Achilles' heel of Grapevine Downy Mildew

The Contractile Vacuole as Target for Potential Control Strategies in Viticulture

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Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation mit dem Titel „Achilles' heel of Grapevine Downy Mildew: The Contractile Vacuole as Target for Potential Control Strategies in Viticulture" selbständig verfasst habe, und nur die angegebenen Quellen und Hilfsmittel verwendet habe. Alle Inhalte, die wörtlich oder sinngemäß aus anderen Arbeiten entnommen sind, habe ich unter Angabe der Quelle gekennzeichnet.

Zusammenfassung

Der Falsche Mehltau stellt seit dem 19. Jahrhundert eine der schwerwiegendsten Pflanzenkrankheiten im Weinbau dar. Sie wird durch den parasitisch auf Pflanzen lebenden Oomyceten *Plasmopara viticola* hervorgerufen, der von einem Algen-ähnlichen Vorfahr abstammt. Üblicherweise wird der Falsche Mehltau im Weinberg durch wiederholte Spritzungen mit Fungiziden in Schach gehalten, welche giftige Kupfer- und Aluminiumionen oder neuerdings synthetisch hergestellte Chemikalien enthalten, die die Atmungskette von Pilzen und Oomyceten inhibieren. Diese Fungizide sind für eine umweltfreundliche Bekämpfung des Falschen Mehltaus nur bedingt geeignet, da die Metallionen sehr giftig und persistent sind und die synthetischen Fungizide permanent chemisch angepasst werden müssen, da *P. viticola* extrem schnell Resistenzen bildet. Eine alternative Strategie stellt daher die Züchtung resistenter Reben dar, die auf einzelnen Loci des Rebengenoms basieren, welche mit Resistenz gekoppelt sind. Jedoch sind aufgrund der Variabilität des Falschen Mehltaus auch diese Rebsorten nicht stabil resistent und werden bereits innerhalb einer Saison befallen.

Diese Einschränkungen zeigen, dass neue Bekämpfungsstrategien gegen den Falschen Mehltau nötig sind. *P. viticola* hat mit seiner motilen Lebensform, den Zoosporen, eine verletzliche Seite, da sie lediglich von einer Membran umgeben sind und für eine kurze Zeit an der Oberfläche der Weinreben schwimmen. Diese Seite wurde in dieser Arbeit untersucht. Dazu wurden das frühe Infektionsverhalten auf Weinblättern sowie die zellulären Strukturen der Zoosporen untersucht, die zur Stabilität der Zoosporen beitragen. Der Fokus lag auf der Charakterisierung der kontraktilen Vakuole, einem überlebenswichtigen Organell in Zoosporen, welches einströmendes Wasser sammelt und in regelmäßigen Zyklen wieder ausschleust. Ohne diesen Mechanismus würden die Zoosporen platzen.

Die Ergebnisse zeigten, dass Blätter von Weinreben mit einer geringeren Dichteverteilung an Stomata eine geringere Anfälligkeit gegenüber Infektionen durch den Falschen Mehltau aufweisen. Zudem erfolgt das frühe Infektionsverhalten von Zoosporen über Chemotaxis, abhängig von dem Genotyp der Weinrebe und der Größe der befallenen Stomata. Diese Ergebnisse könnten bei zukünftigen Projekten zur Züchtung resistenter Rebsorten, die auf mehreren Genen beruhen, Verwendung finden.

Die Stabilität von Zoosporen, die wie Protoplasten nur von einer Membran umgeben sind, wird durch mehrere ineinandergreifende Strukturen und Prozesse aufrechterhalten, was durch Mikroskopie und funktionelle Assays gezeigt wurde. Die markanteste und wichtigste Struktur ist die kontraktile Vakuole, die das Volumen und das osmotische Gleichgewicht der Zoosporen reguliert. NaCl verändert das osmotische Potential, jedoch passen sich Zoosporen daran an und bleiben weiterhin motil. Mittels Videomikroskopie wurde aufgezeichnet, dass die Vakuole in *P. viticola* bereits im synzytialen Sporangium pulsiert, bevor Zoosporen gebildet werden, was zeigt, wie wichtig die Vakuole für Zoosporen ist. Sie sammelt über kleinere in der Zelle verteilte Vakuolen stetig das einströmende Wasser ein und pumpt es in regelmäßigen Zeitabständen durch Kontraktion wieder aus. Die Funktion der

Vakuole kann durch Kupfer- und Aluminiumionen spezifisch inhibiert werden, die mit Proteinen der Zoosporenmembran interagieren, was zum Platzen der Zoosporen führt. Auch durch RGD-Peptide, die mit dem Plasmalemmaretikulum interagieren, kann die Vakuole inhibiert werden sodass folglich die Zooporen platzen. Es wurde beobachtet, dass sich das endoplasmatische Retikulum während der Kontraktionszyklen dynamisch neu strukturiert. Neben der kontraktilen Vakuole ist auch das Aktinzytoskelett ein wichtiges Strukturelement für die Stabilität der Zoosporen und könnte sogar für die Kontraktion der kontraktilen Vakuolen verantwortlich sein. Es besteht aus sternförmig auseinanderstrahlenden Aktinfilamenten, die über Aktinplaques miteinander vernetzt sind. Das Aktinzytoskelett ist vermutlich über Integrin-ähnliche Proteine mit der Zellmembran und der extrazellulären Matrix verknüpft, was in einem Assay mittels RGD-Peptiden nachgewiesen wurde. RGD-Peptide sind jedoch nicht stabil und zu teuer, um als Fungizid angewendet zu werden.

Eine vielversprechende Alternative zur Mehltaubekämpfung wurde mit dem biologischen Extrakt aus den Wurzeln des Schmetterlingsblütlers *Glycyrrhiza glabra* (*G. glabra*) gefunden. *G. glabra* Extrakt verhindert Infektionen durch den Falschen Mehltau, da er inhibierend auf die Aktivität der kontraktilen Vakuole wirkt und zum Platzen oder verfrühten Enzystieren der Zoosporen führt. Im Gegensatz dazu wirkt *G. glabra* Extrakt stärkend auf das Immunsystem von Weinreben. Dies wurde in dieser Arbeit anhand eines Assays mit Zellkulturen von Pinot Noir gezeigt, bei dem *G. glabra* Extrakt eine extrazelluläre Alkalinisierung im Medium auslöst. Die Hauptwirkkomponente in dem Wurzelextrakt ist Glycyrrhizin, welches ebenfalls inhibierend auf die Vakuole der Zoosporen sowie stärkend auf die Immunabwehr von Weinreben wirkt. Dieser doppelseitige Effekt auf Pflanze und Pathogen macht Glycyrrhizin zu einem vielversprechenden Kandidaten für eine Anwendung im Weinbau zu der Bekämpfung des Falschen Mehltaus.

Der Falsche Mehltau kann sich extrem schnell an veränderte Umweltbedingungen anpassen, was dazu führt, dass er schon binnen einer Vegetationsperiode resistente Rebsorten befallen und Resistenzen gegenüber Fungiziden ausbilden kann. Um geeignetere Maßnahmen als die permanente Abwandlung von bereits bestehenden Fungiziden zur Eindämmung zu finden, könnte zukünftig vermehrt daran geforscht werden, den zugrunde liegenden Mechanismus dieses effizienten Anpassungsprozesses aufzuklären. Weitere Forschungsarbeiten zu Glycyrrhizin und dem Wurzelextrakt aus *G. glabra* könnten beispielsweise die Entwicklung geeigneter Formulierungen sein, die sicherstellen dass Glycyrrhizin auch bei Regen an den Blättern haftet. Dadurch wäre der Wirkstoff schon zum Zeitpunkt des Zoosporenschlupfs auf den Reben und würde im frühen Infektionsstadium durch Interaktion mit der Oberfläche von Zoosporen inhibierend auf die Vakuolenaktivität wirken und die Zoosporen zum Platzen oder verfrühten Enzystieren bringen. Dadurch wäre einer Epidemie durch den Falschen Mehltau auf umweltfreundliche Weise vorgebeugt und die Ernteerträge durch Verwendung von biologischem Glycyrrhizaextrakt gesichert.

Abstract

Downy Mildew is one of the major diseases in viticulture since the 19th century. It is caused by the plant parasitic oomycete *Plasmopara viticola*, which has algal ancestors. Conventionally, Downy Mildew is controlled by multiple applications of fungicides, which contain toxic copper and aluminium ions. Since the nineties fungicides are synthetically produced inhibitors of the respiratory chain of fungi and oomycetes. These fungicides are of limited suitability for Downy Mildew control in an ecologically friendly way. Not only are the metal ions highly toxic and persistent but also the synthetic fungicides require constant chemical adaptions because *P. viticola* rapidly evolves resistances. Therefore, breeding of resistant grapevine cultivars based on single resistance loci within the grapevine genome is an alternative strategy. However, due to the variability of Downy Mildew, these cultivars are not stably resistant and get already infected within one season.

These limitations show that new strategies are required for the control of Downy Mildew. *P. viticola* has a vulnerable side at his motile life cycle stage, the zoospores. Merely surrounded by one membrane, they swim on the surface of grapevines for a short time. This side was analysed in this study. For that purpose, the early infection behaviour on grapevine leaves was investigated as well as the cellular structures of zoospores, accounting for the stability of zoospores. The focus was set on the characterization of the contractile vacuole, a pivotal organelle of zoospores, collecting incoming water and expelling it again in regular cycles. Without this mechanism zoospores would burst.

The results revealed that grapevine leaves which have a lower stomatal density allocation exhibit a lower susceptibility to Downy Mildew infections. In addition, the early infection behaviour of zoospores occurs via chemotaxis, dependent on the grapevine genotype and the size of the infected stomata. These results could be used in future projects for breeding resistant cultivars based on several genes.

The stability of zoospores, which are merely surrounded by a membrane like protoplasts, is maintained by several interdependent structures and processes, shown with microscopy and functional assays. The most prominent and important structure is the contractile vacuole, which is regulating volume and osmotic potential of zoospores. NaCl changes the osmotic potential. However, the zoospores adapt to it and remain motile. Using video microscopy it was shown that the vacuole in *P. viticola* is already pulsing in the syncycial sporangium, before cellularization into zoospores, indicating the importance of the vacuole to zoospores. The vacuole collects the constantly incoming water via smaller vacuoles, distributed in the cell, and pumps it out again by frequent contractions.

The function of the vacuole can be specifically inhibited by copper and aluminium ions, which interact with proteins within the membrane of the zoospores, resulting in the burst of the zoospores. As well, the vacuole can be inhibited with RGD peptides, which lead to disintegration of the zoospores due to interactions with the plasmalemmal reticulum. It was observed, that the endoplasmatic reticulum gets

dynamically restructured during the contraction cycles. Besides the contractile vacuole, the actin cytoskeleton is an important structural element for the stability of the zoospores and could even be responsible for the contractions of the contractile vacuole. It is formed out of star-like radiating actin filaments, interconnected with actin plaques. The actin cytoskeleton is presumably connected with the cell membrane and the extracellular matrix by integrin-like proteins, which was shown in an assay using RGD peptides. However, RGD peptides are not stable and too expensive for being used as fungicides.

A promising alternative for the control of Downy Mildew was found with the biological extract of roots of the pea family member *Glycyrrhiza glabra* (*G. glabra*). The extract of *G. glabra* can prevent Downy Mildew infections due to its inhibiting effect on the activity of the contractile vacuole, leading to disintegration or premature encystment of the zoospores. In contrast, the extract of *G. glabra* has a tonic effect on the grapevine immune system. This was shown in this study in an assay using Pinot Noir cultured cells, in which *G. glabra* extract triggers an extracellular alkalinisation response. The main active compound in the root extract is glycyrrhizin, which has as well an inhibitory effect on the vacuole of the zoospores and a tonic effect on the immune response of grapevine cells. This double sided effect on plant and pathogen makes glycyrrhizin to a promising candidate for the application in viticulture for the control of Downy Mildew.

Downy Mildew can rapidly adapt to changing environmental conditions, resulting in the infection of resistant grapevine cultivars after only one vegetation period and development of resistances to fungicides. To find more suitable control measures than the constant adaption of already existing fungicides, increased future research could be done to elucidate the mechanism behind this efficient adaption process. Further projects regarding glycyrrhizin and the root extract of *G. glabra* could for example identify appropriate formulations, which ensure adhesion of glycyrrhizin on leaves during rain. Thus, the active component would be present on the grapevine already when zoospores emerge. The activity of the vacuole would be inhibited during early infection state by interaction with the zoospore surface, resulting in burst or premature encystment. The use of the biological extract of *G. glabra* would prevent an epidemic spread of Downy Mildew ecologically friendly and protect crop yields.

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1 Introduction

Grapevine (*Vitis*) is a common crop plant cultivated in the temperate zones all over the world, including Europe, Asia, the Southern Hemisphere and the USA. Besides its use for direct consumption it is used for the production of vine. The global wine production of 2011 was 267,000 million hectolitre (International Organisation of Vine and Wine, 2013). The three major wine producing countries - France, Italy and Spain - produced almost 50% of this amount. The genus *Vitis* consists of many species, cultivars and accessions. They show a spectrum of properties, not only qualities relevant for the market, like fruit colour and taste, but also those relevant for the production, like fruit productivity and resistance level to pathogens. In Europe, *Vitis vinifera* ssp. *vinifera* is the main crop in viticulture, characterized by an intensive taste but also a high susceptibility to diseases like Powdery Mildew and Downy Mildew, the major diseases worldwide in viticulture. To combat these diseases, which can cause total yield losses (Wille, 1927) and tremendous financial losses in viticulture, a high amount of pesticides is used (Gessler et al., 2011). At the beginning of viticulture in Europe, a copper sulphate and hydrated lime containing aqueous solution, called Bordeaux mixture, was successfully used (Millardet, 1885). Later many adjustments have been developed, regarding copper amount in the mixture, the timing of pesticide applications depending on the infection status of Mildews, the weather conditions, the season or the spray apparatus, which is reviewed in detail in (Gessler et al., 2011). Due to the high toxicity of metal ions, it is recommended to find alternatives for the control of Downy Mildew. Only with an efficient control, the aim of the global wine industry can be achieved – the sustainable production of high quality grapes and wines in high yield and with a minimum of pesticide usage. To provide new strategies for a more sustainable control, it is important to investigate the Downy Mildew disease in more detail. In this study, cellular properties of Downy Mildew were investigated in high resolution and functional details were explored. The results will provide a new base for future control strategies that are far more sustainable than the current methods, saving the environment as well as the human health using non-toxic compounds for treatments against Downy Mildew.

1.1 Biological view on grapevine Downy Mildew

Downy Mildew is caused by *Plasmopara viticola* (*P. viticola*), an obligate biotrophic parasitic protist, belonging to the class of Oomycetes or biotrophic Peronosporomycetes (Spring & Thines, 2004). Oomycetes occur in various environments including freshwater, marine water, soil or they live as sapronts or parasites on plant or animal hosts. Some plant parasitic Oomycetes have agricultural and economic importance such as the order of Peronosporales, containing not only *Plasmopara* but also the genera *Bremia*, *Peronospora* and *Phytophtora*. Famous members besides Downy Mildew of grapevine are the Downy Mildew of potato (*Phytophtora infestans*), which caused the Great Irish Famine in 1845 as well as the Downy Mildew of more than 100 different species of trees and ornamental plants worldwide (*Phytophtora ramorum),* which causes the Sudden Oak Death nowadays. Oomycetes are pseudo-fungi. Originally considered as true fungi, they are meanwhile classified into the phylum of biflagellate algae, called Heterokontophyta (Beakes et al., 2012), due to several deviating properties. Oomycetes are diplonts, not haplonts. Their cell wall components are glucan-cellulose (Hardham, 2007; Kortekamp, 2005) and rarely minor amounts of chitin (Werner et al., 2002). They pass through a characteristic motile stage during their life cycle (see Fig. 1.2), which enables the zoospores to swim in a defined direction by two differently shaped flagella. During non-motile stages, the Oomycete thallus is growing unicellular to mycelia, mainly without septa, which is another difference to fungi. Also the inner organelles show differences: Oomycetes possess mitochondria with tubular christae, like slime moulds, in contrast to fungi which possess plate-like christae as animal and plant cells.

During sexual reproduction, ploidy of the diploid gametes is changing into haploid cells (Carlile et al., 2001). Oomycete gametangia are large female oogonia (unfertilized eggs) and small male antheridia, which undergo meiosis to give haploid nuclei (see Fig. 1.2). The haploid antheridia grow on the surface of oogonia and produce fertilization tubes, which penetrate the oogonium. Each tube fuses with one oosphere, providing one single haploid nucleus for fusion with one single haploid nucleus from the oosphere, forming diploid oospores (fertilized eggs). Oospores possess resistant cell walls and thus are well protected against environmental influences like low temperatures. These properties enable Downy Mildew to survive the winter season within foliage and soil (Vercesi et al., 1999). After hibernation, humid and 16-23°C warm spring and summer periods revitalize Downy Mildew and their life cycle continues. The robustly armoured oospores first initiate primary infection and produce a germination peg terminating in primary sporangia (Müller & Sleumer, 1934). Primary sporangia release primary zoospores, which are followed by many cycles of secondary sporangia and zoospores. Numerous secondary sporangia are produced and transported by

wind and rain drops whilst each sporangium evolves to 5-8 motile zoospores at high humidity (Riemann et al., 2002) within 50 minutes. Released zoospores swim towards the plant stomata (see **Fig. 1.1**) and start new infection cycles. Stomata on healthy grapevine tissues can be infected including leaves, stems, inflorescences and also berries. Although Downy Mildew seems to be non-toxic for humans, the losses in yield and quality by the resulting dry berries are substantial.

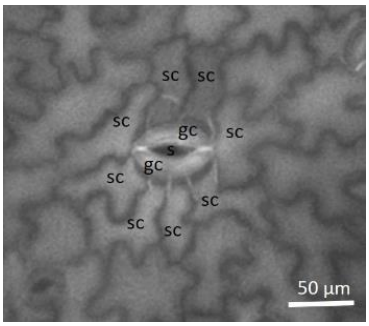


Fig. 1.1 Stomatal complex, found on the lower epidermis of a *Vitis sylvestris* leaf. (s) stoma, (gc) guard cells, (sc) subsidiary cells. Picture shows a glue imprint, seen with bright field microscopy.

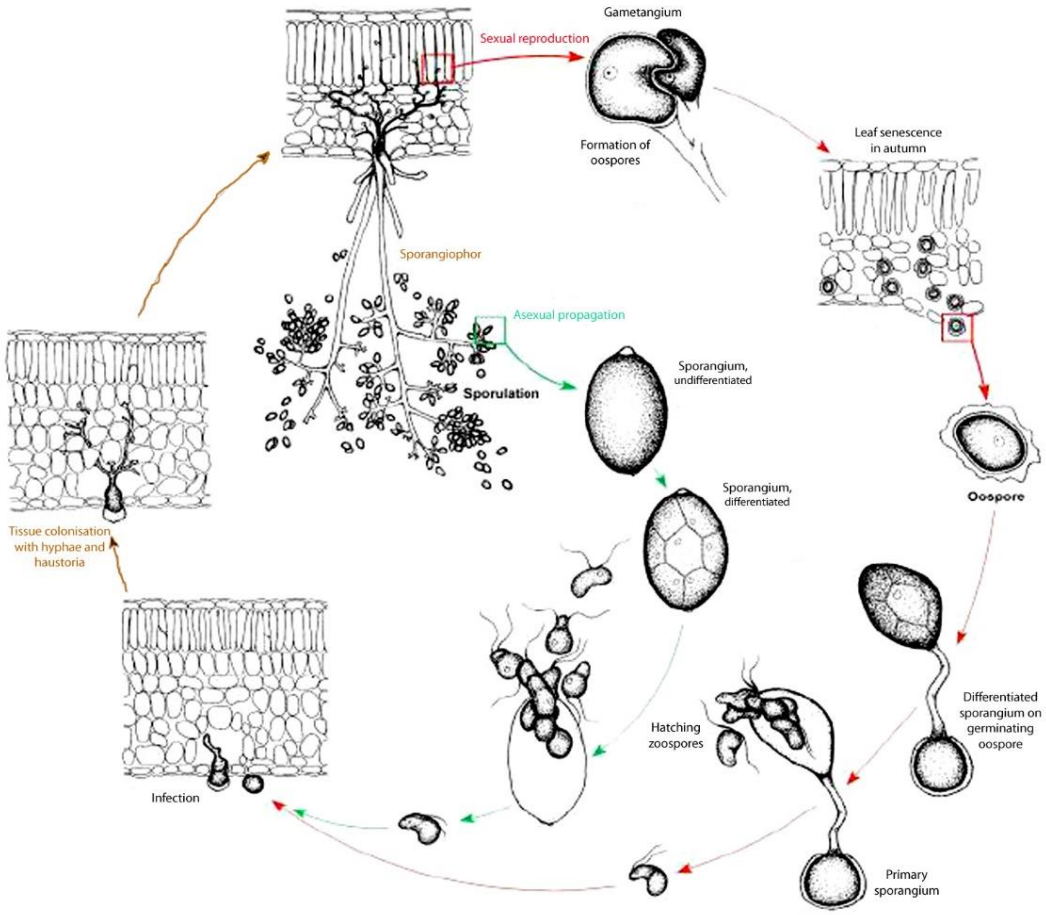


Fig. 1.2 Life cycle of *Plasmopara viticola* (adjusted from Schoepe). Red arrows depict sexual reproduction steps, green arrows describe the asexual reproduction cycles. Shared steps are connected in brown.

The anatomic and molecular details of the infection cycle are specified further to point out interesting characteristics of this efficient plant-pathogen interaction. Zoospores use the plant stomata as entries into the plant host. Stomata evolved around 400 million years ago, enabling land plants to survive in air by regulating their gas exchange during photosynthesis and respiration and preventing desiccation (Peterson et al., 2010). They consist of symmetric guard cells that operate by changing turgor pressure. Together with surrounding subsidiary cells and the stoma they build up the stomatal complex (see

Fig**. 1.1**). Once the biflagellate zoospores have successfully located a stoma, they attach at the guard cells by means of adhesive mucus consisting of a sticky protein (Robold & Hardham, 2005), shed their flagella, and encyst there. During encystation, zoospores undergo massive changes in structure. They round up, rapidly generate a cellulosic cell wall, eject the two flagella, and polarize their cytoskeleton (Riemann et al., 2002). After several hours, cysts germinate and produce a germination peg which can orient itself and reaches into the substomatal cavity. There, a substomatal vesicle is built from which a primary hypha emerges. The hypha penetrates into the mesophyll cells and forms haustoria to get nutrients from the host plant, without breaching the host membrane.

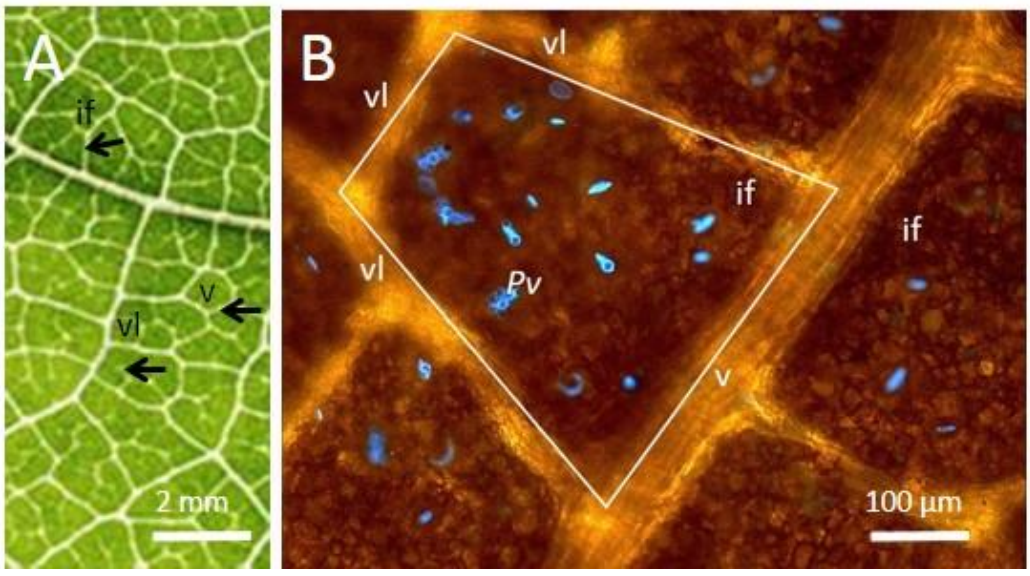


Fig. 1.3 Intercostal fields, found on the lower epidermis of an infected *Vitis sylvestris* leaf disc. **A** View on leaf blade with bright field microscopy. (if) intercostal field, (v) vein, (vl) veinlet. **B** Closer view through a fluorescence microscope, taken after staining structures of *P. viticola* with calcofluor white. Bright field channel in overlay with the DAPI filter. (*Pv) P. viticola* encysted zoospores.

The hyphae develop further into mycelia colonizing the intercellular space of one intercostal field (see Fig. 1.3), the area of a leaf between larger veins and veinlets. At this stage, the infection can be observed with the naked eye as yellow "oil spots" on top of grapevine leaves.

After a few days, the mycelium develops sporangiophores that protrude through the stomata, and form tree-like branched structures, which carry lemon-shaped sporangia. This sporulation is visible as whitish powder on the bottom side of the leaves, eponymous for the name of the disease: Downy Mildew.

Summer periods are characterized by high rates of asexual reproduction, resulting in epidemic spread all over a vineyard within a few days. This efficiency can be exemplified with the order of events after the historical incidence, that *P. viticola*, native to the United States of America, has accidentally been introduced into Europe on grapevine root stocks used as biological control against the pathogenic insect *Phylloxera* in the 19th century (Gessler et al., 2011). In the United States, grapevine cultivars had evolutionary contact with the pathogen. Presumably, American grapes developed preformed defence strategies in co-evolution with Downy Mildew (Jürges et al., 2009). Therefore, Downy Mildew is not a big problem in America. European grapevines never had contact with *P. viticola* before. They were naive hosts without any evolutionary barrier against *P. viticola*, which could propagate successfully and thus rapidly conquered all vineyards in Europe. The economically used species in Europe are susceptible until today. Some exceptions are found in *Vitis sylvestris* species, which will be explained below.

It was proposed that this highly efficient infection cycle is correlated with a high efficiency of zoospores to sense and target stomata (Royle & Thomas, 1973; Kiefer et al., 2002). The impact of light conditions was found to be different for two Oomycete species. Zoospores of *Pseudoperonospora humuli* target stomata faster at light compared to in the dark, whereas zoospores of *P. viticola* also target in the dark and additionally show the effect of targeting stomata in groups (Royle & Thomas, 1973).
The targeting and settling on stomata was described to be achieved by two independent factors, a chemical one and at least for *Pseudoperonospora humuli* a physical one from open stomata (Royle & Thomas, 1973). Therefore, the chemical factor likely derives from the host plant. The importance of host factors for this sensing is illustrated by the fact that zoospores are released more rapidly in presence of the host compared to a host-free system (Kiefer et al., 2002). Moreover, zoospores accumulate at the stomata within several minutes and only rarely attach elsewhere. This stomatal targeting is not observed on the upper leaf surface lacking stomata and is impaired, when stomatal closure is induced by abscisic acid. Also subsequent steps of early pathogen development, such as germ-tube morphogenesis seem to be coordinated by unknown signals from the host (Kiefer et al., 2002).

1.2 Control strategies for Downy Mildew disease

Control of Downy Mildew in the highly susceptible European grapevine species *Vitis vinifera* is difficult since most of the life cycle proceeds inside the host. In fields without fungicide applications, yield losses can reach 100% (Wille, 1927). Traditional fungicide applications are based on copper and aluminium, nevertheless they are toxic to many organisms. Aluminium has highly toxic influence on plants in several manners. It inhibits root growth by reducing endocytosis and membrane fluidity and causes changed root morphology as well as it has a stabilizing effect on cortical microtubules (Krtkováa et al., 2012). Phospholipid degrading enzyme phospholipase D plays an important role in regulation of cytoskeleton remodelling in both animals and plants. It was shown that phospholipid pathway and the cytoskeleton are influenced by Al^{3+} (Pejchara et al., 2007). Copper affects biological diversity in the soil and impairs the ground water (reviewed e.g. in Van-Zwieten et al. 2004). Copper ions are blocking enzymes in microorganisms in very low concentrations (Kovacic et al., 2013) and produce highly reactive oxygen species (Stenersen, 2004). Meanwhile, copper is only admitted for organic viticulture as alternative for conventional fungicides. However, this practice is seen progressively as problematic. Conventional fungicides against Downy Mildew include the strobilurins, for example azoxystrobin (Bartlett et al., 2001) and associated compounds belonging to the group of QoI (quinone outside inhibitors) fungicides. These fungicides act by inhibiting mitochondrial respiration at complex III (ubiquinol oxidation) in fungi and Oomycetes, blocking electron transfer between cytochrome b and cytochrome c1. Thus, the energy cycle is disrupted by halting the production of ATP. However, already after 10 years of usage, *P. viticola* strains have acquired resistance against these compounds and have spread all over Europe (Chen et al., 2007; Toffolatti et al., 2011). Whether the resistance against QoI fungicides might be conferred by point mutations in the cytochrome b gene is controversial (Grasso et al., 2006). To increase the efficiency of fungicide applications and to contain spread of fungicide resistance, interactive prediction systems based on epidemiological, meteorological and biological data have been developed (Bleyer et al., 2008; Raynal et al., 2011).

An alternative strategy has been the breeding of resistant cultivars that are based on resistance loci from North American or Siberian grapes, resulting in so-called fungus-resistant "PiWi" grape varieties (from German pilzwiderstandsfähige Rebsorten, PiWi International), for example Regent, Johanniter and Rondo. However, the initial success of this strategy is progressively endangered by the evolution of new strains of *P. viticola* that are able to overcome these resistances and to infect these resistant cultivars (Delmotte et al., 2014). Therefore, breeders are working on an enhancement of this strategy, breeding resistant

cultivars based on combination of several resistance loci. The combination should result in an additive resistance effect, called pyramidization. An adaption of *P. viticola* to the resistance mechanism should get more and more difficult with each added resistance factor. This would result in a more stable resistance over longer time (Schwander et al., 2012).

The European wild grapevine *Vitis sylvestris* has found to be an exception in the susceptible genus *Vitis*. Some of its accessions seem to harbour natural resistance factors against Downy Mildew, despite the lack of co-evolutionary development (Gessler et al., 2011; Schröder et al., 2015), which makes it a promising candidate for breeding. These factors were found by screening a unique collection of the survivors of the European wild grapevine *Vitis sylvestris*, originating from the largest *sylvestris* population in Germany at the Ketsch peninsula in the Rhine river (Duan et al., 2015; Schröder et al., 2015). These Ketsch accessions (Ke) are part of a complete genetic copy for the *sylvestris* genotypes still available in Germany that was established for an ex situ conservation project in the Botanical Garden of the Karlsruhe Institute of Technology, and characterized genetically by microsatellite markers as well as with respect to their infection levels against Downy Mildew, Powdery Mildew and Black rot (Tisch, unpublished data), their morphological traits (Rühle, 2011) and their genetic recourse of stilbenes. One cellular resistance factor was found - the density of stomata per square millimetre, a factor dependent from environmental factors influencing growth, like for example age of the observed leaf as well as light, soil and water conditions. It slightly correlated with infection level to Downy Mildew (Rühle, 2011). To specify this correlation, the wild grape collection was screened in this study for stomatal cellular density (stomata per epidermal cell). This factor has the advantage that the number is genetically regulated and therefore independent from leaf size and environmental influence.

1.3 Potential "Achilles' heel" of the Downy Mildew disease

Achilles was described as a powerful warrior in the ancient Greek literature (Homer's *Ilias*). His only weakness was his heel, through which he was finally killed. As well, *P. viticola* can be regarded as powerful warrior, which might have a weak point where it might be killed easily. The limitations in chemical or genetic control of Downy Mildew described in the previous section demonstrate that alternative, weaker targets are warranted. During most of its life cycle, the pathogen is well protected against fungicides. Either it is not accessible because it is hidden inside the host leaf or it is protected by cell walls, as in the case of sporangiophores, sporangia and cysts. Due to the cellular composition, which is similar to plant cell walls (Kortekamp, 2005; Hardham, 2007), it is complicated to investigate fungicides specific for Oomycetes, not impairing plants. There is only a very short phase in the life cycle, where the pathogen is really vulnerable: between hatching from the sporangium and the encystment at the stoma. This short phase represents something like the "Achilles' heel" of the pathogen and might be used as target for chemical plant protection. Zoospores provide at least two targets that are interesting: they need to locate the stomata, and they need to maintain their integrity during this mobile phase (see Fig. 1.4).

It has been implicated by a comparative infection study that the stomatal targeting of mobile zoospores might contribute to differences of susceptibility between different grapevines. Grapevine species from North America, Asia and Europe revealed differences in the response to *P. viticola* infection (Jürges et al., 2009). European species are successfully colonized, whereas species from North America and one species from Siberia can arrest the colonization of *P. viticola* briefly after the formation of a germ tube. In several Asiatic species, a third response pattern was observed, where stomatal targeting was impaired. As a consequence, a small and aberrant mycelium is produced on the surface of the leaf that is not leading to a successful infection (Jürges et al., 2009). The fact that failed targeting of zoospores correlates with a failure of colonization suggests that specific (host) signals control the interaction between host and pathogen. Chemotaxis of zoospores has also been reported for other Oomycetes, for instance, for the guiding of *Phytophthora cinnamomi* to the root (Hardham, 1987). In this study, stomatal targeting was investigated using pictures of *P. viticola* encysted zoospores like in Fig. 1.4 A from single sporangial strains (Gómez-Zeledón et al., 2013), evolved by a single isolated sporangium and propagating the hatching zoospores on a fresh grapevine leaf. Usually *P. viticola* material is collected in fields and further propagated on grapevine tissue. Experiments usually showed highly variable results. This can be explained by the high variability of genotypes in field isolates, which also explains the ability of *P. viticola* to adapt rapidly to its environment and host. The genotypes of zoospores within

one sporangium are limited. Thus, the single sporangial strains showed more stable properties and they could be characterized regarding their infection behaviour on different *Vitis* genotypes and their resistance against fungicides as well (Gómez-Zeledón et al., 2013). The advantage of these strains is higher reproducibility of experiments due to their defined genotype.

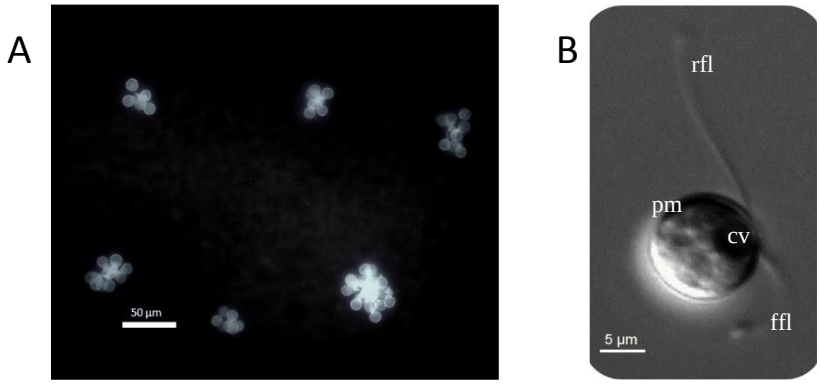


Fig. 1.4 Zoospores of *P. viticola* as potential "Achilles' heel" of Downy Mildew of grapevine. **A** Stomatal targeting revealed gathering effect of several zoospores per stoma. Six successfully targeted and infected stomata with cysts and germinated cysts 60 minutes after inoculation of *Vitis sylvestris*. **B** Zoospore, lacking cell walls, seen in DIC channel of bright field microscope. (rfl) rear whiplash flagellum, (pm) plasma membrane, (cv) contractile vacuole, (ffl) front tinsel flagellum.

The second target is linked with the fact that zoospores lack cell walls. They are only protected by a cell membrane, making them fragile like protoplasts during the time interval of 15-30 min, while they are swimming unprotected over the leaf surface (Fig. 1.4 B). Probably attracted by plant substances, released from the stomata, they target stomata, encyst there and reacquire a cell wall that safeguards the cell against environmental influences. Before encystation, the zoospores have to cope with a constant influx of water. To avoid bursting, zoospores are endowed with contractile vacuoles, collecting water and expulsing it in regularly cycles. Contractile vacuoles are pivotal for the regulation of cell volume and osmotic potential in freshwater protists (Patterson, 1980). The contractile vacuole represents an interesting potential target for chemical plant protection because this structure is specific for Oomycetes. Therefore, inhibition of the vacuole should leave the host cells untouched.

Nevertheless, knowledge about volume control of plant cells might contribute to understand the physiology and regulation of the contractile vacuole. In fact, also plant protoplasts, although lacking a contractile vacuole, are able to adjust cell volume to a certain extent in response to osmotic challenge by releasing or recycling membrane material from internal stores (Liu et al., 2013). This volume control is linked with a dynamic remodelling of submembraneous actin. Thus, actin must physically interact with the cell membrane and integrate mechanic load acting upon the membrane (reviewed in Nick, 2011). Although plants

lack canonical integrins that convey this function in mammalian cells, there are hints to functional analogues. For instance, the involvement of integrins or integrin-like proteins in adhesions was tested in animal cells by adding specific heptapeptides (YGRGDSP, usually referred to as RGD peptides) that compete for integrin-binding sites on ECM molecules (Schindler et al. 1989; Wayne et al. 1992). This titration of the interaction of integrins with the extracellular matrix is also active in plant cells and leads to disrupted adhesion (Gronowicz & Derome, 1994; Canut et al., 1998; Ruoslahti, 1996; Zaban et al., 2013; Ruoslahti, 1996)). Recently, green algal integrins were described (Becker et al., 2015).

In addition to this functional analogy, there are structural analogies as well. A meshwork of adhesion sites at the plasma membrane, connecting membrane with cortical cytoplasm, termed the plasmalemmal reticulum (reviewed in (Pickard, 2008) shares morphological and molecular similarities with adhesion sites of mammalian cells and has been implicated with mechanosensory ion channels, controlling cellular rise of Ca^{2+}.

Whether Oomycetes are endowed with a plasmalemmal reticulum is not known. However, the observation of fungal RGD-mediated adhesion (Hostetter, 2000) and specific effects of RGD peptides in *Achlya* and *Saprolegnia* (Chitcholtan & Garrill, 2005; Kaminskyj & Heath, 1995) indicates that functional integrin analogues also span the plasma membrane in Oomycetes and might integrate the extracellular matrix with intracellular actin.

The actin cytoskeleton of *P. viticola* is organized in stage-specific patterns forming granulous plaque-like structures, typical for Oomycetes (Heath, 1990), along with filaments in encysted and germinated spores and longitudinal strands in germ tubes (Riemann, 2000). Oomycete actin is strongly polarized at the site of germ tube emergence in *Phytophthora cinnamomi* (Hyde & Hardham, 1993) and in the germ tube itself (Riemann et al., 2002). Actin plaques are assumed to be organization centres, related to the reorganization of cell structure and the transition of the cell into a new stage (Riemann et al., 2002).

An involvement of actin filaments and analogues or homologues of a plasmalemmal reticulum for the function of the contractile vacuole are likely, but the structural detection of these components *in vivo* is far from trivial, since the zoospores are very small and moving vividly. However, it is possible to probe the functionality of the contractile vacuole by quantifying zoospore burst in response to compounds supposedly interfering with the plasmalemmal reticulum (RGD peptides), osmotic potential (NaCl) or apoplastic oxidative burst (Cu and Al ions) as important signal for osmosensing (reviewed in Ismail et al. 2014). Burst zoospores are characterized by break up of their cell membrane, expulsion and diffusion of the intracellular vacuoles and organelles due to loss of turgor pressure, and a remaining constant

branched meshwork structure. These functional data of the contractile vacuole were combined with kinetic microscopy at high-resolution by both differential interference contrast and after fluorescent visualization of actin and endomembrane system. The developmental sequence of the contractile vacuole was followed from its genesis during cellularization of the syncytial sporangium until its disintegration upon encystment.

1.4 New biological alternative multi-site control strategy using a medicinal plant

In search of ecological alternatives for sustainable organic farming, biological extracts from different plants are investigated for their potential effects on plant health against diseases (Thuerig et al., 2015). A promising discovery succeeded with the liquorice plant *Glycyrrhiza glabra* (Fig. 1.5 A) from the family of Fabaceae few years ago. It was revealed that a raw extract of the roots of *G. glabra* (Fig. 1.5 B) possesses a positive influence on vegetables against different Downy Mildews (*Pseudoperonospora cubensis*, *Peronospora destructor* and *Bremia lactucae*) (Schuster et al., 2010; Schmitt et al., 2011) in a government-funded research program (BÖLN). In greenhouse trials infections could be reduced at 70-100% using 3-5% solution of *G. glabra* extract. In contrast, in field applications impact was reduced, presumably due to rainfastness and UV-stability.

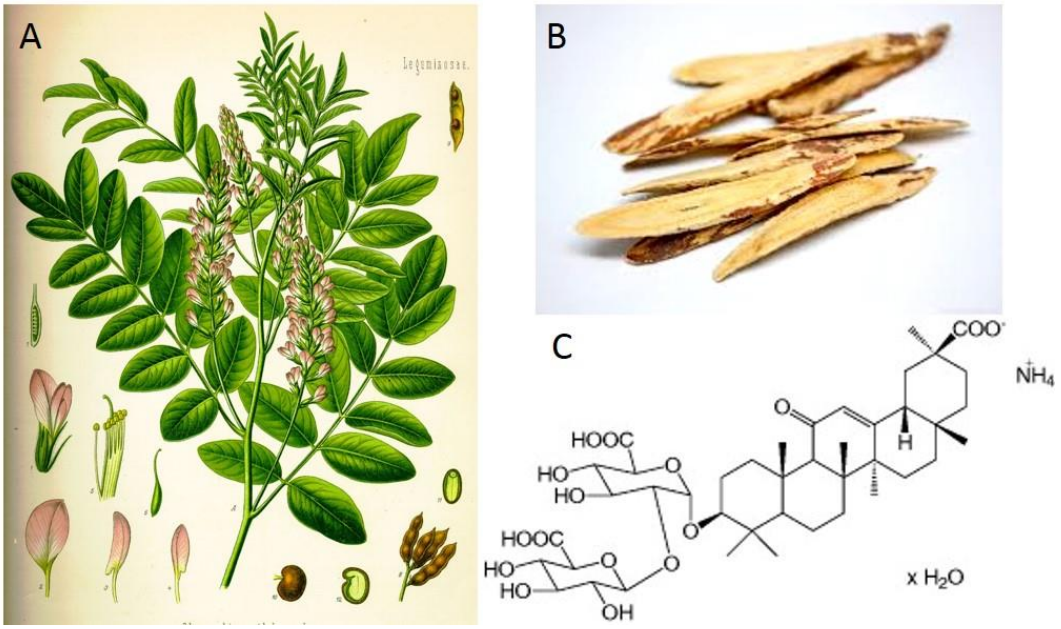


Fig. 1.5 Liquorice, its organs and main ingredient. **A** *Glycyrrhiza glabra* (drawing by Franz Eugen Köhler, Köhlers Medizinal-Pflanzen in naturgetreuen Abbildungen und kurz erläuterndem Texte, image from http://caliban.mpiz-koeln.mpg.de/koehler/SUESSHOLZ.jpg). **B** Slices from *G. glabra* root (image from http://orionherbs.com/wp-content/uploads/2012/05/Gan-cao2.jpg). **C** Structure of glycyrrhizin (glycyrrhizinate ammonium salt, Carl Roth, Germany).

Traditionally, liquorice is used as medicinal plant in many cultures worldwide with a wide range of beneficial properties for human health. The roots of the two species *Glycyrrhiza glabra* and *Glycyrrhiza uralensis* are used for the production of liquorice or for medicinal purposes, for example as powder or crushed to a tea ingredient (Fig. 1.5 B). For instance, the anti-inflammatory action of liquorice extract is approved, showing significant antiradical activity, protective effect against lipid peroxidation of liposomal membrane and inhibitory effect on whole blood reactive oxygen species (ROS) liberation (Racková et al.,

2007). Anti-bacterial properties of liquorice were shown in a µM range (Gupta et al., 2008). As well, anti-viral properties were confirmed, inhibiting the replication of flaviviruses (Crance et al., 2003).

The main active compound in liquorice is glycyrrhizin (Fig. 1.5 C). As a tribasic acid, glycyrrhizin can form a variety of salts. In liquorice roots it occurs as calcium and potassium salts. Also compounds with one or no sugar molecule appear in liquorice roots (Fig. 2.1). It was demonstrated, that the aglycon component β-glycyrrhetinic acid also has anti-bacterial activity against *Bacillus subtilis* and *Staphylococcus epidermidis* (Astaf'eva & Sukhenko, 2014). It has no haemolytic properties on human erythrocytes. Confocal microscopy showed that β-glycyrrhetinic acid was located within the bacteria, but had not caused membrane disruption. Glycyrrhetinic acid was shown to act as a dual inhibitor of two ABC-transporters, proteins which export a wide variety of compounds over the plasma membrane. ABC-transporters are also present in plants and Oomycetes (Latijnhouwers et al., 2003). Comparing to the mechanism in bacteria, the authors suggested in this review that Oomycetes as well evolved a mechanism to export anti-microbial host compounds, presumably using ABC-transporters. This could be one explanation for the anti-microbial activity of liquorice extract.

1.5 Scope of this study

P. viticola is one of the biggest challenges in viticulture since it was introduced from America to Europe in the 19th century. Many strategies have been invented to save yields, mostly defending epidemic spread of the pathogen with chemical applications but also improving grapevine plants by breeding or adapted cultivation techniques. Due to restrictions of fungicides, organic fields are treated with traditional copper solutions. However, none of these strategies provides a method for long-term and sustainable control of Downy Mildew. Therefore, new effective strategies are required. To target the pathogen with more precision, a detailed characterization of its structures is required. To improve grapevine properties, a detailed characterization of all possible traits is inevitable. This study builds on prior characterizations of grapevine and its pathogen *P. viticola* and follows both approaches, providing the base for new strategies for both sides for a sustainable organic viticulture. Based on a detailed screening of genetic and morphological properties of European wild grapevine *Vitis sylvestris* (Rühle, 2011; Schröder et al., 2015), this study investigated one cellular promising detail of grapevine leaves, the stomatal density. This factor was found to correlate with lower susceptibility to Downy Mildew infections. Due to its genetic determination it might be a tool for future breeding strategies.

On the pathogen side, the zoospores are regarded as the most vulnerable life cycle stage of *P. viticola*, providing two interesting targets, which have been followed in this study: the process of chemotaxis to stomata and the need to maintain zoospore integrity during their mobile phase. Stomatal targeting of mobile zoospores has been investigated in infection studies and microscopic evaluation and quantification. The second target, the fragility during mobile phase, relies on the activity of contractile vacuoles of zoospores. The vacuole activity represents an interesting potential target for chemical plant protection. The cellular details and the functionality of the contractile vacuole have been investigated in detail. The function may rely on the participation of cytoskeleton and indeed actin filaments have already been detected in zoospores. The cell membrane is regarded as an additional important factor. Therefore, actin filaments as well as analogues or homologues of a plasmalemmal reticulum as connector between cell membrane and cytoskeleton are investigated in this study.

In summary, the aim of this study is to create a base for new control strategies against Downy Mildew of grapevine, targeting the unprotected fragile zoospore state of *P. viticola* and to find alternatives for copper based fungicides. Additionally, *Vitis sylvestris* is investigated for its suitability for breeding new cultivars with lower susceptibility against Downy Mildew.

2 Materials and methods

2.1 Plant material

2.1.1 Vitis vinifera L.cv. Müller-Thurgau

The highly susceptible *Vitis vinifera* L.cv. Müller-Thurgau was used for propagation of the pathogen material. Plants were grown in the greenhouse in pots with day light and additional lamps during the day for 14 hours (400 W, Vialox NAV-T (SON-T); Osram, Munich, Federal Republic of Germany). Fully expanded leaves were harvested and used for the pathogen propagation in petri dishes.

2.1.2 *Vitis vinifera* ssp. *sylvestris* Ketsch Collection

The *Vitis vinifera* ssp. *sylvestris* plants used in this dissertation originate from an *ex-situ* collection as cuttings from the Rhine peninsula Ketsch in the south of Germany (Nick, 2014). This peninsula harbours the largest natural population of European wild grapes in Central Europe. The collected accessions were named with 'K' and a number and are maintained in the field area of the Botanical Garden of Karlsruhe Institute of Technology. The entire collection was previously characterized morphologically (Rühle, 2011). One morphological trait of *Vitis* leaves was mapped further in this thesis for the entire collection, the stomatal density. Exemplarily, the accessions K51 and K103 were selected for a comparative analysis of the early infection behaviour of zoospores of *P. viticola*.

2.1.3 *Vitis vinifera* cv. 'Pinot noir' cell culture

Suspension cell culture of *V. vinifera* cv. 'Pinot Noir' generated from leaves (Seibicke, 2002) was used in this experiment to assess whether liquorice root extract can be detected by *Vitis* cells. Extracellular alkalinisation measurements were performed and were used as readout for activation of immune system in grapevine cells. Cells were grown in Erlenmeyer flasks containing liquid medium (4.3 g l^{-1} Murashige and Skoog salts (Duchefa, Netherlands), 30 g l^{-1} sucrose, 200 mg l^{-1} KH_2PO_4, 100 mg l^{-1} myo-inositol, 1 mg l^{-1} thiamine, and 0.2 mg l^{-1} 2.4-dichlorophenoxyacetic acid (2.4-D)) and pH was adjusted to 5.8. Flasks were incubated on an orbital shaker (KS250 basic, IKA Labortechnik, Germany) in darkness at 27°C at 150 rpm. The cells were sub-cultivated in weekly intervals, using 5 ml of stationary cell suspension per 30 ml of fresh medium. Assays were conducted at day 4 to 8 after subculture. To induce cellular response, the culture was treated with different concentrations of liquorice extract or glycyrrhizin, as described below (see 2.52.5.4 and 2.5.5).

2.2 Pathogen material

2.2.1 *Plasmopara viticola* (Berk. & Curtis) Berl. & DeToni

Plasmopara viticola (Berk. & Curtis) Berl. & De Toni was isolated from the field from the host cultivar Lemberger in Lauffen in Germany and selected into single sporangial strains (Gómez-Zeledón *et al*. 2013). In this study the single sporangial strains 1191_B11 and 1191_B15 were used, both infecting Müller-Thurgau intensively. They differ in their infection behaviour on the American wild grapevine species *Vitis riparia*, which has a high tolerance against Downy Mildew. Strain B15 is infecting *V. riparia* very strongly, whereas strain B11 does not. The strains were propagated on single leaves of the highly susceptible cultivar Müller-Thurgau. Fully expanded leaves were placed with the lower surface on an aqueous suspension of sporangia in a petri dish (14 cm diameter) and incubated overnight (12-24 h) in the dark (wrapped with black foil) in a Percival plant incubator at 16°C. The plant incubator was equipped with full spectrum lamps (Philips Master TL-D Super 80 18W/840, Cool White, Eindhoven). Light intensity was adjusted to 25 µmol $m^{-2}s^{-1}$ (Williams et al., 2007) during day cycles of 14 hours and 0 µmol $m^{-2}s^{-1}$ during night cycles of 10 hours, both at a constant temperature of 16°C. At the following day the leaf was removed from the suspension and placed with the inoculated abaxial side up on a wet tissue paper in a fresh petri dish (150 mm in diameter). Sporangia emerged after 4-7 days. Successful infection was recognized as a dense white sporulation layer consisting of tree-like sporangiophores and many sporangia.

Unused sporangia can be stored at -80 °C up to 12 month. Harvest of the sporangia was conducted on completely dry leaves with a small self-made vacuum cleaner (a sleeve with a pipette tip on a waterjet) (personal communication, Buchholz, G.). For experiments exclusively freshly harvested sporangia were used.

2.2.2 Preparing a sporangial solution

The basic procedure was to harvest sporangia and adjust the solution to a concentration of approximately 50,000 cells per ml to ensure optimal germination rate (Kortekamp, A. and Tisch, C., personal communication). Infected grapevine leaves were immersed in water in a Schott flask or for smaller volumes a 1 cm x 1 cm leaf part was cut out and immersed in water in Eppendorf test tubes in 600 µl – 1 ml volume and mixed gently. Leaf or leaf parts were removed directly afterwards. Solution was incubated at room temperature or 16°C in a climate chamber in the dark to let zoospores emerge.

Table 2.1 Plant and pathogen material used in this study.

Abbreviation	Name	Description	Property
Mth	*V. vinifera* ssp. *vinifera* Müller-Thurgau	European cultivated grape	Intermediate stomatal density
K51	*V. vinifera* ssp. *sylvestris*	European wild grape	High stomatal density
K103	*V. vinifera* ssp. *sylvestris*	European wild grape	Low stomatal density
1191_B11	*Plasmopara viticola*	Single sporangial strain	Weak
1191_B15	*Plasmopara viticola*	Single sporangial strain	Aggressive

2.3 Cell biological methods

2.3.1 Determination of stomatal density

To evaluate the stomatal density of each accession of the European wild grapevine *ex-situ* collection Ketsch (Nick, 2014), glue imprints of fully expanded healthy fresh leaves, harvested from plants grown in the greenhouse of the Botanical Garden of the KIT, were used. Glue imprints were obtained using the lower, abaxial, leaf surfaces of four different leaves of each accession as template. A drop of glue (UHU Hart, Germany) was placed on the respective leaf region near the leaf base. To allow for high-quality imaging, intercostal fields (see Fig. 1.3) with a sufficiently planar surface were used in the region between the midrib and lateral vein, and covered by a thin and homogenous layer, distributing the glue with the fingertip. 5-10 minutes after, the glue cures to a thin film, conserving an imprint of the leaf surface. This imprint could then be removed using a pair of tweezers and were placed on an object slide in a drop of distilled water. Grey-scale microscopic images were collected from these glue imprints with differential interference contrast (DIC) using a digital imaging system (Zeiss AxioScope, equipped with a CCD-camera AxioCam). Pictures were recorded at 20-fold magnification with 2720 x 2048 pixels and saved as RGB colour tif-files for evaluation with ImageJ (Rasband, W.S., National Institutes of Health, USA). All stomata and epidermal cells on the picture were quantified using the plugin “cell counter”. A part of the pictures was counted by Jennifer Welschhoff during her time as trainee at the Botanical Institute 1.

Stomatal cellular density was defined as the ratio of the stomata per epidermal cells. This Parameter was found to be independent of leaf expansion, leaf differentiation, and year. Between 200 and 600 stomata were scored with epidermal pavement cells to determine the stomatal density. Values represent mean values from four independent samples collected over two subsequent vegetation periods.

2.3.2 Determination of "gathering" and stomatal length

Early infection behaviour of zoospores was investigated comparatively on *Vitis sylvestris* accessions K51 (high stomatal density) and K103 (low stomatal density) from the Ketsch *ex-situ* collection (Nick, 2014). *P. viticola* was grown on detached leaves of *Vitis vinifera* cv. Müller-Thurgau in incubation chamber at 16°C, with 14 h of day light and 10 h night cycle in darkness. For the experiments sporangia were harvested and hatched in vitro. Gathering behaviour of zoospores was observed with 100 µl zoospore suspension ($80x10^3$ sporangia per ml) per leaf disc of 13 mm in diameter. After the indicated incubation time, infection was stopped by incubation of the discs in 70% ethanol for 1 minute. After drying with a tissue paper, leaf discs were placed on object slides for staining of the infected area with a 30 µl droplet of 0.05% calcofluor white (Sigma, Germany) and a droplet of 0.5% Triton X to ensure equal dilution of stain on the leaf surface for 10 minutes. Leaf discs were washed with water and observed under a fluorescence microscope (Zeiss AxioScope, equipped with a CCD-camera AxioCam), UV-light excitation and DAPI filter settings. For each time step four leaf discs were used (around 4000 stomata) and 13 time steps were assessed between 2.5 and 45 minutes after inoculation. Pictures were taken at 10x magnification with different illumination times (55 – 200 milliseconds) and double saturation, 1,04 contrast of gamma and 2.0x gain. Evaluation was done with ImageJ software (Rasband, W.S., National Institutes of Health, USA), using the cell counter tool. Number of cysts per stoma were evaluated in 11 categories ranging continuously from 1 (one cyst per stoma) up to 10 (ten cysts per stoma). Category 11 includes cases with crowded cyst numbers so that they were assessed as "more than 10". Sum of infected stomata was calculated.

Length of stomata was measured with ImageJ software (Rasband, W.S., National Institutes of Health, USA), using the measure tool. Each data point shows average size of 15-30 evaluated stomata.

2.4 Visualization of cell structures

2.4.1 Staining of *P. viticola* structures on leaf surface with aniline blue

P. viticola structures on the surface of infected grapevine leaves are sporangia and mycelium and were stained with aniline blue after KOH bleaching. This method allowed to investigate the infection structures over time and to compare dry and swollen sporangia. Leaf discs were cut out from *P. viticola* infected grapevine leaf tissue with a cork borer (6 mm diameter) 8 days after inoculation. Discs were pre-treated by autoclaving them in 2 ml of 1M KOH for 15 minutes at 121 °C in glass vials in order to de-stain them to avoid auto-fluorescence of chlorophyll. The resulting brownish whitish leaf discs were washed in distilled water and stained with a drop per leaf disc of 0.05% aniline blue in 0.067 M K_2HPO_4 (pH 9). After 5 minutes fluorescence on the leaf discs was observed under a fluorescence microscope using excitation at 395–440 nm, a beam splitter at 460 nm and a 470 nm low-pass barrier filter on an epifluorescence microscope (AxioPhot, Carl Zeiss, Germany). Pictures were taken with AxioCam MRm (Carl Zeiss, Germany) and observed with AxioVision SE64 Rel. 4.9 software (Carl Zeiss, Germany).

2.4.2 Staining of *P. viticola* actin filaments by fluorescent phalloidin

Actin microfilament structures and distribution of actin plaques of *P. viticola* sporangia was visualized similar to the protocol for Downy Mildew spores of lettuce (Cohen et al., 2008). Zoospore solution was fixed for 10 minutes with formaldehyde (3.7% formaldehyde (PFA) in PBS, pH 7.4) in reaction tubes. After slight centrifugation two washing steps were done with 1 ml distilled water each step. Zoospores were stained after down centrifugation and supernatant removal to 100 µl for 10 minutes with 66 nM Alexa Fluor® 488 Phalloidin (Invitrogen, Molecular Probes, USA, 6.6 µM in methanol). Zoospores were directly observed with spinning disc confocal microscope (Carl Zeiss, Germany), equipped with a 63x objective magnification and 488 nm line of an Ar-Kr laser and GFP filter set with excitation wavelength of 488 nm and emission wavelength of 518 nm. The data were collected by Tabea Setzer and Christina Manz during a practical course in May 2013.

2.4.3 Staining of *P. viticola* endoplasmatic reticulum by $DiOC_6$ in vivo

To visualize structure, size and distribution of the endoplasmatic reticulum (ER) and nuclear envelope of *P. viticola* sporangia and zoospores, $DiOC_6$ (Invitrogen) staining was performed with minor modifications according to (Koning et al., 1993). Concentration of cells and dye was reduced by a factor of 1000.

1 µl of 10 µg ml^{-1} $DiOC_6$ in DMSO stock was added to 1 ml of zoospore suspension, resulting in a final concentration of 10 ng ml^{-1}, mixed gently by inverting and incubated for 30 minutes. Afterwards the stained zoospores were observed under spinning disk confocal microscope (Carl Zeiss, Germany), equipped with a 63x objective magnification and 488 nm line of an Ar-Kr laser and GFP filter set of excitation wavelength of 488 nm and emission wavelength of 518 nm. The data were collected by Tabea Setzer and Christina Manz during a practical course in May 2013.

2.4.4 Endocytosis tracker FM4-64 staining of sporangia and zoospore membranes

Endocytosis tracker FM® 4-64 (Invitrogen molecular probes) enters cells via endocytosis and stains cell membranes, vesicle membranes and vacuole membranes of *P .viticola* sporangia and zoospores. Final concentration of 1 µM FM® 4-64 aqueous solution was used, based on suggestions of (Bolte et al., 2004). Dye was added to a sporangial suspension prior to differentiation to zoospores. After incubation time of 30 minutes sporangia and zoospores were observed under spinning disc confocal microscope (Carl Zeiss, Germany), equipped with a 63x objective magnification and 561 nm line of an Ar-Kr laser and RFP filter set of excitation wavelength of 561 nm and emission wavelength of 670 nm. The data were collected by Tabea Setzer and Christina Manz during a practical course in May 2013.

2.5 Functional analysis

2.5.1 Alkalinisation response of cell cultures

To measure the alkalinisation response, 2.5 ml aliquots of the cell suspension were pre-incubated for one hour in open vials on a rotary shaker at 150 cycles per min to ensure a stable ΔpH course. Values of pH in the medium were continuously measured with small combined pH electrodes (Mettler Toledo, LoT403-M8-S7/120) and recorded using a video-graphic data recorder (VR06; MF Instuments GmbH, Germany) at 1 s intervals. Addition of a substrate (different concentrations of *Glycyrrhiza* root extract (3-333 mg ml^{-1}) or glycyrrhizin solution (0.005-5 mg $^{-1}$)) was done carefully into the vials on rotating shaker to avoid pH changes due to stopping the shaker and pH change was recorded for 60-90 minutes. Maximal pH shifts occurred typically 30 minutes after substrate addition. Each concentration was measured two times and the mean values of the maximal pH shifts were evaluated. The pH data were exported from the recorder via SD card to Microsoft Office Excel by data acquisition software Observer II_V2.35 (MF Instruments GmbH). The data were collected by Anna Pecina during a practical course in May 2014.

2.5.2 Treatment with aluminium ions, copper ions, sodium ions

Aqueous sporangial suspension of *P. viticola* was adjusted to a concentration of 15.000 sporangia per ml. Suspensions were treated with salt solutions of 1 mM stock to following final concentrations: 5 µM, 10 µM, 20 µM, 40 µM. The salts used in this study were copper II sulfate ($Cu^{2+}SO_4^{2-}$), aluminium chloride, ($Al^{3+}Cl_3^-$), and sodium chloride (Na^+Cl^-). The zoospore behaviour was observed under a bright field microscope immediately or after respective incubation times. Pictures were taken and evaluated afterwards. For two experimental series the numbers of sporangia within 10 big squares of a counting chamber were counted and mean values and standard errors for burst zoospores per ml were calculated. Counted volume was 3.2 µl, corresponding to 48 sporangia and 384 zoospores after 1 hour of incubation. The data were collected by Tabea Setzer during her Bachelor thesis in July 2013.

2.5.3 Peptide treatments

P. viticola sporangial suspension was adjusted to a concentration of 1.5 x 10^4 sporangia ml^{-1} to get a zoospore concentration of 1.2 x 10^5 zoospores ml^{-1} after one hour of incubation. For each experiment an untreated control was used as internal standard. Values give the incidence of bursting zoospores relative to the internal standard at the final time step of the experiment

(30 minutes in case of metal ions, 90 minutes in case of ECM-interacting peptides). Values represent means and standard errors from two independent experimental series. Counted volume was 3.2 µl, corresponding to 384 zoospores. The number of burst zoospores within 10 big squares on counting chamber were counted and mean values of the number of burst zoospores were calculated. The heptapeptides YGRGDSP ("RGD") and the reverse sequence peptide PSDGRGY ("RGD") (Panatecs, Germany, purity > 75%), as well as protamine sulphate (Carl Roth, Germany) were prepared in this study as stock solutions of 100 ng ml^{-1}. The solutions were diluted in the treatment to stepwise final concentrations of 10, 20, 30 and 50 ng ml^{-1}. After incubation of the zoospore suspension with peptides for 1.5 hours pictures were taken from zoospores on object slides with a light microscope and counting was performed. The data were collected by Tabea Setzer during her Bachelor thesis in July 2013.

2.5.4 *Glycyrrhiza* root extract treatment

Glycyrrhiza roots **Fig. 1.5** or an aqueous or alcoholic extract from the roots is used in traditional Chinese medicine and known to have several benefits for human health. Surprisingly, it was found to be active against different Downy Mildews of crops and showed positive effect on the plant itself (Schuster et al., 2010; Schmitt et al., 2011; Scherf, 2011). In this study, the influence of liquorice extract on Downy Mildew of grapevine was tested and its influence on grapevine cells. Additionally, the influence of its main compound glycyrrhizin was tested in parallel on both, pathogen and plant cells. The extract originated from roots of *Glycyrrhiza* and was ordered as powder based on cellulose granules (PhÿtoComm®, Germany), called *Glycyrrhizae* radix et rhizoma praeparata 118A (PhÿtoComm®, Germany), containing root extract powder on base of cellulose granules. 1 g extract was dissolved in 3 ml distilled water. 1 g of the powder consists of 50% of water soluble extract and 50% non-dissolvable cellulose granules. The brown-white suspension was filtrated in several steps to remove cellulose. The resulting solution was approximately 166.6 mg ml^{-1} *Glycyrrhizae* radix extract. Assuming, that 5-12% of the extract are glycyrrhizin Fig. 2.1 1) (Chen & Sheu, 1998; Yokozawa et al., 2000), the maximal concentration of the extract used in this study would contain 8-20 mg ml^{-1} glycyrrhizin. This concentration was examined for creating a corresponding solution of glycyrrhizin. Due to progressive gel formation at high concentrations, only freshly prepared *Glycyrrhiza* extract filtrates were used (maximum 1 week old). The data were collected by Jana Fesseler, Corinna Schmider and Thomas Hirth in a practical course in April 2014 and Anna Pecina in May 2014.

2.5.5 Glycyrrhizin treatments

Glycyrrhizinate (Carl Roth, Germany) was dissolved in distilled water to a stock of 15 mg in 3 ml to a final concentration of 5 mg ml^{-1}. Due to progressive gel formation at high concentrations, only fresh prepared glycyrrhizin solutions were used (maximum 1 day old). Chen and Sheu 1993 published the results from measurements of glycyrrhizin content in *Glycyrrhizae* radix preparations by capillary chromatography (Fig. 2.1). They found 52.06 mg glycyrrhizin per gram radix preparation. This is around 5.2% of glycyrrhizin in the *Glycyrrhizae* radix extract. Another group published a glycyrrhizin content of 11.9% after high-performance liquid chromatography (Yokozawa et al., 2000).

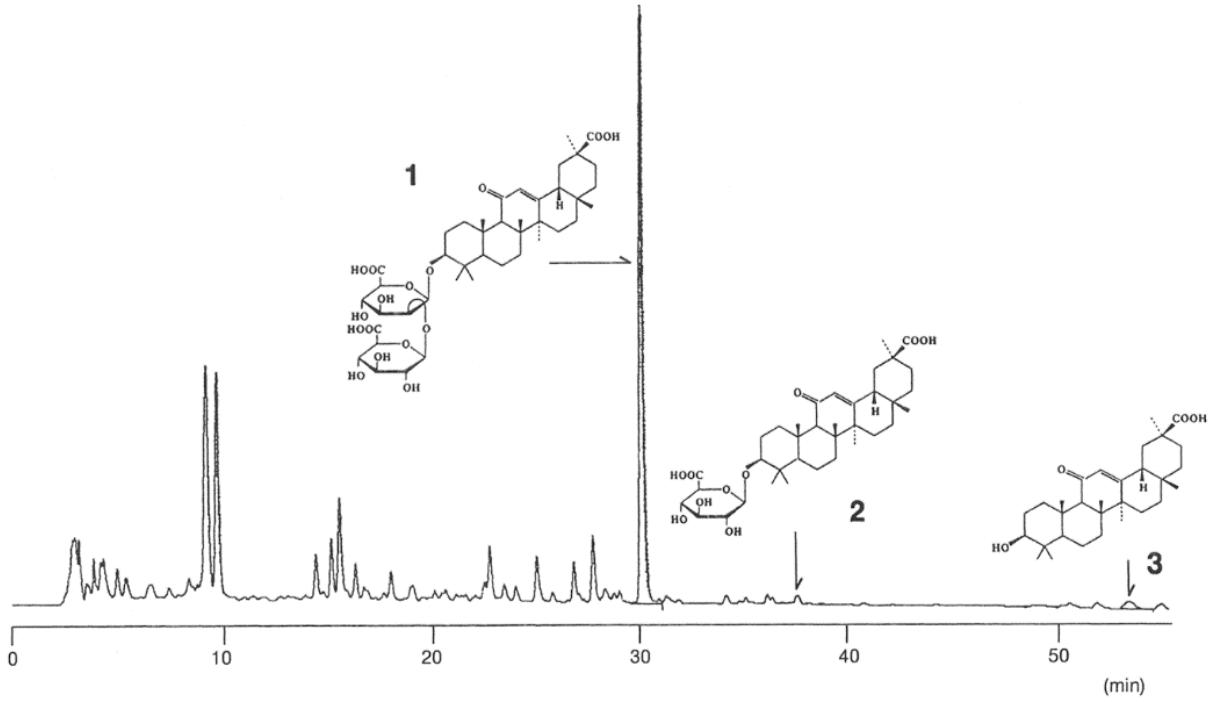


Fig. 2.1 High-performance liquid chromatogram of Glycyrrhizae radix water extract (Yokozawa et al. 2000). **1** Glycyrrhizin, **2** 3-glycyrrhetinic monodesmoside, **3** glycyrrhetinic acid

Time course studies of the behaviour of zoospores were performed with microscopic pictures as output data. In order to facilitate the evaluation of the zoospore state, zoospores were slightly stained with Evans blue dye (aqueous solution of 0.25% (w/v), 1 µl added to 80 µl zoospore suspension, final concentration was 0.0031%). Zoospores were evaluated in three categories: vital swimming, encysted or burst. Vital swimming cells and cysts transport the incoming colour out again. Burst cells accumulate the colour and are visible as dark blue spots. The data were collected by Jana Fesseler, Corinna Schmider and Thomas Hirth in a practical course in April 2014 and Anna Pecina in May 2014.

2.6 Microscopy

2.6.1 Light microscopy

Light microscopy was performed with differential interference contrast microscopy (AxioImager Z.1, Carl Zeiss, Germany). Pictures were taken with AxioCam MRm (Carl Zeiss, Germany) and observed with AxioVision SE64 Rel. 4.9 software (Carl Zeiss, Germany).

2.6.2 Confocal microscopy by spinning disc

Stained *P. viticola* sporangia and zoospores were viewed under a spinning disc confocal microscope (Zeiss, Jena, Germany) with 63x oil objective. Confocal z-stacks were recorded with an AxioObserver.Z1 (Zeiss, Jena, Germany). Differential interference contrast (DIC) objective (NA 1.3), with 561 nm or 488 nm line of Ar-Kr laser. Images were analyzed with Zeiss Zen software.

Filter sets for GFP (excitation at 488 nm, emission at 518 nm) were used for viewing $DiOC_6$ and Alexa Fluor 488 Phalloidin. Filter sets for red fluorescence (excitation at 561 nm, emission at 670 nm) were used for viewing Fm4-64.

2.6.3 Time-lapse series of contractile vacuoles

1 cm^2 of a well sporulated grapevine leaf was cut out and dispensed in 600 µl of distilled water. After inverting a few times for suspending the sporangia, leaf material was removed and sporangia suspension was incubated in the dark at 16°C in a plant incubator (I-30 BLL, Percival Scientific, USA) for 1-2 hours. After zoospores were released, 80 µl of zoospore suspension were observed on object slides specially prepared with a spacer made of adhesive tape to prevent zoospore bursting. For the video microscopy AxioImager Z.1 microscope (Carl Zeiss, Germany) with an ApoTome microscope slider for optical sectioning was used. Vital moving zoospores were observed under 63× LCI- Neofluar Imm Corr DIC objective (NA 1.3) and digital CCD camera (AxioCam MRm, Carl Zeiss Germany) and pictures were taken using "time-lapse" mode with an interval of 250 ms, producing a series of 100 pictures within 25 s. Videos were analyzed with AxioVision SE64 Rel. 4.9 software (Carl Zeiss, Germany). The data were collected by Thomas Hirth during a practical course in July 2014.

2.6.4 Transmission electron microscopy

To get insight into the membrane dynamics during cellularization, different stages of sporangium germination till the hatching of zoospores were investigated by transmission electron microscopy of *P. viticola* single sporangial strain 1191_B11. The ultrastructure of zoospores and sporangia was visualized during differentiation. Protocol was based on (Heumann, 1992). Freshly harvested sporangia were incubated in water for one hour in the dark. After sedimentation, pellet of zoospores and sporangia was resuspended in a part of the supernatant. 1.5 ml fixation solution (equal volume of 2.5% glutaraldehyde, buffered with 0.1 M cacodylat buffer, pH 7.2) was added and the microwave fixation protocol was followed (Heumann, 1992). Secondary fixation was performed with OsO4. The samples were dehydrated in solutions of increasing ethanol concentrations (30%, 50%, 70%, 80%, 90%, 100% ethanol). Epon embedded samples were cut with an ultra-microtome (Leica Ultracut R) with handmade glass knifes or with diamond knife (DiATOME). Ultra-thin slides were put on pioloform coated grids and kept dry in a grid box. After staining with 10% methanolic uranyl acetate and lead citrate samples were viewed in a Zeiss electron microscope at 80 kV. The pictures were collected with Mohammad Fotouhi Ardakani at the Laboratory for Electron Microscopy, Karlsruhe Institute of Technology (KIT). Pictures were viewed and processed with Adobe Photoshop CS software, using the tool Photomerge for combination of partial pictures to picture of a full sporangium.

3 Results

This study aims to find a base for new strategies for a sustainable control of Downy Mildew disease of grapevine. Therefore, structural and functional studies were conducted on both, the host and the pathogen, to reveal the cellular properties which influence pathogenicity. The results might lead to improvements in breeding and disease control for a future viticulture with more stably resistant cultivars and less toxic treatments.

The first part of the result section deals with the outcome of the morphological characterization of stomatal densities of wild grapevines (*Vitis vinifera* ssp. *Sylvestris*), collected on the Rhine peninsula Ketsch (Nick, 2014). The stomatal cellular densities were assessed to get values independent from environmental factors. The results show, that stomatal cellular densities are a highly conserved factor and independent from environmental conditions and can be used as a factor for comparing different genotypes. Stomatal cellular densities vary between a minimal and maximal value, describing "low stomatal density" and "high stomatal density". These were correlated with the results of a comprehensive infection study, investigating infection strength of Downy Mildew on all wild grapevine accessions (Duan et al., 2015). The results revealed a correlation, indicating that less stomatal cellular density contributes to a higher resistance level against *P. viticola*.

In the second section, the early infection behaviour of *P. viticola* zoospores on wild grapevine leaves were examined in more detail. The results show, that zoospores are able to find the stomata and encyst there within less than 2.5 minutes. The zoospores show a gathering pattern because several zoospores can infect one single stoma. Hereby zoospore number correlates with size of the infected stoma. Different strains show similar behaviour to infect different grapes, but stomatal density seems not to be an important factor for a high gathering pattern.

The third section describes cellular details of the pulsating vacuole during development of *P. viticola* sporangia and zoospores. The results show that dry sporangia have a shrunken shape and crinkled cell wall structure before taking up water to evolve to mature sporangia with smooth cell wall and round lemon shaped structure. Contractile vacuoles are constructed already in syncycial sporangia. The mature sporangia differ in vacuolar composition and contain mature dense body vesicles, looking like a slice of a cooked egg, consisting of a clear larger vesicle including an electron dense inner vesicle with separate membrane. In comparison, early dense body vesicles of dry and not mature sporangia are equally intermediate electron dense and contain homogenous material, which might be lipids. Another cellular characteristic of the mature sporangium are star-shaped actin plaques, connected with actin cables. During sporangial development the contractile vacuole activity is linked to a highly dynamic remodelling of ER. Mature sporangia are highly active in exocytosis and

produce new membranes and flagella for future zoospore individuation. Contractile vacuole activity in free zoospores is accelerated and linked with massive endocytosis.

The fourth section deals with the outcome of several functional studies with the contractile vacuole. It was shown that the vacuole can be inhibited by metal ions and RGD peptides. As well, inhibition was seen after treatment with a biological extract of *Glycyrrhiza* roots and its main compound glycyrrhizin. Furthermore, it was shown that these compounds influence *Vitis* suspension cells by activating the plant immune system.

3.1 Infection rate increases with stomatal densities of *Vitis sylvestris*

Plasmopara viticola infects grapevine through stomata. The number of available stomata might contribute to the grade of infection success. To investigate this possibility, the wild *V. vinifera* ssp. *sylvestris* Ketsch population (Fig. 3.1 A) was screened for stomatal density on leaves. Previous studies investigated stomatal density per square millimetre (Rühle, 2011). This factor is variable because environmental fluctuations can influence the leaf growth. Therefore, the relative number of stomata over the entire number of epidermal cells was assessed using pictures of glue imprints of the lower epidermal leaf surface (Fig. 3.1 B, C). Counting was done with the plugin "cell counter" of ImageJ and ratios were calculated. The ratios of stomata per epidermal cells resulted between the lowest stomatal density of 0.5 and the highest stomatal density of 0.16 (see Table 6.1 in the appendix). All Ketsch species as well as some cultivars were assessed and range equally between these numbers.
Stomatal cellular density is a constant marker, independent from variations due to cell expansions. The values for this relative stomatal density were found to be very stable over two vegetation periods and dependent on the genotype.

Correlation study was performed between stomatal density values and *P. viticola* infection levels, including data about stilbene contents of the grapevine accessions. Stilbenes are secondary metabolites of grapevine and play a fundamental role in basal immunity of plants. They belong to the large group of antimicrobial plant defence compounds of phytoalexins which have been found to play a role in pathogen defence against *P. viticola* as well. It might be that they are released from stomata. As expected, a higher stomatal cellular density correlated with higher infection level. Low stomatal density grapevines showed less penetration events. Infection rate was significantly reduced in the grapevine group with low stomatal density compared to the average of entire population and compared to the high stomatal group (Duan et al., 2015). Interestingly, the group of European grapevine *V. sylvestris* differed in stilbene content, classified in two groups dependent on stilbene content, and could be correlated with infection level as well. The smaller group of high stilbene producing grapevine accessions showed significantly less infections compared to the large subset of low stilbene grapevine accessions (Duan et al., 2015). The stilbene composition differed in the two groups. The group with high stilbene production was characterized by quick and strong accumulation of stilbenes after inoculation, almost exclusively in the form of non-glycosylated resveratrol and viniferin. In contrast, the group with low stilbene production accumulated fewer stilbenes and relatively high proportions of piceatannol and the glycosylated piceid. Both investigated traits can be useful for future breeding strategies creating less susceptible *Vitis vinifera* grapevines.

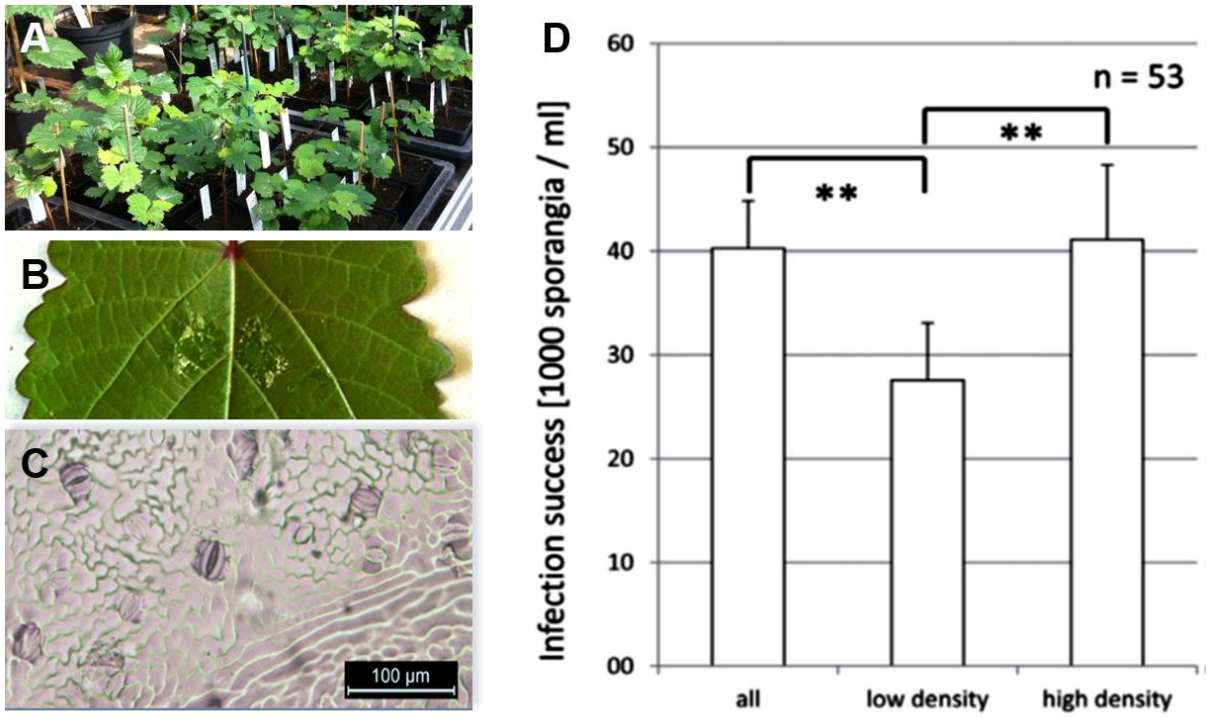


Fig. 3.1 Stomatal densities of grapevine collection of *Vitis vinifera* ssp. *sylvestris*, Ketsch. **A** Greenhouse plants in the Botanical Garden. **B** Leaf with thin layer of glue covering several intercostal fields around the middle vein and **C** Glue imprint of the lower epidermis of one *Vitis vinifera* ssp. *sylvestris* leaf, exemplarily. **D** Average infection levels of *Vitis sylvestris* leaves with different stomatal densities, evaluated as sporangia per ml. All, low (below the median of the population) and high (above the median of the population) densities of stomata. ** indicate differences that are statistically significant at the P <0.01 level. The data represent means and standard errors from three independent biological replicas obtained from at least two different years.

3.2 Early infection behaviour reveals zoospore gathering of *Plasmopara* on *Vitis sylvestris*

P. viticola zoospores can sense stomata and swim to them rapidly. The mechanism behind is called chemotaxis (Arens, 1929; Royle & Thomas, 1973) and might be the first event during the process of infection. The time span of early infection events is important for infection success. The earlier zoospores enter stomata and start infection process, the less time is left for the plant to defend. To find out if stomatal density might correlate with early zoospore infection behaviour, zoospores of two different single sporangial strains were used in a comparative assay to evaluate their early infection behaviour dependent on different stomatal densities of *Vitis sylvestris* accessions. Zoospores of the two single sporangial strains, the weaker 1191_B11 and the more aggressive growing 1191_B15 (Gómez-Zeledón et al., 2013), were hatched in vitro, inoculated on different wild grape leave discs and evaluated microscopically after the indicated time and stained with calcofluor white staining. Interestingly, zoospore early infection is characterized by a very fast gathering of zoospores on few stomata. Already 2.5 minutes after inoculation, almost all zoospores were found to be encysted and attached to stomatal guard cells. Evaluation of gathering was performed counting the cysts in stomata or attached to stomatal guard cells and ratios were calculated of the number of counted encysted zoospores per stomata. Average gathering levels were calculated. In a control assay on the upper epidermis of leaves, lacking stomata, no gathering was seen. Instead, zoospores were spread equally.

Zoospores of strain 1191_B11 showed a clear gathering behaviour. They gathered in a different manner on the lower epidermis of the tested host genotypes. On low stomatal density accession K103 gathering was obviously higher, with a value of 5.27 after 35 minutes, compared to that on Müller-Thurgau (2.21) and K51 (2.03) (Fig. 3.2 D). On Fig. 3.2 B and C the difference is shown in fluorescence pictures of calcofluor white stained cysts of strain 1191_B11 gathered strongly on stomata of *Vitis sylvestris* K103 (panel B) and less severe gathering on *Vitis vinifera* Müller-Thurgau (panel C).

P. viticola strain 1191_B15 showed no pronounced gathering behaviour. B15 zoospores infected the low stomatal density accession K103 in a similar pattern as Müller-Thurgau. Zoospores per stomata values were low, ranging from 0.09 to 1.27. It could be observed, that in the first 12.5 minutes "gathering" raised up to 1.27, decreasing again afterwards. It might be, that the range was lower after 12.5 because new zoospores hatched out of sporangia and started new infections. High stomatal density accession K51 was infected slightly stronger from 1191_B15, with ratios ranging from 0.45 after 2.5 minutes up to 2.64 after 40 minutes.

It was observed that some stomata were infected and others (mostly small ones) not. Therefore, the sizes of the infected stomata were evaluated in correlation with number of gathering cysts. Indeed, the results show a correlation between the amount of gathered zoospores and stomatal size (Fig. 3.2 A). With increasing length, more zoospores are gathered together. Small and closed stomata were not infected, indicating that zoospores are attracted by host factors released out of stomatal cavity of mature stomata. Fig. 3.2 (B, C) shows two representative pictures of stomata which were infected with equally dense zoospore solutions but show a different density of attached zoospores.

The stomata of the grapevine leaves of *Vitis vinifera* L.cv. Müller-Thurgau (Mth) and the *Vitis sylvestris* Ketsch accessions K103 and K51 have almost the same mean size (20.72 µm – 24.37 µm), which is the same with the average stomatal size of the plants of the entire Ketsch collection (24.33 µm) (Rühle, 2011). This makes no significant difference regarding gathering effect (see Fig. 3.2 A). The biggest gathering effect was detected with strain B11 on the low stomatal density *Vitis* accession K103, which has the same average stomatal size as Mth. Concluding, the early zoospore infection behaviour of the strong gathering strain B11 shows a gathering effect correlating reciprocally with stomatal density.

Table 3.1 Overview about gathering effects of the two tested strains on the three tested *Vitis* accessions compared to their stomatal sizes and densities.

	Gathering B11	Stomatal size	Stomatal density	Gathering B15
K103	5.27	24.37	0.05	1.27
Mth	2.21	24.01	0.09	1.27
K51	2.03	20.72	0.13	2.64
Ketsch mean value	-	24.33	0.09	-

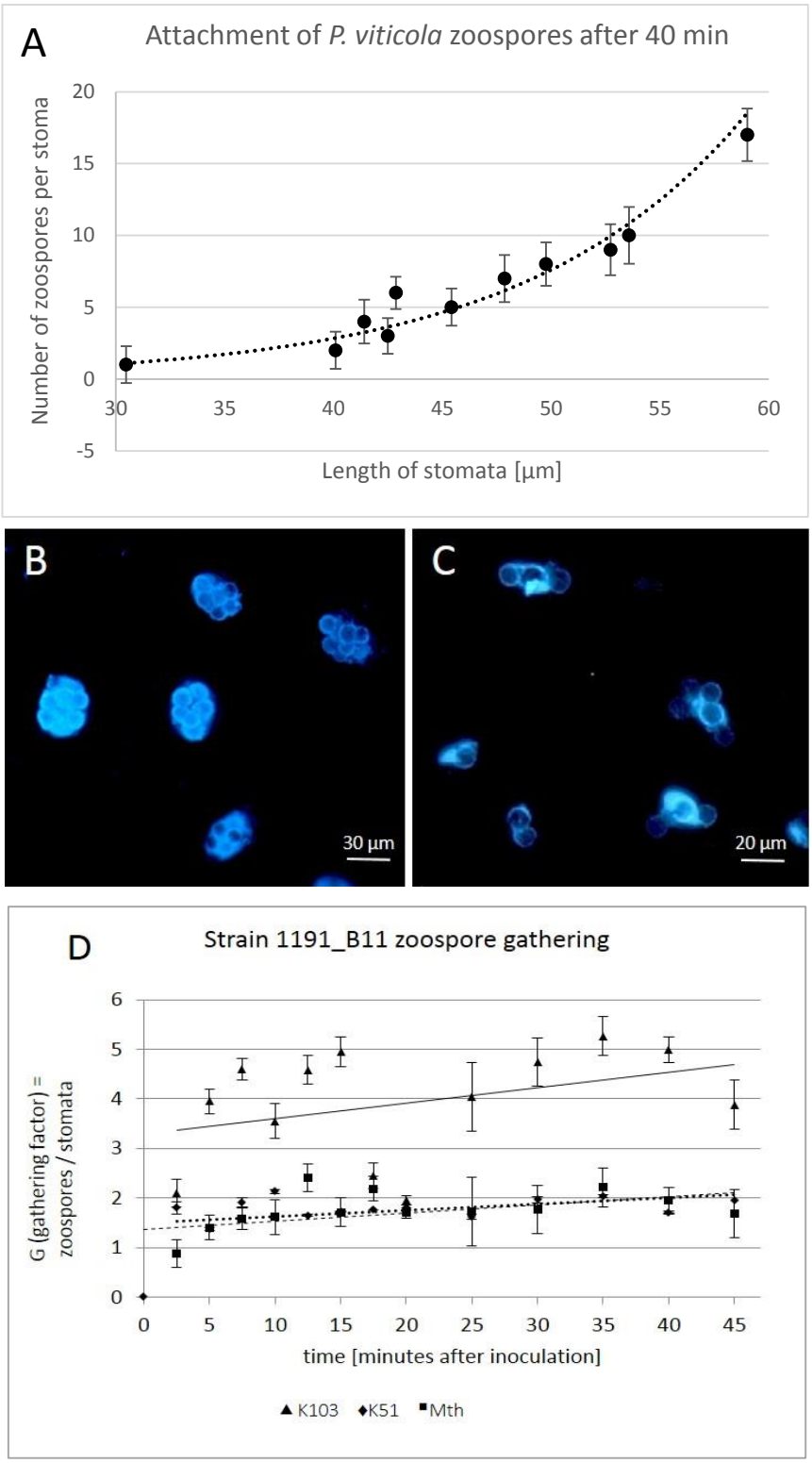


Fig. 3.2 Gathering effect of zoospores getting attached on stomata. **A** Correlation of stomatal length with number of zoospores per stomata 40 minutes after inoculation, exemplarily for *P. viticola* strain 1191_B15 on *Vitis sylvestris* Ketsch K51. Each data point shows average length of 15-30 evaluated stomata. **B,C** Microscopic pictures of encysted gathered zoospores on stomata of *Vitis vinifera* ssp. *sylvestris* K103 (B) and ssp. *vinifera* Müller-Thurgau (C) 40 minutes after inoculation, stained with calcofluor white, irradiated with UV light. **D** Evaluation of a time series was done with ImageJ software for gathered *P. viticola* zoospores of strain 1191_B11 on *Vitis sylvestris* Ketsch K103, Ketsch 51 and Müller-Thurgau. Each data point for G shows average value of 400-500 infected stomata, bars depict standard error of mean.

3.3 Observation of cellular details of zoospore individuation reveals - Contractile vacuoles are first

The developmental stage of *P. viticola*, during which contractile vacuoles do exist, extends over few hours, beginning with water uptake into the dry sporangium and ending with the formation of a cyst at the stoma (Fig. 3.2 B, C). The time span of this phase in the life cycle can vary between 45 minutes and 3 hours, depending on environmental factors such as light conditions, temperature, ions, and some unknown host factors (Kiefer et al., 2002). During this time interval, the cytoplasm experiences several sharp changes in water potential, and to get insight into the cellular details of these transitions, we followed changes of ultrastructure by transmission electron microscopy following chemical fixation (Fig. 3.3): Whereas the dry sporangia show a crinkled raisin-shape (Fig. 3.3 A) they immediately swell to lemon-shaped balloons (Fig. 3.3 C) upon contact with water, indicating that the water potential of the cytoplasm in the dry sporangium must be very negative. Already prior to swelling, the cytoplasm is in close physical contact with the sporangial wall (Fig. 3.3 B, spw), such that the increase in volume will build up a considerable turgor within a few seconds. The partitioning of the syncytial cytoplasm into individual zoospores prior to hatching (Fig. 1.2, centre) requires that the cytoplasm has to detach from the sporangial wall (Fig. 3.3 E, G), such that the turgor component of the water potential will return to zero, and this situation will persist throughout the entire mobile phase. This will only change, when zoospores encyst and generate their own individual cell wall (Fig. 1.4 A; Fig. 3.2 B, C), the negative water potential of the cytoplasm will be again compensated by turgor pressure.

These sharp changes of water potential are accompanied by dramatic remodelling of the cytoplasm (Fig. **3.3**). Prior to swelling, when the sporangial wall is still wrinkled (Fig. 3.3 B, spw) numerous small early dense-body vesicles (Fig. 3.3 B, edbv) are observed. Their grey colour may indicate lipids, which have been proposed as energy source for the syncytium (Gay et al., 1971). These early dense-body vesicles are also seen in fully turgescent sporangia. There they have an increased size and appear lighter in colour instead (Fig. 3.3 D, edbv). Additional organelles, such as individual nuclei (Fig. 3.3 B, nu) or mitochondria (Fig. 3.3 D, mt) can be encountered as well. At later stages, the maturating sporangium contains numerous mature dense-body vesicles (Fig. 3.3 E, F, mdbv), progressively emerging and replacing the grey early dense-body vesicles. Hereby, transition stages can be seen, where a smaller and denser structure, clearly delineated by a membrane, is seen inside a larger, white vesicle that is by itself lined by a membrane. With progressive maturation of the sporangium, a clear gap between sporangial wall and cell membrane develops (Fig. 3.3 F, G), where granular material seems to be deposited by exocytotic vesicles (Fig. 3.3 F (large), exv). Moreover, longer sheets

lined by two membranes in parallel can be found that probably correspond to prospective plasma membranes between future zoospores in different stages of individuation (Fig. 3.3 F, prpm; H, pm). Occasionally, pairs of oval structures with electron dense parallel stripes are encountered in some sections (Fig. 3.3 G, H, bb), probably representing the basal bodies of the two flagellae.

Sporangia are syncytial cells, containing several nuclei in the cytoplasm. It could be observed that contractile vacuoles were already active in the syncytial cytoplasm prior to cellularization into single zoospores (Fig. 3.4). A contraction cycle comprised phases, where the vacuole was bifurcated (Fig. 3.4 A) followed by phases, where the two chambers fused to one larger vacuole (Fig. 3 B). This sequence lasted around 20-30 sec and could be followed repeatedly, showing that the activity of contractile vacuoles starts prior to cellularization of the syncytium. When this activity was investigated at high temporal resolution (4 frames per second), further details became observable: The bifurcated state persisted for the first 6 seconds (Fig. 3.4 C1-3), starting with two vacuole chambers of comparable size (Fig. 3.4 C1), followed by shrinkage of one chamber (Fig. 3.4 C2), and fusion of both parts (Fig. 3.4 C3) to a larger single vacuole (Fig. 3.4 C4). This single vacuole was seen over the next 6 seconds (Fig. 3.4 C 4-6), but underwent phases of expansion and shrinkage (Fig. 3.4 C4, 6), when it appeared rough and granular and dynamic, whereas the fully turgescent vacuole appeared translucent lined by a membrane that was clearly visible in the differential interference contrast (Fig. 3.4 C5). Then the next cycle started with the bifurcated initial situation (Fig. 3.4 C7).

Cellularization proceeds by formation of membranes in a star-like manner in centrifugal direction (Fig. 3.4 D). The cytoplasm is partitioned such that one contractile vacuole is assigned to each prospective zoospore.

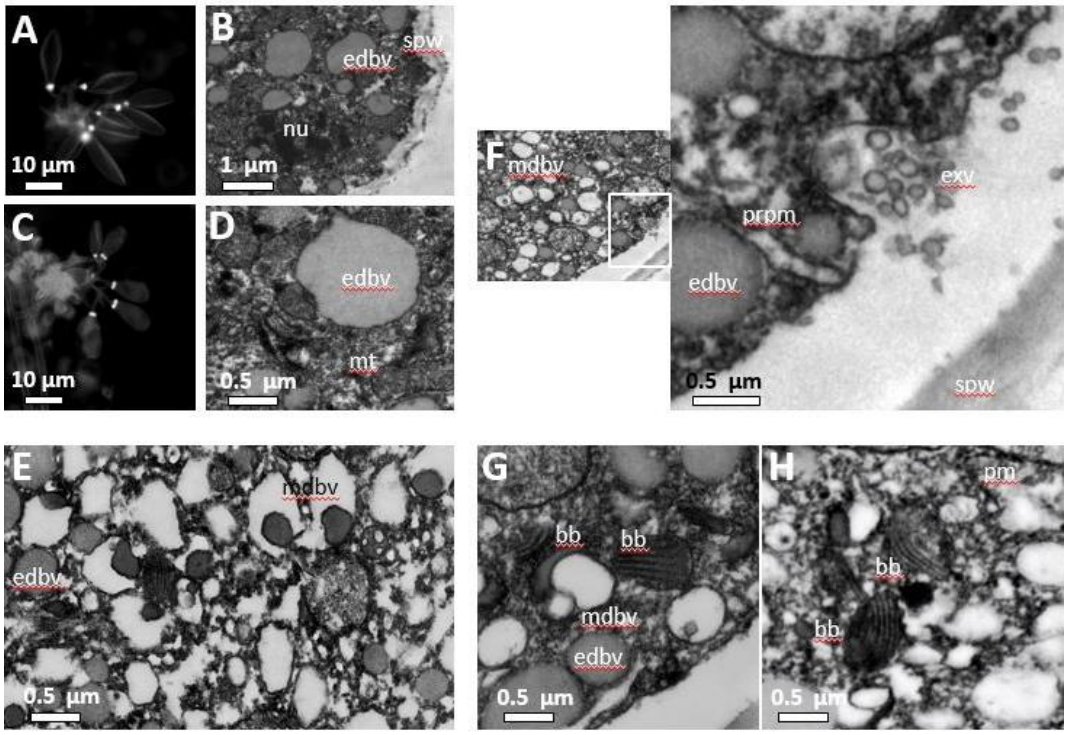


Fig. 3.3 Ultrastructural details of sporangial swelling and zoospore individuation followed by transmission electron microscopy. Dry sporangia prior to swelling (**A**, **B**) in comparison with fully turgescent sporangia (**C**, **D**) after aniline blue staining (**A**, **C**) and in the TEM (**B**, **D**). nu nucleus, edbv early dense-body vesicles, mt mitochondrion, note the still wrinkled sporangial wall (spw) in B. **E** Emergence of numerous mature dense-body vesicles (mdbv) progressively replacing the early dense-body vesicles (edbv) in a maturating sporangium. **F** secretion of electron-dense material in a mature sporangium with partially completed separation of zoospores. prpm prospective plasma membrane, exv exocytotic vacuole. Note that the zoospore has already detached from the sporangial wall (spw). **G**, **H** putative basal bodies (bb) in mature sporangia with almost completed individuation of zoospores, note the contiguous plasma membrane (pm) in H.

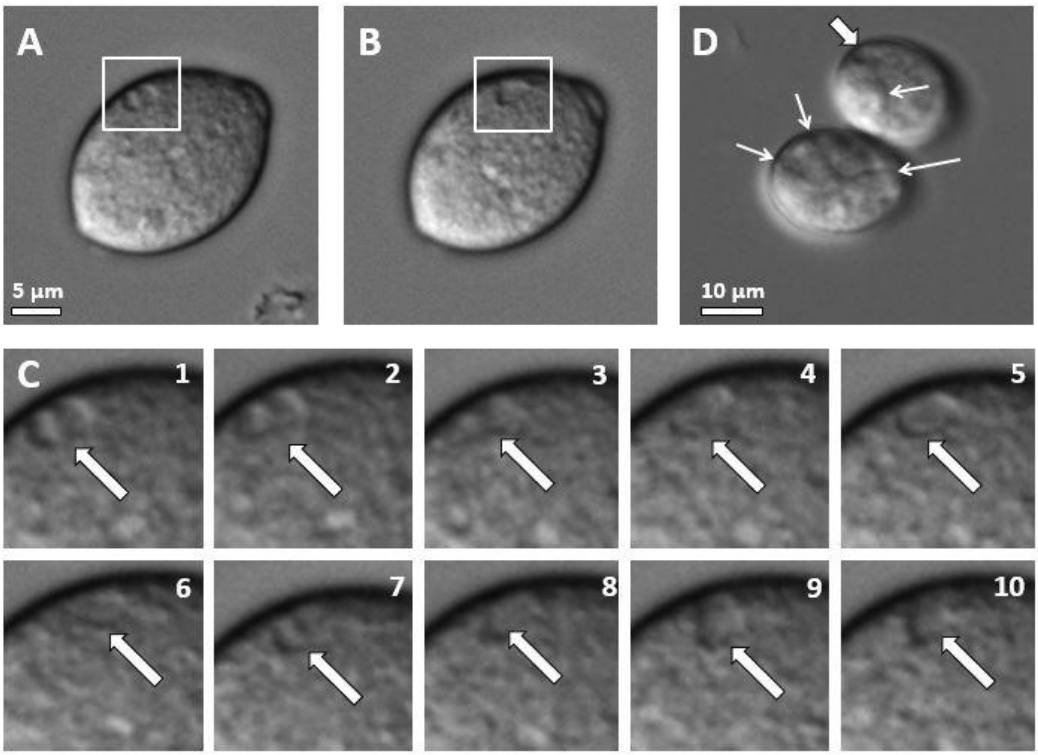


Fig. 3.4 Activity of contractile vacuole prior to cellularization of the syncytium. **A**, **B** Mature sporangium with bifurcated contractile vacuole at the beginning (A) and end (B) of a recorded time-lapse series over 40 sec. **C** Individual frames from a time-lapse series recorded at a frequency of 4 frames per second, zoom-in indicated by the white square in A and B, time interval between the shown frames is 2 seconds. White arrowheads indicate the position of the contractile vacuole. **D** Two sporangia at incipient cellularization, the ensuing cell membranes are indicated by white arrows, note the contractile vacuole in the upper sporangium (white arrowhead).

3.4 Contractile vacuole activity is linked with a dynamic remodelling of the ER

To investigate the cellular structures around the contractile vacuole within sporangia, actin and the endoplasmatic reticulum (ER) were observed after staining with fluorescent markers. Actin observation was done after cell fixation and allowed to see the cell structures in detail. The observation of the ER was performed in living cells and allowed observation of the cellular structures with life cell imaging. Combining these staining methods allows to study details of the contractile vacuole that would be hard to observe in the rapidly moving zoospores.

The structure of actin in turgescent syncycial sporangia was visualized with fluorescent phalloidin after chemical fixation (Fig. 3.5 A). When subsequent sections from confocal z-stacks were followed, the mature sporangium was found to be filled with star-like, brightly fluorescent, actin plaques, from which connecting actin cables emanated, consistent with previous observations (Riemann et al., 2002).

Actin dynamic is important for vital zoospores, which was shown in an assay using the cytoskeleton drug latrunculin B to treat zoospores. The results indicate, that latrunculin B specifically inhibits zoospores and leads mainly to cell burst (see appendix, Fig. 6.5). Latrunculin B blocks the actin monomers and prevents their integration in growing actin filaments. The ongoing actin-turnover leads to a decrease of filaments which then stay shrinking due to the blocked monomers. Shrunk actin filaments lead rapidly to burst zoospores and therefore seem to be an important factor for zoospore stability.

The potential dynamic changes within the cells was observed with stained ER *in vivo* using the fluorescent dye $DiOC_6$. The dynamics are showed in a movie, which is attached in the supplemental CD of this study (Fig. 3.5 B, supplemental movie S1). When the fluorescent $DiOC_6$ signal in individual sections from a confocal z-stack was followed over time in comparison with the corresponding image collected by Differential Interference Contrast (DIC), two prominent arrays were observed during the phase, when the contractile vacuole was rough and granular (Fig. 3.5 B1, B2): A star-like ER structure (asterisks in Fig. 3.5 B1, B2) subtending the contractile vacuole and consisting of multiple ER cisternae separated the chambers of the contractile vacuole and disappeared upon fusion of the chambers. Rosette-like structures consisting of a central small cavity surrounded by several smaller or larger cavities were seen around the site of release (arrows in Fig. 3.5 B1) and would fuse with one ray of the ER star during the same phase (arrows in Fig. 3.5 B1, B2). During the subsequent phase of the contractile cycle, when the vacuole appeared smooth and translucent, the ER did not exhibit a pattern that was obviously linked with the contractile vacuole. It should be

mentioned that the fluorescent signal appears to be especially strong at the poles of the sporangium.

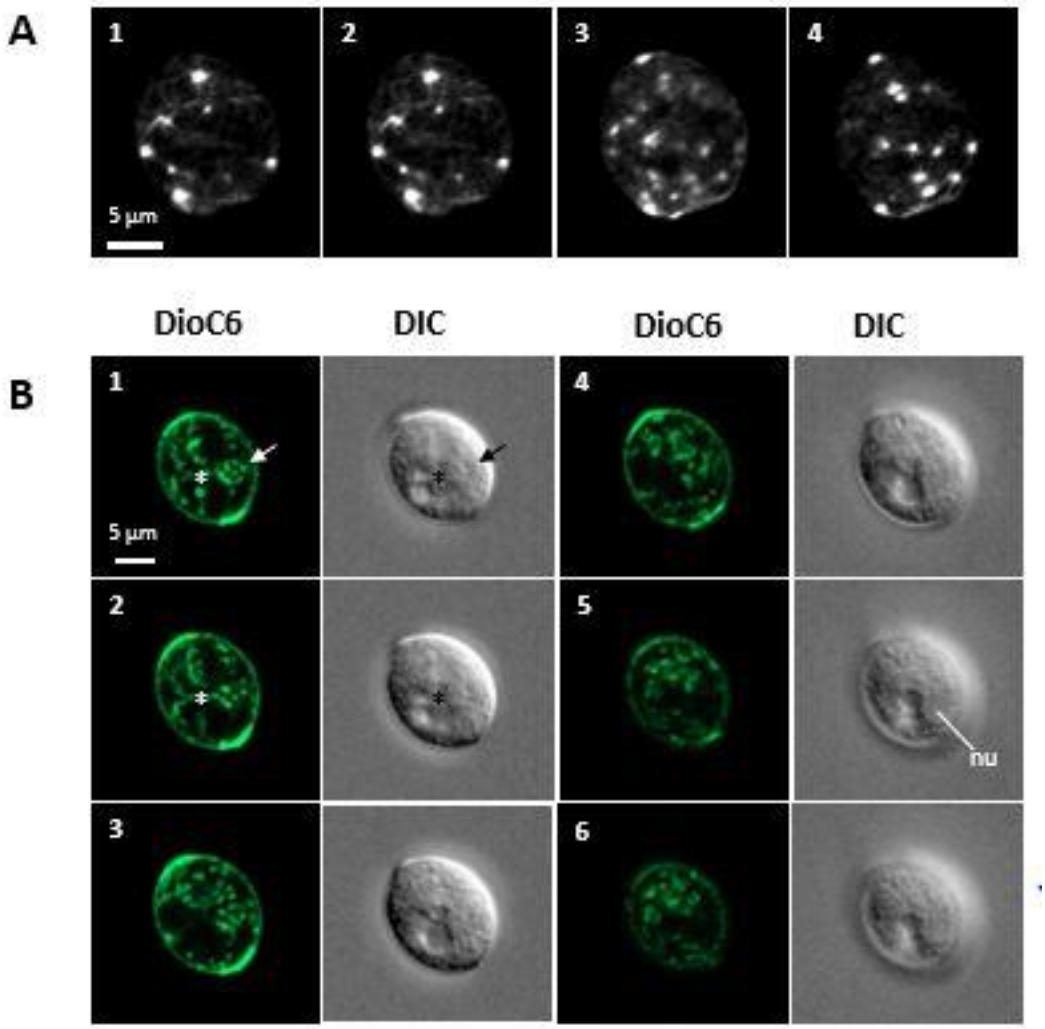


Fig. 3.5 Cytology of the turgescent sporangium. **A** Actin visualized by fluorescent phalloidin after chemical fixation. Subsequent sections from a confocal z-stack. **B** ER visualized by $DiOC_6$ in vivo. Subsequent sections from a confocal z-stack. The $DiOC_6$ signal is shown along with the corresponding image by Differential Interference Contrast (DIC). Arrows indicate a release site of contractile vacuoles, asterisks indicate a star-like ER structure subtending the contractile vacuole, (nu) nucleus.

3.5 Contractile vacuole activity in free zoospores is accelerated and linked with massive endocytosis

In this part of the study, the membrane structures were observed to follow the cellular dynamics in zoospores. After the individuation of the syncytial sporangium into single zoospores, the individual zoospores are already motile within the sporangium and are swimming actively prior to hatching (Fig. **3.6** A, the time interval between the frames 1-8 was 250 milliseconds). Upon staining with the membrane impermeable endocytosis tracker FM4-64, the cell interior was strongly stained within a few minutes indicating a high intensity of endocytotic uptake (Fig. 3.6 B). This was also observed for the mature, but still syncytial sporangium. Already in the pre-hatching state, the individual zoospores showed active contractile vacuoles. In the free zoospores, the contractile vacuole was observed in proximity of the flagellar roots (Fig. 3.6 C).

To characterize functional and cytological aspects of contractile vacuoles of *P. viticola*, single zoospores were followed on their way by video microscopy and time series were recorded by differential interference contrast (shown representatively in Fig. 3.7 A, supplemental movie S2). The entire cycle lasted around 3 seconds (3.28±0.24 s, n=42) and thus, was strongly accelerated over the situation in the mature sporangium. The contractile vacuole was established during the first ~20% of the cycle, remained stable till ~90% of the cycle, and was then rapidly released during the last ~10% of the cycle. During the granular phase of the cycle, numerous vesicles could be seen to merge with the vacuole, which were replaced by radial channels in the translucent phase (Fig. 3.7 B). These channels were dynamic as well, and persisted only a short time after emergence.

Assuming a spherical shape for both vacuole and cell, the mean volume flow per second was estimated to be 3.8% (±0.30%) of total volume, which means that in around 25 cycles (around 75 sec) one entire cell volume of water must be secreted to prevent the zoospore from bursting.

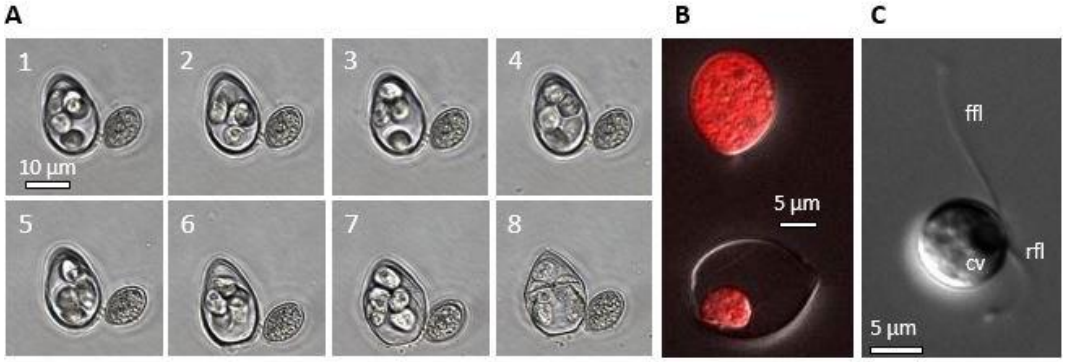


Fig. 3.6 Cytology of zoospores. **A** Zoospores are released from turgor prior to hatching. **B** Intensive staining of syncytial sporangium (upper cell) and hatching zoospore (lower cell) by the endocytosis tracker FM4-64. **C** Mobile zoospore recorded by differential interference contrast. (ffl) front flagellum, (rfl) rear flagellum, (cv) contractile vacuole.

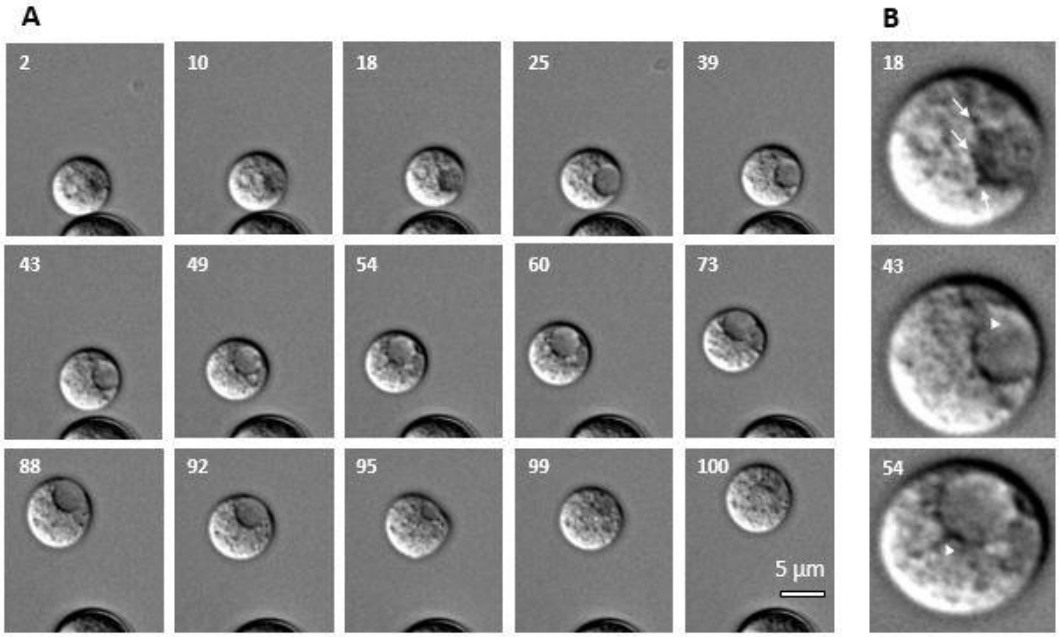


Fig. 3.7 Functional and cytological details of the contractile vacuole in a free zoospore. **A** Time series over one contraction cycle recorded by differential interference contrast. The entire cycle lasted 3 s, numbers indicate the relative time of the respective frame in % of the entire cycle (100% corresponding to one completed cycle). The contractile vacuole is established during the first ~20% of the cycle, remains stable till ~90% of the cycle, and is then rapidly released during the last ~10% of the cycle. **B** Magnification of the contractile vacuole from the time series shown in A to highlight cytological details. Numbers as described in A. White arrows indicate vesicular structures that fuse during the early phase of the contractile cycle, white arrowheads indicate channels of changing width that appear and disappear during the stable phase of the contractile cycle.

3.6 Inhibition of the contractile vacuole by ions and RGD peptides

Due to the massive secretion of water necessary to preserve integrity of a motile zoospore, even minor perturbation of the contractile vacuole is expected to result in zoospore burst. Therefore, the incidence of zoospore burst in response to different compounds can be used as readout for the rate of impairment of the contractile vacuole function.

At reference-conditions, no zoospore burst was observed up to 20 min. After 30 min a low number of burst cells (200 cases) could be observed and this value increased to 400 cells at 90 min - in a total population of 120,000 cells. The variability in this number between biological replicates was 21%. The addition of 10 µM of sodium chloride increased the incidence of zoospore burst by a factor of 4 (

Fig. **3.8** A). In contrast, the addition of the same concentration of either copper sulfate or aluminum chloride drastically enhanced the incidence of zoospore burst to more than two orders of magnitude within 30 min compared to the control. A dose-response curve recorded at 30 min after the onset of the treatment (

Fig. **3.8** B) showed that the effect was saturated from 20 µM in case of aluminium ions, whereas in case of copper, similar values were reached for 40 µM. For sodium, from 20 µM, a plateau at around 25-fold increase compared to the plateau was observed.

The heptapeptide motif YGRGDSP (abbreviated as RGD) represents a motif on the extracellular matrix proteins fibronectin and vitronectin, which is recognized by animal integrins. The binding confers mutual adhesion between animal cells, or their interaction with the extracellular matrix. To explore, whether analogues of integrins are relevant for the function of the contractile vacuole, the influence of RGD peptides was tested along with the biologically inactive, but equally charged invert YGDGRSP (DGR), and the non-specific, charged peptide polyamine sulphate. Time-courses of zoospore burst were recorded for these peptides (

Fig. **3.8** C) using a peptide concentration of 20 ng ml^{-1}. After a lag phase of 20 minutes, zoospores progressively burst reaching a plateau of 10-fold increase compared to control from 40 min (polyamine sulphate), or 80 min (RGD). In contrast, the DGR peptides were found to be mostly ineffective. Dose-response curves assessed at 90 minutes after addition of the peptides (

Fig. **3.8** D) showed a strong increase of burst with increasing concentrations for the RGD peptide, resulting in almost 20-fold higher values compared to control, whereas there was little effect of DGR even at the highest concentration (50 ng ml^{-1}). Polyamine sulfate levelled at a

plateau that was around half the value observed for the RGD peptide. These observations indicate that RGD specifically can impair the function of the contractile vacuole and that a part of this effect can be due to the unspecific, positively charged polyamine sulphate.

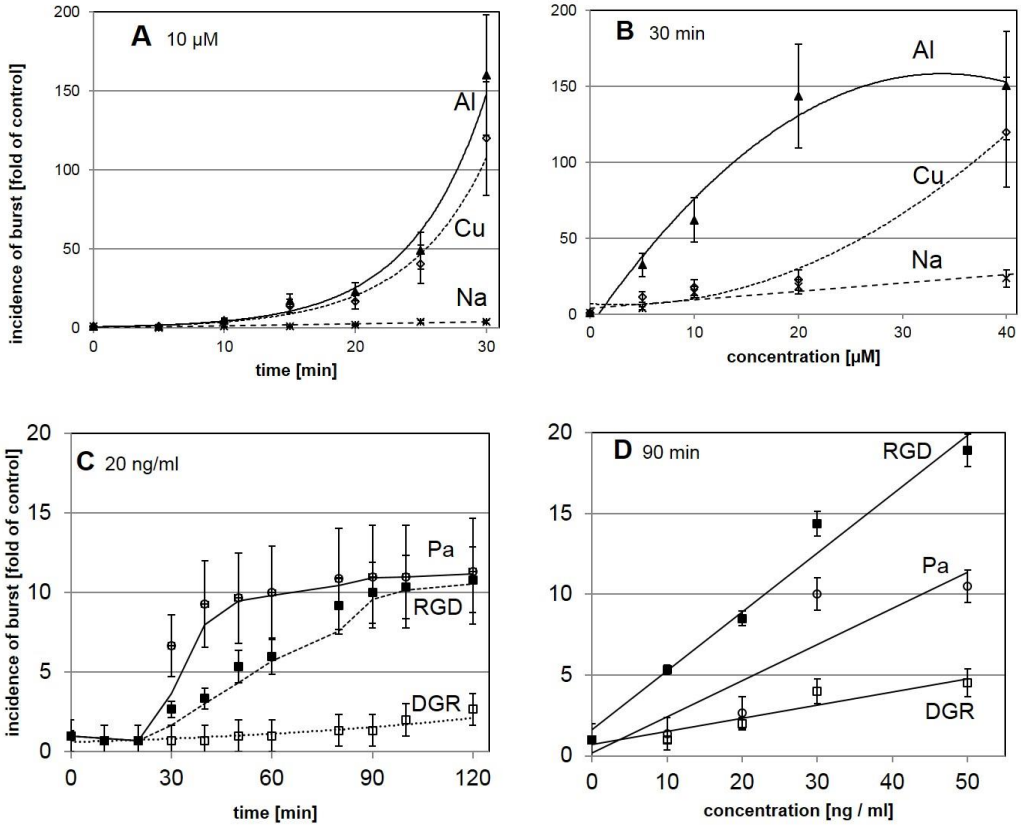


Fig. 3.8 Functional analysis of contractile vacuole of motile zoospores. Influence of copper, aluminium and sodium (**A**, **B**) as well as peptides interacting with the extracellular matrix (**C**, **D**) on burst incidence of zoospores as readout for impaired function of the contractile vacuole. Time courses (A, C) and dose-response curves for a fixed time of treatment (B, D) are shown. Evaluated area was within 10 big squares in counting chamber (Fig. 1, Fig. 2). Identical concentrations of 1.5 x 10^4 sporangia ml^{-1} were used, and each experiment was accompanied by an untreated control as internal standard. Values give the incidence of bursting zoospores relative to this internal standard at the final time step of the experiment (30 minutes in case of metal ions, 90 minutes in case of ECM-interacting peptides). Values represent means and standard errors from two independent experimental series, under control conditions, no zoospore burst was observed up to 20 min, a low number (200 per ml of sporangial suspension) was observed at 30 minutes. This value increased to 400 per ml at 90 min. The variability in this number between biological replicas was 21%.

3.7 Glycyrrhizin and *Glycyrrhiza* root extract inhibit zoospores and promote *Vitis* Pinot Noir cell immunity

To explore the influence of biological extracts on zoospore stability, the medicinal plant *Glycyrrhiza glabra* was selected. In former field studies the effectiveness of this extract against Downy Mildew was discovered (Schmitt et al., 2011). In this study effect of *Glycyrrhiza* on *P. viticola* zoospores was investigated microscopically and functionally in more detail and the active component was found to be glycyrrhizin. Zoospore solution was evaluated microscopically for three possible zoospore forms (motile, encysted or burst) after treatment with respective concentrations of *G. glabra* extract solution or glycyrrhizin for 10 minutes.

Fig. 3.9 shows dose-response relations of the two solutions in rising concentrations up to 5 mg ml^{-1}. The percentage of the counted burst zoospores increased to approximately 30% with rising concentrations of the liquorice solutions. The number of the motile zoospores decreased. The dilution to 0.05 mg ml^{-1} extract showed same results as water control and therefore seems to have no effect. Interestingly, the predominant part of zoospores treated with 0.5-5 mg ml^{-1} was found to be encysted (data not shown). Encysted zoospores have already constructed a cell wall, characteristic for the following life cycle step. In lower concentration, *G. glabra* extract was found to cause a higher amount of bursting zoospores than glycyrrhizin. High concentration of both solutions resulted in the same effect regarding burst and encysted zoospores.

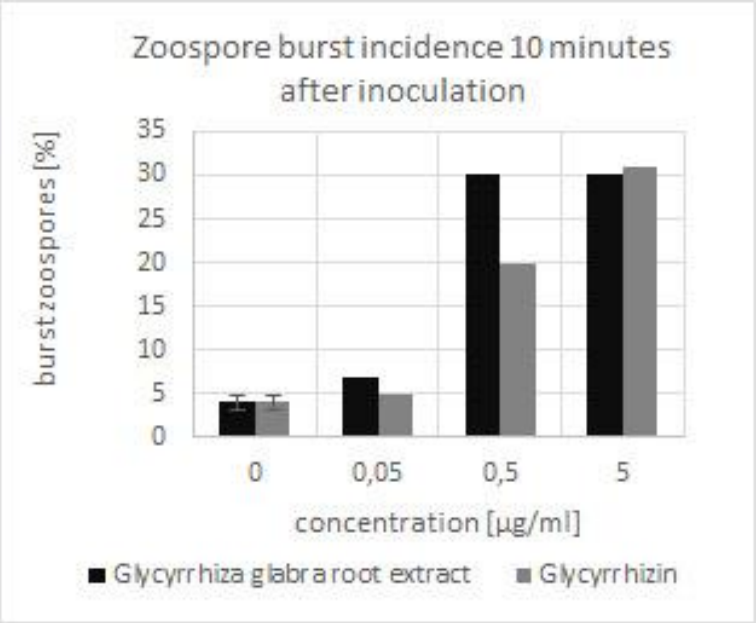


Fig. 3.9 Dose-response relation of *Glycyrrhiza glabra* root extract and glycyrrhizin on zoospores. Amount of burst zoospores are indicated as percentage of all evaluated zoospores. Concentrations ranging up to 5 mg ml^{-1}. Evaluated cells 300-500. Error bars represent standard deviations of two independent experiments, exclusively conducted for the water treated control group.

To reveal, whether plant cells also can sense glycyrrhizin or *G. glabra* extract and activate their immune system, a pH assay was conducted, measuring the apoplastic alkalinisation of suspension cells after addition of the substances. This assay is based on measuring the extracellular pH of cell culture medium after stimulation of cells with elicitor or tested compounds of interest. Changes of pH into alkaline are indicators for the stimulation of the plant immune system (Felix et al., 1999). Fig. 3.10 sums up the results of pH measurements after addition of different concentrations of *G. glabra* extract and glycyrrhizin. Each point represents the maximal shift of the pH into the alkaline 30 minutes after substrate addition.

Glycyrrhizin and the *G. glabra* extract caused extracellular alkalinisation in the *Vitis* cell culture. The effect seems stronger with glycyrrhizin, but should be regarded as qualitative result, due to possible differences of the glycyrrhizin content in the extract and the pure glycyrrhizin solutions.

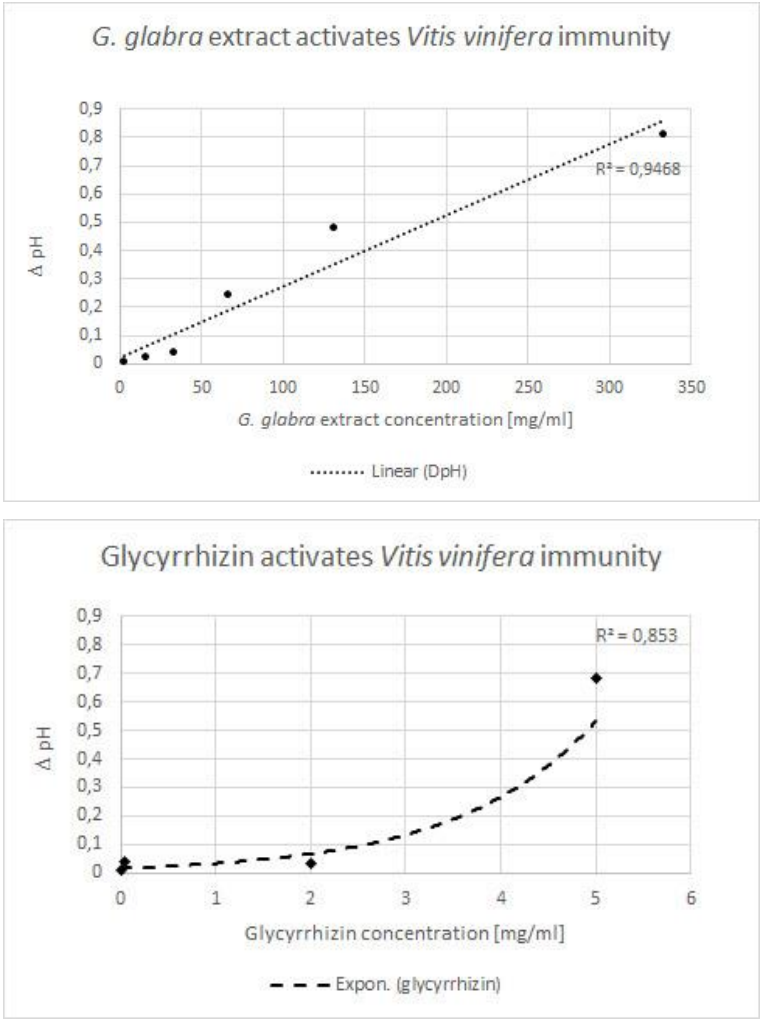


Fig. 3.10 Dose-response of *G. glabra* root extract (upper figure) and glycyrrhizin (bottom figure) after 30 minutes on *Vitis vinifera* Pinot noir cultured cells. Figure shows extracellular alkalinisation of the medium (ΔpH) as readout for enhanced immunity. Dotted line represents simple linear regression line. Dashed line represents exponential regression line.

3.8 Summary

The general aim of this study was to reveal cellular details of a globally important disease, the Downy Mildew of grapevine to investigate strategies towards a more sustainable viticulture. Both sides were considered, host and pathogen. In the European wild grapevine *V. sylvestris* one possible resistance factor was elucidated, which could be provided for future breeding programs. The weakness of *P. viticola*, the causative organism of Downy Mildew of grapevine, was investigated. Knowledge about the cellular details and the function of the contractile vacuole would enable a less toxic control of Downy Mildew.

The first part of this study describes one crucial factor of the morphology of *Vitis sylvestris* grapevine leaves, the stomatal density allocations. Each accession of the Ketsch collection was analysed for its stomatal density and its level of susceptibility to Downy Mildew. The stomatal densities range from the lowest density of 0.8 stomata per epidermal cells to the highest stomatal density of 1.6 stomata per epidermal cells. The results show, that stomatal density is correlating with the infection rate. More stomata correlate with higher infection rate. Higher stomatal density confers higher susceptibility of the grapevine accession to Downy Mildew, which is published in Duan et al., 2015. A correlation study with all wild grapevine plants of the Ketsch collection and different Downy Mildew strains in three subsequent years is listed there. In the opposite the results indicate, that a lower stomatal density contributes to a higher resistance level against *P. viticola*.

In a second part, the cellular and functional properties of *P. viticola* were examined besides its early infection behaviour, with special focus on one fundamental organelle, specific for sporangia and zoospores, the contractile vacuole. The vacuole is pivotal for zoospores, maintaining cellular stability via osmoregulation, and was discovered to be formed already in the sporangia upon contact with water. Due to the importance of the vacuole and its occurrence in the very fragile zoospore state of *P. viticola*, it was regarded as potential convenient target for more efficient applications for disease control. High-resolution electron microscopy combined with video-microscopy of sporangia and zoospores revealed developmental changes of the contractile vacuole during these defined life cycle steps. In summary, an extract of the root of liquorice plant *G. glabra* was shown to be active against zoospores and additionally stimulate plant immune system, which makes it an interesting candidate for treatments in viticulture. As well, the main component glycyrrhizin shows these effects.

The early infection behaviour of *P. viticola* zoospores was investigated by observing stomatal infection levels after different incubation times. Therefore, the number of infected stomata and the number of zoospores on each stoma were counted and the results were displayed as zoospores per infected stoma. The resulting factor reveals the gathering level of different

strains on different plants. A time course was assessed from 2.5 minutes up to 45 minutes after inoculation. The results indicate the remarkable fastness and affinity of zoospores to host stomata. They are able to detect the stomata, approach them by swimming and encyst there within less than 2.5 minutes. At a later time, interestingly, the gathering pattern was relatively constant. Several zoospores can infect one stoma, depending on the stomatal size, resulting in a so called "gathering pattern". The time course gathering pattern of zoospores shows the ratios of number of zoospores per number of stomata in the first 45 minutes after inoculation. The values obtained in this approach could be seen as an indicator for *P. viticola* infection strength and epidemic strength. This pattern was observed in both tested wild grapevines as well as in Müller-Thurgau. On Müller-Thurgau both tested strains were constantly infecting with average of 2 zoospores per stoma, which is a quite low gathering effect. Same low gathering level was observed with the "weaker" B11 zoospores on high stomatal density accession K51. And also the zoospores of the aggressive strain B15 gathered in a low level on low stomatal density accession K103. A stronger gathering pattern was found with the aggressive B15 strain on the high stomatal density accession K51, with average of 2-4 zoospores per stoma. The highest gathering occurred with the "weaker" *P. viticola* strain B11 on the low stomatal density accession K103, with an average of 4-5 zoospores per stomata. In this combination, many stomata have been occupied with more than 10 encysted zoospores, in some cases 20 cysts have been counted and presumably more have been covered under the upper layer of cysts, which is comparable with the literature (Royle & Thomas, 1973).
P. viticola infection is starting with the attachment to and entry in stomata, which is necessary for zoospores to infect. The gathering effect is higher in accessions with lower stomatal density. Therefore, the strategy against Downy Mildew should target *P. viticola* in its early stage as zoospores during the contractile vacuole is active. Before encystment and entry of germination peg in the plant tissue only the cell membrane is protecting the Downy Mildew. The vacuole is an ideal target for combat Downy Mildew and therefore tests were conducted if and how the vacuole could be inhibited and how inhibition of the vacuole is influencing zoospore fate and infection rate.

The cellular development of *P. viticola* sporangia and zoospores was investigated in deeper detail and functional assays inhibiting the contractile vacuole in zoospores were conducted. The results show how dry sporangia evolve in water to mature sporangia and which cellular changes occur until functional zoospores are emerging and hatching. Cellular structures are described on high resolution microscopy pictures as well as functional studies were undertaken to understand the function of the contractile vacuole regarding volume and osmoregulation in zoospores. The functionality of the contractile vacuole could be probed by quantification of zoospore burst in response to different compounds, supposedly interfering with the

plasmalemmal reticulum (RGD peptides), osmotic potential (NaCl), or apoplastic oxidative burst (Cu and Al ions) as important signal for osmosensing. The data suggest a mode of action for the traditional, copper-based control of Downy Mildew. RGD peptides were shown to result in impairment of the functionality of the contractile vacuole. As well, the main ingredient in roots of the liquorice plant, glycyrrhizin, can impair function of the contractile vacuole and cause burst of zoospores. To reveal, whether the biological extract or glycyrrhizin are suitable candidates for treatment of grapevines, their influence on plants should be investigated. In this study, the influence on cultured grapevine cells (Pinot noir) were examined. It was found, that they stimulate plant cell immunity.

The functional data of the contractile vacuole have been combined with microscopical data. Specially, video-microscopy at high-resolution has been conducted by both differential interference contrast and after fluorescent visualization of actin and endomembrane system. With this application, the developmental sequence of the contractile vacuole could be followed from its genesis in sporangia during cellularization into zoospores until vacuole disintegration during encystment of zoospores. Additionally, the membrane dynamics during cellularization could be investigated by transmission electron microscopy of different stages of sporangium germination.

4 Discussion

Effective disease management of Downy Mildew of grapevine is urgently needed since control strategies, especially in organic viticulture, face several limitations (Gessler et al., 2011). Traditional fungicide applications include suspensions with toxic metal ions, influencing organisms in the soil and ground water (Kovacic et al., 2013). Modern fungicides work with single side targets on *P. viticola*, at the complex III of respiratory chain (Bartlett et al., 2001), which are shown to be rapidly overcome by the pathogen, which adapt resistances against these fungicides (Toffolatti et al., 2011). Even genetic control strategies, using resistant varieties, turned out to be limited. *P. viticola* is able to rapidly adapt to resistant varieties (Delmotte et al., 2014). Breeding of stably resistant cultivars is an ongoing journey. A new strategy is based on the idea to insert several resistance factors to gain a more stable resistance, which is more difficult for *P. viticola* to adapt (Schwander et al., 2012). Treatments with toxic copper ions are still used in organic viticulture and need to be exchanged by other substances that could be used more effectively for a more sustainable and economically friendly viticulture. Either plants should be protected more efficiently or the defence strategy against the pathogen should turn into less toxic influence on the environment.

Two approaches were followed in this study. Since *P. viticola* needs plant stomata to enter and grow further, stomata were in focus on the plant side and the early infection behaviour of zoospores on stomata. First, a relevant morphological trait of *Vitis sylvestris* was investigated, the stomatal density, to find out if the amount of stomata is influencing infection rate with zoospores. The results showed, that a higher stomatal density confers a higher infection rate with *P. viticola* and thus a higher susceptibility to Downy Mildew. Secondly, a weak point of *P. viticola* should be defined. This knowledge would allow to use less amount of fungicides in applications or use of less toxic compounds. The focus was situated on the zoospore state, which was regarded as suitable target due to two reasons. Zoospores lack cell walls and they appear at the plant surface, in contrast to all other life cycle stages of *P. viticola*, which possess cell walls and are widely growing inside the host plant, like the hyphae. This facts make zoospores especially vulnerable to many environmental influences and therefore give the chance to applicate less toxic compounds, used in the appropriate moment. However, zoospores rely on the proper activity of contractile vacuoles, collecting invading water and expulsing it in regularly cycles to the outside of the cell. Therefore, the focus was set on investigating the shape, the formation, and the function of contractile vacuoles of *P. viticola*. The approach to achieve this was a combination of different microscopical investigations of the vacuole in sporangia and zoospores and functional studies on the contractile vacuole of zoospores with different compounds. The results revealed that zoospores are very vulnerable

and fragile. The contractile vacuoles are formed already inside the syncytial sporangia before zoospores are formed. They remain stable during the zoospore state until encystment of zoospores. The vacuole pumping cycles were quantified and their frequency were found to be comparable to those in Phytophtora. Additionally, the cycles show larger frequencies before zoospores burst. Treatment of zoospores with ECM-interacting RGD peptides revealed possible integrin-like proteins in *P. vitic*ola. The results of treatments with metal ions Cu and Al revealed specific impairment of the activity of the contractile vacuoles which caused zoospore burst. An extract of *G. glabra* roots was used to elucidate if its positive influence on plants against Downy Mildew (Schuster et al., 2010) is impairing *P. viticola* on the zoospore state due to impairment of the contractile vacuole. It could be shown that this extract impairs zoospores and additionally is elevating plant cell immunity. The same effect could be shown with the main compound of *G. glabra* root extract in glycyrrhizin.

The Achilles' heel of Downy Mildew can be seen in the unprotected zoospores and their dependency on a functional vacuole. This fragile life cycle stage can be easily impaired with glycyrrhizin as well as with *G. glabra* root extract, which therefore might be suitable compounds for a sustainable viticulture.

4.1 Stomatal density

The first aim of this study focused on one detail of the host side, characterizing the morphological structures of *Vitis* leaf surface, the place where the infection process of *P. viticola* starts. One morphological factor was found to correlate with *P. viticola* infection strength, the stomatal cellular densities (Rühle, 2011). To further characterize this factor, a microscope screening study was applied. Therefore, glue imprints of the leaves of the European wild grapevines of a conservation population (*Vitis vinifera* ssp. *sylvestris* Ketsch) in the Botanical Garden of KIT were created and analysed for their number of stomata and epidermal cells. The resulting stomatal densities were correlated with the results of a screening for infection levels in the same *Vitis sylvestris* collection with *P. viticola*.

The results indicate, that a lower stomatal density contributes to a higher resistance level against *P. viticola*. This reveals a possible new trait for breeders for future breeding projects. For this purpose, it would be necessary to find out the genetic background of stomatal density and distribution in *V. sylvestris* accessions with a low stomatal density. This would enable breeders to select new varieties with a lower stomatal density, which might contribute to a more sustainable strategy to protect grapevine stomata against Downy Mildew infection.

Stomatal distribution and development depends on the interplay of several genes. Stomata emerge out of epidermal cells which are able to divide asymmetrically a process also involving cell-cell signalling (Nadeau & Sack, 2002). The distribution of stomata is tightly regulated with several transcription factors and signalling pathways, defining orientation of the asymmetric cell divisions and preventing overproduction and clustering of stomata (Peterson et al., 2010) and at least in *Arabidopsis* three proteins are found to be basic for this, SPEECHLESS, MUTE and FAMA as well as partner proteins SCREAM and SCREAM2. Also the proteins TOO MANY MOUTH, STOMATAL DENSITY AND DISTRIBUTION1 and FOUR LIPS were found to be important for the regulation of stomatal formation (Nadeau & Sack, 2002). The corresponding genes and proteins in grapevine could be analysed for correlation with the stomatal densities of the different *V. sylvestris* accessions, for example the high stomatal density accession K51 and the low stomatal density accession K103 described in this study. Candidate genes can be examined for pyramidisation strategy in breeding, shifting stomatal regulation towards a lower stomatal density.

It was found out in a large screening study, that stomatal density inversely correlates with atmospheric CO_2 concentration and this factor is plant species specific (Royer, 2001). They found out that CO_2 appeared to inversely affect stomatal initiation, but the mechanism may involve genetic adaptation. Therefore, the focus should be set on the genetic manipulation of grapevine by breeding new varieties based on *Vitis sylvestris* accessions with a low stomatal

density. But, at least for the aim of lower stomatal density in grapevines, the rising atmospheric CO_2 concentration might be one little advantageous factor. It was analysed, that the stomatal density is dependent from the atmospheric CO_2 concentration (Serna & Fenoll, 2000).

It was discovered, that the distribution of stomata is tightly regulated and following a rule that at least one epidermal cell needs to be located between two stomatal complexes (Peterson et al., 2010). As decision-makers in *Arabidopsis* the three proteins SPEECHLESS, MUTE and FAMA and their partner proteins SCREAM and SCREAM2 are found. Also the protein STOMATAL DENSITY AND DISTRIBUTION1 was found to be important for the negative regulation of stomatal formation throughout the shoot and encodes a processing protease that may function in intercellular communication (Nadeau & Sack, 2002). Obviously, *Vitis sylvestris* accessions have slight differences in stomatal densities and might be founded to different regulation processes or due to genetic variances in these proteins. If it would be possible to define stomatal genetic background and regulation, it could be a promising factor for future breeding strategies producing grapevines with lower stomatal densities for control of Downy Mildew.

4.2 Early infection behaviour on the host

The second aim of this study was to reveal the weak point of the pathogenic Oomycete *P. viticola*, predominantly growing well-protected inside of the plant, which makes it difficult to reach it with treatments from outside. To provide new ways for creation of sustainable treatment applications, the zoospore state was regarded as interesting target and discussed as weak point due to the fragility of zoospores, which lack cell walls, and their appearance on the leaf surface at the same time. The first step of the early infection behaviour is the targeting of stomata. Therefore, the first goal was to investigate zoospores regarding their need to target stomata during early stage of infection process.

Stomatal targeting was defined as attached cysts per stoma. The approach was to evaluate the number of zoospores per stoma on zoospore solution inoculated *V. sylvestris* leaf discs. Different indicated time spans were applied before quantifying. The gathering factors of different genotype combinations of grapevine and *P. viticola* strains were compared.

Stomatal targeting turned out to be a very rapid and effective process on *Vitis sylvestris*, suggesting that host factors serve as guiding factors like described already for *P. viticola* (Kiefer et al., 2002) and *Achlya* (Thomas & Peterson, 1990). Several different other factor may contribute to the efficient targeting, for example oxygen, released out of stomata, ionic fields around stomata, or the light gradient between stomata and epidermal cells. It was shown, that *P. viticola* zoospores can find stomata independently from light (Royle & Thomas, 1973), which was shown in infection experiments in the dark. As well, a water current can be assumed as recognition factor due to gas exchange at the stomata. Also chemical gradients, for example with calcium ions, around stomata can be a factor affecting stomatal targeting. Another factor is the different composition of guard cell walls in comparison to epidermal cell walls. It was found, that Oomycete zoospores can accumulate selectively on different surfaces (cellulose and chitin), showing that zoospores can recognize surface details and suggesting, that surface recognition mediates zoospore accumulation (Mitchell & Deacon, 1986). Since the cell walls of guard cells and epidermal cells differ in their polysaccharide composition, it might be possible that surface recognition influences the targeting. For example, stomata contain large amounts of molecules bearing terminal fucose, predominantly in ventral and lateral guard cell walls (Majewska-Sawka et al., 2002), and they have thicker cell walls (Zhao & Sack, 1999) and a collective distributions of cellulose (Nadeau & Sack, 2002). These differences could explain the final targeting and encystation, but the long distance perception of the directed movement to stomata likely depends on other factors mentioned before.

In this study, gathering was found to be correlating with stomatal size. Stomata can have different sizes due to their characteristic growth, starting from a small meristemoid mother

cell, which builds a meristemoid cell after asymmetric cell division, which itself divides asymmetrically to guard mother cell, from which a young small guard cell emerges after symmetrical cell division (Nadeau & Sack, 2002).

The stomatal size could fortify the surface recognition factor mentioned before due to a higher surface. And, since adult stomata seem to be preferred by zoospores, the bigger stomata presumably do release attracting factors more efficiently, maybe in higher concentration or in earlier stages of their growth. It might be possible, that zoospores could perceive the stomatal cells due to their different cell wall composition mentioned before. In a control experiment which was conducted in this study on the upper leaf epidermis (data not shown), zoospore distribution was found to be equally spread over the surface without any gathering effect. This observation shows that zoospores do not attract themselves in a mutualistic way during swimming. However, it might be that they start to attract other zoospores after they found a stoma and settled there as cysts, maybe by releasing factors which emerge during encystation, or by amplifying the host signals (Kiefer et al., 2002). This could be described as selective adelphotaxis. The host factors might result from a water soluble signal from open *Vitis* stomata.

As outlook on future experiments, in vivo video microscopy could be performed directly after inoculation of a leaf disc with a stained zoospore suspension. The life observation of stomatal targeting would bring light in the early infection process of zoospores on the grapevine leaf surface. As calcofluor exclusively stains cysts it would visualize the progress of targeting at the encystation state and would enable to follow and quantify the progress of infection. Due to the facts that the surface of grapevine leaves is arched and zoospores are moving rapidly, which makes it unclear where exactly they gather, it would be necessary to observe an area on the leaf surface. These proposed investigations would be facilitated by using a digital microscope. The advantage of such microscopes is that they produce pictures which are fully in focus, which would enable to screen the infected leaf surface in high throughput. Furthermore, it would allow to test and evaluate several different zoospore suspension concentrations in parallel, to investigate if the density of the zoospore suspension influences the gathering process.

Zoospore cell integrity was found to be maintained by several integrative processes and structures. They are based on the morphology and activity of the contractile vacuole in zoospores. They were investigated in functional assays and kinetic microscopy. As well, a comparison with plant cell integrity was done. These outcomes are discussed in more detail in the following chapters.

Another need of zoospores is to retain their cell integrity within water during their unprotected time as protoplast-like cells on the leaf surface. This is achieved by their water pumping organelle, the contractile vacuole. The following chapter focusses on this organelle.

4.3 Developmental sequence of the contractile vacuole of *P. viticola* from genesis to disintegration

The aim of this part of the study was to visualize the contractile vacuole to find out characteristics of its life cycle. This information would show whether the vacuole might be a potential target for future treatments against Downy Mildew. Because of their rapid movement, it seemed to be necessary to decelerate the zoospores for microscopical observation. However, deceleration seemed to be toxic to zoospores. Therefore, the stainings were performed with fixed zoospores or sporangia.

First, kinetic microscopy was performed after staining of filamentous actin in a zoospore suspension. Observations with the DIC channel and the fluorescence channel revealed that *P. viticola* sporangia are filled with a fine structured actin network, interconnected with star-shaped actin plaques through the whole cell. Actin plaques have already been described as putative signs for life cycle transitions of *P. viticola (Riemann* et al., *2002)*. Actin was mentioned in Liu et al., 2013 to be important for volume control in plant protoplasts and a comparison reveals several functional and structural analogies. As the zoospores lack cell walls, they might physically behave like protoplasts. Plant protoplasts are able to adjust cell volume during change of osmotic pressure by swelling, which is performed by releasing membrane material from internal stores (Liu et al., 2013). Possible substrate could be the cytoskeleton-plasma membrane-cell wall continuum, which is connected with the water expulsion vacuole. Based on the fact that stability of plant membranes depends on submembraneous actin, the authors demonstrated that submembraneous actin cytoskeleton controls the release of intracellular membrane stores during regulatory volume change (Liu et al., 2013). It might be that the opposite process is existing in zoospores which regulate cell volume by internalization of membrane material into cellular stores.

Secondly, microscopy of different stacks of $DiOC_6$ stained ER in sporangia revealed a dynamic remodelling of ER cisternae over time.

Thirdly, ultrastructural details of membrane distribution and dynamics during cellularization of sporangia were observed using transmission electron microscopy. The results showed high rates of endocytosis and membrane recycling. It is reported, that a continuous flow of secretion vesicles is essential for a polarized growth of fungi, providing material for cell wall and membrane extension (Fischer et al., 2008). It might be, that a similar process, possibly involving actin and microtubule cytoskeleton, is occurring in sporangia. Additionally it might be, that this process is regulating membrane dynamics in zoospores during the cycling of the contractile vacuole. For proper function of the vacuole, which constantly changes its volume, a tight volume regulation is needed.

Morphological observations in this study revealed, that the contractile vacuole is formed already before zoospore individuation within evolving sporangia upon contact to water, in contrast to the finding in *Phytophtora*, where contractile vacuole activity was observed after zoospore individuation in individual zoospores within the sporangial shell before release from the sporangium (Mitchell & Hardham, 1998). The vacuole remains active until zoospore encystment, which goes along with cell wall construction conferring cell stability. The developmental steps of *P. viticola* are already described in the literature (Kiefer et al., 2002) and are similar to other Oomycete life cycles, for example *Plasmopara haldstedii* (Bouterige et al., 2003) and *Phytophtora* (Hardham, 1992). Cycling of the vacuole in a sporangium was observed to consist of a bifurcated state and a fully expanded single bladder state, both contributing to a cycle interval of 8-12 seconds. The cycling was observed to be accelerated in free zoospores.

In summary, the visualization of the contractile vacuole of *P. viticola* was performed indirectly using fluorescent staining of the surrounding cytoplasmic structures actin and ER, which were observed in parallel with the pulsating vacuole in the DIC channel over time. Besides a star-like actin cytoskeleton, which might be in tight contact with the vacuole and perform the contractions for expelling water to maintain cell stability, dynamic change of ER cisternae was observed.

Electron microscopy confirmed the finding, which was received from the microscopy of the stained membranes, that zoospores and sporangia have a high endo- and exocytotic turnover.

4.4 *P. viticola* contractile vacuole is a central organ for zoospore cell integrity

The aim of this part of the study was to further characterize the contractile vacuole of *P. viticola* during its appearance within zoospores using video microscopy, followed by quantification of the cycle activity. Cycles of the evaluated zoospore vacuoles were shown to last between 1 and 8 seconds. This spread might be explained with the different zoospore ages, fitness and nutrition states. It was observed, that long lasting cycles occurred in more slowly swimming zoospores shortly before burst. In contrast, rapid cycling vacuoles were observed in vital zoospores which were rapidly swimming. They could be observed over a long time period.

However, the times are comparable with those observed in *Phytophtora*. There, a mean vacuole cycle time of 6.1 seconds was evaluated in a *Phytophtora nicotianae* zoospore, which was temporarily immobilized between two hyphae, and a time of 5.7 seconds was measured in hyphae (Mitchell & Hardham, 1998). This indicates, that vital Oomycete zoospores and hyphae obtain similar cycle times of contractile vacuoles. Thus, vacuolar expulsion rhythm seems to be old conserved mechanism in Oomycetes, which is similar in different organs as well as in different orders of the subclass of Peronosporomycetidae.

In summary, these findings indicate the importance of a functional contractile vacuole for *P. viticola* zoospores, which rely on the activity of their contractile vacuoles, efficiently collecting incoming water and contracting in regular cycles for pumping water out of the cell to retain stable cell volume and integrity (Patterson, 1980).

Due to the differences in the cycling depending on the zoospore situation - vital zoospores swim rapidly through the water and are thus very difficult to observe - the zoospores found in this study are likely a selection of the slower zoospores, representing zoospores shortly before encystation or burst. Several experiments to slow down zoospores resulted in zoospore burst, indicating that also the rapid movement seems to be necessary for zoospore vitality.

To sum up, assuming that it is necessary for zoospores to have a rapid cycling vacuole and a rapid swimming movement, it is possible to impair zoospores by impairing the function of the contractile vacuole or the flagellar movement. Therefore, flagellar function could be regarded as a target of applications.

4.5 Contractile vacuole is anchored with integrin-like proteins

The aim of this part of the study was to investigate the cellular details of cell wall and cell membrane of *P. viticola* zoospores, regarding their connection to the intracellular cytoskeleton which presumably has connections to the contractile vacuole. Additionally, the osmotic stability of zoospores was investigated. The approaches were to investigate the zoospore fate after addition of RGD peptides and after the addition of NaCl salt ions, as well.

RGD peptides in concentrations of ng ml^{-1} resulted in a 10.8-fold increased incidence of burst zoospores after 120 minutes compared to control. The time course shows a sigmoidal curve of increase of burst zoospores per time and bursting started 20 minutes after RGD incubation. This kinetics indicates that RGD peptides act on zoospores already in low concentrations in a comparably slow way which involves presumably several steps. The output appears as bursting. Thus, addition of RGD peptides is toxic to zoospores.

RGD heptapeptides contain a motif, which naturally occurs in animal proteins and serves as a signal motif, which is recognized by animal integrin proteins (Ruoslahti, 1996). Adhesion and the involvement of integrins or integrin-like proteins in adhesion can be tested by adding RGD peptides. The additionally added RGD peptides act at the plasma membrane surface, competing for integrin-binding sites on ECM molecules in animal cells (Schindler et al. 1989; Wayne et al. 1992) and cause de-connection of cell membrane, cytoskeleton and ECM. As well, additional RGD peptides can modulate cytoskeletal organization by interfering with the so-called cytoskeleton plasma membrane cell wall continuum (Pickard, 2008). Plants lack integrins. However, in plants RGD peptides were shown to interact with unknown plant components that mediate interactions of cytoplasmic strands with the extracellular matrix (Canut et al., 1998). The same group showed, that the reverse peptide DGR, with reverse amino acid order, is not interacting and therefore inactive. It was shown before, that RGD peptides act on plant protoplasts (Zaban et al., 2013). It is assumed that analogous binding proteins do exist in plants and fungi. For some fungal pathogens RGD-meadiated adhesion was described already (Hostetter, 2000). Also Recently, green algal integrins were described (Becker et al., 2015) as predicted linkers between scales and the cytoskeleton in many green algae. *Plasmopara* lacks scales, but has a complex actin cytoskeleton as shown in Fig. 3.5 A, which is consistent with former results where the actin cytoskeleton was visualized with immune staining (Riemann et al., 2002).

The results from this study, described above, suggest that *P. viticola* membrane possibly contains integrin-like proteins, which released their naturally RGD-binding site containing proteins for binding externally added RGD peptides. Integrin-like proteins could confer adhesion between zoospore membrane and intracellular and extracellular matrix. This idea is

supported by a recent finding, shown for *Arabidopsis*, that the recognition of a protein of *Phytophtora* was performed by a RGD-sequence (Senchou et al., 2004).

For comparing the specificity of these short peptides to zoospores, the same zoospore experiment was performed with the inverted amino acid sequence peptide DGR. The DGR peptides showed only 3-fold higher burst incidence compared to control within 120 minutes. This lower effect was also seen in plant protoplast experiments (Zaban et al., 2013). This suggests, that RGD specifically interacts with integrin-like proteins in *P. viticola* zoospore membrane and the reverse peptide DGR does not interact or just in an insignificant low amount.
Interestingly, the charged protamine sulfate caused 11.3-fold higher burst incidence compared to control after 120 minutes. This is the same burst incidence level than after RGD treatment. Polyamine sulfate (Pa) and RGD peptide showed a sigmoidal dose-response curve, Pa with half maximal burst at a concentration of 20 ng ml^{-1}. This suggests, that both ingredients bind to a receptor or maybe integrin-like protein which has an activity state dependent on substrate concentration, and is maybe regulated allosterically.

Pa is positively charged and interacts nonspecifically with negatively charged structures for example cell outer surface of the membrane.

RGD is not suitable for viticulture due to its general toxicity for all kind of organisms. Further efforts should be conducted for finding useful biological exctracts, which are impairing Oomycetes but in contrast are raising the plant immune system. Several extracts are already found to have these properties e.g. *Salvia officinalis* extract (Dagostin et al., 2010), and *Chloris virgata*, *Dalbergia hupeana*, *Pinus massoniana*, *Paeonia suffruticosa* and *Robinia pseudoacacia* (Chen et al., 2002). Their usage is limited by high costs, availability in large quantities, low persistence, and low level of rainfastness (Dagostin et al., 2010). One promising solution can be the combination of biological extracts with gel formulations which persist rain, or directly biological extracts and compounds which build gels, like glycyrrhizin (Koga et al., 2004).

The second part deals with the results of the assay investigating the osmoregulation of zoospores. The rate of burst zoospores was evaluated microscopically after treatment of a zoospore suspension with NaCl, a salt interfering with osmotic potential. The result was a negligible low rate of burst zoospores. This result indicates, that zoospores have a stable osmoregulation. Salt ions might retain water in the outer medium and slow down water influx into the cells. Nevertheless, salt ions do almost not impair zoospores and thus zoospores seem to have a tight regulation of water efflux. In plants, the answer to salt stress is tightly regulated. In Ismail et al.(2012) it was shown, that cultured cells from grapevine plants, which differ in

their salt tolerance level, differ in their gene regulation and that salt tolerance is mediated by the pathway of the plant hormone jasmonate.

In summary this part of the study showed that it is likely that *P. viticola* zoospores maintain their stability with RGD peptide mediated adhesion and a good osmoregulation. The following chapter discusses the factors which specifically inhibit zoospore stability.

4.6 The metal ions copper and aluminum

The anti-oomycete effect is reported intensively since the introduction of Downy Mildew into Europe in the 19th century (Gessler et al., 2011), but the exact mechanism is still not elucidated. The aim of this part of the study was to investigate the cellular influence of the metal ions used in traditional fungicide applications, copper and aluminium, on *P. viticola* zoospores and try to explore the mechanism behind their anti-oomycete activity. The approach used for this part was to treat a zoospore solution with the indicated concentrations of the metal ions, followed by the microscopical analysis of the burst incidence. The results can be summarized as highly increased zoospore burst rates after addition of minimal amounts of metal ions.

Presumably, the metal ions inhibit the mentioned connection between the cytoskeleton, the plasma membrane, and the extracellular matrix of zoospores. Copper ions were shown to inhibit many targets and processes in cells, like for example enzymes of the respiratory chain, protein synthesis, cell membrane activity, organelles and they produce reactive oxygen species (Kovacic et al., 2013). Aluminum was shown to have negative influences on the fluidity of the cell membrane, endocytotic processes, phospholipase D (PLD) and cell wall synthesis (Pejchara et al., 2007; Dercks & Buchenauer, 1987). PLD is an important enzyme, producing the signalling molecule phosphatidic acid from the cell membrane compound phosphatidyl choline, which is involved in several cellular processes (Billah et al., 1989). The enzyme was detected in *Phytophtora* and was shown to be secreted into extracellular medium and involved in zoospore encystment (Latijnhouwers et al., 2002; Meijer et al., 2005; Meijer et al., 2011). An interesting structure was described in protoplasts in Pickard (2008), the *plasmalemmal reticulum*, which links cytoplasmic structures to the cell wall and can control cellular rise of Ca^{2+}. Treatment with inhibitors confirmed that Ca^{2+} rising is mediated by mechano-sensory channels (Pickard, 2008). Thus, it might be that the reticulum or a related structure is also present in zoospores, linking the cytoplasmic structures to the cell membrane and leading to osmotic regulation.

Cell membrane activity and cell wall synthesis are important traits for Oomycete zoospores and activity of metal ions influence both, resulting in burst zoospores and encystments.

Encystation process might be conferred due to the influence of metal ions on the PLD (Latijnhouwers et al., 2002). One question can be investigated further, how the encystated zoospores evolve. Do they enter cell cycle arrest or are they able to germinate and infect stomata? This can be tested by observing cysts for several hours after encystation.

In summary, the results show that the copper and aluminium ions, used in conventional viticulture, interact specifically and strongly in several manners with the membrane surface of *P. viticola* zoospores. They interrupt the membrane fluidity and lead to rapid cell burst. Due to the toxicity of the ions to many organisms their use in viticulture has limited benefit. Thus, alternative substances are required which inhibit *P. viticola* without harming the grapevine and other organisms. The following part deals with the results of the investigations with *G. glabra* root extract and glycyrrhizin on zoospores and grapevine.

4.7 Bio-extract of *Glycyrrhiza* and its active compound glycyrrhizin stimulate immune system in plants and destabilize contractile vacuoles

In previous studies, a biological extract of roots of the liquorice plant *Glycyrrhiza glabra* was found to be active against several Downy Mildews of vegetables (Schmitt et al., 2011). Based on this interesting finding, this part of this study aimed to reveal whether a *G. glabra* extract affects Downy Mildew of grapevine as well, especially the zoospores. This was verified in a leaf disc assay, using grapevine leaves (see Fig. 6.4). The results showed a clear effect of *G. glabra* extract against Downy Mildew infection. Therefore, the further goals were elucidating the molecular reasons for this activity against Downy Mildew and finding potential active substances within the extract. Furthermore, it was aimed to investigate the molecular effects of *G. glabra* especially on *P. viticola* zoospores. Within the *G. glabra* extract, its main compound glycyrrhizin was revealed to be the active compound. For the purposes, a microscopical approach was applied, counting burst incidence under the microscope after treatment with the extract or glycyrrhizin. Additionally, it was tested if extract and glycyrrhizin have an influence on *Vitis* plant cells.

First, the effect of *G. glabra* extract and glycyrrhizin on Downy Mildew infection on grapevine was verified, using *Vitis vinifera* Müller-Thurgau leaf discs, inoculated with a zoospore suspension and treated with the liquorice solutions. The results were obtained during a practical course in April 2014 by Thomas Hirth, Jana Fesseler and Corinna Schmider. The results showed, that infections occurred to 0% on the discs treated with different concentrations of the *G. glabra* extract or the compound glycyrrhizin, compared to a water treated control (see Fig. 6.4). The control showed high infection rate with a dense mycelium and numerous sporangia. This first result was comparable to the results of other Downy Mildews of vegetables in Schmitt et al., 2011. In the following, the molecular mechanism behind this inhibition is analysed.

The influence of the liquorice treatments on zoospores was investigated in detail by observation of zoospores on the microscope after treatment with the indicated concentrations of the *G. glabra* extract or glycyrrhizin.

Both, the biological raw extract and the compound glycyrrhizin showed a strong effect on zoospores in concentrations of 0.5 mg ml^{-1} or higher. The impact was quantified as burst incidence. Interestingly, liquorice resulted in encystation processes as well. The fact that both treatments resulted in the same amount of burst and encystation rates, indicates that glycyrrhizin is the active compound against zoospores. The short time, within less than

10 minutes, further shows, that the liquorice treatments lead rapidly to cell burst or encystations. Presumably, this is an indication that glycyrrhizin inhibits zoospores specifically by surface interactions, which change the stability of the zoospore cells. Finally, this leads either to encystation, the more stable constitution of zoospores, or to a deconnection of the plasma membrane from the cytoskeleton and thus to a disabled contractile vacuole.

Slight differences in the effect of extract and glycyrrhizin could be explained with the fact, that the extract might contain several other active compounds which cause zoospore burst. Several candidate ingredients are described already, having anti-Oomycete properties, like licoflavanon, pinocembrin and glabranin (Gupta et al., 2008; Scherf, 2011). As well, it might be that the concentration of glycyrrhizin in the extract and the raw solution differed in our experiment.

Glycyrrhizin is an amphiphilic triterpene glycoside. This amphiphilic character could lead to entry of glycyrrhizin molecules into cell membranes and change membrane the properties. Indeed, it was shown in a biochemical study, that glycyrrhizin causes decreased membrane fluidity and proposed that molecules can be incorporated in the lipid bilayer of cell membranes (Harada, 2005). Moreover, it was shown that glycyrrhizin alters cell membrane integrity and changes the levels of receptor signalling (Schröfelbauer et al., 2009). These findings suggest, that the cell membrane of *P. viticola* zoospores possibly also incorporate glycyrrhizin molecules, leading to altered membrane fluidity and signalling, resulting in cell burst or zoospore encystment. Zoospore burst would reduce the infection material. Also, the premature encystment possibly reduces infections, due to unripe cysts randomly spread over the leaf surface in distance to stomata. It possibly interferes with stomatal targeting, preventing that zoospores can smell stomata and swim to them rapidly or preventing germination and invasion into the stomata. The time span is an important factor for infection success of Downy Mildew or defence success of the plant. The faster zoospores enter stomata and start infection process, the less time for the plant to defend (Kiefer et al., 2002). This means, the earlier Downy Mildew can be attacked, the better an epidemic spread could be prevented. Glycyrrhizin is incorporated in membranes, resulting in rapid cell burst and premature encystments of zoospores, and therefore might be an appropriate substance against Downy Mildew. Additionally, its molecular properties reveal an interesting fact of this compound. Glycyrrhizin forms a gel at high concentrations in an aqueous solution, which is described already (Koga et al., 2004). In water, secondary forces act in glycyrrhizin molecules, such as Van deer Waals bonds between hydrophobic parts and hydrogen bonds between hydrophilic parts, causing assembly of glycyrrhizin monomers into a non-covalently bonded network. As the network grows, it exhibits gel-like physical properties, building an “organogel” via “self-assembly”, presumably comparable to the lectin organogel (Kumar & Katare, 2005), consisting of tubes which have an outer surface of hydrophilic negative charge and might interact with positively

charged proteins in cells like enzymes, proteins, DNA and RNA, and a hydrophobic inside. The fibers may include hydrophobic compounds in a dry inner area. It would be interesting to test if glycyrrhizin gel has different influence on zoospores than the liquid solution containing monomers and additionally if these compounds affect other *P. viticola* organs as well. This information could help finding an optimized concentration and formulation for field applications. Especially it is informative to know, that higher concentrations of glycyrrhizin can be gained by addition of clove oil, which prevents glycyrrhizin gel formation in aqueous solution (Koga et al., 2004), resulting in an emulsified glycyrrhizin solution. As well, a phosphate buffer liquidizes glycyrrhizin and prevents gel formation because glycyrrhizin charge is lower, preventing hydrogen bonds. The buffer has the advantage that the osmotic pressure and pH can be adjusted for the application (Koga et al., 2004). In combination, the resulting glycyrrhizin emulsion consists of vesicles filled with buffer, surrounded by glycyrrhizin containing lipid membranes.

On the other hand, glycyrrhizin as gel formulation might be more stable on grapevine plants in the field during rain. Additionally, the sticky stomatal guard cells would be coated by the anti-Oomycete polymer. Zoospores would presumably not find stomata anymore and attach randomly somewhere.

To test weather glycyrrhizin activates a ROS dependent signalling pathway, a blocker of ROS producing NADPH oxidase, diphenylene iodonium (DPI), was used (data not shown). After addition of DPI, a synergistic effect on zoospores has been observed, resulting in elevated numbers of burst zoospores, indicating that the response might be independent from ROS signaling pathways. The NADPH oxidase seems not to be a target of glycyrrhizin.

To assess the possible influence of liquorice also on plant cells, the activation of plant immunity was tested in cultured grapevine cells. For this, the change of the pH in the medium over time after inoculation with the liquorice compounds *G. glabra* root extract and glycyrrhizin was measured, comparable to cell culture assays with elicitors (Felix et al., 1993). The grapevine cells used for this study were *Vitis* Pinot Noir cultured cells.

Both, the *G. glabra* extract and the glycyrrhizin resulted in remarkably extracellular alkalinisation of the grapevine cultured cells. This shows first of all, that glycyrrhizin is the active compound within the root extract. The comparison of the results should be regarded qualitatively. One reason is the fact, the composition of biological material usually fluctuates. The calculation of the used glycyrrhizin concentration was based on data of the literature of the glycyrrhizin contents in *Glycyrrhiza* roots. It might be that this concentration was different from the glycyrrhizin content in the root extract used in this study. Therefore, it might be that

the concentration of glycyrrhizin in the pure solution and the extract differed, leading to different output in the experiments.

Besides the described effect of activation of plant cell immunity, glycyrrhizin has another beneficial effect on plants. It was found that treated plants appear greener (Scherf, 2011), indicating a stimulating effect on photosynthesis.

The exact mode of action of glycyrrhizin remains widely unknown (Schröfelbauer et al., 2009). But in a molecular study it was found, that glycyrrhizin has a broad anti-inflammatory activity in human cells, which is mediated by the interaction with the lipid bilayer, thereby attenuating receptor-mediated signalling (Schröfelbauer et al., 2009). The identified signalling ways which are influenced by glycyrrhizin include the NF-kappaB (nuclear factor kappaB) pathway and the MAPK (mitogen-activated protein kinase) signalling cascade, where glycyrrhizin blocked inflammatory proteins and attenuated inflammatory responses. Furthermore, the group showed that glycyrrhizin treatment decreased cellular attachment. The processes triggered by glycyrrhizin in animal cells might be analogous to the processes in zoospores and grapevine cells. Interaction with the zoospore cell membrane and with receptor-mediated signalling are likely the reasons for zoospore burst, blocking the adhesion of cell membrane with extracellular matrix and intracellular cytosceleton. As well, these mentioned interactions of glycyrrhizin might lead to plant cell activated immunity due to its interference with signalling cascades. The possible signalling pathways which might be influenced, are MAPK and Ca^{2+} signalling. For plant cells it was shown that elicitors lead to activation of Ca^{2+} channels in the plasma membrane, leading to Ca^{2+}/H^{+} influx, further activating MAPK cascade, which is activating gene expression of phytoalexins for example resveratrol and thus leading to a defence reaction (Kurusu et al., 2005). For parsley cell culture it was shown, that addition of a fungal elicitor lead to rapid protein phosphorylation (Dietrich et al., 1990), which is a basic signalling component. For tomato cell culture it was shown, that pathogen defence response was activated by a Ca^{2+} influx, caused in response to an elicitor (White, 2000). Furthermore, White (2000) lists the localizations of plant calcium-permeable channels: in the plasma membrane, tonoplast, endoplasmic reticulum, chloroplast and nuclear membranes. Opening of Ca^{2+} channels and the resulting increased cytoplasmic Ca^{2+} concentration cause spatial and temporal variations which elicit specific physiological responses to diverse biotic and abiotic stimuli (White, 2000). These findings all together suggest, that glycyrrhizin specifically activates the plant immune system.

Another family of signalling molecules are the reactive oxygen species (ROS). ROSes are important signals in plant defence (Wojtaszek, 1997), built as early signals in plants after

contact of a plant with a pathogen or an elicitor. Both described effects of glycyrrhizin, bursting of zoospores and immune stimulation in plant cells, are possibly linked with the introduction of ROSes.

ROSes have been shown to cause bundling of actin (Chang & Nick, 2012). In zoospores, bundling of actin would lead to a rigid cytoskeleton, lacking its turnover, which would lead to the recognized cell burst. Presumably, the rigidity of actin cytoskeleton also blocks the proper action of the contractile vacuole, leading to burst due to constant water influx without the expulsion after contraction. A rigid actin cytoskeleton presumably does not contract. *G. glabra* root extract and glycyrrhizin are shown to be direct or indirect oxygen radical scavenging agents (Yokozawa et al. 2000) and thus might interfere in ROS signalling in zoospores and plants.

NADPH oxidases (respiratory burst oxidase homologues, RBOHs) appear as key proteins in pathogenesis-related ROS production (Trujillo et al., 2006). RBOHs are plasma membrane resident proteins that produce extracellular $O_2^{\cdot-}$, which is subsequently converted to H_2O_2 (Garrido et al., 2012). The influence on the NADPH oxidase dependent ROS signalling was tested using DPI, an inhibitor of the ROS producing NADPH oxidase. The results showed no difference between DPI treated and non-DPI treated samples, suggesting that the NADPH oxidase plays no role in zoospores in the function of the contractile vacuole, and no role in plant cell immunity activation.

It can be summarized, that biological extracts from *Glycyrrhiza* roots or pure glycyrrhizin are useful candidates for control of Downy Mildew of grapevine during the zoospore state. Due to the interaction with the plasma membrane, with structure proteins and signalling components, zoospore stability is changed. This is presumably caused by indirect destabilization of the contractile vacuole in zoospores, which are an unprotected motile life cycle stage of *P. viticola* on the leaf surface, resulting in burst or premature encystment of the zoospores, which finally results in infection free leaves.

Additionally, glycyrrhizin is specifically interacting with the cell walls of grapevine cells, resulting in activation of the immune system, which was seen in apoplastic alkalinisation of the cell culture medium after addition of these biological compounds. These findings show, that glycyrrhizin seems to be a promising candidate for alternative, ecologically safer control of Downy Mildew in viticulture.

4.8 Summary and future challenges in sustainable viticulture

The aim of this study was to reveal a base for new control strategies of Downy Mildew, one of the major diseases in viticulture, caused by the plant parasitic oomycete *Plasmopara viticola*. The parasite starts infection of susceptible grapevine plants by invading plant stomata, the plant organs for gas exchange and transpiration. In this study, the early infection behaviour of zoospores was observed on different grapevine hosts in addition to the morphological characteristic of stomatal density. The results revealed that a lower stomatal density is correlated to a lower susceptibility to Downy Mildew infection. Additionally, the results support the idea that zoospores are an ideal target for treatments with less toxic compounds because they are easily attainable, swimming unprotected at the host surface, and need to target the stomata.

This explains why both interacting organisms – the host plant grapevine and the Downy Mildew causing pathogen – should be considered when exploring new control strategies. Sustainability would be conferred by strategies which avoid the application of toxic compounds, influencing a broad range of organisms in the environment, and on the other hand avoid the possibility of creation of new resistant pathogen strains. Instead, the focus should be set on non-toxic biological compounds, which act directly on zoospores, the weak point of Downy Mildew. Biological extracts were found to enhance the plant immune system and therefore act double sided against plant diseases, for example the *G. glabra* root extract or the main active compound glycyrrhizin, presented in Schuster et al. (2010) and in this study. Intensified research in this direction would open promising new possibilities in viticulture.

The molecular strategy applied in modern viticulture is to breed resistant grapevine cultivars, based on several different resistance loci, which should maintain the resistance against Downy Mildew for a longer time. One possible target for resistance breeding could be found in the stomatal density, which was shown to correlate with lower susceptibility against Downy Mildew in Duan et al. (2015) and in this study. Breeding for lower stomatal density could be a promising factor for future pyramidization breeding strategies in addition with further resistance loci because stomatal density relies on the interplay of several genes and proteins. Therefore, this trait might contribute to a more stable breeding success.

On the pathogen side, the contractile vacuole of *P. viticola* zoospores was found to be a promising target for treatment applications. The contractile vacuole is a pivotal organelle in zoospores, which are very fragile and occur unprotected on the host plant surface. Only with vital vacuole the zoospores are able to swim vividly and target stomata. Is the vacuole impaired during the fragile zoospores state, zoospores rapidly burst or prematurely encyst. Both effects lead to interrupted infection. This effects have been achieved in this study by the biological

extract of roots of liquorice plant *G. glabra.* Also, its main compound glycyrrhizin showed to have the same effect.

Both substances were shown to possess a positive influence on the plant, stimulating the plant immunity. In the literature it was described that also photosynthesis of the plant is enhanced by *Glycyrrhiza glabra* extract (Scherf, 2011). In contrast, glycyrrhizin and the *G. glabra* extract have a negative influence on *P. viticola* zoospores, causing premature encystment and cell burst. Both effects result in prevention or reduction of Downy Mildew symptoms on grapevines, which was observed in a practical course in this study as well as in a study with different Downy Mildews on vegetables (Schmitt et al., 2011).

Zoospores maintain their cell integrity by several integrative structures and processes. In this study it was explained, that one key process for stability is the osmoregulation via the water expulsion vacuole. Besides its general need to expel the incoming water to avoid bursting, the zoospore cell is able to regulate this process. Zoospores could regulate their osmotic potential very tightly, which was quantified in an assay by using NaCl.

The vacuole was proposed to be in tight contact with the actin cytoskeleton. The actin cytoskeleton was seen to possess star-like interconnections. These filaments and plaques are necessary for zoospore stability and vitality, which was seen in an experiment using the actin drug latrunculin B. Therefore, it might be that the vacuole contractions are performed by actin filaments. The connections of intracellular structures with the cell membrane and extracellular matrix are conferred by integrin-like proteins, shown in an assay using the ECM interacting heptapeptides RGD. A model of the contractile vacuole of a *P. viticola* zoospore with its structural components which confer cell stability is shown in Fig. 4.1, completed by RGD and glycyrrhizin, which impair cell stability by interactions with the cell surface, resulting in zoospore burst.

Zoospore cell integrity is accompanied by the dynamic change of the structure of ER cisternae as well as a highly dynamic endo- and exocytosis processes during the zoospore state. The cytosol dynamics enable the proper function of the contractile vacuole, which increases in size during collection of incoming water and contraction to expelled water in regular cycles. The cell volume remains constant.

The zoospores can be impaired in several different ways. Impairing the vacuole activity has a big advantage regarding acquiring of new resistance of Downy Mildew disease. Due to their severe toxicity, metal ions like copper and aluminium have to be exchanged. A, alternative was found with the biological compound glycyrrhizin, derived from the root of *Glycyrrhiza glabra*. They interact with the cell surface of *P. viticola* zoospores, leading to rapid cell burst and premature encystments. In plants, they activate the immune system, shown in this study

using an assay measuring extracellular alkalinisation. This activation is presumably conferred due to cell surface interaction (Schröfelbauer et al., 2009), influencing Ca^{2+} channels and signalling cascades, resulting in the expression of genes related to plant defence. In this study it was tested whether the ROS signalling pathway is involved in the glycyrrhizin effects. Therefore, the inhibitor of the NADPH oxidase, DPI, was used. Grapevine cells were shown to activate their immune system independently from ROS signalling. Zoospores were shown to burst after treatment with DPI and glycyrrhizin in a synergistic way. This result might indicate, that ROS signalling is important for maintaining zoospore stability.

In summary, both, the root extract and glycyrrhizin, might be promising candidates for future treatments against Downy Mildew. Research has to be done regarding the best formulation, which has an appropriate concentration active against Downy Mildew, but also the right consistency for application with fungicide machines and an appropriate stability in rain. Possibly, synergistic effects can be achieved by mixtures with other compounds.

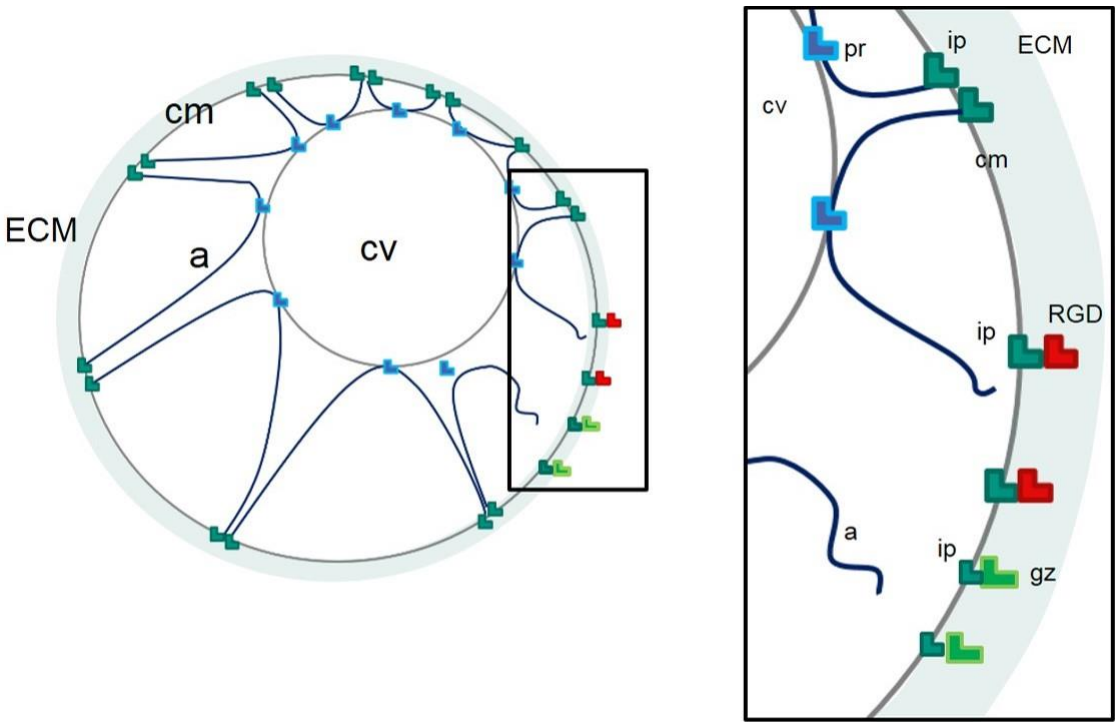


Fig. 4.1 Model of the contractile vacuole of a *P. viticola* zoospore and the structural components conferring stabiliy. RGD heptapeptide and glycyrrhizin (gz) lead to zoospore burst by interactions with the zoospore cell surface proteins. (cm) cell membrane, (cv) contractile vacuole, (a) actin filament, (ECM) extracellular matrix, (pr) plasmalemmal reticulum, (ip) integrin-like protein.

5 List of abbreviations

B11 *Plasmopara viticola* single sporangial strain 1191_B11
B15 *Plasmopara viticola* single sporangial strain 1191_B15

DIC *Differential Interference Contrast*

ECM extracellular matrix

K103 *Vitis sylvestris* Ketsch K103
K51 *Vitis sylvestris* Ketsch K51

Mth *Vitis vinifera* L.cv. Müller Thurgau

P. viticola Plasmopara viticola

QoI Strobilurin

RGD Heptapeptid motif YGRGDSP

V. vinifera Vitis vinifera

6 Appendix

Table 6.1 Mean stomatal densities (sd), calculated as number of stomata per epidermal cells, providing a gentotype dependent ratio for each accession of the European wild grapevin *V. sylvestris* and some selected other grapevine cultivars. Table elements containing high stomatal densities (> 0.11) are highlighted in light grey. Table elements containing low stomatal densities (< 0.8) are highlighted in dark grey.

Vitis	mean sd	*Vitis*	mean sd	*Vitis*	mean sd
K103	0,05	**K2DLR**	0,08	**3**	0,11
K114	0,06	**K17(2)**	0,08	**13**	0,11
K71	0,06	**K2**	0,08	***V. riparia***	0,11
K81	0,06	**K16**	0,09	**K77**	0,11
K92	0,06	**K110**	0,09	**K32**	0,11
K100	0,06	**K83**	0,09	**10**	0,11
K75	0,06	**Riesling**	0,09	**K30**	0,11
K47	0,06	**K35**	0,09	**Hö29**	0,11
K116	0,07	**K48**	0,09	**Hö17**	0,11
K07	0,07	**K106**	0,09	**Augster Weiss**	0,11
K28b	0,07	**K27**	0,09	**H9**	0,12
K88	0,07	**K57**	0,09	**K89bc**	0,12
K54	0,07	**K26**	0,09	**Hö29**	0,12
K66	0,07	**K64**	0,09	**K44a**	0,12
K58	0,07	**K108**	0,09	**K63**	0,12
K34	0,07	**K44**	0,09	**2**	0,12
4	0,07	**K65**	0,09	**K61**	0,12
7	0,07	**K67**	0,09	**K115**	0,12
Regent	0,07	**K17(1)**	0,09	**K56**	0,12
K90	0,07	**K15**	0,09	**K74**	0,13
K118	0,07	**K24**	0,09	**K18**	0,13
k38	0,07	**K22**	0,09	**K68**	0,13
K10	0,07	**14**	0,09	**K51**	0,13
K91	0,08	**rosa**	0,09	**K61**	0,14
9	0,08	**K112**	0,09	**Börner**	0,14
K28a	0,08	**M. Thurgau**	0,09	**Solaris**	0,15
K62	0,08	**K55**	0,10		
K36	0,08	**8**	0,10		
K119	0,08	**K13**	0,10		
K109	0,08	**12**	0,10		
K42	0,08	**blau**	0,10		
1	0,08	**Ö11**	0,10		
K33	0,08	***V. amurensis***	0,10		
Klaus	0,08	***V. rupestris***	0,10		
K59	0,08	**K60**	0,10		
11	0,08	**K53**	0,10		
K86	0,08	**Ö7**	0,10		
K96	0,08	**CO5**	0,10		
K84	0,08	**Ö10**	0,10		

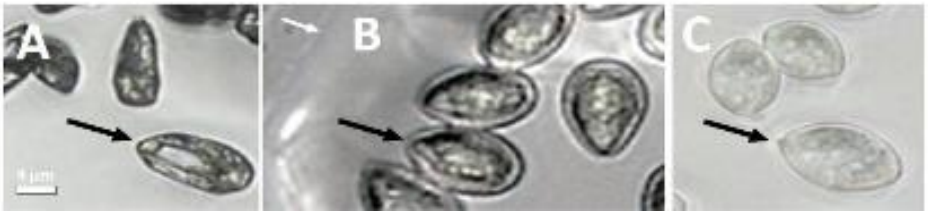
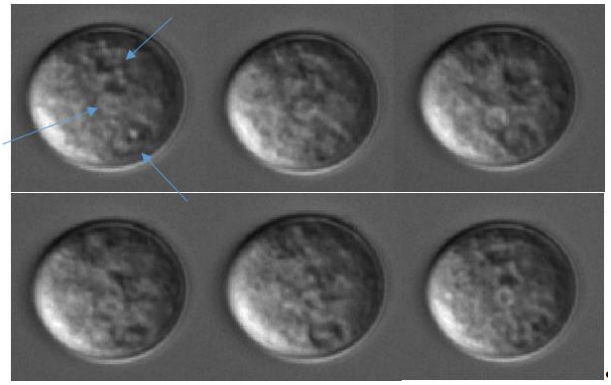


Fig. 6.1 Dry (A) and partially (B) versus fully swollen sporangia (C). Dry and partially swollen sporangia appear dark in bright field microscopy. Partially swollen sporangia seem to have a space between sporangial cell wall and syncytial cytoplasm. Fully swollen sporangia appear translucent and cytoplasm has contact to the sporangial cell wall.



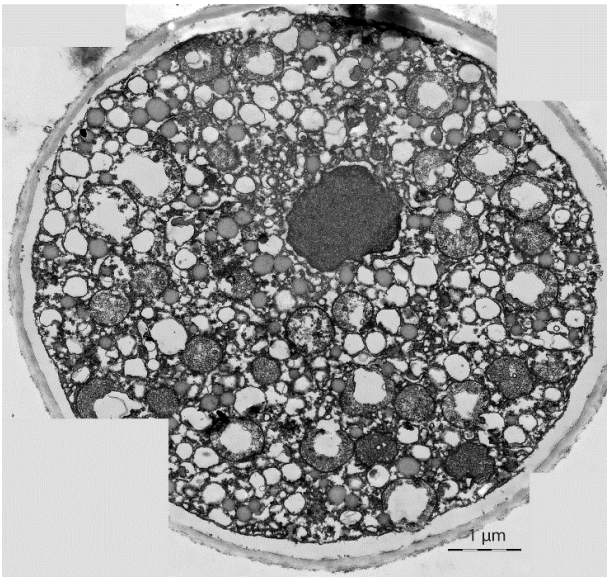


Fig. 6.2 Upper picture shows a sequence of an encysted zoospore during vacuolar break-down, seen with DIC channel of an Apotome microscope. Lower picture shows a slice through a zoospore after encystment, surrounded by a cell wall and containing one nucleus in the cell center. No bladder is seen. Instead, serveral vacuoles are visible. Picture was taken with transmission electron microscope.

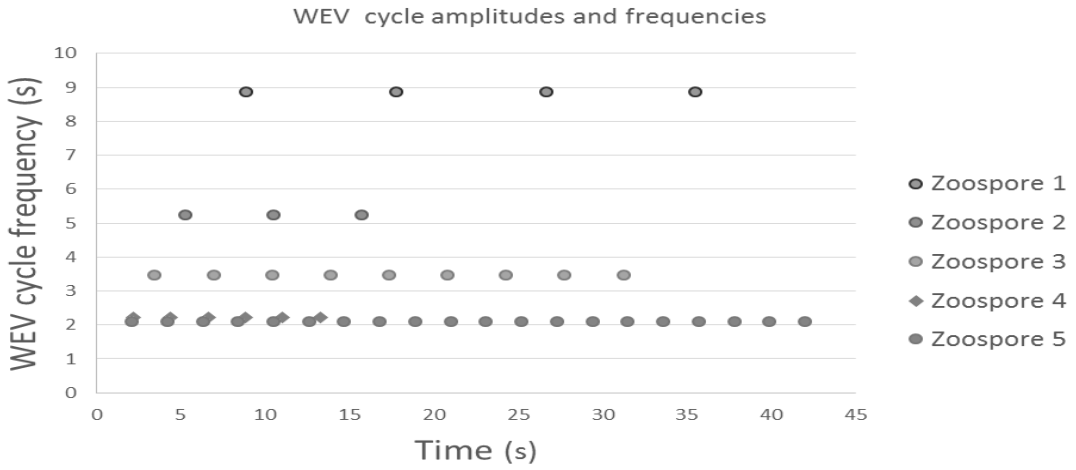


Fig. 6.3 Results of counting number of cycles and their cycling frequency of the contractile vacuoles of five individual zoopores, counting was finished with burst of the zoospore.

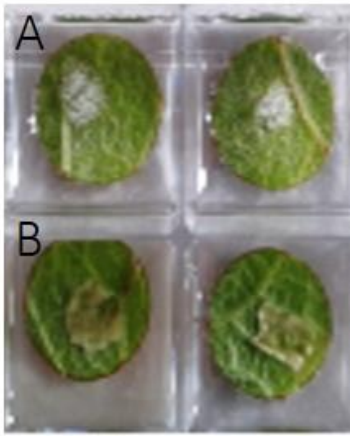


Fig. 6.4 *Vitis vinifera* leaf disc infection assay. **A** Sporulated leaf discs, 7 days after infection with an aqueous solution of *P. viticola*. **B** Leaf discs without sporulation, 7 days after infection with *P. viticola* solution containing *Glycyrrhizae* radix extract, visible as brownish gel.

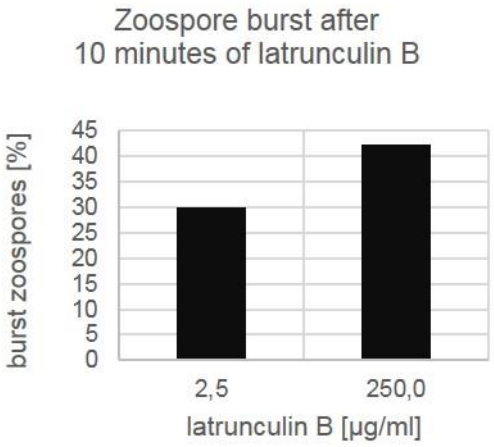


Fig. 6.5 Influence of latrunculin B on vital zoospores of *P. viticola*. Colums represent the burst incidence of 150-250 evaluated zoospores compared to the water treated control. Data were collected by Thomas Hirth during a practical course in July 2014.

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9 Publications

Research Paper

- Genetic diversity of stilbene metabolism in *Vitis sylvestris*

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Poster Presentations

- Tröster, V., 2014. Early Cellular Events during Grapevine Downy Mildew Infection. 7th Grapevine Downy and Powdery Mildew Workshop. Vitoria-Gasteiz, Spain, June/July 2014.

- Tröster, V., 2014. Early Cellular Events during Grapevine Downy Mildew Infection. 10th Rhein-Wein Symposium. Karlsruhe Institute for Technology. Karlsruhe, Germany, May 2015.

- Tröster, V., Nick, P. 2015. Achilles‘ heel of a Grapevine Pathogen - Early Cellular Events during Downy Mildew Infection. Congress of DPG Workgroup Mycology and Host Parasite Relationships. German Phytomedicin Society. Martin-Luther-Universität Halle-Wittenberg. Halle, Germany, March 2015.

Supervision

- Tabea Setzer, B.Sc. (2014), The water expulsion vacuole - weak point of *Plasmopara viticola* Microscopic study and analysis of the effect of toxic substances on *Plasmopara viticola*