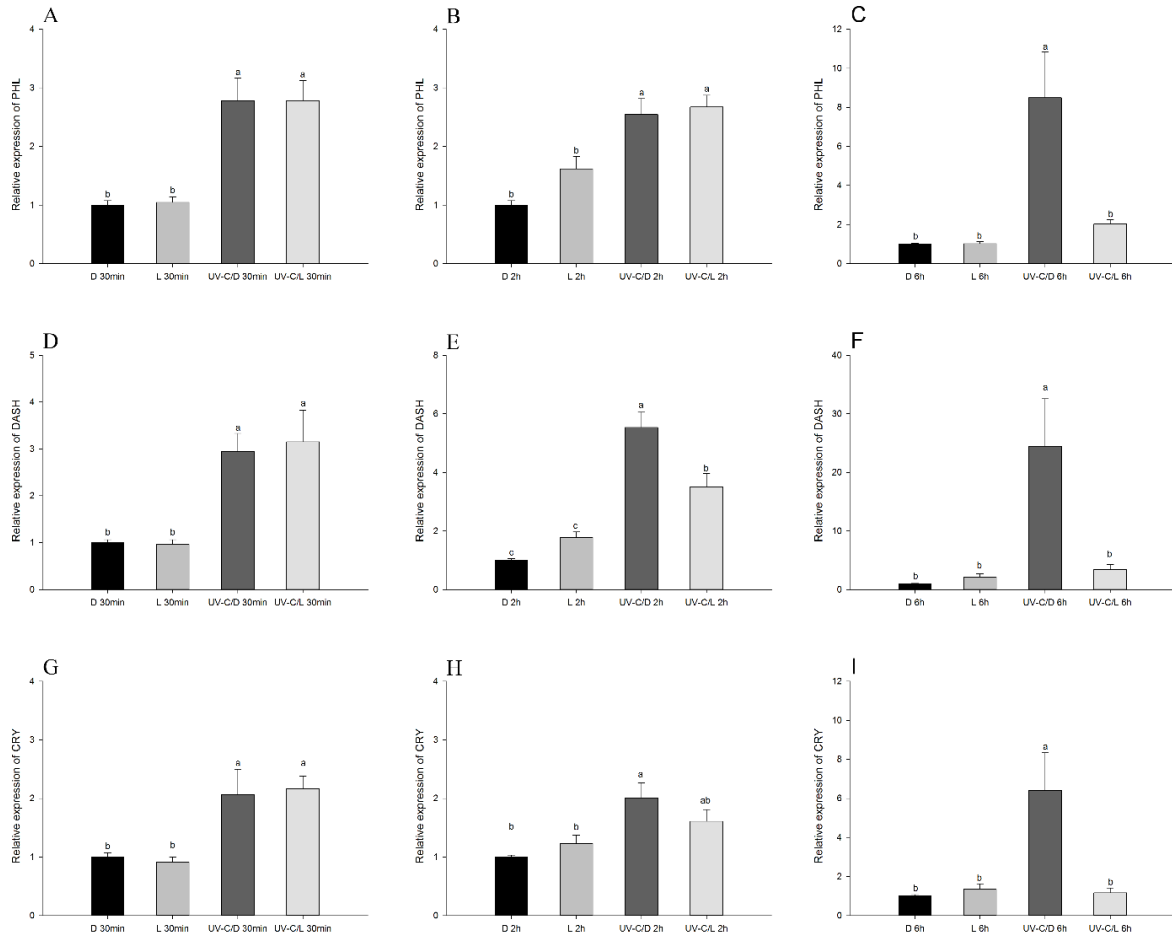
Figure 4.7 RT-qPCR analysis of putative photolyase gene expression in *Blumeria graminis* f. sp. *hordei* conidia after UV-C exposure *in vitro*. (A, B, C) Relative transcript levels of the putative CPD-photolyase gene BGHDH14_bgh00666 (PHL), (D, E, F) of the putative Cry-DASH encoding gene BGHDH14_bghG00112900000100 (DASH), and (G, H, I) of the putative 6-4 DNA photolyase gene BGHDH14_bgh01427 (CRY). Conidia on Formvar®/barley wax coated glass slides were irradiated or not with UV-C light (100 J m^{-2}) and then incubated with/without white light exposure (234 µmol m^{-2} s^{-1}) for (A, D, G) 30 min, (B, E, H) 2 h or (C, F, I) 6 h. D: dark; L: white light. GAPDH gene was employed as the reference gene. Each value is given as mean ± SE of three independent biological experiments. Significant differences were determined in a One-way ANOVA test, with *post hoc* Tukey test: different letters indicate significant differences (P < 0.05).

4.2.7 Transcription of photolyase genes in *B. graminis* were not induced by blue light

It is well known that blue light is the major source utilized by photolyases in photoreactivation (Mei & Dvornyk, 2015). In order to determine whether the transcription of photolyase genes was induced by blue light, conidia were treated with/without UV-C light (100 J m^{-2}) and then incubated in darkness or under blue light (wavelength peak, 475 nm) conditions. In addition, an intermediate incubation time – 2 h was selected (**Figure 4.8**). For BGHDH14_bgh00666, almost the same pattern was obtained as for the previous 30 min and 2 h white light-incubation. Transcripts were significantly up-regulated upon UV-C irradiation irrespective of light conditions (**Figure 4.8** A). For BGHDH14_bgh01427 and BGHDH14_bghG001129000001001 incubation in darkness after UV-C irradiation resulted in significantly increased values (twofold and eightfold, respectively). The blue light incubation, however, resulted in a distinct decrease of transcripts to levels, which were not significantly different with the non-UV/darkness controls (**Figure 4.8** B and C). LED blue light irradiation did not result in a significant transcriptional increase of the putative *B. graminis* photolyase genes.
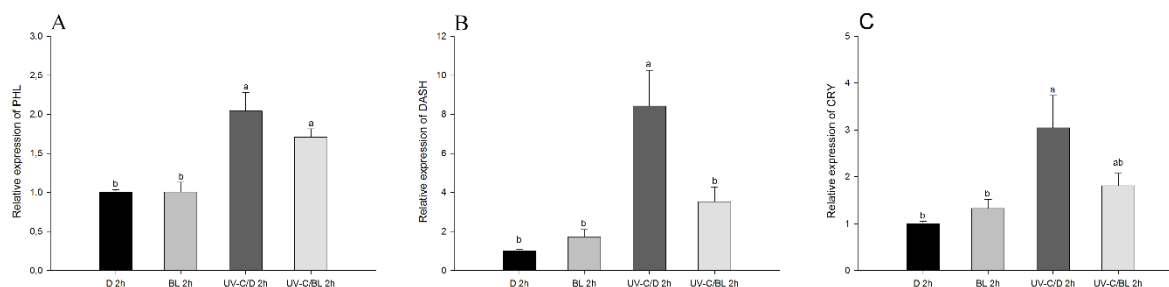
Figure 4.8 RT-qPCR analysis of putative photolyase gene expression in *Blumeria graminis* f. sp. *hordei* conidia *in vitro* on Formvar®/barley wax coated glass slides after UV-C exposure (100 J m^{-2}) followed by 2 h irradiation with/without blue light (80 µmol m^{-2} s^{-1}). Relative transcript levels of (A) the putative CPD-photolyase gene BGHDH14_bgh00666, (B) of the putative Cry-DASH encoding gene BGHDH14_bghG001129000001001, and (C) of the putative 6-4 DNA photolyase gene BGHDH14_bgh01427. D: darkness; BL: blue light. Each value is given as mean ± SE of three independent biological experiments. Significant differences were determined in a One-way ANOVA test, with *post hoc* Tukey test: different letters indicate significant differences (P < 0.05).

4.3 Discussion

The suppression effect of UV irradiation on development of several powdery mildews was reported in previous studies (Buxton *et al.*, 1957; Willocquet *et al.*, 1996; Suthaparan *et al.*, 2012a; Suthaparan *et al.*, 2014; Janisiewicz *et al.*, 2016b; Suthaparan *et al.*, 2016a). However, to date, the effects of UV irradiation on the differentiation of appressorial germ tubes and formation of functional appressoria in *Blumeria graminis* are not clear.

In this study, it was demonstrated that when treated with UV-C light, conidial development *in vitro* and *in vivo* was suppressed in a dosage-dependent manner, indicating the potential of UV-C irradiation to control *B. graminis*. It was shown that powdery mildew in vineyard could be suppressed by employing UV-C emission device (Michaloski, 1991). Recently, it was reported that the application of UV-C light was efficiently able to control strawberry and tomato powdery mildew fungi (Janisiewicz *et al.*, 2016b; Suthaparan *et al.*, 2016b). The emerging interest of controlling powdery mildew by UV-C irradiation is probably due to the trend that powdery mildew is becoming more and more selectively developing resistant to various antifungal agents, which are very costly and non-environment-friendly (McGrath, 2001). The application of UV-C treatment shows an attractive alternative method to control *B. graminis in vitro* and *in vivo* because the UV-irradiation is a transient exposure process and does not leave any residue harmful to the environment after application (Janisiewicz *et al.*, 2016a).

4.3.1 UV-C mediated suppression of conidial prepenetration processes *in vitro* and *in vivo* after dark incubation

In previous studies, it was reported that UV light irradiation ranging from 125 to 500 J m^{-2} could enhance the resistance of plant against pathogens (Nigro *et al.*, 1998; Urban *et al.*, 2018). In this study, besides *in vivo* assays, the Formvar®-based *in vitro* system additionally was applied. This system is an effective tool for analyzing the direct effects of UV-C irradiation on conidial prepenetration processes of *B. graminis.* In addition, the platform can remote the influence of other plant factors during assays. When conidia were treated with UV-C irradiation and incubated in darkness for 18 h or 48 h, rather similar suppression effects were found *in vitro* and *in vivo.* The proportions of nongerminated conidia were increased and the percentages of differentiated conidia were decreased by increasing the doses of UV-C applied. The conidia, which were treated with 100 J m^{-2} of UV-C irradiation and incubated for 48 h in darkness, showed significantly lower percentages of nongermination when compared with proportions of irradiated conidia that were incubated in darkness for 18 h. In line with this, it might suggest that, like many organisms (Hu & Adar, 2017), a dark repair mechanism was processed in the

prepenetration phases of irradiated powdery mildew conidia. Nevertheless, 100 J m^{-2} of UV-C irradiation were capable of efficiently suppressing the germination, differentiation and infection of *B. graminis* conidia incubated in darkness (**Figure 4.2** and **4.3**).

It was shown that the growth of *B. cinerea* on strawberries was significantly retarded when the fungus was treated with more than 50 J m^{-2} of UV-C irradiance (Marquenie *et al.*, 2002). However, developments of *Monilinia fructigena* on sweet cherry was illustrated to be impaired only when applied UV-C irradiance at the doses of ≥10000 J m^{-2} (Marquenie *et al.*, 2002). Janisiewicz, *et al.* (2016) reported that it might be useful in controlling *B. cinerea* by irradiating strawberry with UV-C for 60 s (12.36 J m^{-2} of UV-C). However, it was demonstrated that strawberry powdery mildew – *Podosphaera aphanis* was significantly reduced by being exposed UV-C for 15 s (3.09 J m^{-2} of UV-C) (Janisiewicz *et al.*, 2016b). Doses of UV-C applied in various studies differed among fungal species might due to the facts that, on one hand, some fungal spores have melanin-containing dark pigmented cell walls (Begum *et al.*, 2009) and, on the other hand, UV-C irradiation can induce the plant tissue resistance against pathogens (Nigro *et al.*, 1998; Mercier *et al.*, 2000; Pombo *et al.*, 2011; Urban *et al.*, 2018). In this study, the dose of UV-C light efficiently suppressing *B. graminis* conidial germination and differentiation was ≥ 100 J m^{-2}, which was higher than the dose used (3.09 J m^{-2}) for controlling *P. aphanis* (Janisiewicz *et al.*, 2016b). The notable differences of UV-C doses might be due to that *B. graminis* conidia contain 3-hydroxykynurenine, which is hypothesized as a UV protectant (Wilson *et al.*, 2003). More study is needed determining the function of anti-UV pigment in powdery mildews to provide more information for designing and managing the application against phytopathogens.

4.3.2 Photoreactivation in Bgh conidial prepenetration processes

It is well known that photolyases of many organisms can utilize white light/ blue light to repair UV-induced damaged DNA (Mei & Dvornyk, 2015). This light-dependent DNA repairing mechanism – the so-called photoreactivation processes - was shown in several phytopathogenic fungi (Alejandre-Duran *et al.*, 2003; Brych *et al.*, 2016; Cohrs & Schumacher, 2017). To date, however, there is only scarce information on the photoreactivation in powdery mildew fungi (Suthaparan *et al.*, 2018). It was reported that *Oidium neolycopersici* conidia could process photo-recovery under light condition (Suthaparan *et al.*, 2018). In order to investigate the photoreactivation of *B. graminis*, conidia *in vitro* and *in vivo* were treated with doses of UV-C light and incubated under photoperiod condition (white light/dark, 2 h/ 16 h) (**Figure 4.4**). After a photo-incubation period, more than 75 % of the irradiated conidia were able to germinate. In addition, the appressorium formation was in a UV-C dose-dependent

manner. This might indicate that (a) photolyase(s) of *B. graminis* conidia utilized light energy to repair the damage caused by UV-C irradiation.

It is well known that *B. graminis* conidial germination and appressorium formation were triggered by very-long-chain aldehydes, the constituents in plant cuticular wax (Zabka *et al.*, 2008; Ringelmann *et al.*, 2009; Hansjakob *et al.*, 2010; Zhu *et al.*, 2017). In this study, the recovered conidia *in vivo* and *in vitro* were capable of perceiving signals from the host and artificial surfaces to germinate and then to differentiate (**Figure 7.1**). These might suggest that UV-C irradiation has no/little effect on very-long-chain aldehydes *in vivo* and *in vitro*. In line with this, it might indicate that the Formvar®-based *in vitro* system was a suitable and stable platform to study conidial prepenetration processes of *B. graminis*.

The results illustrated that, like many fungi (Braga *et al.*, 2015), the irradiated *B. graminis* conidia could process the photoreactivation under a photoperiod condition (white light/dark, 2 h/16 h) (**Figure 4.4**). However, it is not clear whether *B. graminis* conidia can operate the photoreactivation after an incubation period in darkness. In practice, the UV treatment applied in the nighttime showed a successful and efficient management against powdery mildews (Suthaparan *et al.*, 2012a; Suthaparan *et al.*, 2012b; Suthaparan *et al.*, 2014). In line with this, conidia were treated with UV-C irradiation (100 J m^{-2} or 200 J m^{-2}) and incubated in photoperiod conditions (dark/ white light, 6 h/12 h or 6 h/18 h) to investigate the ability of photo-repair of photolyase. Interestingly, it was found that after the initial incubation in darkness for 6 h, conidia could repair UV-induced damage by photoreactivation. However, the percentages of differentiated conidia were still less than 20 %. Moreover, there was rarely penetration of appressoria of recovered conidia. It was shown that the irradiated conidia of *B. cinerea* were not able to cause the infection in apple wounds when the samples were initially incubated in darkness for 4 h (Janisiewicz *et al.*, 2016a). It was reported that the colonization of UV-treated *P. aphanis* was negatively affected by dark incubations (≥ 4 h)(Janisiewicz *et al.*, 2016b). Suthaparan, *et al.* (2017) reported that there was a significant effect of photo-incubation on the germination of irradiated conidia of *O. neolycopersici*. So far, it is not known whether *B. graminis* can infect hosts if conidia are treated with the dose of ≥ 100 J m^{-2} of UV-C irradiation and then incubated in darkness for < 6 h. More research is needed following the (pre)penetration processes of the irradiated conidia of powdery mildew under different photoperiod conditions to expand the knowledge of photoreactivation in this powdery mildew.

It was reported that the visible symptoms and conidial production of *P. aphanis* on strawberry plants were dramatically reduced after the UV-C application (Janisiewicz *et al.*, 2016b). In this study, rather similar results were found when the irradiated conidia of *B. graminis* were incubated under a photoperiod (dark/ white light, 6 h/ 18 h) condition for 5 d (**Figure 4.6**). By macroscopy analyses, no obvious pustule symptoms of the irradiated samples

were observed. However, plants in control (no irradiation) were highly infected and showed white powder like pustules (the formation of conidia-chains). With microscopy study, the recovered conidia showed the capability of forming appressoria, which infected host epidermal cells and formed mycelium. However, the young colonies were not able to propagate. Moreover, the recovered conidia showed rarely infection and propagation abilities, indicating the doses of UV-C (≥ 100 J m^{-2}) applied in this study was sufficient to control *B. graminis* infection and propagation.

4.3.3 UV-C light, neither white light nor blue light, induced up-regulation of photolyase genes in *B. graminis*

The genome of *B. graminis* is sequenced and published (Spanu *et al.*, 2010; Wicker *et al.*, 2013). In the database (e.g. NCBI), it is shown that there are three putative genes encoding photolyases in *B. graminis*. The photo-recover processes might indicate that (a) photolyase(s) utilized blue light to repair the UV-induced damage in *B. graminis.* However, to date, in powdery mildew fungi, the transcriptional regulation of photolyase genes is not known. In line with this, RT-qPCR analyses were applied, for the first time, to investigate the transcriptional changes of these putative photolyase genes in conidia of powdery mildew under different irradiation conditions (**Figure 4.7** and **4.8**). Transcriptional analyses showed that the transcription of photolyase genes in the irradiated conidia was significantly up-regulated after 30 min incubation under the UV-C irradiation condition. This suggested that the photolyase gene transcription was mediated in a UV-C-treatment-dependent manner. After an incubation period of 6 h under the white light condition, the expression levels were not significant between the non-irradiated and exposed conidia. Compared to control, by contrast, the transcript levels of conidia incubated in darkness were still remarkably higher 6 h post-inoculation. In accordance with the results that the irradiated conidia preliminarily incubated in darkness and then under white light conditions can repair UV-induced damage, the data indicated that *B. graminis* was still capable of operating photo-repair processes even after 6 h. This similar phenomenon was described in a previous study, which showed after an incubation period of 8 h in darkness the irradiated conidia of tomato powdery mildew fungus - *O. neolycopersici* could process the photo-recover under light conditions (Suthaparan *et al.*, 2018). Furthermore, it was reported that the transcription of photolyase genes in some fungi (*Trichoderma atroviride, Cercospora zeae-maydis* and *Ustilago maydis*) were significantly up-regulated by blue light or white light (Berrocal-Tito *et al.*, 2007; Bluhm & Dunkle, 2008; Brych *et al.*, 2016). Interestingly, unlike those fungi, the transcription of photolyase genes in powdery mildew was induced by neither white light nor blue light.

4.4 Conclusions

Powdery mildew fungus, *B. graminis,* is one of the most destructive fungal pathogens in crop production (Dean *et al.*, 2012). The results in combination with previous data (Buxton et al. 1957) confirm that – like many other powdery mildews (Newsham *et al.*, 2000; Suthaparan *et al.*, 2012a; Suthaparan *et al.*, 2014; Suthaparan *et al.*, 2016a) – the germination and differentiation of *B. graminis* conidia can be efficiently suppressed by applying UV-C irradiation. In the absence of light, conidial prepenetration and infection processes are inhibited by UV-C irradiation in a dose-dependent manner. The data also suggests that it is efficient to hamper conidial germination and differentiation of *B. graminis* f. sp. *hordei* by employing with ≥100 J m^{-2} of UV-C irradiation. These findings demonstrate that UV-C can be employed as the suppressor factor against *B. graminis*.

Photolyases, the enzymes utilize blue light or near UV light to repair UV-induced damage in photoreactivation, are well known in many organisms (Mei & Dvornyk, 2015). The results suggest that under light conditions, the irradiated conidia can process photo-recovery (photoreactivation). Additionally, the data, for the first time, illustrates that, in contrast to other fungi (Berrocal-Tito *et al.*, 2007; Bluhm & Dunkle, 2008; Brych *et al.*, 2016), the transcripts of photolyase genes in this powdery mildew fungus is up regulated only under the UV-C irradiation condition, indicating the transcription is induced in a UV-C-mediated-dependent manner. Neither white light nor blue light can significantly induce the transcriptional changes of photolyase genes in *B. graminis*. On one hand, the ability of repair UV-induced damage under the white/blue light condition gives this powdery mildew fungus an obvious biological advantage by enhancing the physiological amplitude facilitating survives and subsequent infection of the host plant even when conidia are exposed to UV light. On the other hand, the capability of photoreactivation of powdery mildew should be taken into consideration when applying the UV-mediated management in fungal pathogen controls. The results demonstrate that the specific optimization of dose of UV-C light (e.g. ≥ 100 J m^{-2}), right application time (e.g. 6h dark period following irradiation) and frequency (e.g. every 3 days) can enhance the efficiency of UV-C-mediated management against powdery mildew on the agriculturally important crops wheat and barley.

5 Chapter 3 Influence of light conditions on conidial germination, differentiation, appressorial elongation and penetration processes of powdery mildew fungus, *Blumeria graminis*

5.1 Introduction

The grass powdery mildew fungus *Blumeria graminis* is among the most prevalent fungal plant pathogens (Dean *et al.*, 2012). After initial contact with the host surface, the wind-distributed asexual conidia of this obligate biotrophic ascomycete germinate by producing a primary germ tube, which then attaches to the leaf surface forcing a short peg through the host cuticle, probably enabling nutrient uptake and water from the host apoplast (Carver & Bushnell, 1983; Edwards, 2002; Yamaoka *et al.*, 2006). Subsequently, a secondary germ tube elongates that swells and differentiates to an appressorial germ tube, which matures and becomes a lobed, apical appressorium with a penetration peg formed to pierce both host cuticle and cell wall. These so-called prepenetration processes of *B. graminis* are completed 12-15 h post-inoculation. Among the Erysiphales, *B. graminis* is unique in producing a primary germ tube before a second germ tube that finally differentiates to the appressorium.

Several studies have illustrated that, specifically very long-chain aldehydes (C_{22} -C_{30}), present in many plant cuticular waxes, promote germination and appressorium differentiation of *B. graminis* conidia (Tsuba *et al.*, 2002; Zabka *et al.*, 2008; Ringelmann *et al.*, 2009; Hansjakob *et al.*, 2010; Hansjakob *et al.*, 2011; Hansjakob *et al.*, 2012). Besides the chemical composition of the plant surface, a multitude of environmental factors, such as surface hydrophobicity, atmospheric humidity, temperature and light can significantly affect the prepenetration processes (Buxton *et al.*, 1957; Masri & Ellingboe 1966; Carver *et al.*, 1990 Zabka *et al.*, 2008 Ringelmann *et al.*, 2009).

It was reported that the improved development of powdery mildews in shade than in full natural sunlight (Yarwood, 1957; Schnathorst, 1965). However, at least in part, this stimulating shade effect could be due to a decreased host surface temperature and increased humidity rather than a direct consequence of decreased irradiation. Similarly, the greater severity of powdery mildew disease with close than with more distant spacing of plants under field conditions could be explained by a lower light intensity per plant but also might be primarily due to other microclimatic causes (Tompkins *et al.*, 1992).

However, so far most studies focused on the effect of light on the later stages of *B. graminis* infection such as formation of haustoria and conidiogenesis (Carver *et al.*, 1994; Nakamura *et al.*, 2007), Masri & Ellingboe (1966) reported that light distinctly affected the maturation of *B. graminis* f. sp. *tritici* appressoria on wheat leaves. Maturation was favored by low illumination intensity (2.6 klx) and inhibited by high intensity (30 klx) and in darkness. Whereas, within 10 hours after inoculation, all light treatments, including darkness, resulted in more than 85 % conidia that had formed mature appressoria. Likewise, continuous lighting (at approx. 30 µW m^{-2} s^{-1}) during the first 12 h of infection resulted in a retardation of *B. graminis* f. sp. *hordei* appressorium formation (Edwards, 1993). On oat leaves, however, conidia of *B. graminis* f. sp. *avenae* did not show any reduction in germination and differentiation under conditions of continuous lighting with 150 µmoles photons m^{-2} s^{-1} (Carver *et al.*, 1994).

Besides light intensity, spectral quality was illustrated to affect *B. graminis* infection (Edwards, 1993). White and blue light resulted in retarded fungal development, whereas development was faster under red light as in dark treatments. Using the closely related powdery mildew fungi *Erysiphe pisi* and *Erysiphe polygoni*, it was found that white light and shorter wavelengths stimulated germination and germ tube development (Singh & Singh, 1981; Ayres, 1983). For *E. pisi*, however, positive phototropism of germ tubes in response to white light and negative phototropism in response to green light were demonstrated (Singh & Singh, 1981). While white and green light treatment had the reverse effect on *E. polygoni* (Yarwood, 1957). *B. graminis* was reported to be totally insensitive to light with respect to germ tube emergence (Fujita *et al.*, 2004). Nevertheless, not only a direct effect of light (e.g. light intensity and/or duration), but also the resulting physiological status of the underlying host tissue might have a significant impact on the outcome of the powdery mildew pre- and postpenetration processes as was demonstrated for the differentiation of *E. pisi* appressoria on albino mutants of pea (Singh *et al.*, 2000) and the formation of *B. graminis* haustoria on barley coleoptiles(Carver & Jones, 1988).

However, the studies that have dealt so far with the impact of light on the prepenetration processes of *B. graminis* suffer from applying different experimental systems, experimental conditions and particularly considering essentially different light sources and lighting parameters (such as illuminance, photosynthetic photon flux density or irradiance), which makes it rather difficult or even impossible to directly convert or compare presented data.

Formvar®-based *in vitro* system that facilitates studying *B. graminis* prepenetration processes independent of the host plant (Hansjakob *et al.*, 2010; Hansjakob *et al.*, 2012; Zhu *et al.*, 2017) was applied in this study. The system was combined with a precise monitoring of temperature and lighting conditions to pursue a comprehensive approach analyzing the objective to determine the effects of light on the prepenetration processes of *B. graminis*.

5.2 Results

5.2.1 Conidial prepenetration processes of *B. graminis* f. sp. *hordei* are impeded by white light *in vitro*

In initial experiments conidia of *B. graminis* f. sp. *hordei* (Bgh) were inoculated onto artificial surfaces. Then the samples were incubated under the conditions of different intensities of white light. The germination and differentiation of conidia was studied 18 h after inoculation (**Figure 5.1** A and B). *In vitro* white light affected conidial germination and differentiation in an intensity-dependent manner. While in darkness 9 ± 1 % of conidia on Formvar® /barley wax coated slides and 5 ± 3 % of conidia Formvar® /barley wax/agar coated slides remained non-germinated, the numbers were substantially increased up to 26 ± 5 % and 17 ± 2 %, respectively under the conditions of white light. Consequently, on Formvar® /barley wax coated slides the proportions of conidia with primary germ tube significantly increased from 9 ± 6 % to 34 ± 2 %. However, on Formvar® /barley wax/agar coated slides there is no significant difference between the percentages of conidia with primary germ tube in darkness and under the conditions of white light. The numbers of differentiated conidia on Formvar® /barley wax coated slides were significantly decreased from 59 ± 3 % (in darkness) to 6 ± 4 % (white light condition, 234 µmol m^{-2} s^{-1}). The percentages of differentiated conidia on Formvar® /barley wax/agar coated slides were notably decreased from 66 ± 2 % (in darkness) to 30 ± 5 % (white light condition, 234 µmol m^{-2} s^{-1}).

To determine whether white light influence Bgh conidial germination and differentiation *in vivo*, conidia were inoculated onto barley leaves and then incubated under the conditions of different intensities of white light for 18 h (**Figure 5.1** C). Samples incubated in darkness were performed as the control. Interestingly, in contrast to the situation *in vitro*, development of conidia on leaf surfaces was not notably affected by white light. Neither the proportions of germinated conidia nor the numbers of appressorium formation significantly changed under the conditions of different intensities of white light.
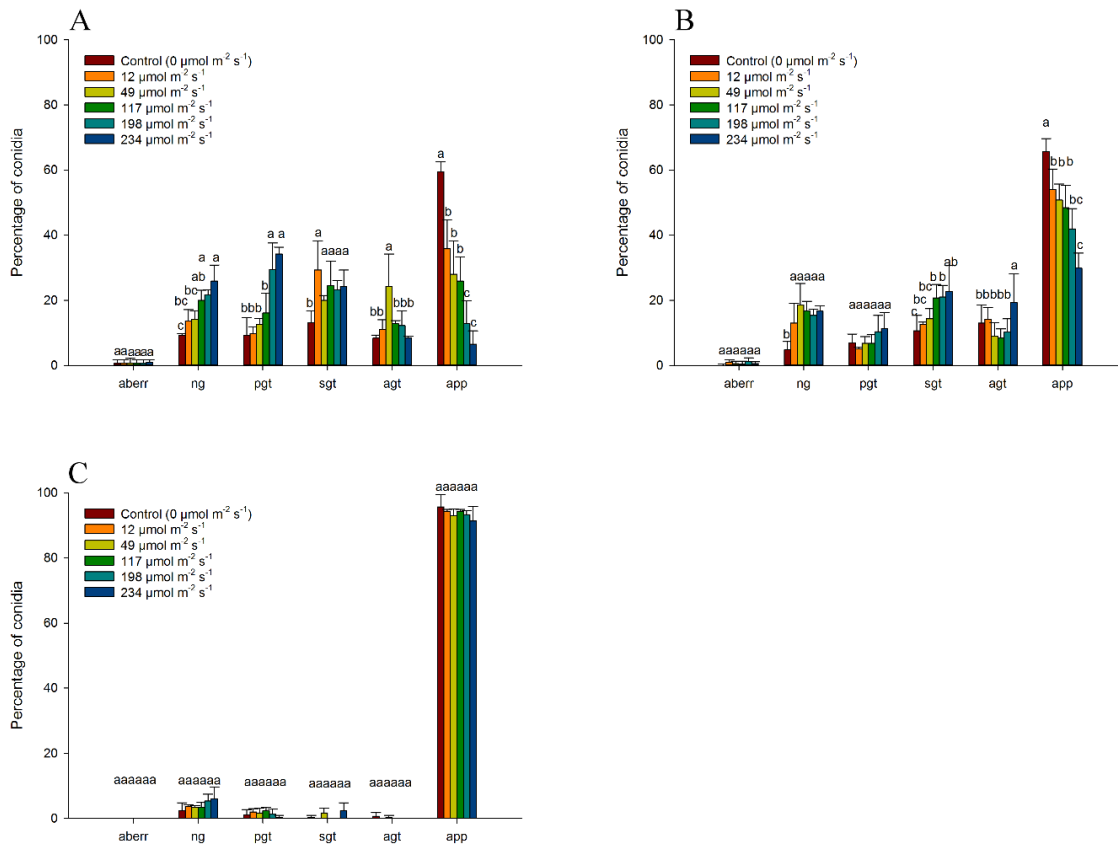
Figure 5.1 Development of *Blumeria graminis* f. sp. *hordei* conidia on (A) Formvar®/barley wax coated glass slides, (B) Formvar®/barley wax/agar coated glass slides and (C) adaxial surface of barley leaf after an incubation period of 18 h under white light conditions. Prepenetration developmental stages of *B. graminis* conidia: aberr, burst or dead conidia; ng, non-germinated conidium; pgt, germinated conidium with a primary germ tube only; sgt, conidium with an elongated secondary germ tube; agt, conidium with a swollen appressorial germ tube; app, conidium with a fully differentiated appressorium. In (A) and (B), each value is given as mean ± SD of 3 replicates with 100 conidia each of three independent experiments. In (C), each value is given as mean ± SD of 3 independent experiments with 100 conidia. Significant differences were determined in One-way ANOVA followed by a Tukey *post hoc* test: different letters within a column indicate significant differences ($P < 0.05$).

In order to exclude the potential impact of UV-A irradiation produced by white light tubes, the similar *in vitro* experiments were performed with white light filtered by UV protection film (**Figure 5.2**). The same influence of white light on conidial germination and differentiation was found. Despite slightly decreased intensities of white light, conidia under the conditions of white light or filtered white light conditions showed almost the same proportions of germination and differentiation (**Figure 5.1** and **Figure 5.2**).
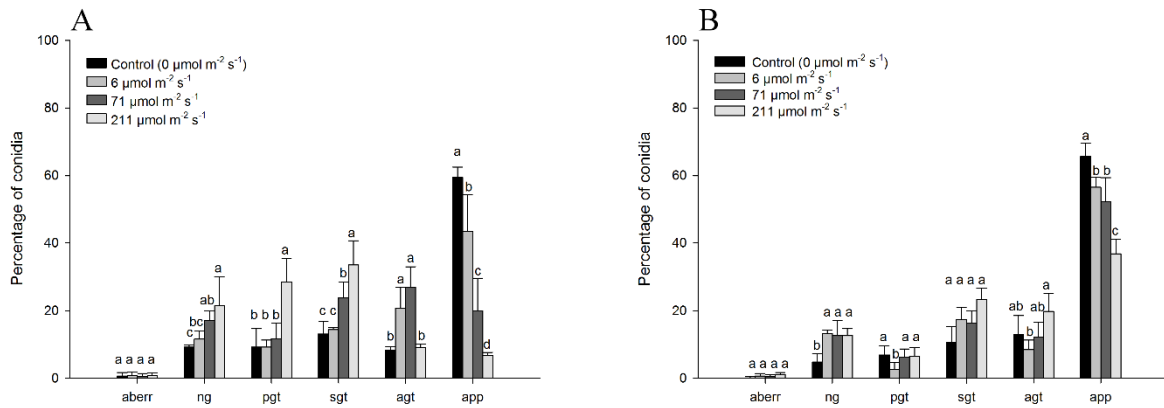
Figure 5.2 Development of *Blumeria graminis* f. sp. *hordei* conidia on (A) Formvar®/barley wax coated glass slides and (B) Formvar®/barley wax/agar coated glass slides after an incubation period of 18 h under white light condition. White light was filtered by UV-protection-film. Light intensity was 211 µmol m^{-2} s^{-1}. Prepenetration developmental stages of *B. graminis* conidia: aberr, burst or dead conidia; ng, non-germinated conidium; pgt, germinated conidium with a primary germ tube only; sgt, conidium with an elongated secondary germ tube; agt, conidium with a swollen appressorial germ tube; app, conidium with a fully differentiated appressorium. Each value is given as mean ± SD of 3 replicates with 100 conidia each of three independent experiments. Significant differences were determined in One-way ANOVA followed by a Tukey *post hoc* test: different letters within a column indicate significant differences ($P < 0.05$).

5.2.2 Differentiation of *B. graminis* f. sp. *tritici* conidia is impaired by white light

To find out whether white light also affects prepenetration processes of another *forma specialis* of *B. graminis* - *B. graminis* f. sp. *tritici* (Bgt), conidia were inoculated onto artificial and native host surfaces. Then the samples were incubated in darkness or under white light conditions (intensity, 234 µmol m^{-2} s^{-1}) for 18 h (**Figure 5.3**). Interestingly, *in vitro* white light only significantly impeded appressorial formation but not conidial germination. While 56 ± 9 % of conidia on Formvar® /barley wax coated slides and 60 ± 7 % of conidia Formvar® /barley wax/agar coated slides were able to form appressoria in darkness, the proportions were significantly decreased to 4 ± 3 % and 36 ± 9 %, respectively, under the conditions of white light. Consequently, on Formvar® /barley wax coated slides the proportions of conidia with primary germ tube significantly increased from 12 ± 2 % to 59 ± 9 %. However, on Formvar® /barley wax/agar coated slides there is no significant difference between the percentages of conidia with primary germ tube in darkness and under the conditions of white light. *In vivo*, conidial development was not notably affected. More than 95 % of conidia germinated and about 90 % of spores formed appressoria.
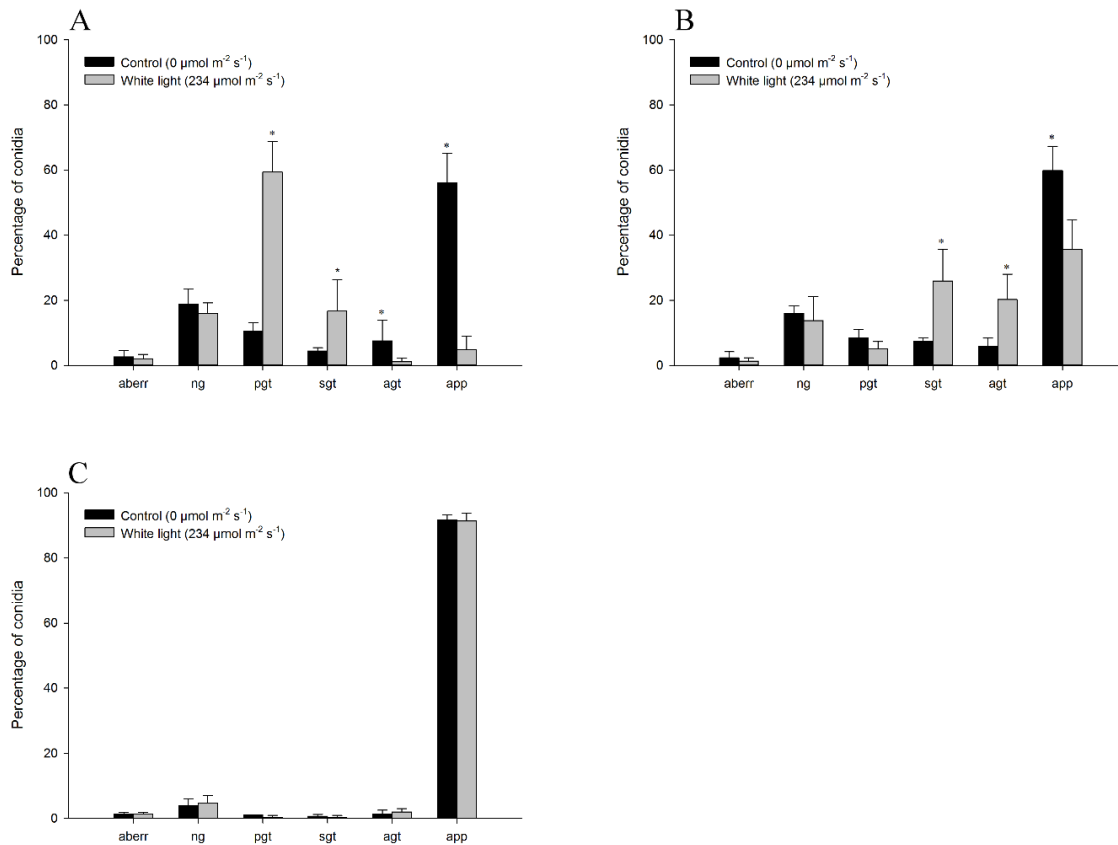
Figure 5.3 Development of *Blumeria graminis* f. sp. *tritici* (Wue1) conidia on (A) Formvar®/wheat wax coated glass slides, (B) Formvar®/wheat wax/agar coated glass slides and (C) adaxial surface of wheat leaf after an incubation period of 18 h under white light condition. Light intensity was 234 µmol m^{-2} s^{-1}. Prepenetration developmental stages of *B. graminis* conidia: aberr, burst or dead conidia; ng, non-germinated conidium; pgt, germinated conidium with a primary germ tube only; sgt, conidium with an elongated secondary germ tube; agt, conidium with a swollen appressorial germ tube; app, conidium with a fully differentiated appressorium. In (A) and (B), each value is given as mean ± SD of 3 replicates with 100 conidia each of three independent experiments. In (C), each value is given as mean ± SD of 3 independent experiments with 100 conidia. Significant differences were determined in Student's T test: * significant differences ($P < 0.05$).

5.2.3 *In vitro* and *in vivo* white light promoted appressorial elongation of *B. graminis*

Intriguingly, *in vitro* and *in vivo* the lengths of appressoria formed by conidia under white light condition was dramatically higher than that produced by conidia in darkness (**Figure 5.4**). In order to quantitatively compare the lengths of appressoria, conidia *in vitro* and *in vivo* were incubated under photoperiod conditions. Then the average lengths of appressoria were determined 18 h after inoculation (**Table 5.1**). In general, in darkness the average length of appressoria produced by conidia of Bgh and Bgt was about 29 µm. However, under photoperiod condition (dark /light, 6 h/12 h) the average length of appressoria significantly increased up to 35 µm. While conidia under light/dark (6 h/12 h) photoperiod conditions and those under continuous light conditions formed almost the same average length of appressoria (about 45 – 49 µm), which increased by roughly 50 % when compared with the average length of appressoria produced by conidia in darkness.
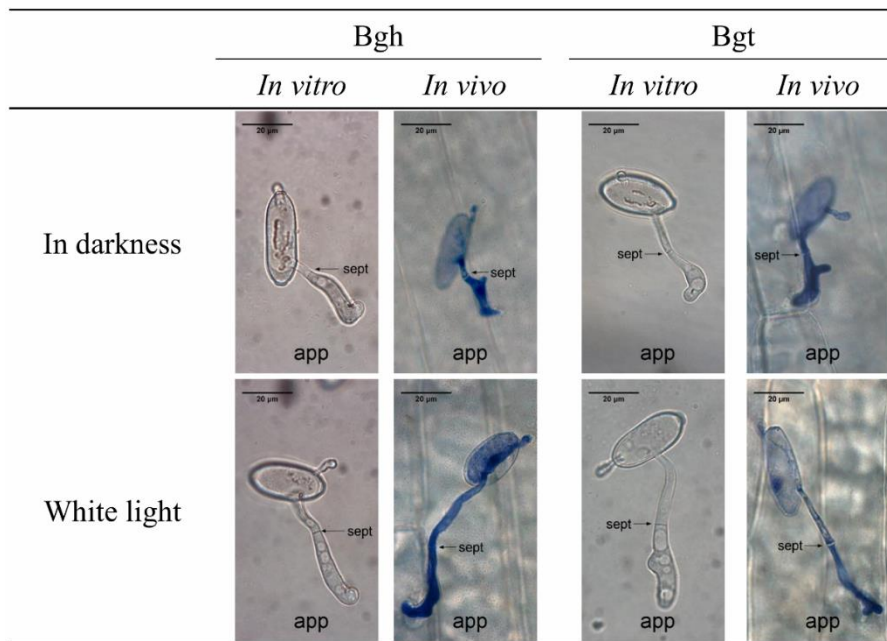
Figure 5.4 *In vitro* and *in vivo* appressorium formation of *Blumeria graminis* f. sp. *hordei* (Bgh) and *Blumeria graminis* f. sp. *tritici* (Bgt) conidia after an incubation of 18 h in darkness or under white light condition. The intensity of white light was 234 µmol m^{-2} s^{-1}.

Table 5.1 *In vitro* and *in vivo* appressorium length of *Blumeria graminis* f. sp. *hordei* (Bgh) and *Blumeria graminis* f. sp. *tritici* (Bgt) conidia after an incubation of 18 h in darkness or under photoperiod conditions.

	Bgh		Bgt	
	In vitro	*In vivo*	*In vitro*	*In vivo*
Dark (D)	29.41±6.10a	26.84±6.23a	31.00±7.63a	29.38 ± 6.78a
Light (L) (18 h)	45.43±13.71b	41.42±15.03b	47.86±17.95b	49.24 ± 12.59b
D/L (6/12h)	33.98±10.72c	29.82±7.32c	35.52±9.68c	35.08±8.52c
L/D (6/12h)	45.41±14.89b	40.54±15.79b	45.82±13.22b	48.58±15.34b

Note, white light intensity was 234 µmol m^{-2} s^{-1}. Each value is given as means ± SD of 100 conidia. Significant differences were determined in Welch ANOVA followed by a Games-Howell *post hoc* test: different letters within a column indicate significant differences ($P < 0.05$).

5.2.4 Interaction between *B. graminis* conidia and host cells under photoperiod conditions

There are significant differences between the dimensions of barley and wheat epidermal cells (**Table 5.2**). In general, A cells (the host cells which lay adjacent to stomata) of barley leaf exhibited around 50 % greater width than those of wheat leaf. B cell (bulliform cell) width of barley leaf increased by roughly 30 % when compared with the width of B cells of wheat leaf.

Table 5.2 Length and width of barley and wheat epidermal cells.

	A cells		B cells		V cells	
	L	W	L	W	L	W
Barley	188 ± 41a	46 ± 7a	229 ± 28a	46 ± 5a	n.m	35 ± 6a
wheat	174 ± 43a	29 ± 5b	196 ± 47b	36 ± 6b	n.m	33 ± 5a

Note, the highest dimensions of length (L) and width (W) of plant epidermal cells were measured. A cells, the host cells which lay adjacent to stomata; B cells, bulliform cells; V cells, the host cells which overlaying vascular bundles. N.m, not measured. Each value is given as means ± SD of 20 cells. Different letters within a column indicate significant differences (P < 0.05) determined in Mann–Whitney U-test.

Accordingly, the conidia with longer appressorium showed higher potential to interact with more on host cells (**Table 5.3** and **Figure 5.5**). In general, under the conditions of dark and 6h dark/18h light, more than 60 % of Bgh conidia interacted with only one host cell (type 1) by a primary germ tube (pgt) and an appressorium (app). However, 51 ± 4 % of Bgh conidia interacted with two host cells (type 2) by conidial structures under the 6h light/18h dark photoperiod condition. More than 70 % of Bgh conidia contacted with two host cells by a pgt and an app under the condition of 18h light/6h dark. Likewise, most (about 80 %) of Bgt conidia contacted with two host cells by conidial structures under the conditions of dark and 6h dark/18h light. However, the numbers significantly decreased to ca. 50 % under the photoperiod conditions of 6h light/18h dark and18h light/6h dark. Consequently, about 45 % of Bgt conidia formed a pgt and an app that located on 3 host cells.

Table 5.3 Types of interaction between host epidermal cells and *B. graminis* structures
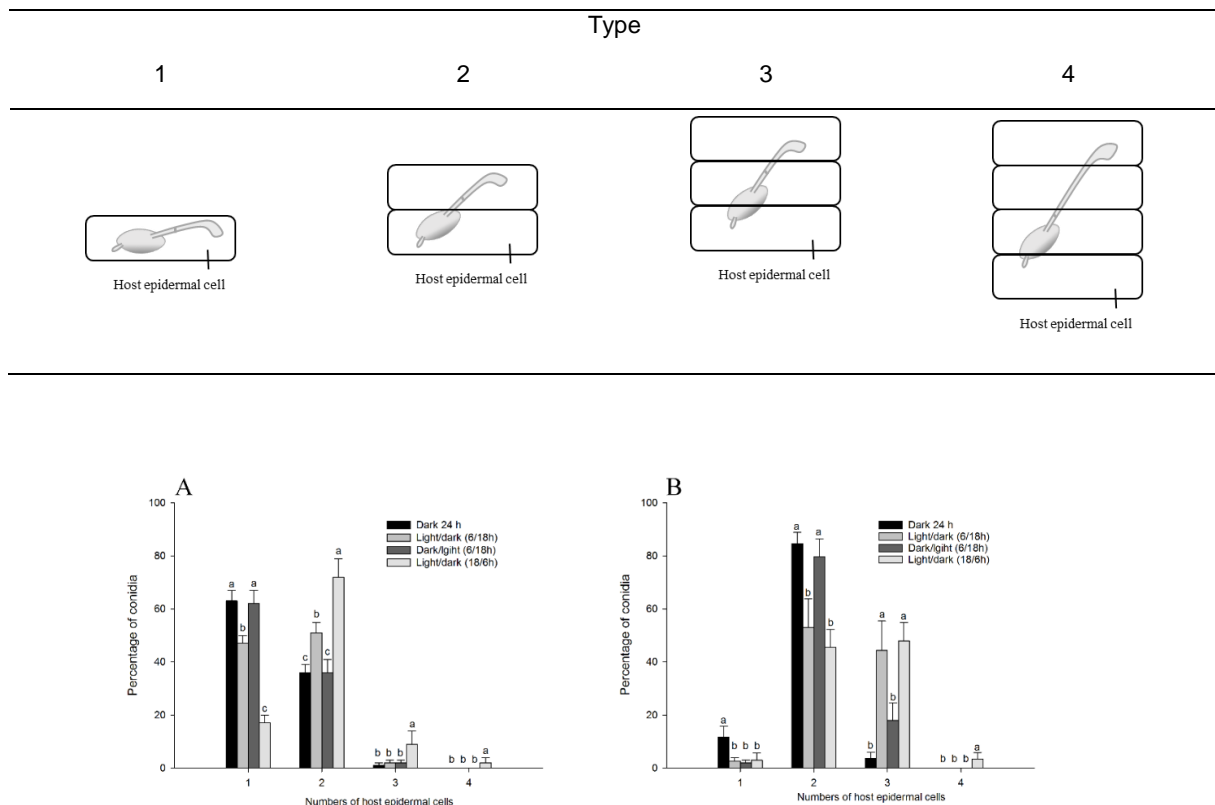
Figure 5.5 Numbers of host cells located by conidial primary germ tube (pgt) and an appressorium (app). *Blumeria graminis* f. sp. *hordei* (A) and *Blumeria graminis* f. sp. *tritici* (B) conidia were inoculated onto host surfaces and incubated for 24 h in darkness or under photoperiod conditions. White light intensity was 234 µmol m^{-2} s^{-1}. Numbers of host cells located by *B. graminis* structures were illustrated in table 3. Each value is given as mean ± SD of three independent experiments with 100 fully differentiated conidia each. Significant differences were determined in One-way ANOVA followed by a Tukey *post hoc* test: different letters within a column indicate significant differences ($P < 0.05$).

5.2.5 *In vitro* and *in vivo* light stimulated *B. graminis* appressorial elongation in a wavelength-dependent manner

To assay the qualitative effects of light on appressorial elongation, conidia *in vitro* and *in vivo* were incubated under the conditions of different qualities of light (intensity, 80 µmol m^{-2} s^{-1}). The lengths of appressoria were determined 18 h after inoculation (**Table 5.4**). Conidia under the condition of blue light produced slightly longer appressoria (29 – 34 µm) when compared with the lengths of appressoria formed by conidia in darkness (27 – 31 µm). However, conidia incubated under the conditions of green and red lights produced significantly longer appressoria. Conidia under green and red light conditions formed roughly 20 % longer appressoria when compared with appressoria produced in darkness.

Table 5.4 *In vitro* and *in vivo* appressorium length of *Blumeria graminis* f. sp. *hordei* (Bgh) and *Blumeria graminis* f. sp. *tritici* (Bgt) conidia after an incubation of 18 h in darkness or under different light conditions.

	Bgh		Bgt	
	In vitro	*In vivo*	*In vitro*	*In vivo*
Dark	29.32±5.75a	27.31±4.38a	30.92±7.51a	29.31±6.84a
Blue light	31.84±7.49a	28.62±5.94a	33.87±6.83a	32.23±5.72a
Green light	37.82±8.22b	32.05±5.54b	39.65±8.92b	37.21±5.82b
Red light	39.16±7.93b	37.65±7.78c	39.73±9.80b	49.56±16.03c

Note, lengths of appressorium were measured after an incubation period of 18 h in dark or under different light conditions (light intensity, 80 µmol m^{-2} s^{-1}). Each value is given as means ± SD of 100 conidia *in vitro* and 50 conidia *in vivo*. Significant differences were determined in Welch ANOVA followed by a Games-Howell *post hoc* test: different letters within a column indicate significant differences ($P < 0.05$).

5.2.6 Penetration of Bgh and Bgt 24 h post-inoculation

It was reported that white light delayed haustorium formation by *B. graminis* f. sp. *avenae* (Carver *et al.*, 1994). To determine whether penetration of Bgh and Bgt was affected by white light, conidia *in vivo* were incubated under photoperiod conditions for 24 h (**Figure 5.6**). Around 20 % of appressoria of Bgh under darkness condition were capable of penetrating cuticle and host epidermal cell wall to form initial haustoria. The number slightly decreased to 13 ± 3 % when conidia were incubated under 6h light/18h dark photoperiod condition. However, the percentages significantly decreased to 10 ± 3 % and 11 ± 3 % under 6h dark/18h light and

18h light/ 6h dark photoperiod conditions, respectively. Rather similar findings were obtained when Bgt conidia were incubated under the same photoperiod conditions. About 15 % of appressoria formed by conidia under darkness condition were able to form haustoria and 14 % of appressoria differentiated by conidia under 6h light/18h dark photoperiod condition were capable of producing haustoria. However, under 6h dark/18h light photoperiod the number significantly decreased to 11 ± 2 %. Only 7 ± 2 % of conidia formed haustoria when incubated under 18h light/ 6h dark photoperiod condition.
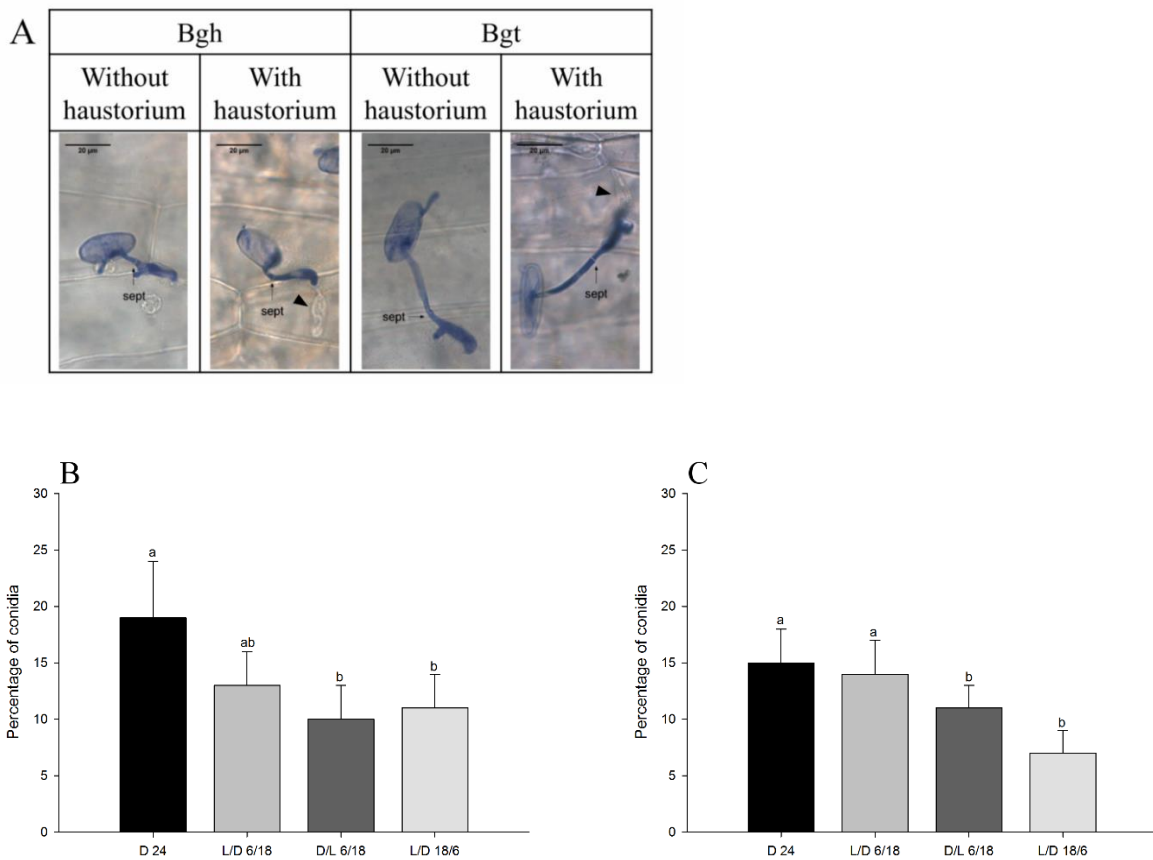
Figure 5.6 Penetration efficiency of conidial appressoria after an incubation period of 24 h under different light conditions. *Blumeria graminis* f. sp. *hordei* (A) and *Blumeria graminis* f. sp. *tritici* (B) conidia were inoculated onto host leaf surface and incubated under photoperiod conditions. D, darkness. L, white light. White light intensity was 234 µmol m^{-2} s^{-1}. Penetration efficiency was quantified by monitoring whether haustoria were formed by differentiated conidia. Primary haustorium was indicated by a black arrowhead. Each value is given as mean ± SD of three independent experiments with 200 fully differentiated conidia each. Significant differences were determined in One-way ANOVA followed by a Tukey *post hoc* test: different letters within a column indicate significant differences ($P < 0.05$).

5.2.7 Penetration efficiency of *B. graminis* conidia under photoperiod conditions 48 h post-inoculation

To find out more about the penetration efficiency of *B. graminis* with photoperiod treatment, conidia *in vivo* were incubated for 48h under the conditions of 6h light/18h dark, 6h dark/18h light or 18h light/ 6h dark. Conidia in darkness were performed as the control (**Figure 5.7**). In darkness 52 ± 2 % of Bgh conidia and 58 ± 4 % of Bgt conidia formed haustoria. However, under photoperiod conditions the proportions significantly decrease to around 45 %. Bgh and Bgt conidia under the condition of 18h light/6h dark showed lowest penetration efficiency (35 ± 4 % and 39 ± 6 %, respectively). However, a significant difference was not determined amongst the penetration efficiencies of conidia under all the photoperiod conditions.
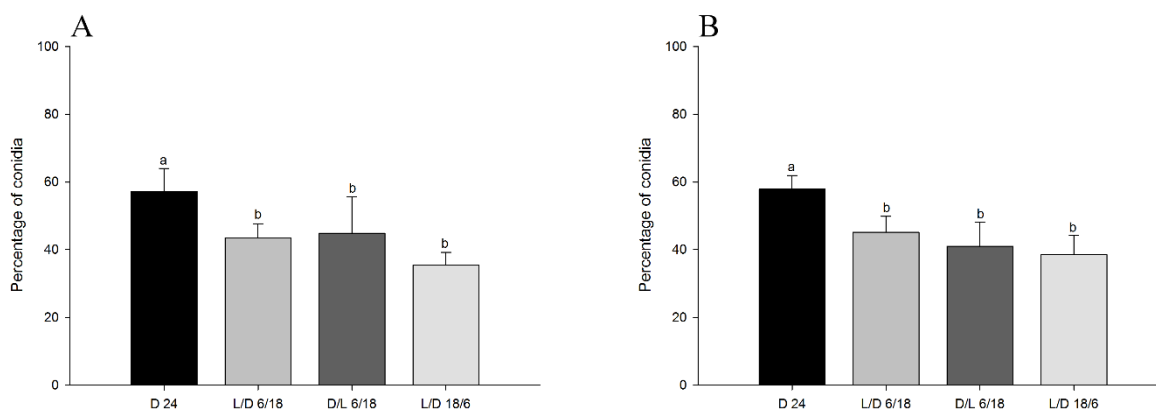
Figure 5.7 Penetration efficiency of conidial appressoria after an incubation period of 48 h under different light conditions. *Blumeria graminis* f. sp. *hordei* (A) and *Blumeria graminis* f. sp. *tritici* (B) conidia were inoculated onto host leaf surface and incubated under photoperiod conditions. D, darkness. L, white light. White light intensity was 234 µmol m^{-2} s^{-1}. Penetration efficiency was quantified by monitoring whether haustoria were formed by differentiated conidia. Each value is given as mean ± SD of three independent experiments with 200 fully differentiated conidia each. Significant differences were determined in One-way ANOVA followed by a Tukey *post hoc* test: different letters within a column indicate significant differences ($P < 0.05$).

5.3 Discussion

5.3.1 White light impaired *B. graminis* prepenetration processes *in vitro*

To date, there is only scarce information available on the effects of light irradiation on conidial development processes of *Blumeria graminis* (Masri & Ellingboe, 1966; Edwards, 1993; Carver *et al.*, 1994). In addition, it is not clear whether the impact of light is mediated by indirect influence on host metabolism/resistance or by direct influence on the powdery mildew (Carver *et al.*, 1994). Because pathosystems (pathogens/hosts) were applied in these studies to determine the developments of the biotrophic fungus under various light irradiation conditions (Masri & Ellingboe, 1966; Edwards, 1993; Carver *et al.*, 1994), the secondary effects from hosts could not be excluded. In line with this, *in vitro* studies were employed to determine the effects on *B. graminis* without living host tissues. when *B. graminis* was irradiated by different intensities of white light, germination and appressorium formation of conidia on artificial surfaces (*in vitro*) was significantly impeded, while the hampering effect of white light was not found when conidia were incubated on host leaf surfaces (*in vivo*) (**Figure 5.1** to **5.3**). The results were in accordance to previous studies (Liebrich, 2008; Reisberg 2009). By applying Formvar®-based *in vitro* systems, the scientific evidence that direct effect of white light irradiation on prepenetration processes of *B. graminis* was found, which is in accordance with the hypothesis demonstrated by Carver et al. (1994).

It was hypothesized that water uptake was one of the crucial functions of primary germ tube (pgt) (Carver & Bushnell, 1983). It was speculated that under unfavorable conditions water uptake by pgt might be involved in the appressorial germ tube formation (Carver & Bushnell, 1983). Under the condition of white light irradiation (intensity, 234 µmol m^{-2} s^{-1}), significantly higher proportions of appressoria formed by conidia on Formvar®/wax/agar coated slides were observed when compared with the numbers of appressoria produced by conidia on Formvar®/wax coated slides (**Figure 5.1** to **5.3**). These might indicate that germinated conidia obtained water source from agar layer via the pgt and then considerably reduced the hampering effect of white light irradiation so that *B. graminis* improved the formation of appressoria.

When compared with the proportions of appressoria produced by *B. graminis* f. sp. *hordei* (Bgh) conidia *in vitro* under white light irradiation conditions (**Figure 5.1**), rather similar results were obtained using *B. graminis* f. sp. *tritici* (Bgt) spores under the corresponding conditions (**Figure 5.2**). This might suggest that the direct effect of white light irradiation on *B. graminis* was not *forma specialis* specific. However, in this study, two *formae speciales* were used to determine the white light influence. More research is needed to explore whether there is the same/similar effect of white light irradiation on different *formae speciales* of *B. graminis*.

It is shown that white light bubbles used in this study can produce a small amount of UV-A (Tong *et al.*, 2008). It was reported that UV-A irradiation did not affect the developments of tomato powdery mildew – *Oidium neoplycopersici* (Suthaparan *et al.*, 2016b). However, the impact of UV-A irradiation on *B. graminis* conidial prepenetration processes was not clear. In order to exclude the potential effect of UV-A light provided by white light bubbles on conidial developments, white light was filtered by UV-protection-film and then employed to irradiate conidia *in vitro* and *in vivo* (**Figure 5.3**). Despite slightly decreased intensities of white light, the results were fully consistent with the findings applying unfiltered white light (**Figure 5.1** and **5.2**). These suggest that the effect of light irradiation is mediated by white light but not by UV-A.

5.3.2 Light-mediated elongation of *B. graminis* appressorium *in vitro* and *in vivo*

The phenomenon that light-mediated appressorial elongation of *B. graminis* was observed when conidia were incubated under a white light condition *in vitro* and *in vivo* (**Figure 5.4**). This is in contrast to the previous report that the formation of first appressorium structure was not affected when conidia were irradiated with white light (Carver *et al.*, 1994). This difference seems to be due to fact that the intensity of white light used in the previous study was 150 μmol m^{-2} s^{-1} (Carver *et al.*, 1994) and the intensity applied in this study was much higher (234 μmol m^{-2} s^{-1}). It was reported that reducing light intensity resulted in the increasing frequency of haustorium formation (Carver *et al.*, 1994), which might indicate the effect of white light on *B. graminis* development was in a light intensity-dependent manner. During prepenetration processes, conidial development *in vitro* was affected in a light intensity-dependent manner (**Figure 5.1** to **5.3**). However, it is not clear whether decreasing white light intensity can significantly reduce the average length of appressoria when compare with the appressorial length formed by conidia under the high intensity of white light conditions. More research is needed to compare the average length of appressoria under different white light intensity conditions in order to determine whether the promoting effect of white light on the appressorial elongation is a light intensity-dependent manner.

Edwards (1993) reported that the appressorium formation was delayed when conidia were treated with light during the first 12 h of incubation. By contrast, it was demonstrated that the timing of prepenetration processes of *B. graminis* was not influenced by lights (Carver *et al.*, 1994). It was found that, in general, the average lengths of elongated appressoria were higher under the conditions of continuous white light or 6h light/12h dark photoperiod when compared with those formed after an incubation period of 18 h under conditions of darkness or 6h dark/12h light photoperiod (**Table 5-1**). This clearly suggests that during prepenetration processes, the appressorial elongation depends on the irradiating duration with white light. It

was illustrated that conidial liberation of *B. graminis* seemed to be correlated with the atmospheric humidity (Glawe, 2008), and mainly started from late afternoons (Hammett & Manners, 1971). Conidia of *B. graminis* may be involved in different strategies to infect hosts by forming different lengths of appressoria under various photoperiod conditions (light/dark cycles).

It was reported that the germ tube elongation of tomato powdery mildew – *O. neolycopersici* was affected by light in a quality (wavelength)-dependent manner (Mieslerová & Lebeda, 2010). However, the influence of light quality on the appressorial elongation of *B. graminis* was not clear. In order to get an idea whether the triggering effect of light irradiation on the appressorium elongation was in a quality-dependent manner, conidia *in vitro* and *in vivo* were incubated under the conditions of darkness, blue light, green light or red light for 18 h. It was found that red light followed by green light was identified to be the most effective in stimulating the appressorial elongation of *B. graminis* (**Table 5-4**). This might indicate that the promoting effect of white light on the appressorial elongation was at least partially dominated by red light. Similarly, it was demonstrated that the average length of germ tube of *O. neoplycopersici* was greater under the red light condition when compared with the average lengths of germ tube formed under white light, blue light or green light conditions (Mieslerová & Lebeda, 2010). Light quality was known to affect the host defense and development of powdery mildews (Schuerger & Brown, 1997; Wang *et al.*, 2010; Kangasjärvi *et al.*, 2012; Fuller *et al.*, 2014; Suzuki *et al.*, 2018). On one hand, it seems that *B. graminis* forms varied lengths of appressoria to cope with host defense under different light irradiation conditions. On the other hand, it appears that red light and green light are stressful factors to control the elongation of appressoria, and that under the blue light condition *B. graminis* is at best slightly affected. Therefore, one important question remains. Is the appressorium elongation process mediated by (a) photoreceptor system(s)? Indeed, in *B. graminis* there are nine potential photoreceptors, which are hypothesized to be involved in regulating the physiological development of this powdery mildew (Schumacher, 2017). However, the functions of these potential photoreceptors are still not clear. More research is needed to study the functions of the potential photoreceptors in *B. graminis*.

5.3.3 Interaction between *B. graminis* and host epidermal cells under different light conditions

In most cases, after the appressorium formation *B. graminis* begins to postpenetration processes (**Figure 1.2**). In order to successfully obtain nutrient and water from host epidermal cell, *B. graminis* needs to forcibly penetrate the host cuticle and the epidermal cell wall by the appressorium and then forms the feeding structure – the haustorium (Pryce *et al.*, 1999; Kuhn

et al., 2016). It was reported that the infection success of *B. graminis* was affected by pathogen-host interaction types and the appressorium length (Woolacott & Archer, 1984). It was found that under photoperiod conditions *B. graminis* formed a pgt and then produced a sgt that differentiated an appressorium in different lengths, which interacted with different numbers of host epidermal cells (**Table 5.3** and **Figure 5.5**). In general, around 60 % of Bgh conidia interacted with 1 host cell and 40% of them interacted with 2 host cells under the conditions of darkness and 6h dark /18h light. Since under 6h light/18h dark and 18h light/6h dark conditions conidia formed relatively longer appressoria, the proportions of conidia that interacted with 2 host cells significantly increased to about 55 % and 70 % respectively. The result for conidia under the 18h light/6h dark photoperiod condition was fully consistent with a previous study illustrating 75 % of Bgh conidia under the condition of 16h light/8h dark interacted with 2 host cells (Woolacott & Archer, 1984).

Due to the fact that wheat epidermal cells showed relatively smaller dimensions when compared to barley epidermal cells (**Table 5.2**), most of Bgt conidia interacted with more than 2 host cells. Similarly, under the conditions of 6h light/18h dark and 18h light/6h dark Bgt showed higher proportions of conidia locating on 3 host epidermal cells when compared with the percentages of conidia incubated in darkness and under the 6h dark/18h light condition. These might suggest that during prepenetration processes *B. graminis* formed varied lengths of appressoria to interact with host epidermal cells under different photoperiod conditions. Because haustoria of *B. graminis* are formed by appressoria, the different lengths of appressoria may relate to the penetration success after the appressorium formation.

5.3.4 Penetration efficiency of *B. graminis* under photoperiod conditions 24 h and 48 h post-inoculation

It was demonstrated that the haustorium formation of *B. graminis* was delayed under continuous light conditions (Edwards, 1993; Carver *et al.*, 1994). Under a continuous light condition conidia formed the greatest lengths of appressoria so that interacted with more host epidermal cells (**Table 5.1** and **Figure 5.5**). In order to evaluate the penetration efficiencies of *B. graminis* under different photoperiod conditions, conidia *in vivo* were monitored. It was observed that 24 h post-inoculation conidia in darkness formed the highest proportions of haustoria. The percentages of haustoria slightly decreased when conidia were incubated under the condition of 6h light/18h dark photoperiod. However, under the 6h dark/18h light or 18h light/6h dark conditions conidia formed least proportions of haustoria. These findings were fully consisted with the previous illustrations showing *B. graminis* under photoperiod conditions formed lower proportions of haustoria than those in darkness (Edwards, 1993; Carver *et al.*, 1994). In addition, 48 h post-inoculation conidia in darkness formed statistically significant

higher percentages of haustoria when compared with proportions of haustoria formed by conidia under photoperiod conditions. Interestingly, under photoperiod conditions there was no notable difference amongst proportions of haustoria. Therefore, the results indicate that 24 h post-inoculation the formation of haustoria under photoperiod conditions was delayed, but was not prevented. Moreover, the results are fully consisted with a previous study reporting white light delayed the haustorium formation of *B. graminis* (Carver *et al.*, 1994). Similarly, Carver et al (1994) demonstrated that 36 h post-inoculation there was no significant difference between proportions of appressoria with haustoria under the dark/light and light/dark photoperiod conditions. However, 60 h post-inoculation conidia formed almost the same numbers of haustoria under darkness or photoperiod conditions (Carver *et al.*, 1994). The different results might be due the facts: 1) different light intensities were applied in different studies (234 µmol m^{-2} s^{-1} (this study), 150 µmol m^{-2} s^{-1} (Carver *et al.*, 1994) and around 30 µmol m^{-2} s^{-1} (Edwards, 1993)) ; 2) Photoperiodic treatments employed in this study were 6h/18h, while those utilized in previous studies were 12h/ 12h (Edwards, 1993; Carver *et al.*, 1994); 3) the differences of pathosystems and the observation time (Carver *et al.*, 1994).

Due to the fact that *B. graminis* has a relatively low number of genes that encoding carbohydrate-active enzymes (Spanu *et al.*, 2010), cell wall-degrading enzymes are regarded to play a minor role in the penetration processes (Kuhn *et al.*, 2016). The penetration of *B. graminis* is considered to mainly depend on turgor pressure (mechanical force) of an appressorium (Pryce *et al.*, 1999; Kuhn *et al.*, 2016). To date, it is not clear whether there is a relationship between the appressorial length and the turgor pressure. Moreover, little is known about the benefit/advantage for *B. graminis* to form different lengths of appressoria under varied photoperiod conditions. More research is needed to analyze the relations between the appressorium development and the penetration processes of *B. graminis*.

As one of the key factors affecting the physiology of organisms, light has been illustrated to regulate plant responses to biotic stress (Karpinski *et al.*, 2003; Roden & Ingle, 2009; Hua, 2013) and to affect developments of plant fungal pathogens (Corrochano, 2007; Tisch & Schmoll, 2010; Schmidt-Heydt *et al.*, 2011; Fuller *et al.*, 2014; Schumacher, 2017). In line with this, it seems that *B. graminis* has evolved in different developmental strategies to successfully infect hosts under various light conditions.

5.4 Conclusions

Blumeria graminis prepenetration processes are severely affected by increasing white light intensities on artificial host wax surfaces that do not provide a source of water beneath germinating conidia. On leaf surfaces, *B. graminis* conidia are slightly affected by increasing white light intensities, which reflects the flexibility and adaptability of *B. graminis* conidia to their leaf surface environment. Comparing to blue light or green light tested, the assayed red light shows a notable triggering impact on the appressorial elongation of conidia, which suggests light quality specifically affects prepenetration processes of *B. graminis*. Under different photoperiod (light/dark cycle) conditions, the haustorium formation of *B. graminis* is delayed 24 h post-inoculation and it is not affected 48 h post-inoculation. These results demonstrated that light directly affects conidial (pre)penetration processes of *B. graminis.*

6 Perspectives

It was found that *Blumeria graminis* ascospores showed variable morphological patterns with the first germ tube differentiating an infection structure (an appressorium) when compared with conidia that produced a primary germ tube before a secondary germ tube, which finally differentiated into an appressorium. It remains unclear, what are the benefits/advantages for ascospores to produce only one germ tube forming an appressorium. Due to the fact that *B. graminis* infects hosts by the appressorium, it would be of highest interest to determine whether there is a difference of the ascosporic infection success between appressoria formed by first germ tube and appressoria formed by secondary germ tube. It would be valuable to analyze the varied morphological patterns of ascospores under different environmental conditions (such as atmospheric humidity and light) to enhance the knowledge of prepenetration biology of the agriculturally and economically relevant plant pathogen *B. graminis*.

It was shown that UV-C could efficiently suppress the development of *B. graminis* conidia. However, there is no information available concerning the effects of UV irradiation on fruiting bodies (chasmothecia) of *B. graminis*. The chasmothecia are dark pigmented. It might be hypothesized that the chasmothecia could be protective structures for *B. graminis* against various abiotic stresses, including UV irradiation. It would be helpful to analyze whether chasmothecia contain anti-UV pigments in further studies to provide more information to understand the functions of chasmothecia.

It was demonstrated that white light (234 µmol m^{-2} s^{-1}) triggered the elongation of *B. graminis* appressoria after an incubation period of 18 h. It remains unclear whether this stimulating effect is in an intensity-dependent manner. The experiments could be performed to determine whether conidia form a reduced appressorial length under the conditions of lower intensities of white light. It would be of particular interest in further studies to find out whether elongated appressoria are more successful under light conditions than their non-elongated counterparts.

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8 Appendix
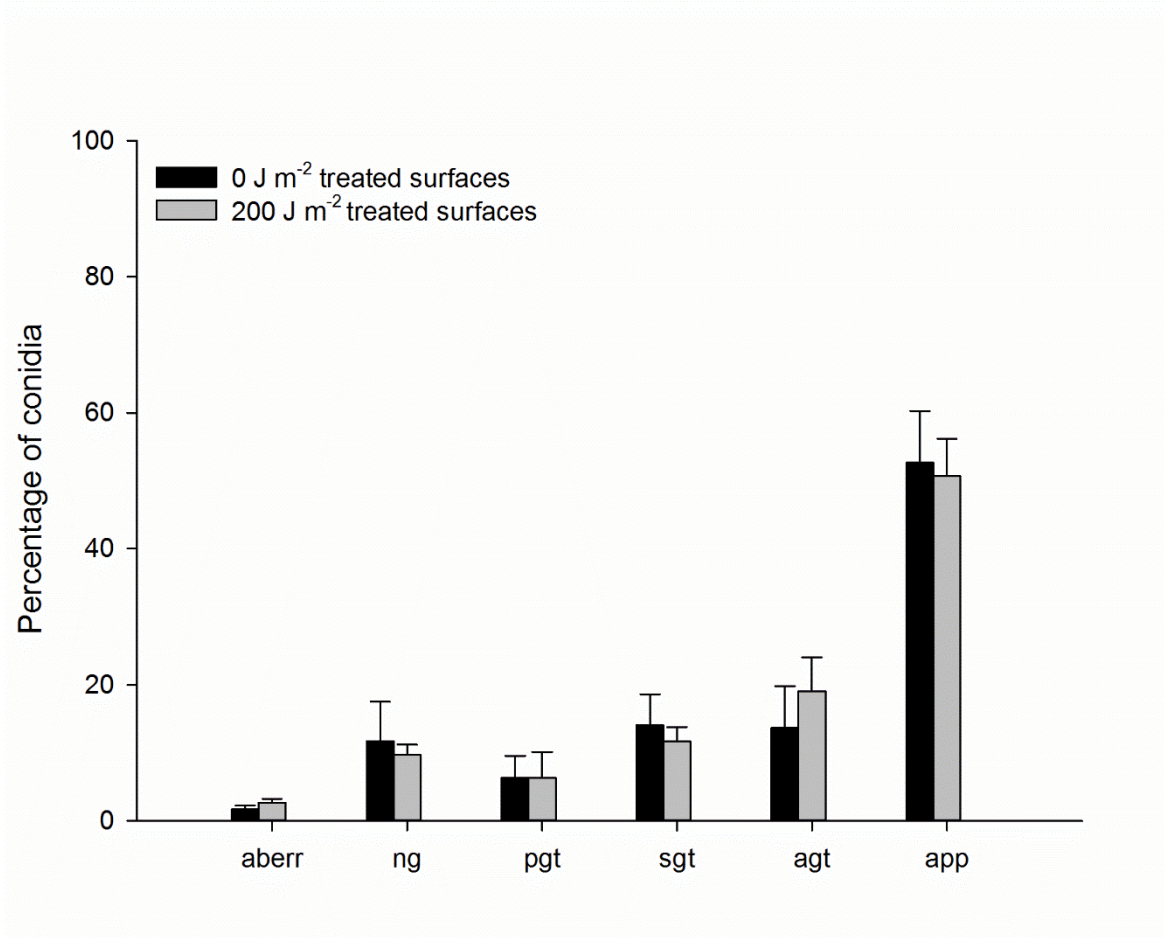
Figure 7.1 Development of *Blumeria graminis* f. sp. *hordei* conidia Formvar®/barley wax/agar coated glass slides pre-treated with or without UV-C irradiation. Developmental stages of *B. graminis* conidia: aberr, burst, dead conidia; ng, non-germinated conidium; pgt, germinated conidium with a primary germ tube only; sgt, conidium with an elongated secondary germ tube; agt, conidium with a swollen appressorial germ tube; app, conidium with a fully differentiated appressorium. Each value is given as mean ± SD of three independent experiments (100 conidia each). Significant differences were determined in Student's T test: * indicates significant differences ($P < 0.05$).

Non-germination

Differentiation
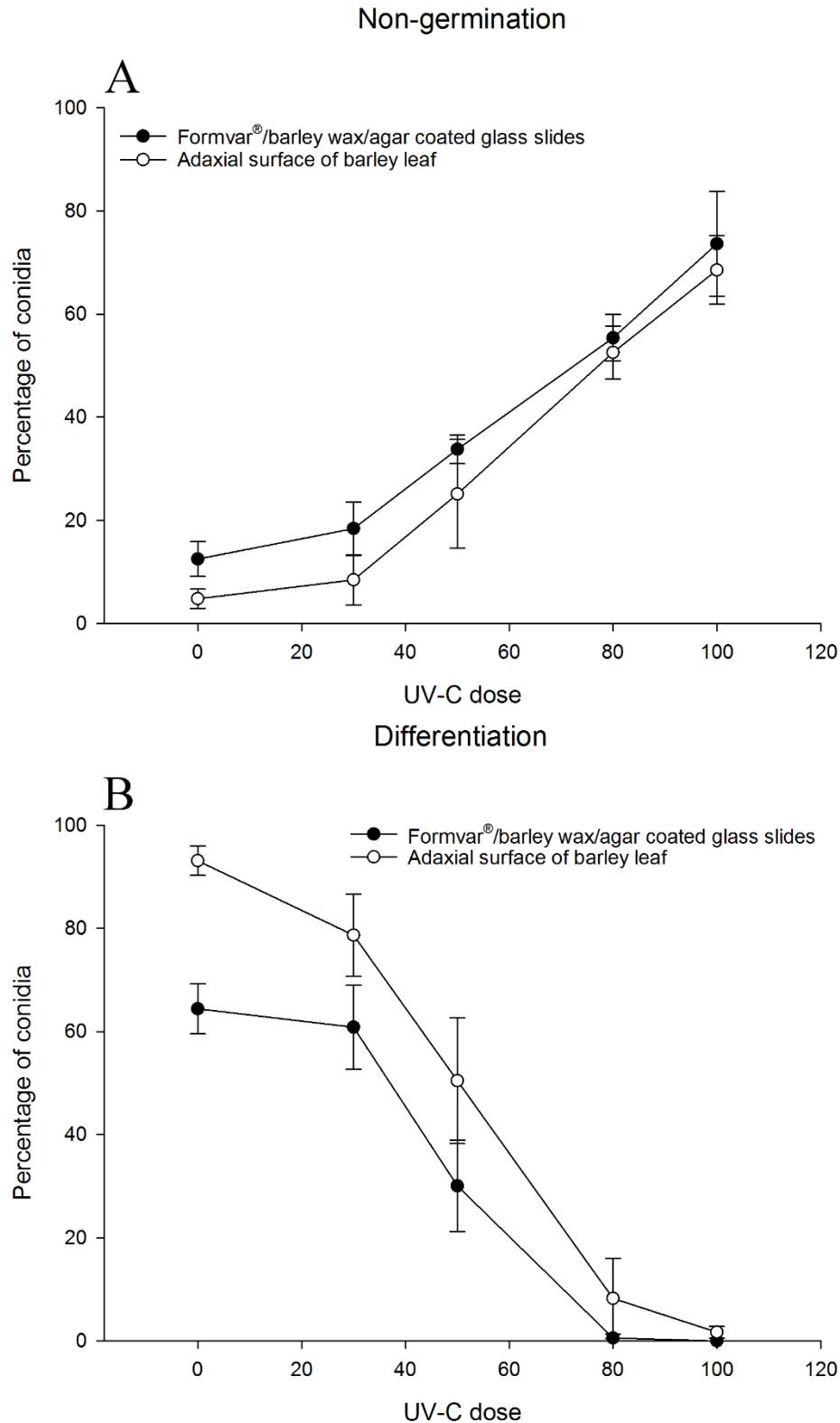
Figure 7.2 Development of *Blumeria graminis* f. sp. *hordei* conidia after UV-C treatment and an incubation period of 18 h in darkness on Formvar®/barley wax/agar coated glass slides and adaxial surface of barley leaf. Dose-response curves showing (A) proportions of non-germinated conidia and (B) of differentiated conidia. Non-germinated, conidia without a germ tube; differentiated, conidia with appressorial germ tube or an appressorium.

Non-germination

Differentiation
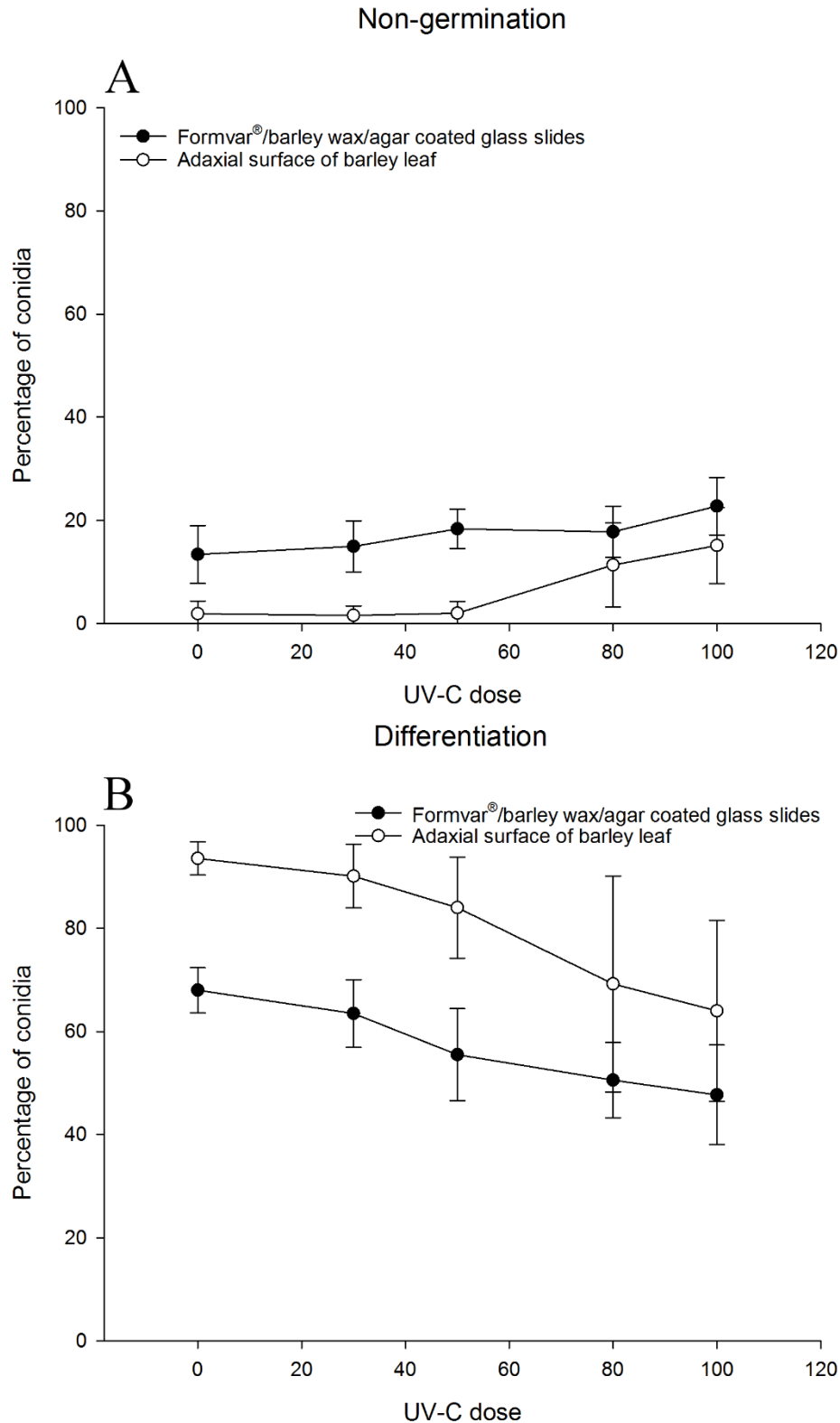
Figure 7.3 Development of *Blumeria graminis* f. sp. *hordei* conidia after UV-C treatment and an incubation period of 18 h in darkness on Formvar®/barley wax/agar coated glass slides and adaxial surface of barley leaf. Dose-response curves showing (A) proportions of non-germinated conidia and (B) of differentiated conidia. Non-germinated, conidia without a germ tube; differentiated, conidia with appressorial germ tube or an appressorium.

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Publications and presentations

Publications

1. **Zhu M**, Riederer M, Hildebrandt U*. Very-long-chain aldehydes induce appressorium formation in ascospores of the wheat powdery mildew fungus *Blumeria graminis* [J]. *Fungal Biology*, 2017, 121(8): 716-728.

2. **Zhu M**, Riederer M, Hildebrandt U*. Determination of UV-C irradiation affecting conidial prepenetration processes and photolyase gene transcription in barley powdery mildew fungus, *Blumeria graminis* f. sp. *hordei* (To be submitted)

3. **Zhu M**, Riederer M, Hildebrandt U*. White light-mediated elongation of appressorium of *Blumeria graminis* conidia. (In preparation)

Presentations

Conferences

1. 10th International Symposium on Phyllosphere Microbiology   07/2015

Poster presentation: *In vitro* white light impairs *Blumeria graminis* prepenetration processes

2. Eureka 10th International Students Symposium   10/2015

Poster presentation: White light impairs *Blumeria graminis* prepenetration processes *in vitro*

3. Eureka 11th International Students Symposium   10/2016

Poster presentation: Very-long chain aldehydes induce germination and differentiation of *Blumeria graminis* f. sp. *tritici* ascospores

4. POWDERY MILDEW GENOMICS WORKSHOP   09/2017

Oral presentation: Very-long-chain aldehydes induce appressorium formation in ascospores of the wheat powdery mildew fungus

Retreats

1. GK1342 Lipid Signaling Retreat   10/2014

2. GK1342 Lipid Signaling Retreat   10/2015 (oral presentation)

3. GSLS Retreat   05/2017 (poster presentation)

4. GSLS Retreat   05/2018 (oral presentation)

CURRICULUM VITAE

Affidavit

Affidavit

I hereby confirm that my thesis entitled "Germination and differentiation of *Blumeria graminis* ascospores and effects of UV-C and white light irradiation on *B. graminis* conidial prepenetration" is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Würzburg

Place, Date   Signature

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation „Keimung und Differenzierung von *Blumeria graminis* Ascosporen und Effekte von UV-C und Weißlichtbestrahlung auf die Konidienpräpenetration von *B. graminis*" eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Würzburg

Ort, Datum   Unterschrift