Licorice, cucumber, downy mildew: tracing the secret

Interactions between the plant extract, the host and the pathogen

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Für Alex und mein Urmel 2.0

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Ich erkläre hiermit ehrenwörtlich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Sämtliche aus fremden Quellen direkt oder indirekt übernommene Gedanken sind als solche kenntlich gemacht. Die Arbeit wurde bisher keiner anderen Prüfungsbehörde vorgelegt und noch nicht veröffentlicht.

Darmstadt, den 06.06.2012

ARBEITEN DIE VON ANDEREN PERSONEN UNTERSTÜTZT BEZIEHUNGSWEISE DURCHGEFÜHRT WURDEN

3. CONTROL OF DOWNY MILDEW (*PSEUDOPERONOSPORA CUBENSIS*) OF GREENHOUSE GROWN CUCUMBERS WITH ALTERNATIVE BIOLOGICAL AGENTS

Dipl. Biol. Christina Schuster plante und führte alle Arbeiten in Zusammenhang mit dem Bakterium *Aneurinibacillus migulanus* durch.

Die Pflanzung der Gurken, die Applikation der Präparate sowie die Tätigkeiten zur Feldhygiene in den Semikommerziellen Versuchen im Plastikgewächshaus wurden von Mitarbeitern der LVG, Heidelberg durchgeführt.

Dr. Annegret Schmitt und Dipl. Biol. Christina Schuster sowie die Technischen Assistentinnen (TA) Karin Bald und Mona von Eitzen-Ritter unterstützten die Bonituren in den Semikommerziellen Versuchen im Plastikgewächshaus.

Dr. Peggy Marx plante, betreute und führte die Semikommerziellen Versuche im Glasgewächshaus durch.

Die TA Karin Bald unterstützte die Durchführung der Bioassays an getopften Pflanzen.

4. INVESTIGATIONS ON THE EFFICACY OF LEAF EXTRACT FRACTIONS OF *GLYCYRRHIZA GLABRA* AGAINST DOWNY MILDEW OF CUCUMBER

Dr. Jonas Treutwein (Trifolio-M GmbH) führte in Zusammenarbeit mit der Universität Giessen die HPLC und1H-NMR-Spectroskopie durch.

Die TA Karin Bald unterstützte die Durchführung der Bioassays an getopften Pflanzen.

5. THE EFFECTS OF AN EXTRACT OF LICORICE LEAVES (*GLYCYRRHIZA GLABRA*) ON CUCUMBER PHOTOSYNTHETIC SYSTEM

Die TA Diana Nagel unterstützte die Durchführung der Bioassays an getopften Pflanzen und die Pigmentextraktion.

All following publications are referred to as chapters in the context of this thesis.

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1. GENERAL INTRODUCTION

1.1. How humans became plant protectors

Around 13,000 to 10,000 years ago, in few places around the world, our ancestors changed their lifestyle from hunter-gatherers to the lifestyle of farmers (Salamini et al. 2002, Doebley et al. 2006, Purugganan and Fuller 2009). The consequences for their cultural development were drastic and had dramatic effects on animals and plants (Mannion 1999) they domesticated.

The domestication led to some morphological and physiological changes in animals and in plants. Because bigger seeds were harvested and stored much easier, plants with bigger seeds (Cook and Evans 1983, Salamini et al. 2002, Purugganan and Fuller 2009) were preferred. But those plants often produced lesser numbers of seeds (Doebley et al. 2006). Also, an inhibition of natural spreading of seeds increased harvest (Salamini et al. 2002, Purugganan and Fuller 2009) and plants with such dysfunctions were selected. Plants with apical dominance were preferred over those without because of their more robust growth (Doebley et al. 2006). Domestication affected also the physiology of plants. For example individuals with a smaller amount of bitter substances, like some alkaloids, were favored (Hondelmann 1984, Doebley et al. 2006).

All selection resulted in a smaller genetic pool in the cultivated specimen compared to the wildtype (Salamini et al. 2002, Zhang et al. 2002) sometimes affecting genes which were not directly selected for (Zhang et al. 2002). Decrease of genetic variability and denser population of domesticated plants sometimes disturbed the equilibrium of pathogens and their host plants (Keane 1997) and made plants more vulnerable to diseases (Reif et al. 2005). In the end, all these changes left domesticated plants depending on the farmer's protection (Doebley et al. 2006). As a consequence, humans had to become protectors of plants and their harvest (Jaskolla 2006).

Because of the lack of writing, first notes and reports of references about cultivation strategies, crop protection and protection of stored harvest date back to around 2000 B.C. (Mayer 1959). The first notations about pest management were found in India and the Mesopotamian area around that time (Mayer 1959). In ancient Egypt stored harvest was protected from invading insects by saline soil on the granary floor (Levinson and Levinson 1998). The insects usually dried out after getting into contact with the dust (Mayer 1959). One of the first reports of using antagonists was made in 304 A.C. when Chinese farmers used ants (*Oecophylla smaragdina*) for protecting their *Citrus* plantations against insect pests (Huang and Yang 1987; van Mele 2008). Despite of a

lack of written proof the assumption can be made, that human beings started to protect their plants from herbivores, right after the beginning of domestication.

The history of using plants as medicine is even longer than their domestication (Ciocan and Bara 2007). Because of the occurrence of medicinal plant use in chimpanzees (Messner and Wrangham 1996) and other animals (Huffman 2003) it was assumed that the medical use of plants in human like beings is even older than the genus Homo (Mahdi et al. 2006). Fossil records of humans utilizing plants as medicine date back to 60,000 years ago (Fabricant and Farnswoth 2001). Particularly in Mesopotamia around 6,000 years ago plants were exploited as drugs. An example for a plant used since this times as medicine is the willow (*Salix* sp.). Bark and leaves were applied against fever and pains (Mahdi et al. 2006). The active compounds of this plant are similar to acetyl-salicylic acid which is still the active compound in Aspirin.

In Europe Hildegard von Bingen (1098-1179) was famous for her herbal medicine. She wrote the scholarly pieces *Liber simplicis medicinae* or *Physica* (1151–1158) and *Liber compositae medicinae* or *Causae et curae.* In these books she described the development and treatment of human diseases. Even the use of licorice roots was described by her in the book *Physica*. Hildegard recommended liquiricium (licorice, Figure 1) as a treatment against hoarseness, coughing, ache of the chest and problems in the digestive and urinary system. Additionally, a soothing effect of licorice was described by her. This might be the first description of licorice as a medical plant in Germany. The cultivation of licorice in fields started in the 18th century in Germany (www.kraeuter-apotheke.net/suessholz.htm) and the most important cultivation area was around Bamberg, Bavaria. Around a hundred years earlier three men, H. and Z. Jansen as well as H. Lippershey discovered that items could be magnified by elongating the tubes of a reversed telescope. Thus they constructed the first compound microscope (Schultheiss and Denil 2002). This invention was necessary for the discovery of microorganisms. Soon scientists all over Europe connected microorganisms with diseases of both, humans and plants (Paulsen 1934). The Frenchman Tillet (1714-1791) for example discovered the fungus *Tilletia caries* as the pathogen causing bunt in wheat (Hammer 2009). One of the first investigations about the control of plant disease with chemicals was done by Glauber (1604-1670), who used sodium sulfate and alcohol as caustic agent for cropseeds (Benzing et al. 1987, Jaskolla 2006). However, not until 1794, when J.J. Planck wrote *Physiologia et Pathologia Plantarum,* a scientific system of plant pathology evolved (Mayer 1959). At that time the combat of microorganisms pathogenic to plants used to be carried out mostly by treatment of seeds with inorganic substances like arsenic and mercury. Plant extracts were used in plant protection at this time only as a defense against arthropods

(Mayer 1959). Initiated by the *Phytophthora infestans* (potato blight) epidemic in potatoes in Western Europe 1845, which was most destructive in Ireland. Three million fallen victim to the famine (Gregory 1983) only in this country or emigrated to the USA. Millet tried to fight the plant pest with a mixture of copper (II) sulfate and hydrated lime called Bordeaux mixture (Ayres 2004). In the midst of the 19th century plant protection became a science itself. One of its pioneers was the German professor Julius Kühn (Hallmann et al. 2010), who wrote *Die Krankheiten der Kulturpflanzen, ihre Ursachen und ihre Verhütung* in 1859 (Mayer 1959). Even though the Russian Metchnikow already worked on the subject of biological control of microorganisms (Mayer 1959) in 1878, the control agents most commonly used were inorganic or organic chemicals. First investigations in selective breeding of disease resistant plants were made at the beginning of the 20th century (Mayer 1959). Defense mechanisms of plants against several stress situations tended to become the focal point of interest in the 1930s (Mayer 1959). At this point the possibility of acquired resistance in plants was discussed already (Mayer 1959). During the second half of the last century the accumulation of chemical control agents in the ground and several animals increasingly became a problem. One of the most prominent cases was the widely spread insecticide DDT (dichloro-diphenyl-trichloro-ethane) which causes eggshell thinning in several birds (van der Trenck et al. 2007). In the 1960s species like the peregrine falcon were nearly extinct in Europe and the USA because all eggs were broken (van der Trenck et al. 2007). An accumulation of DDT was detected in the fat tissue of all animals at the end of the food chain, including humans (Hunter et al. 1963). These observations increased the awareness of people concerning potential risks of chemicals and alternatives to chemical plant products.

In Germany at that time scientific investigations on biological food production began (Vogt 2001). In 2007 in Germany, five percent of the agricultural area used for farming was cultivated under ecological guidelines (www.umweltbundesamt-daten-zur-umwelt.de/). In recent times, a lot of investigations were done on the use of extracts of medicinal plants as biological control agents (Bobbarala et al. 2009; Baka 2010). Ironically, one of the main classes of secondary metabolites of medicinal plants with a high potential for controlling plant diseases are bitter substances, such as alkaloids and glycosides (Ciocan and Bara 2007), which were mostly bred out in cultivated plants. Another group of plants interesting for the field of biological control are those with a high amount of different flavonoids (Ciocan and Bara 2007). Accordingly the focus in the presented work was on one of those medicinal plants – *Glycyrrhiza glabra,* the plant described by Hildegard von Bingen as liquiricium.

Figure 1. Licorice (*Glycyrrhiza glabra*)

Glycyrrhiza glabra (licorice, Figure 1) is a perennial plant (Family Fabaceae, order Fabales), which originates from the Mediterranean area and West Asia (Wiest 1949; Anonymous 2005). This shrub with sticky leaves is growing in ruderal habitats.
Since ancient times licorice roots were used for medical applications (Stepanova and Sampies 1997) for example in ancient Egypt. In central Europe licorice is known since medieval times. First it was only used as a drug against problems of the digestive (Krausse et al. 2004) and urinary system as well as against coughing and hoarsenes. Later on, root extract, blended with sugar, became a popular candy. However, it also is known that excessive ingestion of licorice induces hypertension (Walker and Edwards 1994). Even until now the use of roots has not changed. Furthermore, in several *in vitro* studies an antimicrobial and antifungal effect of licorice leaf extract could be shown (Stepanova and Sampies 1997). Results of preliminary tests at the Julius Kühn – Institute (JKI), Darmstadt, Germany as well as at NAGREF, Patras, Greece showed a high potential of an ethanolic licorice leaf extract against oomycetes (Konstantinidou-Doltsinis and Markellou 2008, Schuster et al. 2010).

Besides products of plants, some antagonistic microorganisms provide a high potential for the biological control of plant diseases. In one part of this work such a bacterium, *Aneurinibacillus migulanus*, was applied and its efficacy compared to that of the plant extracts.
A. migulanus is a gram-positive bacterium of the genus *Bacillus*. Endospores are built in stress situations like heat, drought and presence of chemicals or ionizing radiation. Like other bacilli*, A. migulanus* produces an antifungal metabolite – Gramicidin S. The cyclic

decapeptide is located on the surface of the endospores and has the ability to destabilize cell membranes. It increases the membrane permeability for ions and small metabolites in a concentration-dependent manner (Salgado et al. 2001) thus disturbing the equilibrium of the ion status of affected cells. For that reason the Gramicidin S producer *A. migulanus* is interesting as an antagonist in biological control. Edwards and Seddon (2001) showed that Gramicidin S inhibits for example the germination of the conidiospores of *Botrytis cinerea* as well as the growth of the mycelium.

1.2. Examples of plant extracts in ecological agriculture

Despite the fact that in history mainly inorganic substances were used as pesticides, there are some examples of plant powders and extracts which have a long story of use. One of the most prominent is pyrethrum, an insecticide made from flowers of the genus *Tanacetum*, which was already mentioned in early Chinese history (Glynne-Jones 2001). Nowadays the bio-pesticide is mostly used against houseflies, mosquitoes, cockroaches, fleas and several other insects which are aggravating to humans. But it is also used in agriculture as a bio-insecticide. Examples of successfully used products in organic agriculture based on pyrethrum and its active ingredients pyrethrinin I and II are Spruzit from Neudorff GmbH KG or Bayer Schädlingsfrei.

Another example for a plant with a long history of traditional utilization as a pesticide is the neem tree of India. It was and still is used during the last 2000 years as a medicine but also as a pesticide. In several studies compounds of all parts of the tree were investigated. There are various ingredients to be found with a broad spectrum of activity, like antiinflammatory (e.g. sodium nimbidate, polysaccharides), antiarthritic (nimbidin), antibacterial (e.g. nimbidin, mahmoodin), and antifungal (e.g. gedunin, nimbidin) (Biswas et al. 2002) but also with insecticidal properties (www.bvl.bund.de/infopsm). The latter three activities caused a strong interest of organic farmers in the registered neem products (Bayer Garten Bio-Schädlingsfrei, NeemAzal-T/S) and their main ingredients azadirachtin a and b.

Also a prominent example for a plant with medical as well as bio-control use is sage, which is a part of this investigation.

Figure 2. Sage (*Salvia officinalis*)

S. officinalis (sage, Figure 2) is a shrub, of the family *Lamiaceae*, orders Lamiales. The indeciduous plant with elongated, hairy leaves is a plant of the Mediterranean. In central Europe, sage was known and used as a medical plant since the Middle Ages. It was also employed as a preservative agent for food. Until today the plant is utilized for its antioxidative (Lu and Foo 2001, Tepe et al. 2007), antimicrobial and adstringent properties (Baricevic et al. 2001) in medicine and in addition as a spice.

In ecological agriculture sage is currently available as a plant “strengthener” and is used to enhance the resistance of treated plants against biotic stresses, mainly arthropods (www.bvl.bund.de/pstmzst). In Germany plant strengtheners are a group of plant protection agents, which are assigned to strengthen the plant´s self defense, i.e. to induce resistance in plants. These products are not registered like plant protection agents with direct insecticidal or fungicidal effects but undergo a so called listing procedure.

However, preliminary tests at the Julius Kühn – Institut in Darmstadt showed that the fungicidal effect of sage extract, which is used in human medicine has also a potential for controlling Oomycetes like *Phytophthora infestans* and *Pseudoperonospora cubensis* (Fautz 2006).

Another plant strengthener based on a mixture of plant extracts (*Calendula officinalis*, *Prunus padus* and *Cannabis sativa*) which were used as a standard in this thesis is Elot-Vis® (Prophyta GmbH). This plant strengthener is already commercially used as a control agent against downy mildews (Kofoet and Fischer 2007).

1.3. Possible influence of plant extracts on defense mechanisms and photosynthesis

In its natural environment a plant is often confronted with pathogens of which it is not the proper host. In the case of a successful infection by such an incompatible pathogen several defense mechanisms are started in the plant, which are summarized as the hypersensitive syndrome. A dramatic hallmark of this syndrome is rapid thickening of the cell walls (Kuć 2006) and rapid and localized programmed cell death (hypersensitive reaction, HR) (Ahn et al. 2007) at the infection site. These reactions constitute a more or less physical barrier to further spread of the pathogen. In addition to this passive defense, a signal transduction cascade in the infected plant is started (Ryals et al. 1996) and several metabolites involved in passive and wound responses and various enzymes are activated and up regulated. Table 1 summarizes the defense mechanisms in a plant.

Table 1 Putative defense compounds/systems for disease resistance in plants (Kuć 2006)

Passive and/ or wound responses
Waxes, cutin, phenolic glycosides, phenols, quinines, steroid glycoalkaloids, suberin, terpenoids and proteins
Increases after infection
Phytoalexins, reactive oxygen species/ free radicals, calcium, silicon/ silicates, polyphenoloxidases, peroxidases, phenolic cross-linked cell wall polymers, hydroxyproline and glycine-rich glycoproteins, thionins, antimicrobial proteins and peptides, chitinases, ß-1,3-glucanases, ribonucleases, proteases, callose, lignin, lipoxygenases and phospholipases

An important group of substances involved in induced resistance are so called pathogenesis related proteins (PR proteins). In this class many enzymatic proteins can be found like ß-1.3 glucanase (PR-2) and peroxidase (PR-9) but also PR-1 proteins with antifungal properties (Table 2).

Table 2. Recognized families of pathogenesis related proteins (Tuzun and Somanchi 2006)

Family	Type member	Properties
PR-1	Tobacco PR-1a	antifungal
PR-2	Tobacco PR-2	ß-1,3-glucanase
PR-3	Tobacco P,Q	chitinase type I, II
PR-4	Tobacco R	chitinase type I, II, III, IV, V, VI, VII
PR-5	Tobacco S	thaumatin-like
PR-6	Tomato inhibitor 1	proteinase-inhibitor
PR-7	Tomato P69	endoproteinase
PR-8	Cucumber chitinase	chitinase type III
PR-9	Tobacco `lignin-forming peroxidase´	peroxidase
PR-10	Parsley PR1	ribonuclease-like
PR-11	Tobacco `class V´chitinase	chitinase type I
PR-12	Radish Rs-APP3	defensin
PR-13	Arabidopsis THI2.1	thionin
PR-14	Barley LTP4	lipid-transfer protein
PR-15	Barley OxOa (germin)	oxalate oxidase
PR-16	Barley OxOLP	oxalate oxidase like
PR-17	Tobacco PRp27	unknown

The activated defense mechanisms established after a successfully defended infection protect the plant unspecifically against further infections. This phenomenon is called systemic acquired resistance (SAR).

Some biological control agents also have the ability to induce defense mechanisms and therefore may induce resistance against pathogens to which the plant is susceptible.

A very prominent example of a plant extract based biocontrol agent acting via induced resistance is the plant strengthener Milsana®. Its active ingredients (physcion its glycoside and other unknown compounds) can be found in extracts of *Fallopia sachalinensis (*formerly *Reynoutria sachalinensis)*, the giant knotweed (Schmitt et al. 2005). In several studies it was shown that in Milsana® treated plants, PR-proteins, like peroxidase and β-1.3- glucanase were up-regulated (Herger and Klingauf 1990, Schmitt 2006). This up-regulation occurred faster in induced and infected plants but also in uninfected plants after treatment (Schmitt 2006). This implies that Milsana® does not only have the ability of inducing resistance, but also of priming the plant against a forthcoming infection with a pathogen. Thus Milsana® treated plants are prepared for a coming infection and start its defense mechanisms faster.

Alongside this resistance inducing effect, the plant extract Milsana® also had an effect on the chlorophyll fluorescense of the treated plant. In cucumbers, treatment with

Milsana® caused an increase in total chlorophyll content (Herger and Klingauf 1990; Karaveav et al. 2002) and in chlorophyll fluorescence (Karaveav et al. 2008). Both observations gave evidence for a positive effect of Milsana® on the photosynthetic apparatus of treated plants.

The photosynthetic apparatus of higher plants consists of three redox complexes, photosystem II (PS II), which central chlorophyll can be excited by photons of a wavelength of 680 nm, cytochrome complex and photosystem I (PS I), which central chlorophyll can be excited by photons of a wavelength of 700 nm.

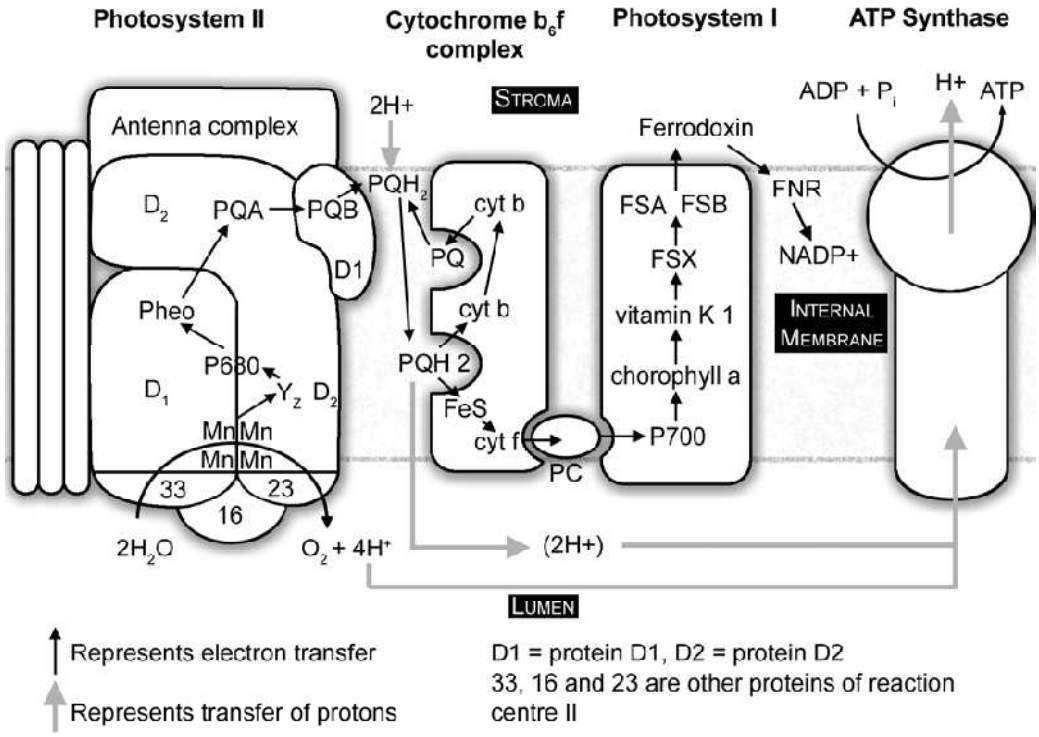

Figure 3. The linear electron transport system of photosynthesis is shown in this figure. Photosystem II, Plastocyanin dissociation and movement are simplified, because PSI is predominantly localized in the stroma lamellae. (Hankamer et al. 1997)

This system is connected via the electron transfer inside the complexes and between them (Figure 3). For a higher absorption rate of light, PS II as well as PS I are surrounded by a light harvesting complex (LHC, antenna), consisting of chlorophyll a, chlorophyll b and carotinoid molecules (Clayton 2002). Those pigments function as antenna molecules (Figure 3). The absorbing molecule (electron donator) leads the energy to pigments with lower energy (electron acceptor) and at the end to the central chlorophyll a (carotinoids → chlorophyll b → chlorophyll a) of the PS II and PS I.

In the case of excessive light or reduced state of the photo systems the energy can be transmitted as heat, dependent of the molecule zeaxanthine, or as fluorescence transmitted from chlorophyll a of the antenna complex (Demmig et al. 1988, Maxwell and Johnson 2000). Heat transmission occurs only in light, because the production of zeaxanthine stops in the dark. The maximal chlorophyll fluorescence can therefore be measured on dark adapted plants. Fluorescence data can be used as an indicator for the efficiency of LHC and by that for the photosynthetic apparatus (Krause and Weis 1991, Maxwell and Johnson 2000). In several ecophysiological works it could be shown that this system reacts to any kind of stress a plant has to deal with (Caspi et al. 1999, Pedros et al. 2008). In these studies two important fluorescence parameters were measured, first the minimum fluorescence (F0) and after a saturating flash of light Fm, the maximal fluorescence (Figure 4). The variable fluorescence (Fv) is the difference between Fm and F0 ((Fm-F0)=Fv). These measurable alterations are part of the fluorescence induction kinetics, first described by Kautsky and Frank (1949) and called Kautsky effect. The ratio between the variable fluorescence and the maximum fluorescence (Fv/Fm) is a good indicator for plant stress (Pedros et al. 2008). In healthy unstressed plants it is 0.83± 0.04 (dimensionless) and rapidly decreases in any kind of stress situation like drought or infection with a pathogen.

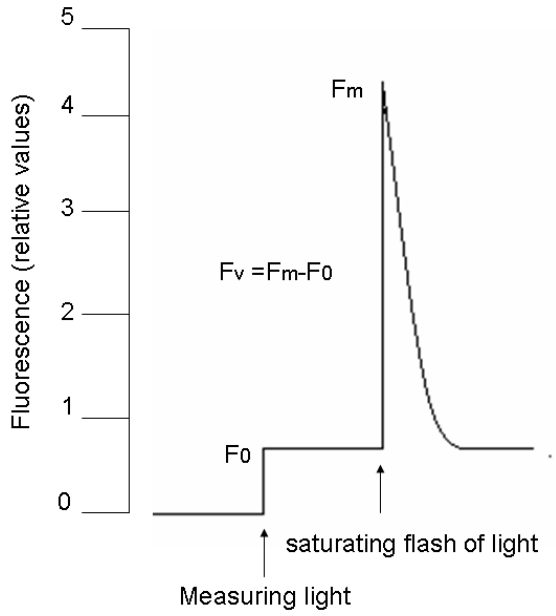

Figure 4: Fluorescence curve (De Ell et al. 1999). Shown are the minimum fluorescence (F0) and the maximum fluorescence (Fm) after a saturating flash of light.

1.4. Cucumber and downy mildew

Nowadays cucumbers are cultivated worldwide. In Germany in 2009 approximately 2,700 ha cucumbers were cultivated under open-field conditions and ca. 270 ha in greenhouses (Statistisches Bundesamt 2009). Of these cucumbers five to eight percent were cultivated following ecological guidelines (www.pan-germany.org).

Figure 5. Cucumber (*Cucumis sativus*)

Cucumber (Figure 5) is a plant of the gourd family (*Cucurbitaceae*, order Cucurbitales). It is a creeping wine which grows up supporting structures by ripping thin spiraling tendrils around. The leaves are large and lobed. The wild form of cucumber*, Cucumis sativus var. hardwickii,* originates from Asia (Renner at al. 2007).

Originally, the cucumber is monoecius with flowers of both genders on one plant. However, most cultivars are gynodioecius meaning that the majority of plants have only female flowers and only a few plants are bisexual. This gender distribution leads to an increase in harvest.

In organic production of cucumbers one of the major problems is the infection with *Pseudoperonospora cubensis,* causing downy mildew of cucumbers (Figure 6).

Figure 6. Symptoms of downy mildew of cucumbers (*Pseudoperonospora cubensis*)

P. cubensis is an obligate parasite of the class Oomycetes (Agrios 2005). The mycelium of oomycetes contains cellulose and glucans (Agrios 2005) instead of chitin, which is typical for the mycelium of true fungi. As resting spores oomycetes produce oospores, as asexual spores they produce zoosporangia or zoospores (Agrios 2005).

In infected plants sporangia of *P. cubensis* develop when relative humidity is high (close to 100%) and temperature is relatively low (maximum 21℃). They emerge on sporangiophores from the stomata, causing a grey mat on the lower side of the leaves. The flagellated zoospores are released from sporangia when a film of water is present. They germinate with a germ tube which penetrates mostly through stomata. The mycelium grows between the cells, sending houstoria into them. Under the right climate conditions the pathogen produces sporangia again (maximum 21℃ and approximately 100% relative humidity).

First symptoms of infection with downy mildew cause dark, translucent spots on the leaves, which become yellow and then necrotic while disease develops. The spots are confined by the leaf veins, which lead to the typical appearance of the disease. The fruits of infected plants remain unharmed. Up to now the most efficient control agents against downy mildew, as well as other oomycetes, in organic farming are copper preparations (Lindenthal 2005). Because of the negative effects on nature (e.g. accumulation in soil) in Germany in 2010 pathogen control with copper preparations in organic cucumber production was banned in greenhouses. Thus it is important to investigate alternative control agents against *P. cubensis* on cucumbers. Because of the known potential of sage extract as a biological control agent, preliminary trials were done at JKI in Darmstadt to investigate its potential of controlling *P. cubensis.* Furthermore, a bacterium, *Aneurinibacillus migulanus,* and ethanolic extract of licorice

leaves *(Glyzhyrriza glabra*) were tested at JKI for control of cucumber downy mildew. All three alternative control agents showed good efficacies in controlling *P. cubensis* in preliminary trials on potted plants and/or a small-scale greenhouse trial. On potted plants, efficacies of up to 100% could be achieved, while in the greenhouse efficacies close to 80% were found (Schmitt, personal communication)

1.5. Aim of this thesis

Based on the positive results achieved in the trials at JKI mentioned above, in this study the potential of the alternative agents, *A. migulanus,* sage extract and licorice leaf extract, in controlling the oomycete, *P. cubensis* (downy mildew) on cucumbers were further investigated. One focus was set on comparative studies of the efficiacy of the three control agents in bioassays and under semi-commercial conditions. Since investigations on *A. migulanus* are part of a different thesis, the results on the bacterium are not discussed here in detail.
Further foci were set on investigations on active ingredients and physiological effects of *G. glabra* (licorice) extract in treated cucumber plants and finally on a first evaluation of its mode of action.

1.6. Literature

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2. MATERIAL

2.1. Cucumber seeds

Cultivar “Chinesische Schlange”
Cultivar “Airbus”
Cultivar “Eminentia”

2.2. Pathogen

Pseudoperonospora cubensis	own conservation breeding

2.3. Biological control agents and plant strengthener

Licorice	NAGREF, Patras
Sage	Alfred Galke GmbH, Gittelde/Harz
Aneurinibacillus migulanus	own conservation breeding
ElotVis	Prophyta GmbH, Malchow/Poel

2.4. Chemicals

Ammonia	Carl Roth GmbH, Karlsruhe
Dichloromethane	Carl Roth GmbH, Karlsruhe
Dioxan	Carl Roth GmbH, Karlsruhe
DAB	Carl Roth GmbH, Karlsruhe
Acetic acid	Carl Roth GmbH, Karlsruhe
Ethanol	Carl Roth GmbH, Karlsruhe
Ethylacetate	Carl Roth GmbH, Karlsruhe
Methanol	Carl Roth GmbH, Karlsruhe
Sodiumhydroxide	Carl Roth GmbH, Karlsruhe
n Hexane	Carl Roth GmbH, Karlsruhe
Phosphoric acid	Carl Roth GmbH, Karlsruhe
Pentane	Carl Roth GmbH, Karlsruhe
Toluene	Carl Roth GmbH, Karlsruhe
Salt acid	Carl Roth GmbH, Karlsruhe
Vanillin	Carl Roth GmbH, Karlsruhe
Agar-Agar	Insula GmbH, Mannheim
Blankophore	Gift from Dr. E. Koch

2.5. Kits

NucleoSpin RNA Plant Kit	Macherey-Nagel GmbH & Co. KG, Düren
iScript cDNA Synthesis Kit	Bio-Rad Laboratories, Inc., München
SYBR Green Master Mix Kit	Fermentas GmbH, St. Leon-Rot
DNeasy Plant Mini Kit	Quiagen GmbH, Hilden

2.6. Incidentals

TLC – Plate silica gel 60 F256	Merck KGaA, Darmstadt
Capillaries	Carl Roth GmbH, Karlsruhe
Pipette tips	Eppendorf AG, Hamburg
Eppendorf tubes	Eppendorf AG, Hamburg
PCR Tubes	Bio-Rad Laboratories, Inc., München plant pots
MR 8x8x8	Hermann Meyer KG, Rellingen
K 15 pot ground	Klasmann-Deilmann GmbH, Geeste
Silica gel	Merck KGaA, Darmstadt

2.7. Equipment

TLC – Chamber	Desaga GmbH, Wiesloch
TLC – Sprayer	Desaga GmbH, Wiesloch
Air condition, Jet cool green	LG Electronics Deutschland GmbH, Willich
Lights, L30W/77	Osram AG, Münschen
Microscope, Axioscope 2	Carl Zeiss AG, Oberkochen
Microscope camera, Moticam 2300	Motic
PH-Meter, SG2	Mettler Toledo GmbH, Giessen
Photometer, BioPhotometer	Eppendorf AG, Hamburg
Pipettes	Eppendorf AG, Hamburg
Analytical balance, AE 163	Mettler Toledo GmbH, Giessen
Analytical balance, 1212 MP	Sartorius AG, Göttingen
Retschmühle	Retsch GmbH, Haan
Rotator evaporator, Rotavapor RII	Büchi Labortechnik GmbH, Essen
Soxhlet – Apparatus	Brand GmbH+CoKG, Wertheim
Water bath, RII	Büchi Labortechnik GmbH, Essen
realTime PCR, DNA Engine Opticon	MJ Research Inc., St Bruno
PCR Cycler, TGradient	Biometra GmbH, Göttingen
Junior Pam	Heinz Walz GmbH, Effeltrich
Centrifuge, 5417R	Eppendorf AG, Hamburg
SPAD 502 Plus	Konica Minolta Business Solutions Deutschland GmbH, Langenhagen
Heating Plate	Schott AG, Mainz

2.8. Software

WinStat 2007.1 and 2009.1 für Excel	R. Fitsch Software, Bad Krozingen
MS Office 2007	Microsoft Deutschland GmbH, Unterschleißheim
Opticon Monitor 1.07	MJ Research Inc., St Bruno
Motic Images Plus 2.0	Motic

2.9. Conservation of the microorganisms

2.9.1. Aneurinibacillus migulanus

Growth and production of *Aneurinibacillus migulanus* was carried out in 1 l flasks with 200 ml Trypton Soya Broth. Before inoculation with over-night-culture (1:100) the medium was autoclaved. Afterwards the flasks were incubated for 5 days at 37 °C and 150 rpm. The cultures were stored in 4°C till they were used for trials.

2.9.2. Pseudoperonospora cubensis

For conservation *Pseudoperonospora cubensis* was bred on young, potted cucumber plants of the cultivar `Chinesische Schlange´.
Inoculation of the lower leaf surface was done with an aqueous sporangia suspension of *P. cubensis*. Fresh downy mildew sporangia were obtained by washing off the leaves of highly infected cucumber plants that were kept overnight at 100% relative humidity. The resulting suspension was counted in a Fuchs-Rosenthal counting chamber. After inoculation, the plants were incubated overnight at 100% relative humidity in the dark. Afterwards these plants were kept at an average temperature of 18.3±2.0°C and an average relative humidity of 47.6±6.7%.

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3. CONTROL OF DOWNY MILDEW (*PSEUDOPERONOSPORA CUBENSIS*) OF GREENHOUSE GROWN CUCUMBERS WITH ALTERNATIVE BIOLOGICAL AGENTS

(Published in Communications in Agricultural and Applied Biological Sciences, **75,** 541-555, 2010)

3.1. Abstract

Plant extracts from *Glycyrrhiza glabra* L. (licorice) and *Salvia officinalis* (sage) as well as cultures of the bacterium *Aneurinibacillus migulanus* were investigated for control of downy mildew in cucumber (*Pseudoperonospora cubensis*) in semi-commercial greenhouse trials in 2008 and 2009. For licorice and sage extract applied at 3% concentration in 7 or 10-day intervals, efficacy reached 68 – 83% and 63 – 74%, respectively. At concentrations of 2.0 or 2.5% and application intervals of 10 or 12 days, *G. glabra* extract resulted in 43 and 64% efficacy while sage extract reached only 34 and 45% efficacy. Treatments with *A. migulanus* at 1:1 dilution of the fermentation culture and applied in 7 or 10 day intervals reached 60 - 80% efficacy, while at a 1:5 dilution efficacy was insufficient. Overall, *G. glabra* extract tended to show best results. Nevertheless, the efficacy of sage extract and *A. migulanus* were in all trials comparable or better than that of the plant strengthener Elot-Vis®.

Keywords:
organic cucumber production, biological control, *Salvia officinalis, Glycyrrhiza glabra*, *Aneurinibacillus migulanus*

3.2. Introduction

In Germany in year 2009 vegetables were cultivated on 116,705 ha. This included cucumbers on 2,746 ha under open-field conditions and 269 ha in greenhouses (Statistisches Bundesamt 2009), of which 5-8% was cultivated following ecological guidelines (www.pan-germany.org).

One of the major problems in organic cucumber production, but also in several other crops is the infection of plants with downy mildew or related diseases like late blight in potato and tomato. Downy mildew in cucumber is caused by the obligatory biotrophic Oomycete *Pseudoperonospora cubensis*. There are years, dependent on temperature and weather, in which infections with this pathogen leads to major harvest loss (Haggag 2002, Urban and Lebeda 2007). For control of this disease and of several other fungi in organic production only copper preparations are available (Urban and Lebeda 2007). However, copper is a heavy metal which accumulates in the soil and can cause negative effects on soil organisms. There are also indications that it causes problems in water organisms (Finckh et al. 2007) and also in birds and mammals (www.oekolandbau.de). For these reasons there are several efforts to reduce the use of copper preparations (Finckh et al. 2007, Wilbois et al. 2009), and copper based plant protection products are not allowed for use in greenhouses in Germany anymore. There are investigations on cultivation strategies, like climate strategy (Marx et al. 2010), time of sowing or planting (Finckh et al. 2008, Wilbois et al. 2009) and also on alternative biological agents (Heungens and Parke 2001) in order to replace copper in the defense of diseases caused by Oomycetes.

In this investigation biological preparations were tested for the control of downy mildew in cucumbers. Based on good results of a licorice extract (*Glycyrrhiza glabra)* against the Oomycete, *Phytophtora infestans,* which causes late blight in potato and tomato (Konstantinidou-Doltsinis and Markellou 2008), and our own preliminary tests in greenhouses with sage extract (*Salvia officinalis*) and *Aneurinibacillus migulanus*, bioassays and semi-commercial trials were done at the Julius Kühn-Institut (JKI) in Darmstadt, Germany, and at the Lehr- und Versuchsanstalt für Gartenbau (LVG) in Heidelberg, Germany. The aim of this work was to investigate the potential of these agents for controlling downy mildew of cucumber in organic production.

3.3. Material and Methods

3.3.1. Plant extracts

The plant extracts were produced from dried and ground leaves and stems of *Glycyrrhiza glabra* L. (licorice; cultivated in Greece) and *S. officinalis* dalmat. (sage; Galke GmbH). For extraction the plant powder was stirred at 60℃ in 70% ethanol (Bläser 1999). These extracts were concentrated afterwards in a rotary-evaporator and were adjusted to a concentration of 25% of licorice (weight of extracted plant material/volume). The concentrated extracts contained 50% ethanol, and were stored at 8℃. For application they were diluted with tap wat er to the given final concentrations.

3.3.2. Growth and production of *Aneurinibacillus migulanus*

Growth and production of *Aneurinibacillus migulanus* was carried out in Tryptone Soya Broth (TSB). Over-night-cultures of *A. migulanus* were produced in 300 ml flasks (baffled) containing 60 ml of the medium. The flasks were incubated at 37℃ and 150 rpm. From this starter culture aliquots of 2 ml were used for inoculation of 1l flasks (un-baffled) containing 200 ml of TSB. The flasks were incubated 5 days at 37℃ and 150 rpm. Afterwards, the cultures were either used fresh or were stored at 4℃ for up to 3 weeks until they were used for trials.

3.3.3. Bioassays on potted plants

For the bioassays cucumbers of the cultivar `Chinesische Schlange´ were cultivated in a mixture of sand with K 15 pot ground (Klasmann-Deilmann GmbH) in a proportion of 1:3. The plants were grown under a 16 hour light (333 µmol quanta μm^{-2} s^{-1}) and 8 hour dark regime at an average temperature of 23.5±2.7℃ and an average relative humidity of 49.5±15.0% until 2 leaves were well developed, the other leaves were pricked out. Both leaves were treated by spraying the agents on the lower surface until run-off. Sage and licorice extract were used in concentrations between 0.31% and 5%, *A. migulanus* cultures were used in dilutions between 1:1 and 1:50.

Inoculation of the lower leaf surface was done a day after treatment with an aqueous sporangia suspension of *P. cubensis*. Fresh downy mildew sporangia were obtained by washing off the leaves of highly infected cucumber plants that were kept overnight at 100% relative humidity. The resulting suspension was counted in a Fuchs-Rosenthal

counting chamber. After inoculation, the plants were incubated overnight at 100% relative humidity in the dark. Afterwards these plants were kept at an average temperature of 18.3±2.0℃ and an average relative h umidity of 47.6±6.7%.

3.3.4. Semi-commercial trials in plastic greenhouses in years 2008 and 2009

3.3.4.1. 2008 Trial

Three-week-old cucumber plants of the cultivars `Eminentia´ and `Airbus´ were planted on May 20th at the Versuchsanstalt für Gartenbau (LVG), Heidelberg. Each cultivar was arranged in a separate area consisting of 3 double rows with 5 plots per double row. Each plot contained 12 plants, which were all treated. Disease rating, however, was done only on the 8 plants in the middle. In each double row one replicate of each treatment was set up. Both areas were surrounded by untreated cucumber plants of the cultivar `Torreon´. Plots were arranged in replicates in randomized design.

The tested agents were ethanolic extracts of *Glycyrrhiza glabra* (licorice), Fabaceae, and *Salvia officinalis* (sage), Lamiaceae, as well as a culture of the microorganism *Aneurinibacillus migulanus.* For comparison a commercially available plant strengthener, Elot-Vis®, was used and, as control, water treatment of plants.

For all applications the agents were diluted in tap water and sprayed with a 15 liter backpack, motor sprayer (approximately 208ml/plant). On the first application day (June 18th 2008) the extracts were sprayed in 5% concentration. In all following applications licorice extract was sprayed in 2.5% and sage extract in 2% concentrations. *A. migulanus* was used as a 1:5 dilution of the harvested culture preparation and Elot-Vis® in 5% concentration (manufacturer instruction). Furthermore all plants were treated with Milsana® 0.5% + Trifolio S forte® to protect them against powdery mildew (Konstantinidou-Doltsinis and Schmitt 1998). The application interval was 10 to 11 days. The day after the first application, the oldest 2 leaves of every plant were inoculated at the lower leaf surface with a suspension of *P. cubensis* ($5*10^3$ – $1*10^4$ sporangia/ ml).

The first disease rating was undertaken on July 9th. The percentage of infected leaf area was evaluated on six leaves. Following ratings were carried out weekly. Cucumber fruits were harvested 3 times per week.

3.3.4.2. 2009 Trial

The trial was carried out at the Versuchsanstalt für Gartenbau (LVG) Heidelberg. In 2009 plants of the cultivar `Airbus´ were planted on June 30th in three double rows with 12 plots per double row. Each plot contained 10 plants of which 6 plants in the middle were rated. There were 4 replications of each treatment, arranged in a randomized design.

Again licorice extract, sage extract and *A. migulanus* were tested. Elot-Vis® 5% was used as a standard and water treatment as control. For the first application (July 21st) licorice extract and sage extract were sprayed in 5% extract concentration. In all following applications the extracts were used in 3% concentration and *A. migulanus* in 1:1 dilution. All plants were treated with Milsana® 0.5% + Trifolio S forte® to protect them against powdery mildew, when necessary. The application interval was 7 and 10 days, respectively, except for *A. migulanus* which was applied every 7 days. For all applications the agents were diluted in tap water and sprayed with a 15 liter backpack, motor sprayer (approximately 375 ml/ plant).

On July 20th downy mildew appeared in the culture by natural infection. The first disease rating took place on July 24th. From then on, 7 leaves were rated weekly by estimation of the percentage of infected leaf area. The following seven disease ratings were carried out weekly. Cucumbers were harvested 3 times per week.

3.3.5. Semi - commercial trials in glass greenhouses (2008 and 2009)

The trials in glass greenhouses were carried out at the JKI Braunschweig in 2008 and 2009. In these trials plants of the cultivar `Airbus´ were planted in 4 single rows with 7 plants per row in each of 4 separate chambers. Each row was treated with one of the tested agents, and there was 1 replication of each treatment in each chamber. In both trials both plant extracts, licorice and sage, were tested as well as bacterium *A. migulanus.* As control water treated plants were used.

The extracts were applied at the concentrations 2% for licorice extract and 2.5% for sage extract in year 2008 and both extracts in 3% in year 2009. The culture broth of the microorganism was applied in year 2008 in 1:5 dilution and 2009 in 1:1 dilution. For all applications the agents were diluted in tap water and sprayed with a 15 liter backpack, motor sprayer (approximately 80 ml/plant). In 2008 the application interval was 12 days, and the first application was carried out on July 16th. In 2009 the interval was 7 days, and the first application was carried out on July 29th. Seven leaves of each of thirteen plants of each replication and treatment were rated by estimation of the percentage of

infected leaf area. The disease rating was carried out weekly. In 2008 there were eight ratings starting on July 29th. In 2009 there were nine disease ratings, starting on July 29th. Cucumber fruits were harvested 2 to 3 times per week in both years.

3.3.6. Statistical analysis

3.3.6.1. Effective concentration (EC_{50}) values

To calculate the effective concentration values of the alternative biological agents resulting in 50% disease reduction (EC_{50}), the fit probit analysis (software MLP Version 3.08) was used. For this, results from trials on potted plants were used. Efficacies were calculated from the medians of disease severity, and used for further processing in the probit analysis (Seifert 1991).

3.3.6.2. AUDPC

For statistical analysis of the semi commercial trials the outlying data were eliminated from the dataset. After this the median of disease severity of the rated leaves per plant, and from this the median per plot was calculated for each treatment. The AUDPC (Area Under Disease Progress Curve) was calculated from medians per replication after Truberg et al. 2008 with the formula:

$$AUDPC = \sum_{i=1}^{n-1} \left(\left(\frac{x_{i+1} + x_i}{2} \right) (t_{i+1} - t_i) \right)$$

x = disease severity

t =time

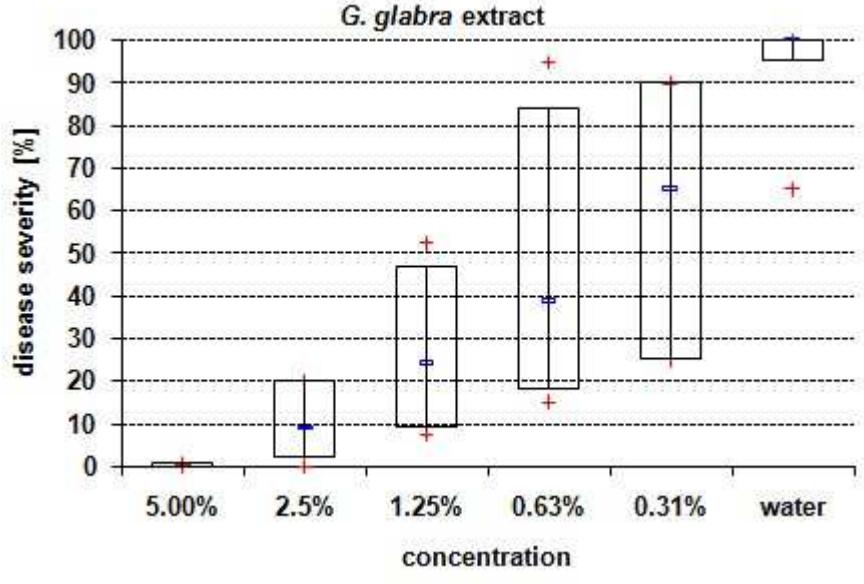
The AUDPC value was finally divided by the number of days of the complete time range.

For the calculation of efficacy as well as the significance analysis (Simulate Test) the AUDPC values were used. Statistical analysis was done with SAS 9.2 (SAS Institute Inc).

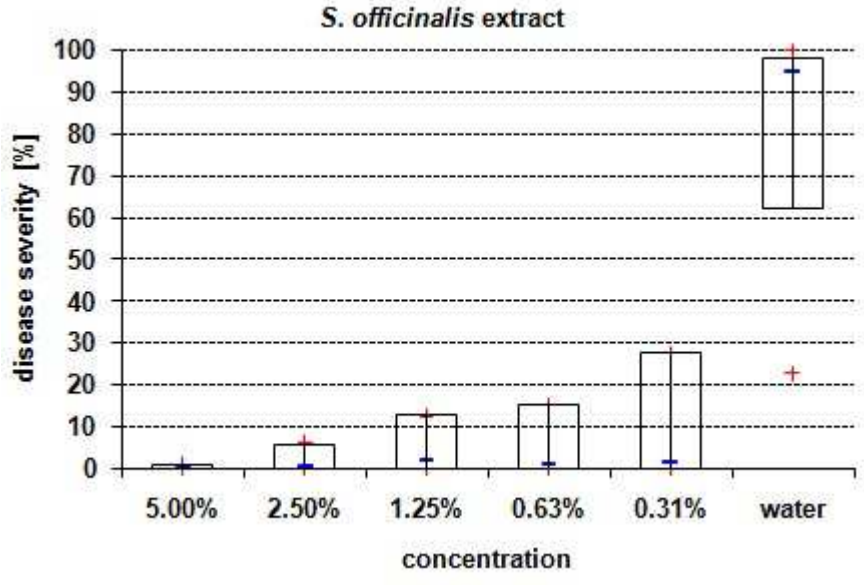
3.4. Results

3.4.1. Bioassays on potted plants

The data of the bioassays with licorice (*G. glabra*) extract showed a strong dependency of disease control of downy mildew on the applied extract concentration (Figure 1 a). An efficacy of 99% was observed for the 5% extract and 35% for the 0.31% extract. The calculated EC_{50} value was approximately 0.5% extract concentration.



a)



b)

Figure 1. Influence of the extracts of licorice (*G. glabra*) and sage (*S. officinalis*) on the disease severity [%] with *P. cubensis* on potted cucumber plants (cv. `Chinesische Schlange´) in a climate room (replication 3-6). Bar = median, crosses = min. and max.; boxes = 25th percentile and 75th percentile (within 50% of the data); whisker = 5th percentile and 95th percentile

In contrast, the data of the bioassays with sage (*S. officinalis*) extract did not show such a dose-efficacy dependency (Figure 1 b). Although the variability of the results was increasing with decreasing concentrations of the extract, the treatments with the sage extract were overall highly effective even at the lowest concentration of 0.31% (efficacy 90%). For that reason a calculation of the EC_{50} value could not be done.

The applied extracts contained up to 10% ethanol. Ethanol in this concentration was not phytotoxic and did not reduce disease severity, as was proven in separate trials (data not shown).

In the bioassays with *A. migulanus* the whole culture broth was diluted in different ratios. Only the lowest dilutions gave high efficacies. The efficacy of the 1:1 dilution was 98% and of the 1:5 dilution 83%. Based on these data, *A. migulanus* was used in a 1:5 dilution for the semi-commercial trials in year 2008. Sage and licorice extract showed a bigger variability of disease severity with extract concentrations below 1.25% and 2.5%, respectively. Taking this into account and the fact that under commercial conditions permanent disease pressure is higher than under controlled conditions, and may thus adversely influence the effectiveness of the extracts, for both plant extracts concentrations of 2% and 2.5% were chosen for the semi-commercial trial 2008.

3.4.2. Semi - commercial trials in plastic greenhouses in years 2008 and 2009

3.4.2.1. 2008 Trial

In the year 2008 plants of two cultivars (cv.), `Eminentia´ and `Airbus´, were treated every 10 days with the alternative biological agents, the plant strengthener Elot-Vis® or water as control. Despite the inoculation of the first two leaves of every plant in this trial, only an intermediate infection with *P. cubensis* (controls 27% and 35%, respectively) developed until the end of the trial.

It appears that licorice extract and Elot-Vis® delayed the downy mildew infection especially at the beginning of the trial in both cultivars (Figure 2 a and b). Sage extract showed an efficacy of 66% in week two in the cultivar `Airbus´ (Figure 2 b). However, other than on this date and in this cultivar the sage extract did not reach efficacies above 50%, neither did treatments with *A. migulanus.* The highest efficacies against *P. cubensis* could be detected for treatments with licorice extract and Elot-Vis® for all disease ratings and in both cultivars (efficacies based on disease severity on single rating dates: licorice extract 55% to 97%, Elot-Vis® 33% to 82%; efficacies based on AUDPC values: licorice extract 64% (cv. `Airbus´) and 74% (cv. `Eminentia´), Elot-Vis® both cultivars 52%; see Table 5).

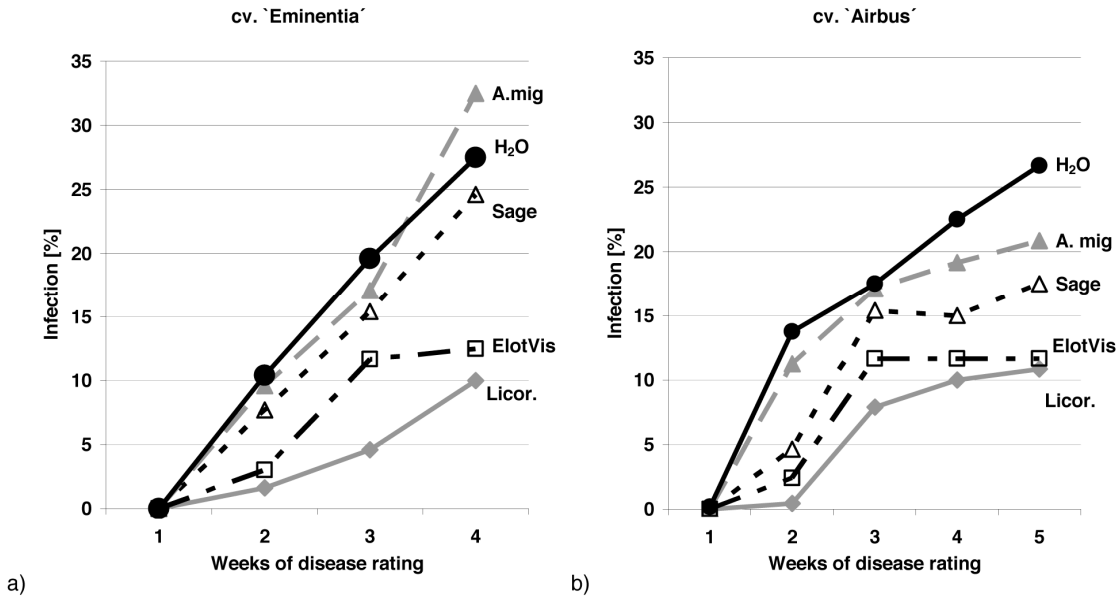


Figure 2. Influence of the extracts of licorice (*G. glabra*) and sage (*S. officinalis*) and the microorganism *A. migulanus* on the disease severity (Infection [%]) of *P. cubensis* on cucumber plants in plastic greenhouse in year 2008. Shown are the progression curves of means of the medians per replication.
Agents and concentrations: Licorice extract (Licor.; 2.5%), sage extract (Sage; 2%), *Aneurinibacillus migulanus* (A. mig.; 1:5 dilution), standard Elot-Vis® and water as control. The agents were tested in a 10 day application interval.

Statistical Analysis via Simulate procedure of the AUDPC values showed significant differences between the licorice extract and the control in both cultivars (Table 1). With the sage extract a significant effect against the downy mildew compared to the control could only be reached in the cultivar `Airbus´. However the effect was significantly lower than that of the licorice extract. In the cultivar `Eminentia´ there was no significant effect of the sage extract, at all. Treatments with *A. migulanus* did not result in significantly different AUDPC values compared to the control in both cultivars. The AUDPC values of the plant strengthener Elot-Vis® was in both cultivars significantly lower compared to the controls, but not to the licorice or the sage extract.

Table 1. Influence of the extracts of licorice (*G. glabra*) and sage (*S. officinalis*) and the microorganism *A. migulanus* on the disease severity (Infection [%]) of *P. cubensis* on cucumber plants in plastic greenhouse in year 2008. Shown are the means per replication of AUDPC value (Area under disease progress curve). Concentrations: Licorice extract (*G. glabra*; 2.5%), sage extract (*S. officinalis*; 2%), *Aneurinibacillus migulanus* (A. mig.; 1:5 dilution), standard Elot-Vis® and water as control. The agents were tested in a 10 day application interval. Also shown are the standard deviation and the results of the Simulate Analysis ($p<0.05$).

	licorice	sage	*A. migulanus*	Elot-Vis®	water
cv. `Eminentia´					
Mean of AUDPC	3.90	12.18	14.78	7.20	14.99
Standard deviation	0.75	2.80	5.08	1.61	4.10
Simulate test	a	b	b	a	b
cv. `Airbus´					
Mean of AUDPC	6.05	11.09	14.60	8.03	16.90
Standard deviation	1.10	4.68	1.60	2.13	5.21
Simulate test	a	b	bc	ab	c

The harvest data were not processed because the main harvest was already finished by the time of the second rating.

3.4.2.2. 2009 Trial

In 2009 the cultivar `Airbus´ was used. No inoculation was necessary because the first symptoms of downy mildew occurred naturally. At the end of the trial the infection with *P. cubensis* of the control plants treated with water in 7 day intervals was around 20% higher than that of cucumber plants with 10 day application interval treatment (Figure 3 a and b). Nevertheless, treatments with the alternative biological agents (licorice and sage extract, *A. migulanus* culture) as well as with the plant strengthener (Elot-Vis®) in both application intervals resulted in a strong disease reduction (Figure 3 a and b) and subsequently high efficacies against downy mildew throughout the whole trial.

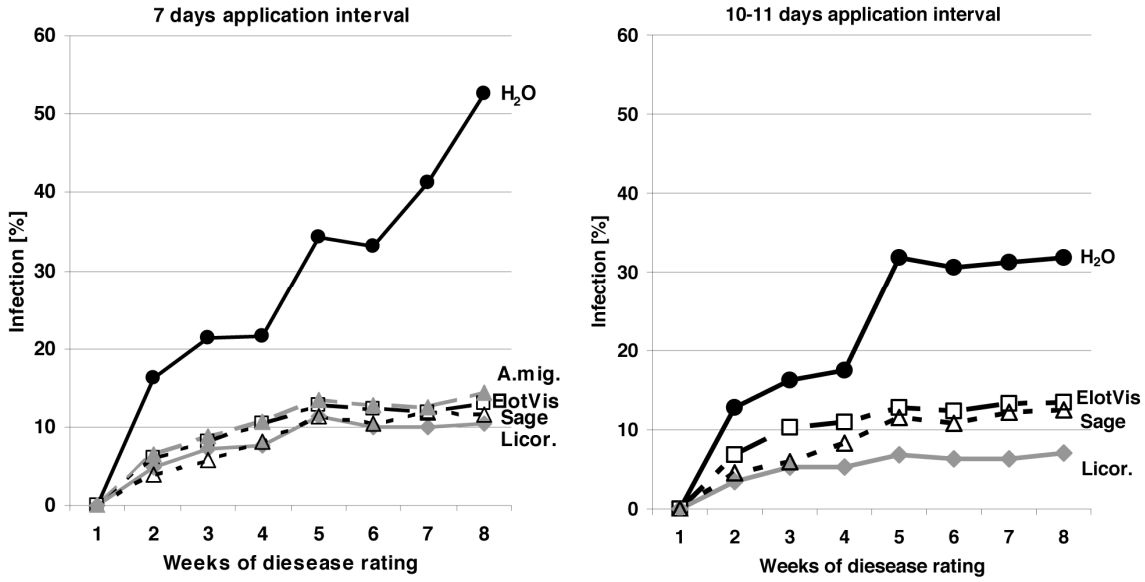


Figure 3. Influence of the extracts of licorice (*G. glabra*) and sage (*S. officinalis*) and the microorganism *A. migulanus* on the disease severity (Infection [%]) with *P. cubensis* on cucumber (cv. `Airbus´) plants in plastic greenhouse in year 2009. Shown are the progression graphs of means of the medians per replication.

Agents and concentrations: Licorice extract (Licor.; 3%), sage extract (Sage; 3%), *Aneurinibacillus migulanus* (A. mig.; 1:1 dilution), standard Elot-Vis® and water as control. The agents were tested in a 7 day application interval (left) and a 10 day application interval (right), respectively.

Statistical analysis via Simulate procedure of the AUDPC values showed significant differences for the licorice extract, sage extract and Elot-Vis® as well as for *A. migulanus* compared to the control for both application intervals (Table 2). There was no significant difference among treatments with the alternative control agents. Efficacies around 70% (based on the AUDPC values) could be reached with the treatment with sage extract in the 7 day application interval and with the application of the licorice extract in 7 and 10 day intervals. For the treatment with *A. migulanus* an efficacy of 59% was calculated (7 day application interval). The efficacy of the plant strengthener Elot-Vis® was higher in the 7 day application interval (62%) than in the 10 day application interval (41%) (Table 5).

Table 2. Influence of the extracts of licorice (*G. glabra*) and sage (*S. officinalis*) and the microorganism *A. migulanus* on the disease severity (Infection [%]) of *P. cubensis* on cucumber (cv. `Airbus´) plants in plastic greenhouse in year 2009. Shown are the means per replication of AUDPC value (Area under disease progress curve). Concentrations: Licorice extract (*G. glabra*; 3%), sage extract (*S. officinalis*; 3%), *Aneurinibacillus migulanus* (A. migulanus; 1:1 dilution), standard Elot-Vis® and water as control. The agents were tested in a 7 day application interval and a 10 day application interval, respectively. Also shown are the standard deviation and the results of the Simulate Analysis ($p<0.05$).

	licorice	**sage**	***A. migulanus***	**water**
7 day application interval				
Mean of AUDPC	7.83	6.38	10.00	24.55
Standard deviation	4.82	3.27	4.26	15.48
Simulate test	a	a	a	b
10 day application interval				
Mean of AUDPC	5.61	6.85	/	18.76
Standard deviation	2.46	4.22	/	9.39
Simulate test	a	a	/	b

There was no significant difference between the fruit yield of the treated plants compared to the control, neither in number nor in weight (data not shown).

3.4.3. Semi-commercial trials in glass greenhouses 2008 and 2009

3.4.3.1. 2008 Trial

In the glass greenhouse the overall development of downy mildew on the plants of the cultivar `Airbus´ was very low in the year 2008, as was already seen in the plastic greenhouse (see above). The agents were applied in a 12 day interval. The average infection with *P. cubensis* of the water treated plants was only 26% at the end of the trial. As presented in Figure 4, all tested agents had an effect on the disease progress. The licorice and the sage extract appeared to be slightly more effective than the microorganism (efficacies at the end of the trial: licorice extract 46%, sage extract 42%, *A. migulanus* 29%).

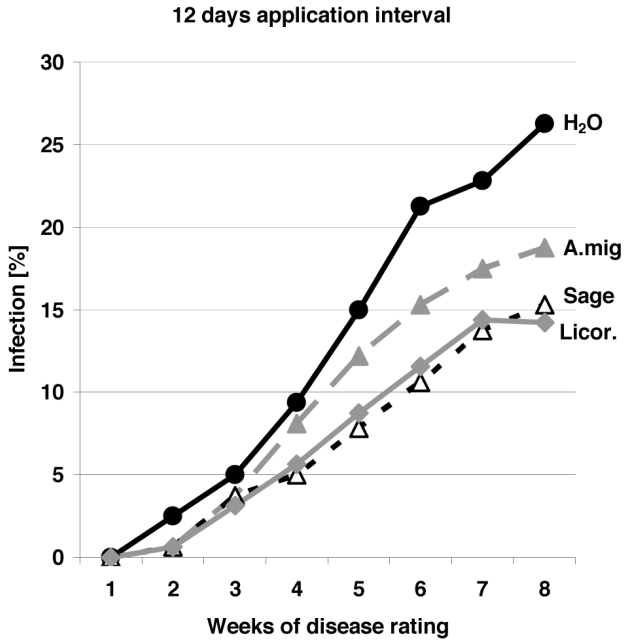


Figure 4. Influence of the extracts of licorice (*G. glabra*) and sage (*S. officinalis*) and the microorganism *A. migulanus* on the disease severity (Infection [%]) of *P. cubensis* on cucumber (cv. `Airbus´) plants in glass greenhouse in year 2008. Shown are the progression graphs of means of the medians per replication.
Agents and concentrations: Licorice extract (Licor.; 2%), sage extract (Sage; 2.5%), *Aneurinibacillus migulanus* (A. mig.; 1:5 dilution), and water as control. The agents were tested in a 7 day application interval.

However, due to large standard deviations, the statistical analysis of the calculated AUDPC values per treatment did not show significant differences, neither among the treatments, nor between them and the control (Table 3).

Table 3. Influence of the extracts of licorice (*G. glabra*) and sage (*S. officinalis*) and the microorganism *A. migulanus* on the disease severity (Infection [%]) of *P. cubensis* on cucumber (cv. `Airbus´) plants in glass greenhouse in year 2008. Shown are the means per replication of AUDPC value (Area under disease progress curve). Concentrations: Licorice extract (*G. glabra*; 2%), sage extract (*S. officinalis*; 2,5%), *Aneurinibacillus migulanus* (A. mig.; 1:5 dilution), and water as control. The agents were tested in a 12 day application.
Also shown are the standard deviation and the results of the Simulate Analysis ($p<0.05$).

	licorice	sage	*A. migulanus*	water
12 day application interval	-	-	-	-
Mean of AUDPC	7.22	6.91	9.49	12.58
Standard deviation	6.98	6.23	5.57	10.84
Simulate test	a	a	a	a

There were no significant differences between the fruit yield of the treated plants compared to the control, neither in number nor in weight.

3.4.3.2. 2009 Trial

The alternative biological agents were applied every 7 days on cucumber cultivar `Airbus´. The disease development of *P. cubensis* in the second half of the trial in the glass greenhouse in year 2009 was stronger than in the year before. On the last rating date the control plants were infested with *P. cubensis* to a degree of 53% while the investigated treatments kept the disease below 10% (Figure 5). Thus, the efficacies of all alternative biological agents at the end of the trial were above 80% (efficacies: licorice extract 93%, sage extract 84%; *A. migulanus* 92%).

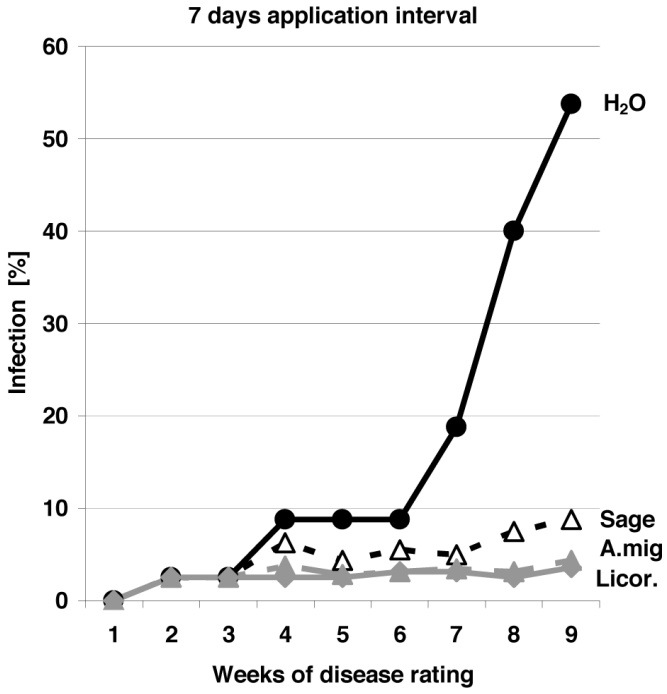


Figure 5. Influence of the extracts of licorice (*G. glabra*) and sage (*S. officinalis*) and the microorganism *A. migulanus* on the disease severity (Infection [%]) of *P. cubensis* on cucumber (cv. `Airbus´) plants in glass greenhouse in year 2009. Shown are the progression graphs of means of the medians per replication. Agents and concentrations: Licorice extract (Licor.; 3%), sage extract (Sage; 3%), *Aneurinibacillus migulanus* (A. mig.; 1:1 dilution), and water as control. The agents were tested in a 7 day application.

The statistical analysis of the calculated AUDPC values showed significantly lower values for the licorice and *A. migulanus* treated compared to the sage treated plants and the control. The AUDPC values of the sage treated plants were also significantly lower than those of the control plants (Table 4).

Table 4. Influence of the extracts of licorice (*G. glabra*) and sage (*S. officinalis*) and the microorganism *A. migulanus* on the disease severity (Infection [%]) of *P. cubensis* on cucumber (cv. `Airbus´) plants in glass greenhouse in year 2009. Shown are the means per replication of AUDPC value (Area under disease progress curve). Concentrations: Licorice extract (*G. glabra*; 3%), sage extract (*S. officinalis*; 3%), *Aneurinibacillus migulanus* (A. migulanus 1:1 dilution), and water as control. The agents were tested in a 7 day application.
Also shown are the standard deviation and the results of the Simulate Analysis ($p<0.05$).

	licorice	sage	*A. migulanus*	water
7 day application interval	-	-	-	-
Mean of AUDPC	2.50	4.64	2.86	14.33
Standard deviation	0.53	0.71	0.57	1.77
Simulate test	a	b	a	c

In this trial the highest efficacy (83%) based on the AUDPC values was calculated for the licorice extract (Table 5). The efficacies for both other alternative biological agents (sage extract and *A. migulanus*) were 68% and 80%, respectively (Table 5).

There were no significant differences between the fruit yield of the treated plants compared to the control, neither in number nor in weight.

Table 5. Efficacies of treatments with the extracts of licorice (*G. glabra*) and sage (*S. officinalis*) and the microorganism *A. migulanus* in semi-commercial trials with *P. cubensis* on cucumber plants (cv. `Airbus´). Shown are efficacies [%] calculated from the AUDPC (Area under disease progress curve) values.

		2008	2008	2009	2009	
		(glass)	(plastic)	(glass)	(plastic)	
		Application Interval				
Agents	**Concentrations**	**12 days**	**10 days**	**7 days**	**10 days**	**7 days**
G. glabra	3%			83	70	68
	2,50%		64			
	2%	43				
S. officinalis	3%			68	63	74
	2,50%	45				
	2%		34			
A. migulanus	1 : 1			80		59
	1 : 5	25	13			
ElotVis®	5%		52		41	62
Disease severity [%] in control at the end of the trial		26	26	53	32	52

3.5. Discussion

In bioassays on potted cucumber plants, treatment with ethanolic extracts (above ground plant parts) of *Glycyrrhiza glabra,* (licorice) and *Salvia officinalis* (sage) showed high efficacies against downy mildew (*Pseudoperonospora cubensis*). The EC_{50} value was calculated to be approximately 0.5% extract concentration for licorice extract, while for sage extract it was even lower. However, exact data cannot be given since the efficacy of the sage extract was higher than 90% even for the lowest concentration (0.31%) tested.

In semi-commercial trials in year 2008 concentrations of 2% and 2.5% of licorice and of sage extract were applied. Because of the high efficacy (83%) of the 1:5 dilution of the microorganism in the assays on potted plants, *Aneurinibacillus migulanus* was applied in this dilution in the trials 2008. Previous data from our laboratory with a preliminary trial in 2007 where 1 to 3 weekly applications of licorice extract were investigated showed that an application interval of 10-12 days was sufficient for effective disease control (unpublished results). Thus, this interval was defined for the semi-commercial trials in year 2008.

However, in the semi-commercial trial on cucumbers in a plastic greenhouse in year 2008, significant diminution of the downy mildew infection could be realized only with the treatment of licorice extract in both tested cultivars. Based on calculations of the AUDPC values, in the cultivar `Airbus´, the extract (2.5% concentration) reached only an efficacy of 64%, which was much lower than expected from the bioassays. Also, the results for sage extract and *A. migulanus* were contrary to the results of the bioassays on potted plants where these treatments showed very high efficacies even in very low concentrations (Figure 1 and results “Bioassays on potted plants”).

In the glass greenhouse in year 2008 both extract treatments, sage and licorice, reduced the disease severity throughout the trial (Figure 4), reaching efficacies at the end of the trial of 42% and 46%. However, due to relatively low disease levels and high standard deviations, there were no significant differences between the control and the treatments based on the AUDPC values (Table 3).

There are many reasons which could lead to the failure of an alternative biological agent under commercial conditions, like the effect of UV light (Buchenauer 1975, Kiss and Virag 2009), the fluctuation in temperature (Steuerbaut 1993) and persistence after rain/overhead sprinkler irrigation (Vincent et al. 2007). In the trials of this study UV stability should not be a crucial factor, due to the roofing of the greenhouses. Since the plants in the plastic greenhouse were sometimes irrigated overhead as a protection against the heat, temperature and rain stability could have had an effect. In year 2006 a

small-scale greenhouse trial with sage extract was undertaken at the JKI in Darmstadt. In this trial the treatment of cucumbers with a 5% sage extract with weekly applications led to an efficacy of 70% compared to the highly infected control plants (infection 92%) (unpublished data). Based on these data, for the year 2008 trial it is suggested that the application interval of ten days combined with the lower extract concentrations of 2% and 2.5% was insufficient. Therefore in the trials in year 2009 a slightly higher concentration (3%) of both extracts was chosen and the culture broth of *A. migulanus* was diluted 1:1. Also in former studies with plant extracts it was shown that the application interval is very critical for the efficacy of an agent (Gupta and Sharma 1981, Latten 1994). Therefore, in order to investigate the influence of the application interval, a comparison of a 7 and a 10-11 day application was done in the trial in year 2009 in the plastic greenhouse. The plants of the trial in the glass greenhouse (2009) were all treated every 7 days. In both trials all tested agents showed good disease reductions (Figure 3 and Figure 5) and efficacies (Table 5) with respect to downy mildew on cucumbers throughout the whole trial. The differences between the application intervals were marginal. These data indicate that the failure in efficacy of the treatment with sage extract and the microorganism in year 2008 was mainly caused by the low concentration used.

Indeed, an overview of the efficacies from the trials in year 2008 and 2009 (Table 5) indicate a dose-efficacy dependency for all alternative biological agents. The efficacies calculated from the AUDPC values were lowest in the trials with the lowest concentration tested (2%). Overall, there was a tendency that licorice extract was the best of the alternative biological agents used. Nevertheless the efficacies of sage extract and *A. migulanus* were at the same level as the efficacies of the commercially used plant strengthener Elot-Vis® or even slightly better.

Similar tendencies in the efficacy of the plant extracts were shown in an open field trial (data unpublished), where all tested agents (licorice and sage extract, and *A. migulanus* culture) tended to slow down the disease development over a period of two weeks. The effect was strongest in the cucumbers treated with licorice extract but at that end of the trial disease severity of all plants was 100%.

Overall abiotic factors, like UV stability and rain fastness are more important for the performance in open field than in protected cultivation. In trials with onions which were treated with licorice extract rain fastness was identified as a factor influencing efficacy (Schmitt et al. 2010). Open field trials with sage extract against *Plasmopara viticola* in grapevine showed the same (Dagostin et al. 2010).

The general impression from these trials in greenhouses is that the overall appearance of treated plants was healthier and in the case of licorice extract the leaves showed a

darker green color. This observation was also made in open field trials, where furthermore the treated plants produced new branches, which could have prolonged the harvest. Unfortunately, final conclusions on the effect of treatments with the alternative biological agents on fruit yield cannot yet be drawn. In none of the greenhouse trials, yield was significantly different between the treatments and the control. Overall in the trials disease appearance was late and further development was slow (Figures 2-5). Thus it is not clear to what extent the disease has influenced yield. Further investigations on the dependency of yield and disease severity are necessary.
In conclusion, all tested alternative biological agents showed a promising potential for control of downy mildew in cucumber in protected cultivation. Most consistent results could be reached by treatments with the licorice extract.

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4. INVESTIGATIONS ON THE EFFICACY OF LEAF EXTRACT FRACTIONS OF *GLYCYRRHIZA GLABRA* AGAINST DOWNY MILDEW OF CUCUMBER

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4.1. Abstract

An ethanolic leaf extract of *Glycyrrhiza glabra* (licorice) was highly effective in former bioassays and semi-commercial trials in controlling cucumber downy mildew (efficacy up to 99.0% in bioassays and 83.0% in semi-commercial trials).

In order to elucidate the active ingredients and the mode of action, licorice leaf extract was fractionated into 6 fractions of defined substance classes, of which the fraction containing acidic substances (F6) showed highest efficacy (97.6%). The calculated EC_{50} values after a probit analysis of concentration series of crude extract and fraction F6 were concentrations of 1.0% (crude extract) and 0.6% (fraction F6). Interestingly, the slopes of the resulting graphs were significantly different. Three flavonoid compounds could be detected. The substances were glabranin, licoflavanon and pinocembrin. All three are known for their antimicrobial and antifungal capacity against plant and human pathogens. Besides these flavanoids, results, such as the different EC_{50} values, indicated that other compounds may be involved in the activity of fraction F6 against *P. cubensis* on cucumber. However, results of probit analysis of the crude extract and the fraction F6 led to the assumption that beside the known direct effect of the licorice extract against downy mildew a resistance inducing effect might play a role.

Keywords:

Biological control, plant extracts, induced resistance

4.2. Introduction

The roots of the Mediterranean Fabaceae *Glycyrrhiza glabra* (licorice) are used in medicine since hundreds of years (Krausse et al. 2004). The application of this drug ranges from mouth ulcers (Krausse et al. 2004) to Crohn´s disease and ileitis (Winston and Maimes 2007). Also, its strong antiviral capacity is used against different diseases caused by herpes viruses (Curreli et al. 2005). Furthermore, there is evidence that preparations of *G. glabra* roots also have effects on HIV (Manfredi et al. 2001). Because of the medical use and the utilization of *G. glabra* for production of candy or as sweetener there are many studies about substances which can be found in the roots of licorice. The substance classes which were studied were mainly flavonoids and isoflavonoids (Hayashi et al. 1996; 2003), and some of them are long known for their antimicrobial (Lester et al. 1983, Fukui et al. 1988) and antifungal (Shain and Miller 1982) activity. Fukui et al. (1988) also discovered antimicrobial and antifungal activity against human as well as plant pathogens in the upper parts of the plant.

In former bioassays and in greenhouses an extract from leaves of *G. glabra* had a high potential for the control of the Oomycete *Pseudoperonospora cubensis,* the causal agent of downy mildew in cucumber (Scherf et al. 2010, see also Chapter 3). This disease is a major problem in organic cucumber production and may lead to total harvest loss dependent on temperature and weather (Haggag 2002, Urban and Lebeda 2007). In semi-commercial trials in greenhouse grown cucumbers, the efficacy of a 3% *G. glabra* extract against downy mildew reached 70 to 83% (Scherf et al. 2010, see also Chapter 3). However, information on active ingredients responsible for the efficacy against *P. cubensis* is lacking.

The aim of this study was therefore to identify fractions and single compounds of the crude *G. glabra* leaf extract which are involved in the activity against downy mildew of cucumbers.

4.3. Material and Methods

4.3.1. Plant extracts

Crude plant extract was prepared from dried and finely ground leaves of *Glycyrrhiza glabra L.* (licorice) harvested in Patras, Greece. The extraction was done with a Soxhlet extractor in 96% ethanol (EtOH). The used amount of ethanol was always 10 times the used amount of plant powder (v/w). Extraction time varied between two hours and ten hours depending on the amount of plant powder. The extract was concentrated to 50%

concentration (w/v), i.e. the extract of 50 g plant powder was adjusted to a final volume of 100 ml.

4.3.2. Fractionation by shake- out procedure

For fractionation, ethanol was removed from the crude licorice extract (50.0% extract concentration) with a rotary evaporator. The dry residue was diluted in equal volumes of CH_2Cl_2 and 5% HCl. Consecutively, emerging phases were parted and washed with fresh portions of the respective other solvent in a separatory funnel until the color of one phase was light and clear.

The pooled CH_2Cl_2 phase was labeled fraction F1. The aqueous phase was alkalinized with ammonia 33% to pH 12 and then washed again with CH_2Cl_2 until one phase was clear. The resulting pooled CH_2Cl_2 phase was labeled fraction F2 (alkaloids). The new aqueous phase was evaporated and the dry residues were washed with ethanol 96%, precipitating salts were discarded. The supernatant was labeled fraction F3 (quarternary alkaloids and sugars). The CH_2Cl_2 phase (F1) of the first fractionation step was washed with portions of caustic soda 5% until the color of one phase was light and clear. The remaining CH_2Cl_2 phase was evaporated and the pellet diluted in equal volumes of methanol 90% and n-hexane. The emerging phases were labeled fraction F4 (terpenoids and sterols) and fraction F5 (lipids and waxes). The caustic soda phase from the step before was acidified with HCl to pH 2 and afterwards washed with portions of fresh CH_2Cl_2. This CH_2Cl_2 phase was labeled fraction F6 (acidic substances). The aqueous phase was discarded. A scheme of the extraction procedure is given in Figure 1. From all fractions solvents were evaporated in a rotary evaporator and the dry residues were afterwards dissolved in ethanol 96%. Final volumes were the same as those of the inserted crude licorice extract. All fractions were tested in bioassays on cucumber against *Pseudoperonospora cubensis*.

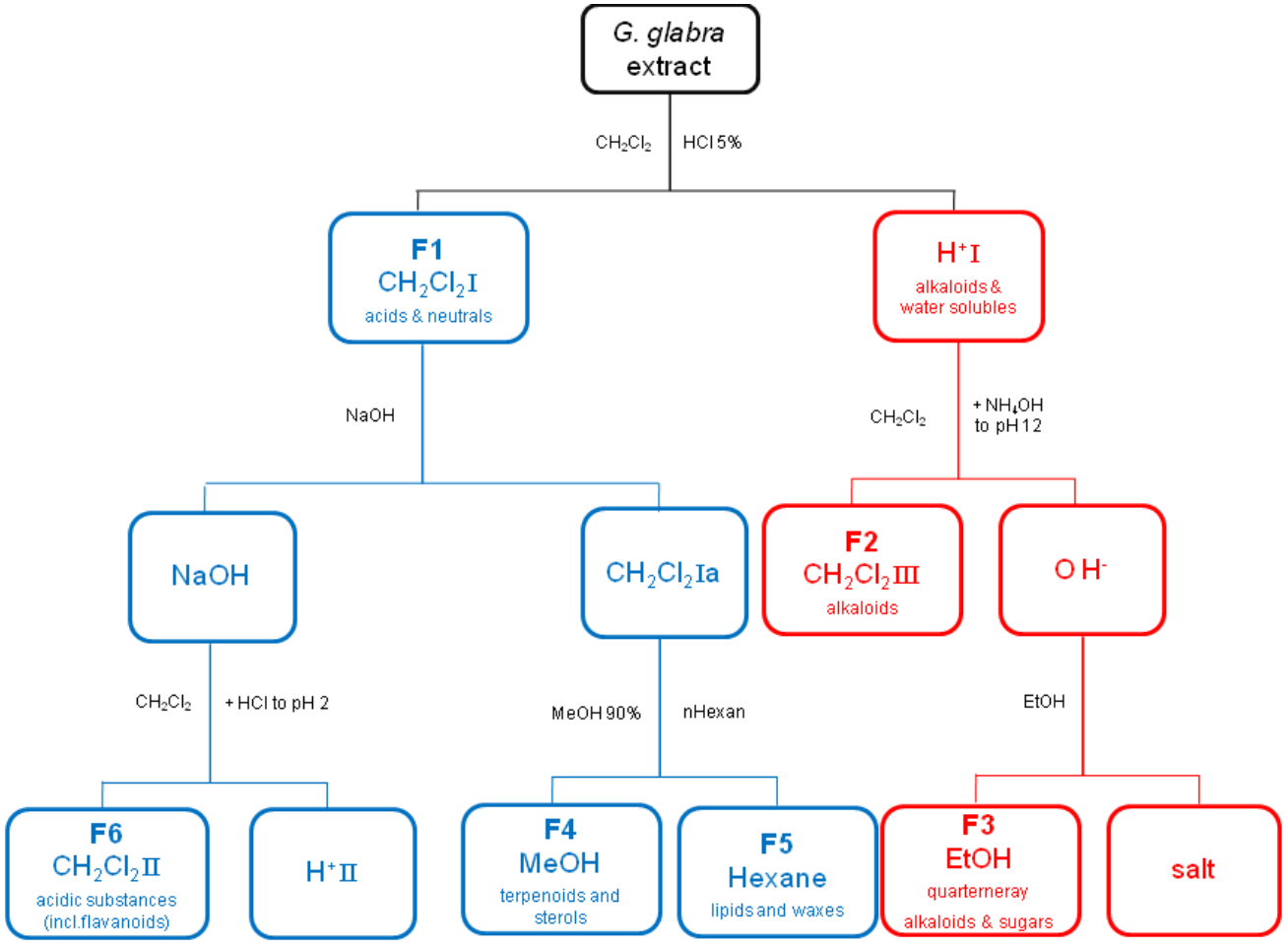


Figure 1. Schematic diagram of the fractionation of crude licorice leaf extract. F1 = acidic substances and neutrals, F2 = alkaloids, F3 = quaternary alkaloids and sugars, F4 = terpenes and sterols, F5 = waxes and lipids, F6 = acidic substances and the cucumber

4.3.3. Chromatography

4.3.3.1. Thin layer chromatography

Thin layer chromatography (TLC) was done on TLC plates (20 cmx20 cm) consisting of aluminum foil covered with silica gel $60F_{254}$ (Merck). Aliquots (2 µl or 5 µl) of fractions and sub-fractions were applied with 2 µl or 5 µl capillaries. TLC plates were dried on air before they were developed in a solvent chamber with toluene, dioxane and acetic acid (20:5:1). TLCs were taken out when the solvent front reached approximately 11 cm height, and dried on air. The spraying reagent (vanillin 10 mg in 100 ml 32% phosphoric acid) was applied with a TLC sprayer (Desaga GmbH). The TLC plate was heated at 110℃ until coloration of the sub-fractions develop ed.

4.3.3.2. Column chromatography

For column chromatography the solvent of fraction F6 (50% extract concentration) was evaporated completely in a rotary evaporator. To the residue the 10-fold amount of silica gel $60F_{254}$ (w/w, Merck KGaA) was added and CH_2Cl_2 was added until the silica gel was covered. The mixture was stirred and finally the solvent was evaporated again. As a result, the fraction´s compounds were fixed to the silica gel. A glass column (Ø 4.5 cm) was filled with a thin layer of quartz sand and then solvent/silica gel $60F_{254}$ (w/w, Merck KGaA) mixture was added. The amount of silica gel was 100-fold the amount of the prepared silica/fraction mixture (w/w). To cast out air bubbles, the column was softly beaten from all sides. Another layer of quartz sand was filled on top and then the silica/fraction mixture was carefully put on top of the prepared column. As eluent pentane/ethyl acetate (6:1) was used. Eluted compounds were collected in at least 200 test tubes (10 ml) before the column was washed with 96% methanol to recover remaining substances which were not soluble in the first eluent. With all test tube samples a TLC was run as described above. Samples which contained the same substances were pooled to sub-fractions and the solvent was evaporated. For the bioassays dry residues were dissolved in ethanol 96% resulting in same volume and concentration as that of the inserted fraction F6. For each bioassay a fresh column chromatography was performed.

4.3.3.3. HPLC and 1H-NMR-Spectroscopy

HPLC was carried out on a Hewlett Packard HP1090 with a photodiode array detector at 292 nm. For sample preparation 30 µl of the 50% extracts were diluted with methanol to 10 ml and filtered through a syringe filter (Nylon, 0.2 nm). The injection volume of each sample was 5 µl. For HPLC a Phenomenex 00G-4337-E0 synergi 4u MAX-RP 80A 250 x 4.6 mm 4 micron column was used. The chromatography was run with a MeCN/H_2O gradient of 70.0% for 2 minutes and afterwards from 70.0% MeCN to 100.0% MeCN in 2 minutes. The flow rate was 0.95 ml per minute and the column temperature was 40.0°C. Identification of the compo unds was done via 1H-NMR-spectroscopy at 400 MHz (Avance II 400 MHz WB (AV 400) Bruker BioSpin GmbH)

4.3.4. Bioassays on potted plants

For the bioassays cucumbers of the cultivar `Chinesische Schlange´ were cultivated in a mixture of sand with K 15 pot ground (Klasmann-Deilmann GmbH) in a proportion of 1:3. The plants were grown under a 16 hour light (333 µmol quanta μm^{-2} s^{-1}) and 8 hour dark regime at an average temperature of 23.5±2.7°C and an average relative humidity of 49.5±15.0% until 2 leaves were well developed, the other leaves were pricked out. Both leaves were treated by spraying the agents on the lower surface until run-off. The crude *G. glabra* extract (50% concentration w/v) as well as the fractions and sub-fractions (50% concentration w/v) were diluted in de-ionized water to the final concentration of 0.3% to 5% (v/v).

Inoculation of the lower leaf surface was done a day after treatment with an aqueous sporangia suspension of *P. cubensis* ($5*10^3$ sporangia/ ml). Downy mildew sporangia were obtained by washing off the leaves of highly infected cucumber plants that were kept overnight at 100% relative humidity. The resulting suspension was counted in a Fuchs-Rosenthal counting chamber. After inoculation, the plants were incubated overnight at 100% relative humidity in the dark. Afterwards these plants were kept at an average temperature of 18.3±2.0°C and an average re lative humidity of 47.6±6.7%.

There were two different designs of bioassays, either (a) the assay was replicated, independently at least four times with varying number of plants per treatment or (b) the assay consists of 4 replications with 2 or 3 plants per treatment in a randomized design.

4.3.5. Statistical analysis

For statistical analysis of bioassays the median disease severity per replication from the median per plant was calculated for each bioassay. Those medians were used for the descriptive statistics (WinStat for Excel 2009.1) pictured in box-plots (bioassays (a) and (b)) and further analysis (biassays (b)).

4.3.5.1. ANOVA with Tukey test

The data of assays with 4 randomized replications with 2 or 3 plants per replication and treatment (b) underwent an ANOVA with Tukey test $p<0.05$. Statistical analysis was done with WinStat for Excel 2009.1.

4.3.5.2. Effective concentration (EC) values

To calculate the effective concentration values of licorice leaf extract and fraction 6 resulting in 50% disease reduction (EC_{50}), probit analysis (software MLP Version 3.08) was used. Efficacies were calculated from the medians of disease severity per replication. Those data were transformed in response data with the formula

response = n*efficacy/100 (n=sample size).

The calculated response data were used for further processing by probit analysis.

4.3.5.3. Chi square (χ^2)

Data of the dose-response curves resulting from the probit analysis underwent a Chi square analysis (software MLP Version 3.08) for position, parallelism and total heterogeneity $p<0.05$.

4.4. Results

4.4.1. Bioassays with extract fractions of *G. glabra* extract on potted cucumbers

Bioassays with cucumber plants and with *P. cubensis* were done with six fractions resulting from a fractionation of crude licorice (*G. glabra*) extract. The main activity was found in the fraction containing acidic substances and neutrals (Figures 1 and 2; F1). Disease severity reached an average of 1.7%, while water treated plants showed an infection of 76.2% with *P. cubensis*. The efficacy of fraction F1 was 97.7% and

comparable to that of crude licorice extract, which reached 98.8% efficacy. In contrast, the fractions which contained alkaloids and water soluble substances (Figure1; F2 and F3) were less effective against *P. cubensis* (Figure 2; F2 and F3). For both mean level of disease severity reached 62.5%.

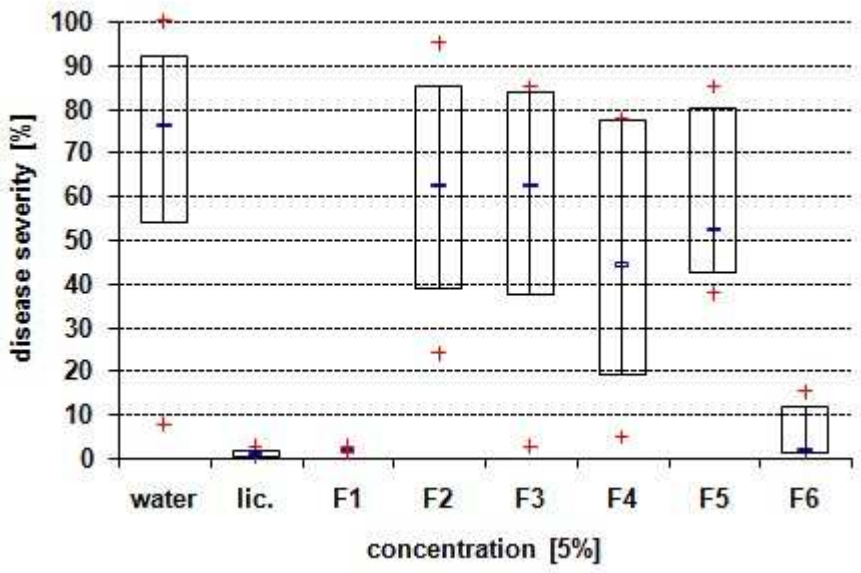


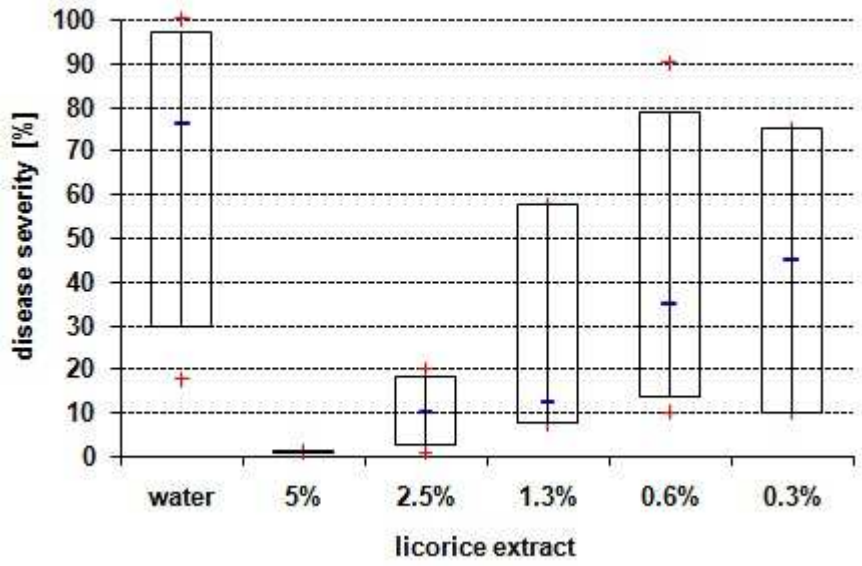
Figure 2. Influence of the extract of licorice (*G. glabra;* lic.) and its fractions (F1-F6) on the disease severity (Infection [%]) of *P. cubensis* on potted cucumber plants (cv. `Chinesische Schlange´) in a climate room (n= 4 – 6 per treatment in 10 independent assays). Box plot: Bar = median, crosses = min. and max.; boxes = 25th percentile and 75th percentile (within 50% of the data); whisker = ^{5th} percentile and 95th percentile

Fractions F4 (terpenoids and sterols) and F5 (waxes and lipids), which originated from F1 (Figure 1), reached efficacies of up to 41.8% and 31.1% efficacy, respectively. They were more effective than fraction F2 and fraction F3 (efficacy 18.0%, both). However, the highest activity of all fractions in controlling downy mildew on cucumber was achieved by fraction F6 (efficacy 97.6%) which also originated from fraction F1 (Figure 1). This fraction which contained acidic substances reached nearly the same efficacy as fraction F1 but the variation in infection levels between single plants was slightly higher (Figure 2).

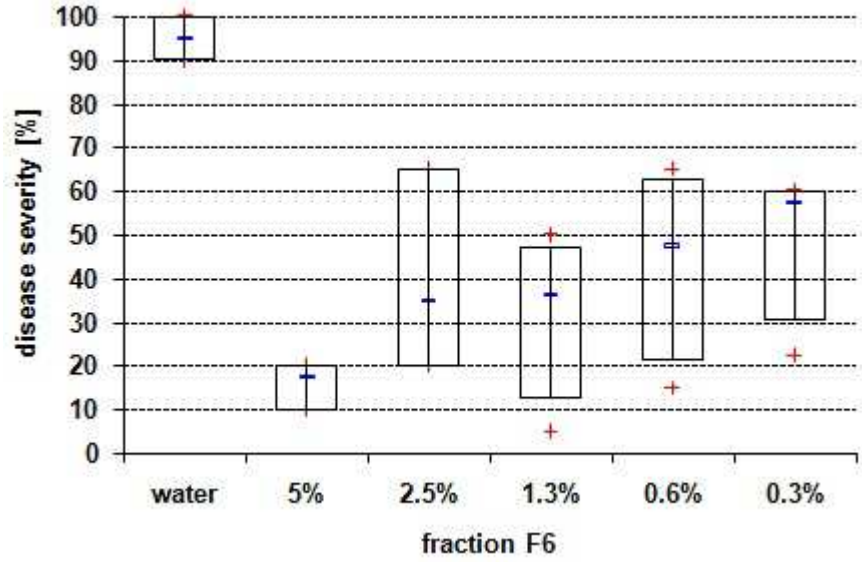
4.4.2. Bioassays and effective concentration of *G. glabra* extract and fraction F6

The data of bioassays with licorice (*G. glabra*) extract showed a strong dependency of disease control of downy mildew on the applied extract concentration (Figure 3a). An efficacy of 98.7% (disease severity 1.0%) was achieved by the 5% extract and 41.0% (disease severity 45.0%) by the 0.3% extract compared to the water treated plants

(disease severity 76.2%). The calculated EC_{50} value was 1.0% extract concentration (Table 1).



a)



b)

Figure 3. Influence of the extract of licorice (*G. glabra; a)* and the fraction F6 b) on the disease severity of *P. cubensis* on potted cucumber plants (cv. `Chinesische Schlange´) in a climate room (n = 6 -16 per treatment in 4 assays). Box plot: Bar = median, crosses = min. and max.; boxes = 25th percentile and 75th percentile (within 50% of the data); whisker = 5th percentile and 95th percentile

Also data of bioassays with fraction F6 from licorice extract showed a strong dependency of disease control of downy mildew on the applied extract concentration (Figure 3b). An efficacy of 81.6% (disease severity 17.5 %) was observed for 5% concentration and 39.5% (disease severity 57.5 %) for 0.3% concentration of this fraction. In water treated cucumber plants the infection level was 95.0%. The calculated EC_{50} value of fraction F6 was 0.6% extract concentration (Table 1).

An effect of the respective ethanol concentrations in the extracts on disease reduction was excluded in separate trials (data not shown).

Tabel 1. EC_{50} value, confidence interval and the slope of the curve resulting from probit analysis of a concentration series of licorice (*G. glabra*) leaf extract and fraction F6 against *P. cubensis* on cucumber.

	EC_{50}value	Confidence interval	Slope
Licorice extract	1.0	0.7-1.2	3.4
Fraction F6	0.6	0.2-1.1	0.9

The graphs resulting from probit analysis (Figure 4) of plants treated with concentration series of fraction F6 and crude licorice extract had different slopes (0.9 and 3.4, respectively) and a significant difference in the parallelism of curves, while position and total heterogeneity were not significantly different from each other (Table 2).

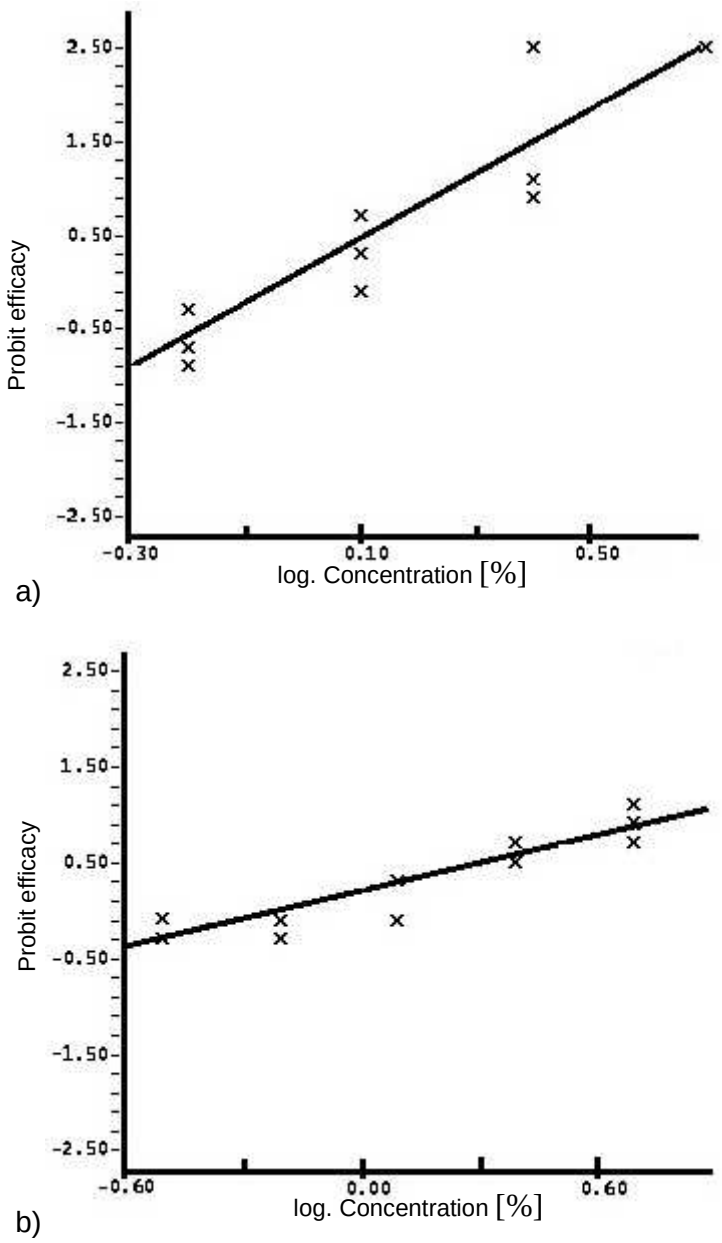


Figure 4. Graphs of the probit analysis of licorice leaf extract a) and the fraction F6 b) based on dose-responses against *P. cubensis* in cucumber

Table 2. Analysis of Chi square (χ^2) for position, parallelism and total heterogeneity of probit analysis of licorice (*G. glabra*) leaf extract and fraction F6 ($p<0.05$).

Analysis of χ^2	χ^2	D.F.
position	0.3	1.0
parallelism	13.9	1.0
total heterogenity	6.0	22.0

4.4.3 Bioassays with single sub-fractions from column chromatography of fraction F6

After column chromatography of fraction F6, a total of five sub-fractions could be separated (B1-B4 and remains) with the given solvent system. The respective R_f values were in the range of 0.4 to 0.65 (Figure 5). These sub-fractions were tested in 3% concentration on cucumbers against *P. cubensis*. For each bioassay separate column chromatographies were done. In both bioassays, a significant effect on the infection with downy mildew was shown for all sub-fractions compared to water treated plants. Water treated plants were infected to 75.0% and 92.5%, respectively. Furthermore, in both assays sub-fraction 3 was significantly less effective than the other sub-fractions 1 and 2 (Figure 6). Treatments with sub-fraction 1 kept disease severity at 6.3% (Figure 6a) and 3.1% (Figure 6b) and on the lowest level of all sub-fractions. In assay a) sub-fraction 4 could not be separated from remains (Figure 5a). However, in assay b) sub-fraction 4 as well as the remains led to a strong reduction (efficacy B4 89.2%, remains 77.0%) of downy mildew compared to water treated plants (disease severity 92.5%). Both were significantly more effective than sub-fraction 3 (Figure 6b).

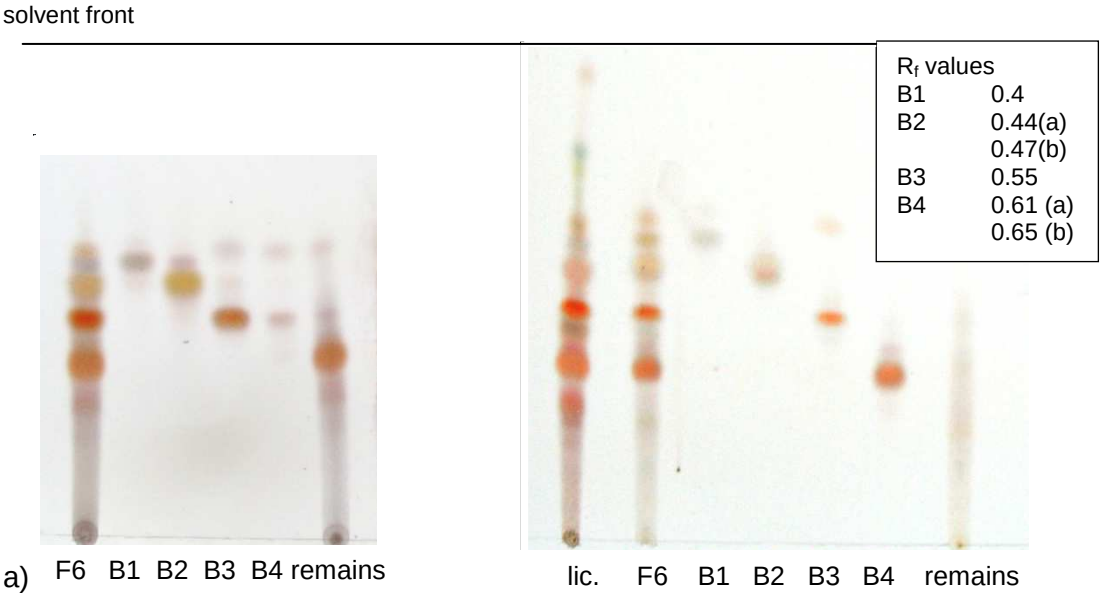
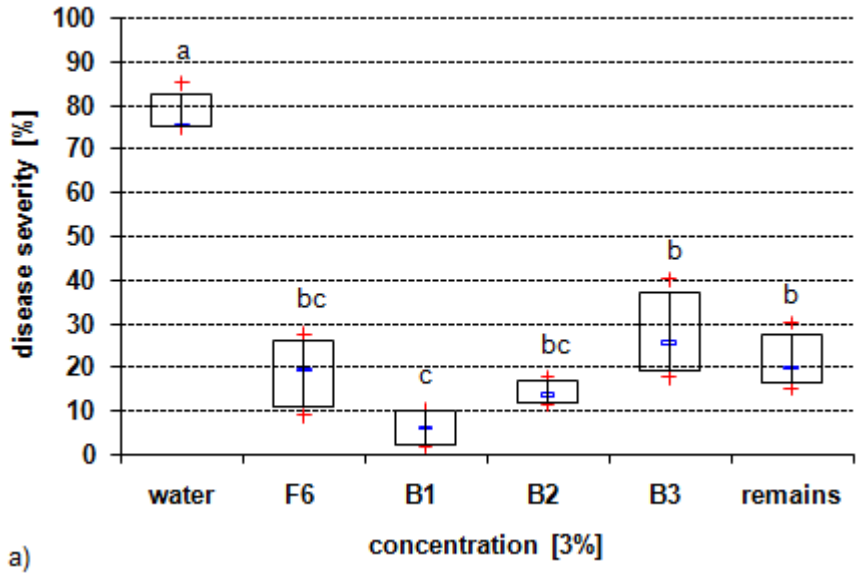
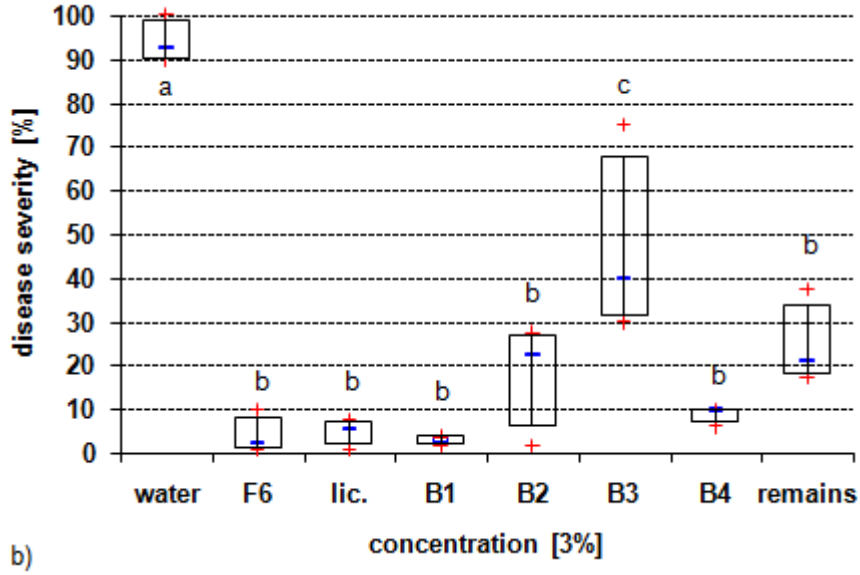


Figure 5. Thin layer chromatograms of separated sub-fractions (B1-B4 and remains; Rem.) resulting from two column chromatographies, (a) and (b), of fraction F6 from leaf extract of licorice (*G. glabra*). For comparison the leaf extract of *G. glabra* (lic.) and fraction F6 were used. Solvent system: Eluent: toluene: dioxan: acetic acid (20:5:1) on silica; Staining with vanillin-phosphoric acid

R_f = Retardation factor



a)



b)

Figure 6. Influence of fraction F6 (F6) from licorice (*G. glabra;* lic.) extract and its sub-fractions(B1-B4 and remains) on disease severity of *P. cubensis* on potted cucumber plants (cv. `Chinesische Schlange´) in two bioassays, a) and b), in a climate room (n= 8-12 per treatment in 4 randomized replications per assay). Different letters indicate significant differences according to Tukey test ($p< 0.05$) Box plot: Bar = median, crosses = min. and max.; boxes = 25th percentile and 75th percentile (within 50% of the data); whisker = ^{5th} percentile and 95th percentile

a) Mean disease severity per treatment out of median per replication: water 93.8%, F6 4%; lic. 4.9%,B1 2.1%, B2 18.6%, B3 46.3%, B4 9.1%, remains 24.4%

b) Mean disease severity per treatment out of median per replication: water77.5%, F6 18.8; B1 6.1%, B2 14.1%, B3 27.2%, remains 21.3

4.4.4. HPLC

The compounds obtained in sub-fractions of fraction F6 were analyzed by HPLC and compared to compounds present in the crude leaf extract of *G. glabra*. Only signals for sub-fractions B1, B2 and B3 were visible, while compounds from sub-fraction B4 and remains were not detectable. In sub-fraction B1 the main compound was identified as glabranin, in B2 the main compound was pinocembrin and the main compound of B3 was licoflavanon. Also, the chromatogram illustrated, that after fractionation of the crude extract the amount of compounds (resembled by the peak height) detectable in fraction F6 and the sub-fractions was notably lower (Figure 7).

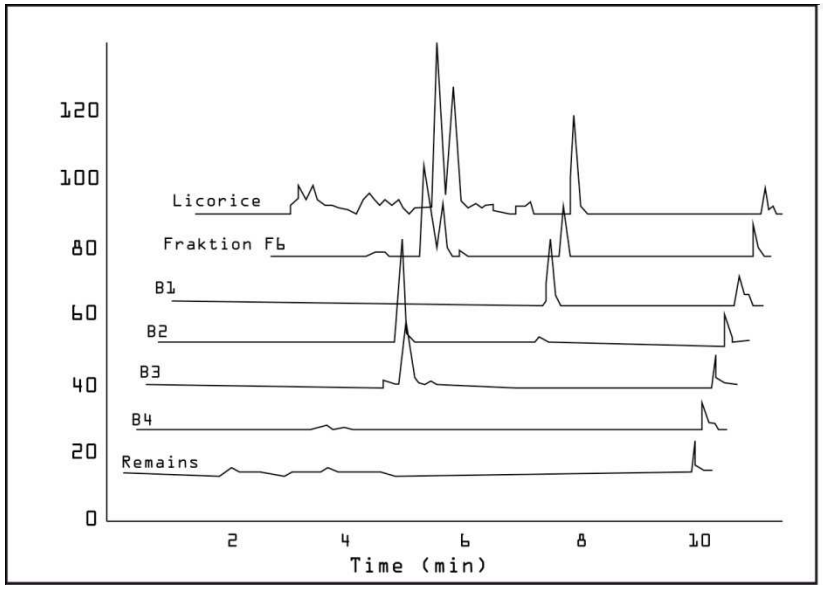


Figure 7. Chromatogram from HPLC of crude licorice (*G. glabra*) extract, fraction F6 and separated sub-fractions (B1-B4 and remains) of fraction F6.

4.5. Discussion

The results of bioassays with different fractions of crude extract from *G. glabra* leaves showed that the activity of the extract against downy mildew on cucumbers is caused by the activity of fraction F6, containing acidic compounds (Figure 2).

With HPLC three dominant compounds of the active fraction F6 could be detected. Those compounds belong to the group of polyphenols and were identified via 1H-NMR-spectroscopy as licoflavanon, pinocembrin and glabranin. Glabranin could be found in sub-fraction 1 (B1), which inhibited the development of downy mildew on cucumber in both trials to a high degree (efficacy assay a 91.7% and assay b 96.6%). The main

component of sub-fraction B2, which was also highly effective against the disease (efficacy 81.7% and 75.7%) was pinocembrin (Figure 6). Although disease levels after treatment with B2 were not significantly different to those after treatment with B1, sub-fraction B2 tended to be less effective. Sub-fraction B3, which contains licoflavanon, was the one with the lowest efficacy against *P. cubensis* from all (assay a 65.8% and assay b 56.8%). All three compounds are well known for their activity against fungi and other microorganisms in humans and plants (Shain and Miller 1982, Lester et al. 1983; Fukui et al. 1988; Fatima et al. 2009). However, none of those substances could be found in the root extract as it could be proven via HPLC and TLC in own experiments (data not shown) and by Hayashi et al. (1996) and therefore cannot be responsible for the impact on human pathogens. Treutwein et al. (2010) reached the same efficacy with a combination of licoflavanon, pinocembrin and glabranin as with crude licorice leaf extract in *in vitro* assays against *Phythophthora infestans.* Those data supported the results of Konstantinidou et al. (2008) and Schuster et al. (2010), which showed that *G. glabra* leaf extract is highly effective against *P. infestans* on tomato plants. Nevertheless, sub-fraction B4 and remains as well reduced the disease significantly compared to the control (Figure 6). Moreover, the infection level with downy mildew after treatment with sub-fraction B4 and remains was not significantly different to that of the highly effective sub-fractions B1 and B2 (Figure 6b). In HPLC, optimized for flavonoids, no peaks could be detected for sub-fraction B4 and remains. However, thin layer chromatography and subsequent bioassays proved that there are more than three active sub-fractions in fraction F6 (Figure 4). This leads to the assumption, that besides the detected three flavanoids also other compounds are present and responsible for the activity of crude licorice leaf extract and its fraction F6 against *P. cubensis* on cucumbers.

The comparison of graphs from probit analysis of dose-responses of plants treated with fraction F6 and crude extract showed a significant difference in their slopes. This observation clearly points to a different mode of action for the two treatments (Bliss 1939). It indicates that besides a direct effect on the pathogen induced resistance plays a role in the mode of action of licorice extract against *P. cubensis*. The compounds involved in the different modes of action may be identical or may differ from each other. In literature it is described that compounds of a single plant extract may interact with each other (Hummelbrunner et al. 2001, Seeram et al. 2004). Thus, it is possible that in crude licorice extract single compounds inhibit or amplify each other in their effects on the pathogen (in case of a direct mode of action) or on the plant (in the case of induced resistance as mode of action). A possible negative interaction between compounds in *G. glabra* leaf extract is supported by the results from the HPLC work. During

fractionation a considerable amount of detectable compounds of the sub-fractions B1-B3 was lost compared to the crude extract (Figure 7). Nevertheless, the EC_{50} value of fraction F6 was with 0.6% extract concentration lower than the EC_{50} value of the crude soxhlet extract of *G. glabra*, which was 1.0% extract concentration. This indicates a reduction in efficacy via inhibition by compounds in the crude extract, which are not present in fraction F6.

Moreover, compounds from sub-fractions B4 and remains were not necessary to resemble *in vitro* activity comparable to that of fraction F6 against *P. infestans.* This can be interpreted in such a way that unknown substances of fraction B4 and remains may play an important role for induced resistance. Otherwise, Strobel and Kuć (1995) described an resistance inducing effect of the fungicide Paraquat applied in low concentrations. Since the concentration of licoflavanon, pinocembrin and glabranin is approximately 50% lower in fraction F6 compared to the crude extract those three substances, which most contributed to the direct effect of licorice extract on *P. cubensis*, may also be responsible for the resistance inducing effect. However, via *in vitro* tests, like those of Treutwein et al. (2010), only the direct effect of a product against the pathogen can be investigated. The resistance inducing effect of an agent in contrast can only be investigated with bioassays on whole plants. The hypothesis of a resistance inducing effect of the crude licorice extract and its active fraction F6, therefore, needs to be proven in further trials.

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5. THE EFFECTS OF AN EXTRACT OF LICORICE LEAVES (*GLYCYRRHIZA GLABRA*) ON CUCUMBER PHOTOSYNTHETIC SYSTEM

To be published in Phytopathology

5.1. Abstract

Chlorophyll fluorescence (Fv/Fm) which is emitted after dark adaptation is an indicator for stress in plants. Treatment of potted cucumber plants with an extract of licorice leaves (*Glycyrrhiza glabra*) and its active fraction (F6, acidic substances) significantly increased the stress tolerance of highly downy mildew (*Pseudoperonospora cubensis*) infected plants (disease severity licorice=25%; F6=92.5%, Fv/Fm values 0.80 and 0.79) compared to water treated plants (disease severity 100%, Fv/Fm values 0.69). A moderate infection (disease severity 45.3%) with downy mildew did not stress the plants, independent of the treatment. Treatment with licorice extract furthermore elevated the chlorophyll content in tendency (water 8.2 mg/ g DW, licorice 10.2 mg/ g DW) and significantly increased anthocyanin content (licorice treated leaves 52.0 Absorbtion/ g DW, water treated leaves 45.4 Absorbtion/ g DW). Both pigments seem to be involved in the dark leaf coloration of licorice extract-treated cucumber plants, while elevated anthocyanin content could play a role in resistance induction by the plant extract.

Keywords:
Chlorophyll fluorescence, plant extract, chlorophyll, anthocyanins

5.2. Introduction

Downy mildew caused by the Oomycete *Pseudoperonospora cubensis*, is one of the major problems in organic cucumber production. An ethanolic leaf extract (3%) of the Fabaceae *Glycyrrhiza glabra* (licorice) showed high efficacies (up to 83.0%) in semi-commercial trials as well as in bioassays on potted cucumber plants (Scherf et al. 2010, see also Chapter 3).

In these bioassays besides the leaf extract its active fraction (acidic substances, fraction F6) exhibited a high potential of controlling downy mildew (*Pseudoperonospora cubensis*) on cucumbers (efficacy licorice extract 98.8% and fraction F6 97.6%). The results of probit analyses of concentration series of both agents led to the assumption that there are two different modes of action involved and that the activity of the licorice extract is not only based on its direct effect on the pathogen (Chapter 4). In this earlier work one of the main substances of the crude extract and its fraction F6 was identified as glabranin. Cespedes et al. 2001 showed that this molecule had a negative effect on the photosynthetic apparatus.

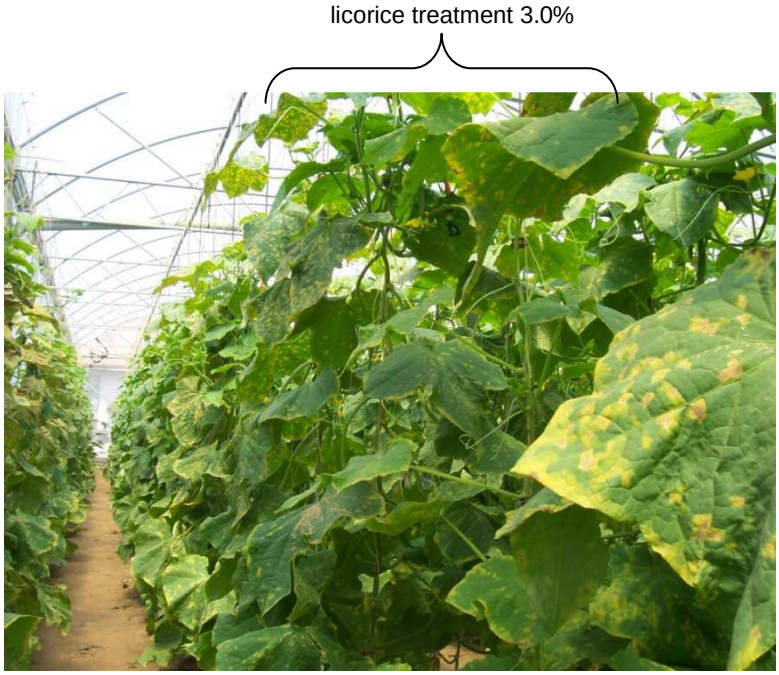


Figure 1. Cucumber plants in the semi-commercial greenhouse trial 2009. Licorice treated plants showed a dark green color

Furthermore, in the semi-commercial trials with the crude licorice extract two observations were made: (i) The cucumber plants treated with *G. glabra* appeared to have a darker green color (Figure. 1) compared to the control plants and (ii) a moderate

infection with downy mildew had no effect on the yield, not even in the water treated control plants (disease severity 52.0%) (data not shown).

Similar observation was done by Daayf et al. (1995) on plants treated with the plant strengthener Milsana®, a plant extract of *Fallopia sachalinensis*. Karavaev et al. (2002) reported that treatment with this plant extract not only induces resistance but also positively influences the photosynthetic system. The Milsana® treated plants showed an increase in chlorophyll content (Schmitt et al. 2005) and an influence on the chlorophyll fluorescence in light conditions (Karavaev et al. 2008). Because of the similarities of the changes in color of plants treated with *G. glabra* and Milsana®, the influence of the licorice extract on the chlorophyll fluorescence of cucumbers was investigated in this work.

The core of the photosystems II and I, which are located in the thylakoid membranes inside the chloroplasts, are two chlorophyll a molecules. Besides those molecules other chlorophylls as well as carotinoids function as antenna, to catch as much photons as possible. Like the central chlorophyll a those pigments act as electron donators, which are transferring the energy from one to another (Clayton 2002). In strong light, the system reaches a limit, where more photons are caught from the antenna in a given time than can be transported from one donor to the next acceptor (Demmig et al. 1988, Krause and Weis 1991, Maxwell and Johnson 2000). The energy, which cannot be used from the system, is emitted as heat or fluorescence. For emission of heat, zeaxanthin is necessary, which is only built by the plant under light conditions (Maxwell and Johnson 2000). Dark adapted plants emit the surplus of energy solely as fluorescence. Under dark conditions quinone, an electron acceptor downstream the central chlorophyll a of photosystem II is reduced and therefore no electrons can be transported (Krause and Weis 1991). This means photosystem II is “closed” and all measurable fluorescence is emitted by the antenna molecules (Krause and Weis 1991). The ratio Fv/Fm (Fv=variable fluorescence; Fm=maximum fluorescence) is discussed to reflect the proportion of well connected antenna molecules in a plant. This value is of high interest for ecophysiological questions, because in a healthy, unstressed plant it is always 0.83± 0.004, independent of age or genus (Krause and Weis 1991, Maxwell and Johnson 2000). The value decreases in any kind of stress situation (Krause and Weis 1991, Angelopoulos et al. 1996, Maxwell and Johnson 2000, Meyer et al. 2001). In an infected plant the Fv/Fm value drops normally before obvious symptoms can be observed (Berger et al. 2007)

In this study chlorophyll fluorescence measurement and particularly the ratio Fv/Fm was measured under dark conditions and used as a tool to investigate the influence of the *G. glabra* leaf extract and its active fraction F6 on plant stress imposed by downy

mildew infection, both with low and high infection intensity. To further investigate possible reasons for the dark green color of the licorice treated plants observed in the semi-commercial trials, the chlorophyll and anthocyanin contents of treated potted plants were measured.

5.3. Material and methods

5.3.1. Plant extract and extract fraction

The crude plant extract was produced from dried and ground leaves of *G. glabra* L. (Licorice) from Greece. The extraction was done with a Soxhlet extractor in 96% ethanol (Scherf et al. 2010, see also Chapter 3). The extract was concentrated to 50% concentration (w/v), i.e. the extract of 50 g plant powder was adjusted to a final volume of 100 ml.
The fractionation of the crude extract was done by a shakeout procedure, with different solvents and acidification as well as alkalinization (Scherf et al. 2010, see also Chapter 3). The fraction F6 (acidic substances) was adjusted to 50% extract concentration (Chapter 4) based on the volume of inserted crude licorice extract.

5.3.2. General set-up of bioassays on potted cucumber plants

For the bioassays cucumbers of the cultivar `Chinesische Schlange´ were cultivated in a mixture of sand with K 15 pot ground (Klasmann-Deilmann GmbH) in a proportion of 1:3. The plants were grown under a 16 hour light (333 µmol quanta μm^{-2} s^{-1}) and 8 hour dark regime at an average temperature of 23.5±2.7℃ and an average relative humidity of 49.5±15.0% until 2 leaves were well developed, the other leaves were pricked out. Both leaves were treated by spraying the agents on the lower surface until run-off. The crude *G. glabra* extract (50% concentration w/v) as well as the fraction F6 (50% concentration w/v) were diluted in de-ionized water to the final concentration of 3% (v/v).

Inoculation of the lower leaf surface was done a day after treatment with an aqueous sporangia suspension of *P. cubensis*. Fresh downy mildew sporangia were obtained by washing off the leaves of highly infected cucumber plants that were kept overnight at 100% relative humidity. The resulting suspension was counted in a Fuchs-Rosenthal counting chamber. After inoculation, the plants were incubated overnight at 100% relative humidity in the dark. Afterwards these plants were kept at an average

temperature of 18.3±2.0℃ and an average relative h umidity of 47.6±6.7%.
For all trials the plants were arranged in a randomized setting. In trial 1 all plants were arranged randomly in all other trials four replications with two plants per replication were arranged in a randomized block design.

5.3.3. Chlorophyll fluorescence measurement on potted plants

5.3.3.1. Trial1

The chlorophyll fluorescence was measured in trial 1 (high infection, inoculum $1.0*10^4$ sporangia/ ml) on 5 randomly arranged plants per treatment on 6 days with a Junior Pam (Walz GmbH). Measuring was done in the morning on dark adapted plants on six defined points of the second leaf (Figure 2) (saturation pulse: 1500 µmol quanta μm^{-2} s^{-1}). The measuring period was finished after 8 days because by then a high proportion of measuring points were infected on the second leaf, as well.
The trial was rated after 11 days by estimation of the percentage of infected leaf area on both leaves of each plant.

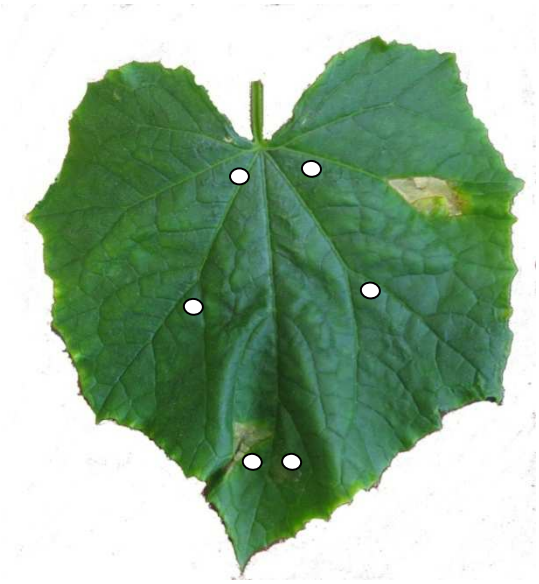


Figure 2. Measuring points for chlorophyll fluorescence measurement on a cucumber leaf (trial 1)

5.3.3.2. Trial 2

In trial 2 (moderate infection, inoculum $5.0*10^3$ sporangia/ ml) on 8 plants per treatment in 4 randomized replications (2 plants/replication) the chlorophyll fluorescence was measured the whole trial period (11 measuring days) with a Junior Pam (Walz GmbH). Measuring was done in the morning on dark adapted plants on four defined points per leaf (Figure 3) (Saturation impulse: 1500 µmol quanta μm^{-2} s^{-1}).

First and second true leaves per plant were measured. The trial was rated after 11 days by estimation of the percentage of infected leaf area on both leaves of each plant.

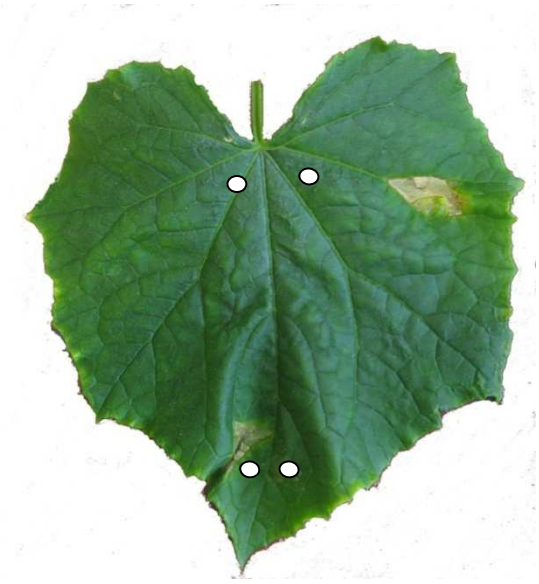


Figure 3. Measuring points for chlorophyll fluorescence measurement on a cucumber leaf (trial 2)

5.3.4. Chlorophyll and anthocyanin content measurement on cucumber plants

The cucumber plants were grown as described above. Three days after the first treatment the plants were treated a second time with licorice extract (3%), ethanol (6%) or water. The day after the second treatment the lower part of the leaf blades was inoculated (Figure 4) with an aqueous sporangia suspension ($3*10^3$ sporangia/ ml) and afterwards incubated as described above. The chlorophyll content was measured 14 days after inoculation with the chlorophyll meter SPAD 502 Plus (Konica-minolta) on 4 defined points on both leaves of 8 plants per treatment (Figure 4).

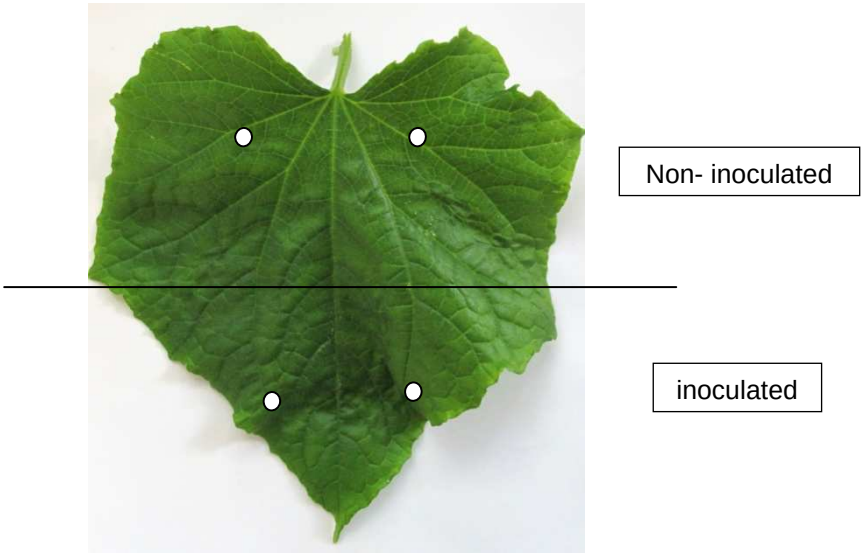


Figure 4. Measuring points for SPAD measurement on a cucumber leaf. Half of the leaf blade was

inoculated

The disease severity of the plants was rated by estimation of the infected leaf area two days before leaf pigment extraction.

The extraction of the leaf pigments was done from 4 leaf discs per plant (2 discs per leaf) cut from the non-inoculated area. The leaf discs of each leaf were extracted in 5 ml acidified methanol (1% HCl) and incubated for 4 hours at 4℃. After incubation the anthocyanin and chlorophyll content of the extract was measured at 532 nm and 652 nm wavelength (after Mancinelli 1983). The leaf discs were dried at 60℃. After 3 days the dry weight of the leaf discs was determined.

The chlorophyll content was then calculated after Arnon (1949):

µg/ g DW = (E_{652}*1000* sample (ml)/ 34.5)/ DW

DW = dry weight

spezific absorption coefficient for wavelength 652nm = 34.5

5.3.5. Statistical analysis

5.3.5.1. Chlorophyll fluorescence measurement

Out of the certain number of measuring points or the disease severity the median per plant based on the median per leaf was calculated for statistical analysis of each trial. In trial 2 the median per replication was than calculated out of the median per plant. For statistical analysis of the fluorescence data the measurements resulting from necrotic tissues were deleted. The Tuckey analysis for data of the last measuring day was run with WinStat for Excel 2009.1 (R. Fitsch software).

5.3.5.2. Pigment extraction

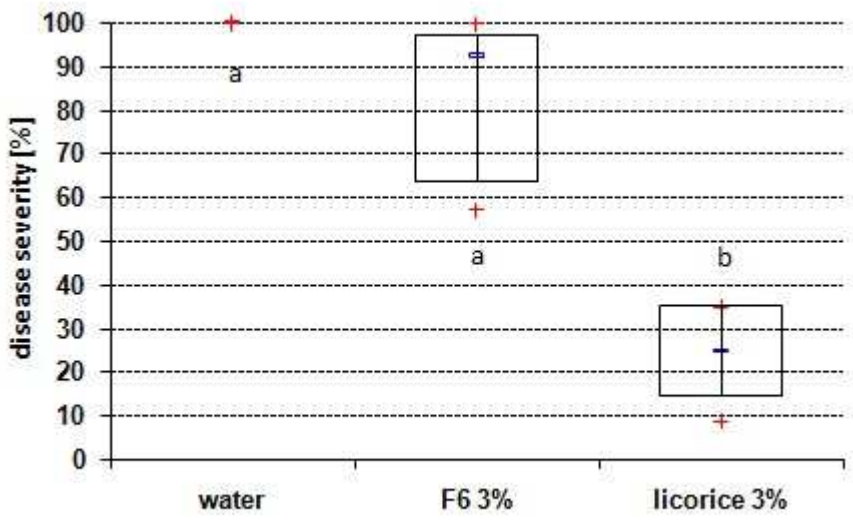
For statistical analysis of the photometric data of the pigment extracts, the measured absorbance per gram dry weight was calculated (Abs/ g DW). Afterwards a Tuckey analysis was run with WinStat for Excel 2009.1 (R. Fitsch software).

5.4. Results

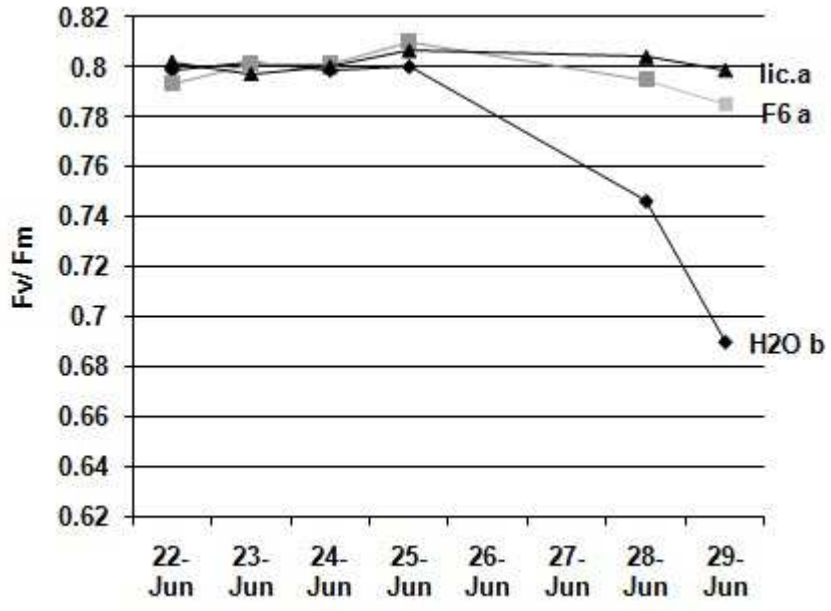
5.4.1. Chlorophyll fluorescence measurement

5.4.1.1. Trial 1

In the first trial the influence of the treatment with crude licorice extract and its fraction F6 on chlorophyll fluorescence was investigated on highly infected cucumber plants.
Eleven days after treatment rating of disease severity of plants was done. As shown in figure 5a water treated plants were 100% infected with *P. cubensis*. Also plants treated with fraction F6 were highly infected. The disease severity of those plants was in most cases above 80%. The infection levels of crude licorice extract treated plants showed a high variation. However, 50% of the plants had infection levels between 14.4% and 35.0%, which were significantly lower than that of water treated and fraction F6 treated plants (disease severity 100% and 92.5% respectively) (Figure 5a).
The results of chlorophyll fluorescence measurement of the first bioassay showed that in water treated plants Fv/Fm started to decrease after the fourth day after inoculation. After 8 days Fv/Fm was significantly decreased from 0.80 to 0.69 (Figure 5b). The Fv/Fm value of the crude licorice or fraction F6 treated plants fluctuated around 0.80 during the whole measuring period. Measurements were stopped due to the intense necrotization of leaf tissue after 8 days.



a)



b)

Figure 5. Trial 1 under high disease pressure

a) Influence of extracts of licorice (*G. glabra*) and its fraction F6 (acidic substances) on disease severity [%] with *P. cubensis* on potted cucumber plants (cv. `Chinesische Schlange´) in a climate room (n = 5) after 11 days. Bar = median, crosses = min. and max.; boxes = 25th percentile and 75th percentile (within 50% of the data); whisker = 5th percentile and 95th percentile. Statistical analysis via Tukey test ($p<0.05$); Significant differences are labeled with different letters. Mean diesease severity per treatment out of median per plant: water 100%, fraction F6 83%, licorice 25%

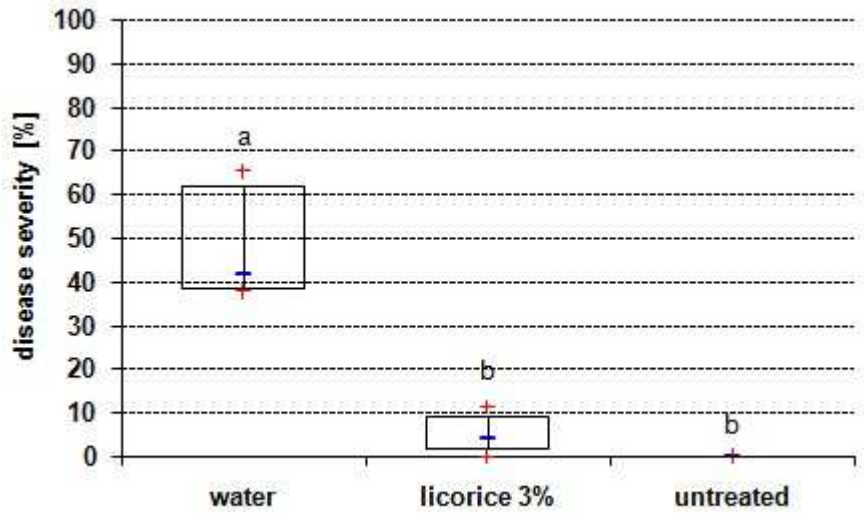
b) Fv/Fm values (medians per treatment) of dark adapted cucumbers in the first week of the same trial. Statistical analysis via Tukey test ($p<0.05$); Significant differences are labeled with different letters. Mean Fv/Fm value per treatment out of median per plant on the last measuring day: water 0.65, fraction F6 0.77, licorice 0.80

5.4.1.2. Trial 2

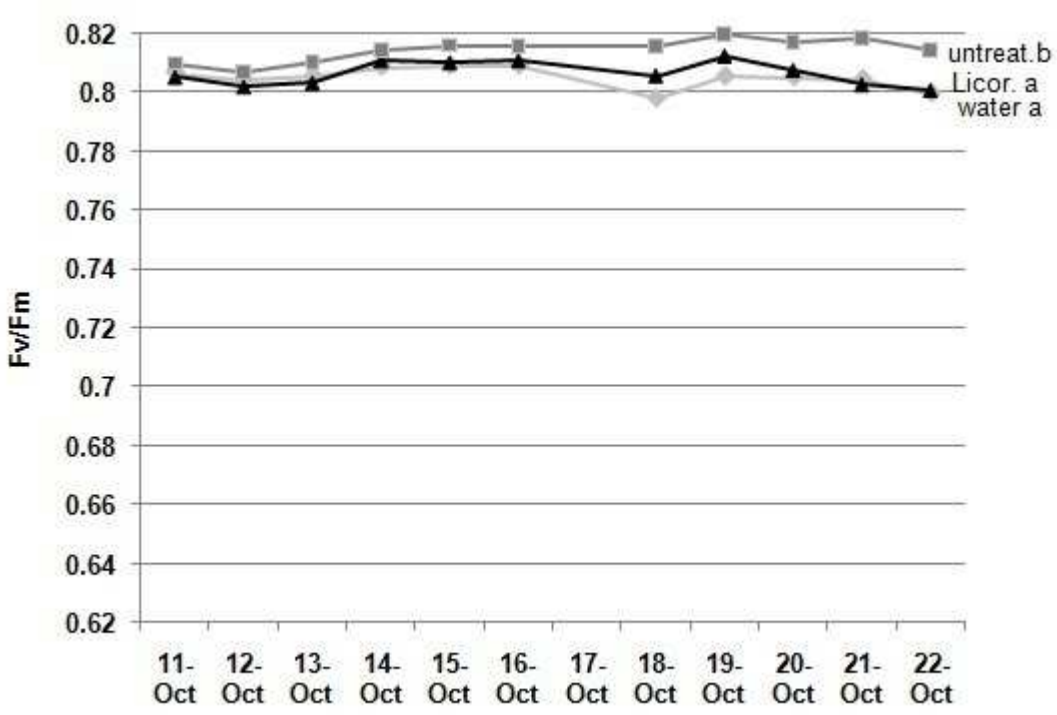
In trial 2 the influence of a moderate infection on the stress indicator Fv/Fm was measured in water treated and licorice extract treated plants and compared to untreated, uninfected plants.

In contrast to the first trial, the infection of water treated plants was only moderate 11 days after inoculation, with 50% of the plants showing a disease severity of *P. cubensis* between 39.2% and 60.3%. The infection of plants treated with licorice extract was low. Fifty percent of the plants showed a disease severity between 2.9% and 6.3% (Figure 6a).

At the same time the Fv/Fm value of water treated as well as licorice extract treated plants was high (0.80 both). However, those values were slightly, but significantly, lower than the values of the untreated and uninfected control (0.81) (Figure 6b). The values of all variants were rather constant over the trial period.



a)



b)

Figure 6. Trial 2 under moderate disease pressure

a) Influence of extracts of licorice (*G. glabra*) on disease severity [%] with *P. cubensis* on potted cucumber plants (cv. `Chinesische Schlange´) in a climate room (n = 8) after 11 days. Bar = median, crosses = min. and max.; boxes = 25th percentile and 75th percentile (within 50% of the data); whisker = 5th percentile and 95th percentile. Statistical analysis via Tukey test ($p<0.05$); Significant differences are labeled with different letters. Mean disease severity per treatment out of median per replication: water 48%, licorice 5%, untreated 0%

b) Fv/Fm values (medians per treatment) of dark adapted cucumbers of the same trial.

Statistical analysis via Tukey test ($p<0.05$); Significant differences are labeled with different letters. Mean Fv/Fm value per treatment out of median per replication on the last measuring day: water 0.80, licorice 0.80, untreated 0.81

5.4.2. Chlorophyll and anthocyanin content in potted plants

Two protective treatments with licorice extract were compared to water and ethanol treatment.

At the end of the trial water treated and ethanol treated plants showed a moderate infection with downy mildew on the inoculated part of the leaves (disease severity 52.5% and 64.4%, respectively), while most of the licorice extract treated plants remained uninfected (Figure 7). Chlorophyll and anthocyanin contents were measured from plant tissue of non-inoculated cucumber leaf bases (Figure 4).

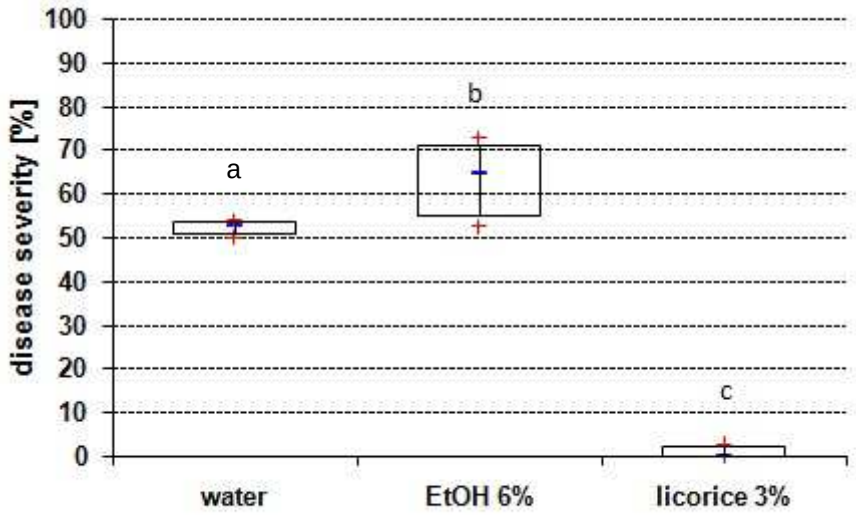


Figure 7. Influence of the extracts of licorice (*G. glabra*) on the disease severity [%] with *P. cubensis* on potted cucumber plants (cv. `Chinesische Schlange´) in a climate room (n=16 leaves). Bar = median, crosses = min. and max.; boxes = 25th percentile and 75th percentile (within 50% of the data); whisker = 5th percentile and 95th percentile. EtOH= ethanol. Statistical analysis via Tukey test ($p<0.05$); Significant differences are labeled with different letters.

Mean disease severity per treatment out of median per replication: water 52%, ethanol 63% and licorice 1%.

Chlorophyll content estimation via SPAD 502 Plus did not show great differences between the three treatments (water, EtOH and licorice extract) after 14 days. Nevertheless, there seemed to be a tendency for an increase in chlorophyll content in licorice treated compared to water treated plants. The median of licorice extract treatment was approximately 4 units higher (Figure 8).

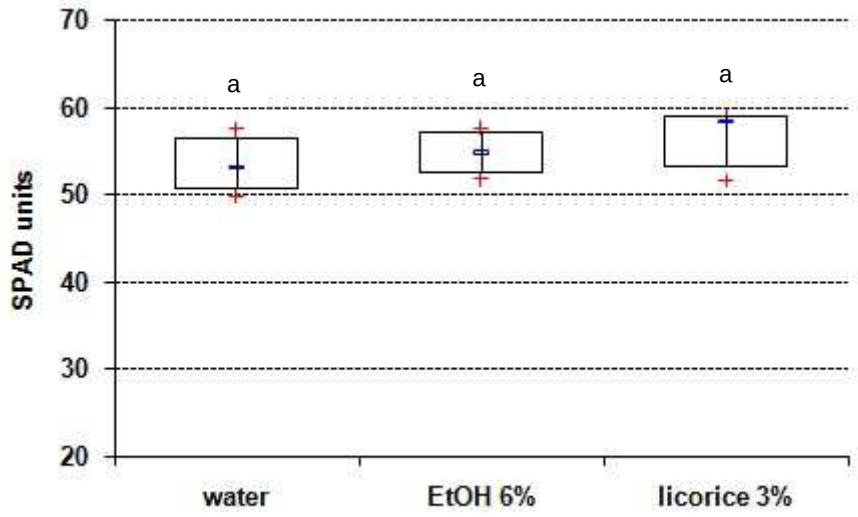


Figure 8. Influence of the extract of licorice (*G. glabra*) on the chlorophyll content of *P. cubensis* infected potted cucumber plants (cv. `Chinesische Schlange´) in a climate room (n= 16 leaves), measured with SPAD 502 Plus at the end of the trial. -EtOH= ethanol, Bar = median, crosses = min. and max.; boxes = 25th percentile and 75th percentile (within 50% of the data); whisker = 5th percentile and 95th percentile. Statistical analysis via Tukey test ($p<0.05$); Significant differences are labeled with different letters. Mean SPAD unit per treatment out of median per replication: water 53, ethanol 55, licorice 57

The basal, non-inoculated parts of the leaves were used for pigment extraction. Measurements were conducted at two wavelengths, 652 nm and 532 nm, in order to determine chlorophyll and anthocyanin contents, respectively. No significant difference in chlorophyll content between the licorice, ethanol and water treated plants was detected. However, the licorice treated plants showed a clear increase in chlorophyll content (10.2 mg/ g DW) compared to water treated and ethanol treated plants (8.4 mg/ g DW and 8.2 mg/ g DW) (Figure 9).

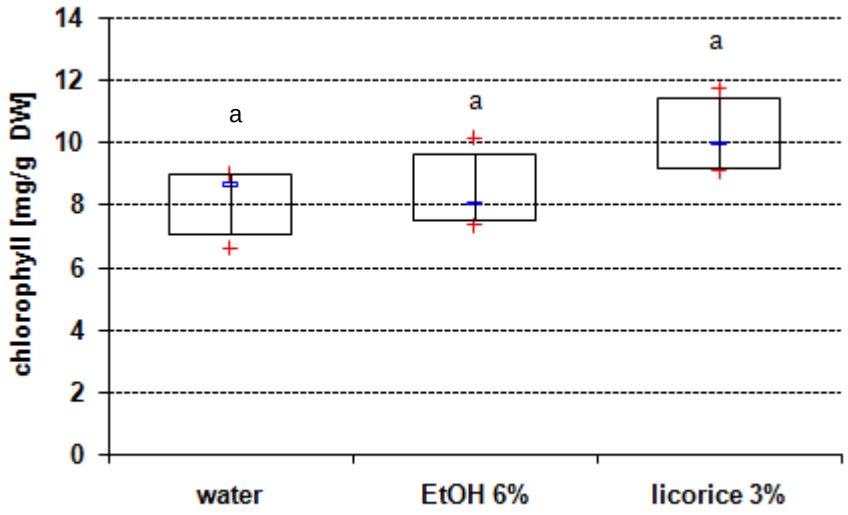


Figure 9. Influence of the extracts of licorice (*G. glabra*) on the chlorophyll content of *P. cubensis* infected potted cucumber plants (cv. `Chinesische Schlange´) in a climate room (n= 16 leaves). Wavelength=652nm, EtOH= ethanol, Bar = median, crosses = min. and max.; boxes = 25th percentile and 75th percentile (within 50% of the data); whisker = 5th percentile and 95th percentile. Statistical analysis via Tukey test ($p<0.05$); Significant differences are labeled with different letters. Mean mg/ g DW per treatment out of median per replication: water 8.2 mg/ g DW, ethanol 8.4 mg/ g DW, licorice 10.2 mg/ g DW

At the same time, licorice treated plants contained significantly more anthocyanin (52.0 Abs/ g DW) than ethanol treated plants (37.8 Abs/ g DW). The anthocyanin content of water treated cucumbers (45.4 Abs/ g DW) was not significantly different from that of ethanol or licorice extract treated plants (Figure 10).

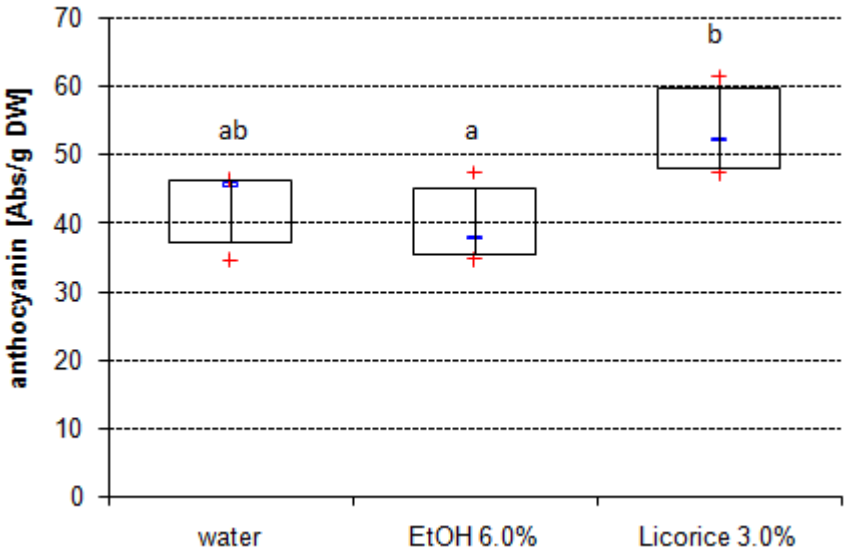


Figure 10. Influence of the extract of licorice (*G. glabra*) on the anthocyanin content of *P. cubensis* infected potted cucumber plants (cv. `Chinesische Schlange´) in a climate room (n=16 leaves). Wavelength= 532nm, Bar = median, crosses = min. and max., boxes = 25th percentile and 75th percentile (within 50% of the data); whisker = 5th percentile and 95th percentile. EtOH=ethanol. Statistical analysis via Tukey test ($p<0.05$); Significant differences are labeled with different letters. Mean Abs/ g DW per treatment out of median per replication: water 42.9 Abs/ g DW, ethanol 39.4 Abs/ g DW, licorice 53.1 Abs/ g DW

5.5. Discussion

In two bioassays the influence of licorice leaf extract and its active fraction F6 (acidic substances) on the photosynthetic system of downy mildew (*P. cubensis*) infected cucumber plants was investigated via chlorophyll fluorescence measurement. In addition, the influence of licorice extract treatment on chlorophyll and anthocyanin contents was determined.

In the first trial, effects were investigated on cucumber plants under high infection intensity. Since the chlorophyll fluorescence value Fv/Fm (approximately 0.83 in unstressed plants) is an indicator for any kind of stress in plants (Pedros et al. 2008, Veres et al. 2009) it was assumed that the Fv/Fm value would decline strongly in highly infected plants. This was true for water treated control plants. Fv/Fm values of those plants (median disease severity 100%) dropped significantly between the fourth and sixth day after inoculation from 0.80 to 0.69 (Figure 5b). Plants treated with fraction F6 were also highly infected (median of 92.5%) at the end of the trial (Figure 5a) but Fv/Fm remained at levels of 0.79 and 0.80 in this period (Figure 5b). This implies that fraction F6 influenced the stress-tolerance of plants in a positive way, even when highly infected with *P. cubensis*. The infection of plants treated with crude licorice extract was far less at the end of the trial, where 50% of the plants showed infection levels from 14.4% to

35.0%. The Fv/Fm values of those plants were fluctuating around 0.80 during the whole measuring period (Figure 5b). This infection level caused no stress in the plants. This result fits an observation made in semi-commercial trials, in which a moderate infection with cucumber downy mildew (disease severity 52.0%) did not result in yield decrease (Scherf et al. 2010, see also Chapter 3). This would indicate that only infection levels of *P. cubensis* above a certain threshold impose stress to the plants. To test this hypothesis and observation the design of the second trial of this study was adapted. In this trial plants were kept under moderate infection pressure and the effect on chlorophyll fluorescence was compared between plants treated with licorice leaf extract, water treated and untreated, uninfected plants. The results matched the observations made in the semi-commercial trials.

Fv/Fm values of the untreated, uninfected plants fluctuated around 0.81 during the whole trial period of two weeks (Figure 6b). This value resembles that of unstressed, healthy plants. The infection of *G. glabra* treated plants was low (disease severity between 2.9% and 6.3%; Figure 6a). This was reflected in Fv/Fm values between 0.80 and 0.81 during the whole trial period (Figure 6b). The infection of water treated plants was moderate (median disease severity 45.3%) but significantly higher than that of licorice extract treated plants (Figure 6a). However, Fv/Fm in water treated plants at the end of the trial was also 0.80 and thus did not differ from the values after licorice extract treatment (Figure 6b). This supports the above mentioned observation in the semi-commercial trials. Moderate infection with *P. cubensis* does not result in measureable plant stress.

The results of the first trial furthermore support results of former bioassays on cucumbers, which compared probit analyses of concentration series of the crude extract and fraction F6. The resulting regression curves had significantly different slopes (Chapter 4). This led to the assumption that the mode of action of fraction F6 may be induced resistance. Most likely the mode of action of the crude licorice extract is a combination of direct effects on the pathogen and resistance inducing effects. Also the results of the present trial 1 can be interpreted by the interaction of two different modes of action. The resistance inducing effect of fraction F6 obviously could not withstand the high disease pressure, so that plants showed a disease severity of 92.5%. The lower infection of the licorice treated plants was due to the antifungal effect of the extract on the pathogen. However, these plants still had a relatively high infection level (median disease severity 25.0%) compared to other bioassays shown in this study (Chapter 4-6), indicating that under high disease pressure also the direct fungicidal activity was less effective. Negative effects of high pathogen pressure on the efficacy of biological control agents is a known phenomenon as described by Daayf et al. (1995) and Berger

et al. (1996).

Again, the positive effect of the crude licorice extract became obvious from the results of chlorophyll content measurement. The SPAD analysis showed a tendency for chlorophyll content to increase in licorice treated compared to water treated leaves (licorice extract approximately 4 units higher; Figure 8). Also, elevated chlorophyll content was detectable in the leaf pigment extract (water 8.2 mg/ g DW, licorice 10.2 mg/ g DW, Figure 9). Furthermore, the anthocyanin content was considerably higher in licorice treated leaves (licorice 52.0 Abs/ g DW, water 45.4 Abs/ g DW; Figure 10). The elevated anthocyanin content is interesting in the context of resistance induction. Lev-Yadun and Gould (2009) suggest a potential of anthocyanins, to protect plants from pathogen infection by their known antiviral, antibacterial and fungicidal activities (Konczak and Zhang 2004, Wrolstad 2004). Thus, this effect may play a role in the licorice extract induced defense mechanisms of the plant. Overall, both leaf pigments seemed to be responsible for the darker leaf coloration of *G. glabra* extract treated plants. Nevertheless, the darker leaf appearance was more intense in the semi-commercial trial than in bioassays, which may be explained by a long term effect caused by the weekly treatment over a period of 8 weeks (Scherf et al. 2010, see also Chapter 3). Since all plants in bioassays as well as in semi-commercial trials respectively had comparable growing conditions, fertilization and light and so on, the darker coloration must have been due to licorice treatment.

The results of the trials presented in this part of the study clearly point to the positive effect of the crude *G. glabra* extract and its active fraction F6 on stress tolerance of treated cucumber plants. Furthermore, the present results also support the assumed resistance inducing effect of the licorice extract and fraction F6.

To stabilize the efficacy of the alternative agent *G. glabra* extract under high pathogen pressure, it is necessary to further investigate the mode of action.

5.6. Literature

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6. Indications for resistance induction by Glycyrrhiza glabra (licorice) leaf extract in cucumbers

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6.1. Abstract

In this study the hypothesis, that induced resistance plays a role in the mode of action of an ethanolic leaf extract of *G. glabra* (licorice) and its active fraction F6 (acidic substances) against cucumber downy mildew (*P. cubensis*), was investigated.
Treatment with licorice extract and fraction F6 (concentration 3% both) strongly inhibited the germination of *P. cubensis* zoospores. The germ tube length on water treated cucumber plants was 17.3-26.6 µm, whereas germ tube length on licorice treated plants was 1.6-6.4 µm. In non-infected cucumber leaf discs it was shown by DAB-staining that treatment with licorice extract and fraction F6 led to an oxidative burst of H_2O_2. Together, these results support the assumption of a resistance inducing effect by the treatments. Strongest evidence for induced resistance gave the analysis of the expression of the antifungal, pathogenesis related protein PR-1. The mRNA of this protein was up-regulated approximately 4 times in licorice treated plants compared to water treated plants.
The results indicated that in addition to the known direct effect of licorice extract on *P. cubensis* induced resistance is a second mode of action.

6.2. Introduction

The infection with *Pseudoperonospora cubensis* (downy mildew) causes major problems and yield losses in organic cucumber production.
In former semi-commercial trials an ethanolic extract of *Glycyrrhiza glabra* (licorice) leaves reached high efficacies (up to 83.0%) against the pathogen (Scherf et al. 2010, see also Chapter 3). The results of bioassays on potted cucumber plants infected with *P. cubensis* indicated that induced resistance may play a role in the mode of action of the licorice extract. A probit analysis of concentration series of the crude extract and its active fraction (acidic substances, F6) clearly showed two different modes of actions. Based on this, it was assumed that the known direct effect (Schuster et al. 2010) of the crude extract is accompanied by a resistance inducing effect. The latter seems to be the

mode of action of fraction F6. In further trials the crude extract as well as fraction F6 had positive effects on the stress indicator Fv/Fm even of highly downy mildew infected cucumber plants (Chapter 5).

Resistance inducing effects are reflected in physiological changes in a treated plant. Those changes are comparable to the mechanisms started in a host plant after infection with a pathogen.

The thickenings of cell walls (Hammerschmitt et al. 1982, Tuzun and Somanchi 2006) as well as the programmed cell death at the infection site are physical barriers against further spread of the pathogen in the infected plant. Often a burst of reactive oxygen species (ROS), like H_2O_2, is the first signal (Chamnongpol et al. 1998; Lin and Ishii 2009) followed by an up-regulation of enzymes, like peroxidases or glucanases, or of metabolites with further signal function (e.g. salicylic acid, jasmonic acid) (Kuć 2006). Some of those substances are known as pathogenesis related proteins (PR proteins) (Tuzun and Somanchi 2006). The antifungal PR-1 proteins have been reported as indicator for defense mechanisms associated with the salicylic acid pathway (Yalpani et al. 1991, Raskin 1992; Yalpani et al. 1993). Inducible resistance is very interesting for use in biocontrol of pathogens in commercially grown crops and vegetables, especially with respect to avoiding resistance of pathogens against chemical control agents and the general reduction of pesticides introduced to the environment. A prominent example of a plant extract, which induces a variety of defense mechanisms, is the plant strengthener Milsana® (Herger and Klingauf 1990, Daayf et al. 1995, Müller 2002).

In order to test the hypothesis that *G. glabra* extract induces resistance in cucumber plants, in this study the effect of licorice leaf extract and its active fraction F6 on production of H_2O_2 and PR-1 mRNA as well as on germination of *P. cubensis* zoospores on cucumber leaves was investigated.

6.3. Material and methods

6.3.1. Plant extract and extract fraction

The crude plant extract was produced from dried and ground leaves of *G. glabra* L. (Licorice) from Greece. The extraction was done with a Soxhlet extractor in 96% ethanol (see Scherf et al. 2010, see also Chapter 3). The extract was concentrated to 50% concentration (w/v), i.e. the extract of 50 g plant powder was adjusted to a final volume of 100 ml.

The fractionation of the crude extract was done by a shakeout procedure, with different solvents and acidification as well as alkalinization (see Scherf et al. 2010, see also

Chapter 3). The fraction F6 (acidic substances) was adjusted to 50% extract concentration (Chapter 4) based on the volume of inserted crude licorice extract.

6.3.2. Bioassays on potted cucumber plants

For the bioassays cucumbers of the cultivar `Chinesische Schlange´ were cultivated in a mixture of sand with K 15 pot ground (Klasmann-Deilmann GmbH) in a proportion of 1:3. The plants were grown under a 16 hour light (333 µmol quanta μm^{-2} s^{-1}) and 8 hour dark regime at an average temperature of 23.5±2.7℃ and an average relative humidity of 49.5±15.0% until 2 leaves were well developed, the other leaves were pricked out.

6.3.3. Germ tube length of *P. cubensis* zoospores on licorice extract treated cucumber leaf discs

For this assay, 9 plants per treatment were grown as described above. Both leaves were treated by spraying the agents (water and 3% licorice extract) on the lower surface of the leaves until run-off. From both leaves of a plant 2 leaf discs (4 per plant) were cut with a cork borer (Ø 1 cm) the day after treatment. Discs were placed with the lower surface up on Petri dishes with water agar (0.5%) and were inoculated with one drop (20 µl) of inoculum suspension ($1*10^4$ sporangia/ ml) containing 70% emerged zoospores. Fresh downy mildew sporangia were obtained by washing off the leaves of highly infected cucumber plants that were kept overnight at 100% relative humidity. The resulting suspension was counted in a Fuchs-Rosenthal counting chamber. The plates were incubated for 9.5 hours at 20℃ in the dark. After incubation leaf discs were stained with the fluorescence dye Blankophore (0.01% in 0.1 M Tris buffer pH 9.0, Ullrich et al. 2009) by dropping the dye with a Pasteur pipette on the discs until they were completely covered. After 5 minutes the dye was washed off by rinsing demineralized water with a Pasteur pipette over the discs. Germinating zoospores on the leaf discs were observed with a microscope (Axioscop 2, Zeiss) and photographed with a Microscope camera (Moticam 2300) (four hundred fold magnification). Afterwards the length of germ tubes was measured on the photographs using the software Motic Images 2.0.

6.3.4. Detection of H_2O_2 in extract treated cucumber leaf discs

Cucumber plants were grown as described above. Leaf discs (Ø 1 cm, 12 or 16 per leaf) of the second true leaf of 10 plants were cut with a cork borer and stored for one night in 0.1% MES buffer pH 6. The next day the discs were transferred into flasks containing licorice extract 3%, fraction F6 3%, EtOH 6%, Milsana® 1% or water. For eight hours, every hour a sample was taken for each treatment (1 disc per plant). These leaf disks were transferred to a solution of 1 mg ml^{-1} 3,3-diaminobenzidin (DAB) HCL, pH 3.8 (Thordal-Christensen et al. 1997) and were incubated 3 hours at room temperature. After staining, the discs were bleached in a mixture of trichloroacetic acid 0.15 g, EtOH 80 ml and $CHCl_3$ 20 ml. The bleaching solution was exchanged against a fresh preparation on the following day and after two days of bleaching the leaf discs were stored in 70% ethanol (Walz and Simon 2009).

6.3.5. Analysis of the expression of PR-1 gene in extract treated cucumbers

Cucumbers (9 plants per treatment) were grown as described above. Both leaves were treated by spraying the agents (water and 3% licorice extract) on the lower surface of the leaves until run-off. Twenty four hours after treatment leaf discs (4 per leaf) of both leaves per plant were cut with a cork borer (Ø 1 cm). Discs of three plants were pooled. Genomic DNA extraction for the standardized dilution series was done with DNeasy plant Mini Kit.

RNA extraction was done with Nucleo Spin RNA plant Kit (Macherey-Nagel) following the protocol. The transcription of mRNA in cDNA was done with iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc.), following the protocol. For realtime qPCR the SyberGreen Master Mix Kit (Fermentas GmbH) was used. The qPCR was run with PR-1 (DQ641122) und actin (AAZ7466) primer pairs from Mycrosynth AG. Actin was used as reference. Sequence data of the primer pairs can be found in the GenBank/EMBL data libraries. Both, primer pairs and the following qPCR protocol were taken from the work of Xia et al. (2009).

Lid Mode: 100.0℃
Denaturation at 95.0℃ for 3 min
Denaturation at 95.0℃ for 30 sec
Annealing at 58.0℃ for 30 sec
Extension at 72.0℃ for 30 sec → 40x
Incubation at 72.0℃ for 7 min
Melting Curve from 50.0℃ to 95.0℃; read every 0.5 ℃
Hold 10 sec between reads
Incubation at 10.0℃ ∞

6.4. Results

In order to test the hypothesis that *G. glabra* extract induces resistance in cucumber, the effect of licorice leaf extract and its active fraction F6 on H_2O_2 and PR-1 production as well as the effects on germination of *P. cubensis* zoospores were investigated.

6.4.1. Germ tube length of *P. cubensis* spores on cucumber leaf discs treated with licorice extract

Germ tubes of downy mildew zoospores were much shorter on licorice extract or fraction F6 (concentration 3% both) treated cucumber leaf discs than on leaf discs of water treated plants, 8 hours after inoculation (Figure 1).
On water treated leaf discs the length of 50% of germ tubes was in the range of 17.3 µm to 26.6 µm, whereas the length of 50% of germ tubes on licorice treated samples varied between 1.6 µm and 6.4 µm (Figure 2, see boxes). Because of the discrepancy in numbers of counted germ tubes on water treated (n=36) and licorice treated (n=10) leaf discs, analysis of statistical significance was not done.

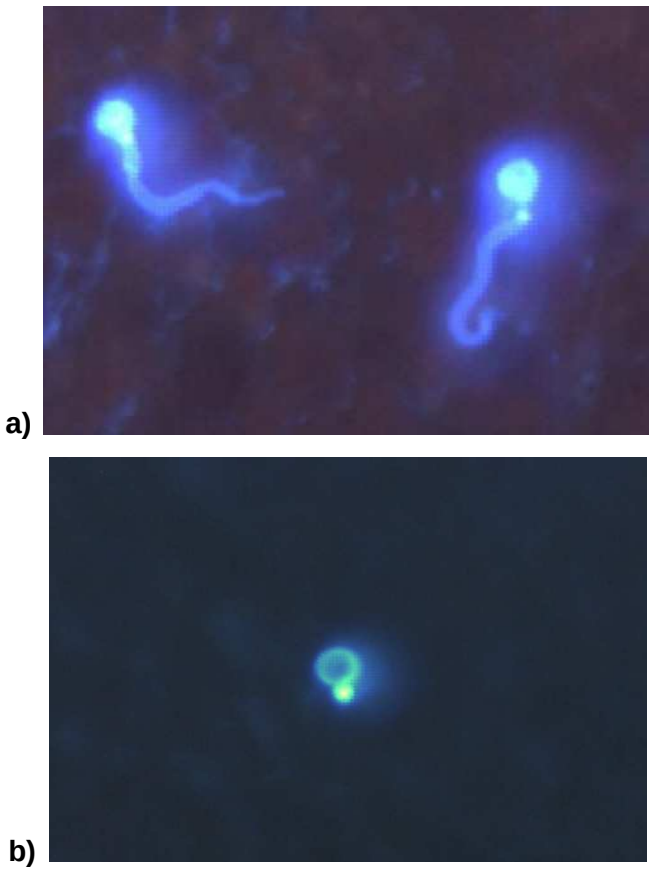

Figure 1. Germinating zoospores of *Pseudoperonospora cubensis* on cucumber leaf discs from plants treated with a) water and b) licorice extract, 8 hours after inoculation. Staining with Blankophore 0.1%, magnification: 400 fold.

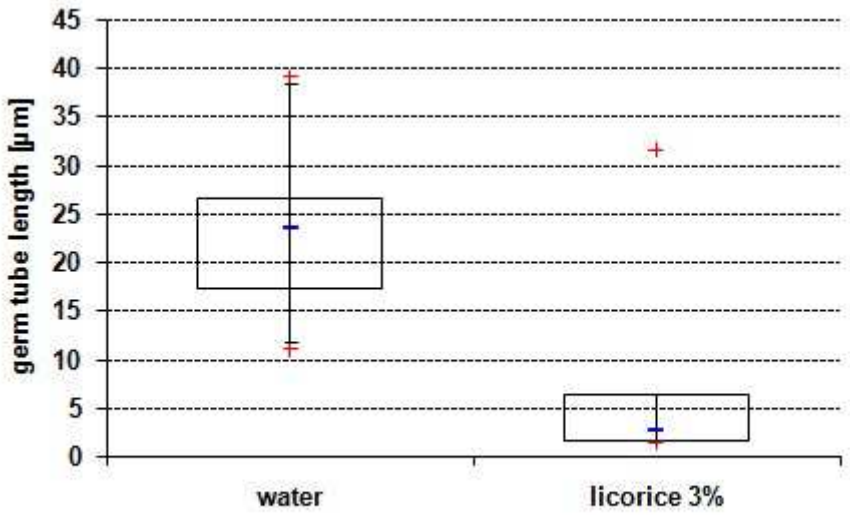

Figure 2. Influence of extract of licorice (*G. glabra*) on germ tube length of *P. cubensis* zoospores on leaf discs of treated cucumber plants (cv. `Chinesische Schlange´) 8 hours after inoculation. Bar = median, crosses = min. and max.; boxes = 25th percentile and 75th percentile (within 50% of the data); whisker = 5th percentile and 95th percentile.

6.4.2. Detection of H_2O_2 in extract treated cucumber leaf discs

In cucumber leaf discs treated with licorice extract, fraction F6 (concentration 3% both) and Milsana® (concentration 1%), development of H_2O_2 could be detected. Licorice extract and fraction F6 treated leaf discs showed in most samples a homogenous golden to brown color, whereas Milsana® treatment resulted in the appearance of dark brown spots (Figure 3). The color of licorice treated leaf discs was lighter compared to that of fraction F6 treated discs. Water and ethanol (concentration 6%) treated discs showed no or only a light color on the cutting edge (Figure 3). The first coloration of leaf discs appeared after 1 hour and was strongest after 6 to 7 hours of treatment in licorice extract or fraction F6. The coloration of Milsana® treated leaf discs was strongest after 8 hours of treatment.

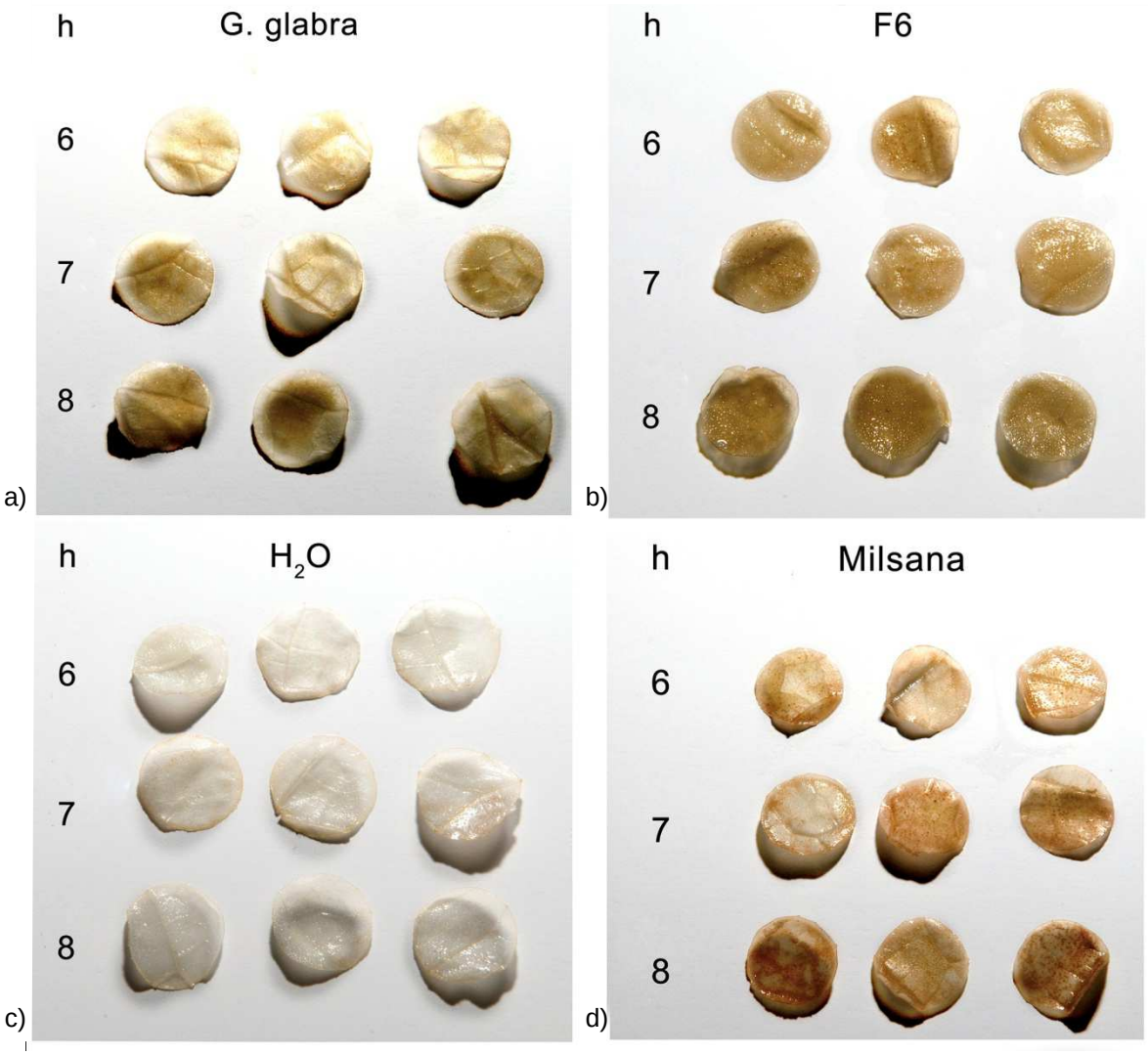

Figure 3. H_2O_2 detection by DAB in cucumber leaf discs, after 6-8 hours of treatment with a) 3% licorice extract, b) 3% fraction F6, c) water, d) 1% Milsana®. Brown coloration = burst of H_2O_2 in the leaf tissue

6.4.3. Analysis of the expression of PR-1 mRNA in extract treated cucumbers

The expression of mRNA of the pathogenesis related protein (PR-1) was up-regulated in cucumber plants 24 hours after treatment with 3% licorice extract (31.6 ng, standard deviation 6.0 ng). It was approximately 4 times higher than in control plants (6.9 ng, standard deviation 2.2 ng; Figure 4).

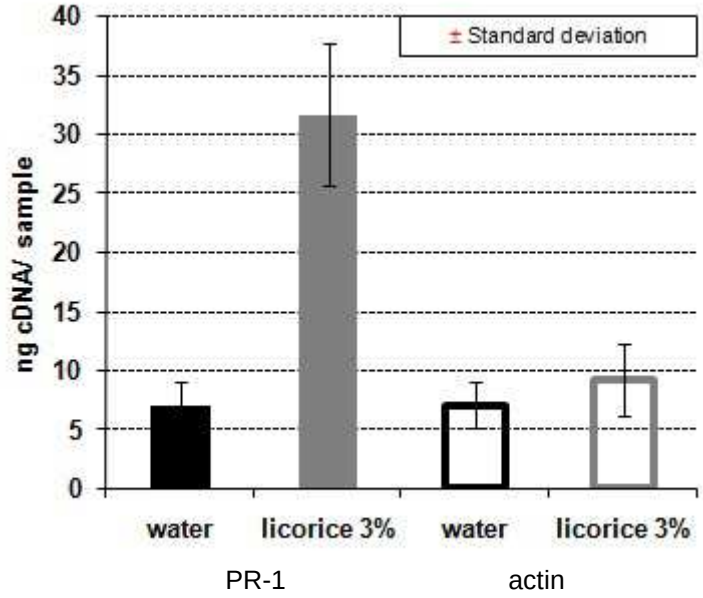

Figure 4. Extrapolated data of the expression of mRNA of a pathogenesis related protein (PR-1) and the standard protein actin in cucumber leaves 24 hours after treatment with licorice extract (3.0%), compared to water treated control plants (n=9 plants).

However, the results are extrapolated data, because the reference actin as well as PR-1 reached amounts above the concentration of the given dilution series of genomic DNA and therefore were not directly comparable. Nevertheless, the amount of the standard actins was almost the same in the samples of the water treated (7.1 ng, standard deviation 2.0 ng) and licorice treated plants (9.2 ng, standard deviation 3.0 ng; Figure 5), indicating that the general mRNA status in all tested plants was the same independent of the treatment.
An analysis of significance was not done, because of the extrapolated data.

6.5. Discussion

In former bioassays on potted cucumber plants protective treatment with an ethanolic leaf extract of *G. glabra* (licorice) or its active fraction (acidic substances, F6) had high

potential for controlling *P. cubensis* (downy mildew). The efficacy was 98.8% and 97.6%, respectively.

In the current study the hypothesis of a resistance inducing effect of the licorice extract and of fraction F6 was tested via the analysis of H_2O_2 and PR-1 production and the influence on germination of *P. cubensis* zoospores *in planta*.

The detection of H_2O_2 is an indicator often used to proof the resistance inducing effect of a substance (Lin and Ishii 2009). In this study leaf discs treated with licorice extract or its fraction F6 and stained with DAB showed a clear golden-brown coloration that was strongest 6 to 7 hours after treatment (Figure 3). The brown color is the product of a reaction of DAB with H_2O_2 (Thordal-Christensen et al. 1997) and therefore a proof of H_2O_2 production in treated leaf discs. The coloration of fraction F6 treated leaf discs was comparable to that of Milsana® treated leaf discs (a resistance-inducing leaf extract of *Fallopia sachalinensis*), but in general it was more homogenously distributed (Figure 3). The coloration of licorice treated discs was less than that of fraction F6 treated discs (Figure 3). Even though with this method no quantification of H_2O_2 in the tissues is possible, the lighter coloration of licorice treated leaf discs indicated a lower H_2O_2 level compared to fraction F6 treated discs.

Also, the inhibition of growth of germ tubes (germ tube length on leaf discs: water treated 17.3 µm - 26.6 µm, licorice treated 1.6 µm - 6.4 µm; Figure 2) may be considered as an indicator for induced resistance (Cantone and Dunkle 1990). At the same time, the elevated H_2O_2 level and the differences in germ tube length could be interpreted in a different way. Production of reactive oxygen species in the plant is also known as a stress reaction on phytotoxic substances (Panda et al. 2003, Mishra et al. 2009). However, several bioassays as well as semi-commercial trials with licorice extract, revealed no observable phytotoxic reaction after as much as 12 weekly treatments (see Scherf et al. 2010, see also Chapter 3) with a 3% extract. Therefore, in the case of *G. glabra* leaf extract and fraction F6, containing the active compounds, the H_2O_2 burst in the plants (Figure 3) seems to be an indicator for induced resistance and not a phytotoxic phenomenon. The same was described by Müller (2004) for Milsana®.

The interpretation for the inhibition of the germ tube growth is more difficult. It is known that the licorice extract has a direct effect on the pathogen (Schuster et al. 2010), since it inhibits the release of zoospores at least for 6 hours *in vitro*. Furthermore, another *in vitro* assay showed that the zoospores were killed in the moment they got in contact with the extract (Schuster et al. 2010). This might be the reason for the fact, that in this work only a small number of zoospores could in total be recovered and counted on extract treated samples. The inhibition of the germ tube growth of the remaining zoospores could be interpreted as a direct effect of the extract. Rhöner et al. (2003)

describe the inhibition of germination of *Phytophtora infestans* zoospores by extracts of *Paeonia suffruticosa* and *Hedera helix.* However, inhibition of germ tube growth could as well be the result of an indirect effect induced in the plant, as reported by He et al. 2002. They found that asparagus plants treated with a nonpathogenic *Fusarium oxysporum* isolate produced antifungal exudates in their roots, which inhibited germination of pathogenic *F. oxysporum* spores.

A strong evidence for the resistance inducing effect of an agent is the up-regulation of the expression of mRNA of pathogenesis related (PR) proteins (Ryals et al. 1996). One of the most prominent of those proteins is the antifungal PR-1 (van Loon and van Stain 1999, Ahn et al. 2007). This molecule seems to be up-regulated approximately 4 times in licorice extract treated cucumber plants compared to water treated control plants (Figure 4). Therefore, despite of the fact that data from realtime PCR had to be extrapolated, the higher amount of PR-1 mRNA in licorice treated cucumber plants is the strongest evidence for a resistance inducing effect of *G. glabra* leaf extract. Due to the antifungal activity of PR-1, it is possible that the inhibition of germ tube growth was caused by the elevated amount of PR-1 in extract treated plants. The up-regulation of PR-1 is part of the salicylic acid pathway (Pieterse and van Loon 2007). This indicates that the mechanism of the resistance inducing effect of licorice extract might be systemic acquired resistance, which key substance is salicylic acid.

However, Poidevin (2010) found that treatment of *Arabidopsis thaliana* plants with licorice extract (concentration 5%) reduced the infection with downy mildew (*Hyaloperonospora parasitica*) significantly, while in *in vitro* assays no antimicrobial activity of the extract could be observed against *Plectospaerella cucumerina*. Interestingly, his investigations on *Arabidopsis* mutants indicated that most of the known pathways for induced resistance (salicylic acid, jamonic acid, ABA.) were not involved in the mode of action of licorice extract. Nevertheless, the results of Poidevin (2010) and of this study clearly support the hypothesis that ethanolic leaf extract of *G. glabra* induces resistance. Furthermore, results of the presented work showed another similarity - besides the influence on the photosynthetic system - between Milsana® and licorice extract. It is known that the extract of *Fallopia sachalinensis* induces resistance before the plant gets in contact with a pathogen. In addition, after pathogen attack, pre-treatment leads to a much faster and greater physiological response in a plant. This phenomenon is called priming (Conrath et al. 2002, Heil and Bueno 2007). Since all above presented data on H_2O_2 and PR-1 resulted from trials with cucumbers not infected with downy mildew, it needs to be analyzed if *G. glabra* extract also primes the plant for defense against subsequent infection.

However, to get final evidence for the resistance inducing effect of the licorice extract

further investigations are necessary. The causal role of H_2O_2 as a first signal in the induction process has to be proven. This could be done by the concomitant use of radical scavengers, for example vitamin C or K preparations. They should lead to a reduction of H_2O_2 levels in the plant and this should result in a loss of efficacy of the extract, given that H_2O_2 plays a significant role in the process. Respective results were shown by Müller (2004) for Milsana® in cucumber and *Podosphaera xanthii* (powdery mildew). Furthermore, qPCR trials have to be replicated to achieve data for a statistical analysis. Also, further investigations with respect to changes in metabolism on licorice extract treated and infected plants as well as further assays with *Arabidopsis* mutants are necessary to get a final picture of the resistance-inducing properties and involved pathways of *G. glabra* extract.

6.6. Literature

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7.1. General discussion

The Fabaceae *Glycyrrhiza glabra* (licorice) is an old medicinal plant. For the control of human diseases only extracts of the radices are used (Stepanova and Sampies 1997). An extract of leaves of the licorice plant showed high efficacies in *in vitro* assays against the Oomycete *Phytophthora infestans*, which causes potato blight (Konstantinidou-Doltsinis and Markellou 2008).
Based on these results in this work an ethanolic leaf extract of *G. glabra* was tested *in vivo* on cucumber plants against one of the most important pathogens in this vegetable, the obligate biotrophic Oomycete *P. cubensis* (downy mildew).

Three questions were addressed in this thesis:

1. Has the extract a potential as alternative control agent, not only in the laboratory, but also under commercial conditions in greenhouses?

2. If it shows a potential, what is the active ingredient?

3. What is the mode of action?

In four semi-commercial trials over two years the licorice extract showed the highest and most stable efficacy (extract concentration 3%, efficacy 68.0%-83.0%) in controlling downy mildew in different concentrations (2%, 2.5% and 3%) and application intervals (11-12 days and 7 days), compared to the other new biological agents sage extract and the bacterium *Aneurinibacillus migulanus* (Scherf et al. 2010, see also Chapter 3). Furthermore, efficacies of licorice extract were even better than those of the commercially used plant strengthener Elot-Vis® (52.0%-62.0%). In organic cucumber production Elot-Vis® is a commonly used agent against *P. cubensis. C*opper preparations are banned from greenhouses, because of the accumulation of this metal in soil and the problems caused by this elevated amount for many organisms (Finckh et al. 2007). Under open field conditions licorice extract delayed the development of the disease in the cucumber crop up to three weeks (Scherf et al. 2010, see also Chapter 3). These results show that licorice extract has the potential to be an adequate alternative for copper preparations. But as future goal the agent should provide high efficacies even under complex open field conditions. Therefore, it was necessary to

investigate active ingredients and to understand the mode of action of the extract.

Schuster et al. (2010) described a direct effect of licorice extract on zoospores of *P. cubensis*. Observations under semi-commercial conditions imply that the extract might have an effect on the plant itself. Treated cucumbers had a darker green color, than water treated control plants and plants treated with the other agents (Scherf et al. 2010, see also Chapters 3, 5).

In the present investigations three substances detected in high amounts were identified as pinocembrin, licoflavanon and glabranin (Chapter 4). They are known for their general antifungal and antimicrobial effect (Shain and Miller 1982, Lester et al. 1983, Fukui et al. 1988, Fatima et al. 2009) and therefore may be responsible for the direct effect of licorice extract on *P. cubensis*. The crude extract was fractionated by a shakeout procedure with different solvents (Chapter 4). The fraction of acidic substances (F6) contained also high amounts of pinocembrin, licoflavanon and glabranin and showed the highest efficacies (97.6%) of all fractions in controlling *P. cubensis* (Chapter 4). A probit analysis of bioassays on potted cucumber plants treated with a concentration series of crude extract and fraction F6 indicated different modes of action for both agents, for the slopes of the two graphs were significantly different (Chapter 4). Besides the known direct effect of crude licorice extract, additional effects (e.g. darker green color) on treated plants were observed. It was assumed that the second mode of action of crude extract and the main mode of action of fraction F6 is induced resistance. However, this implies, pinocembrin, licoflavanon and glabranin might be important for both modes of action of the licorice extract.

The resistance inducing effect and the effect on leaf color of licorice treated cucumbers suggest a comparison between *G. glabra* extract and the commercially used plant strengthener Milsana®. Karavaev et al. (2002) and Schmitt et al. (2005) found an elevated amount of chlorophyll in the Milsana® treated cucumbers as well as an influence on the chlorophyll fluorescence.

Based on these similarities, investigation on the influence of crude licorice extract and its fraction F6 on the chlorophyll fluorescence of licorice treated cucumbers were done. Chlorophyll fluorescence of dark adapted cucumbers was measured. The calculated fluorescence value Fv/Fm (Fv=variable fluorescence, Fm=maximal fluorescence) is an indicator for the stress situation of a plant (Angelopoulos et al. 1996, Meyer et al. 2001) caused for example by a disease. In healthy unstressed plants the value is 0.83 ± 0.004 (Krause and Weis 1991, Maxwell and Johnson 2000). In the presented trial 1, even in highly infected fraction F6 treated plants (disease severity 92.5%) the Fv/Fm value (0.80) was stable during the whole measuring period, whereas the Fv/Fm value of also highly infected water treated plants decreased (disease severity 100.0%) from 0.80 to

0.69 over time. The Fv/Fm values of licorice treated plants fluctuated around 0.80 during the whole measuring period (disease severity 25.0%) (Chapter 5). The high value of fraction F6 treated plants is clearly a positive effect of extract fraction F6 on the stressindicator Fv/Fm. Interestingly, in trial 2 the Fv/Fm value of water treated, only moderately with *P. cubensis* infected plants (disease severity 41.9%) also fluctuated around 0.80 during the measuring time like the Fv/Fm value of licorice treated plants (disease severity 5.6%). These results showed that moderate infection did not mean heavy stress for the cucumber plants. Those results fit an observation made in semi-commercial trials in greenhouses. The cucumbers were only moderately infected with *P. cubensis* at the end of the trial (Serf et al. 2010, see also Chapter 3). This was mainly because of the warm weather conditions in early summer in years 2008 and 2009, which inhibited the development of the pathogen. In both semi- commercial trials no effect on the yield by the moderate infection was found. But it should be noted, in bioassays as well as in semi-commercial trials all stressfactors (biotic and abiotic) beside the investigated infection were widely suppressed. That means under those controlled conditions a moderate infection with downy mildew did not result in heavy stress for the plants. This might be different in years with cooler and more humid growing condition in opend field. Nevertheless, the high Fv/Fm value of the licorice treated plants in trial 1 might have been due to their only moderate infection level. But, the high variability in disease severity of those plants, which is not typical for licorice treatment, indicates another explanation. The results of fraction F6 treated plants showed a failure of its assumed resistance inducing effect in trial 1 (Chapter 5). The same should be true for the crude licorice extract, leaving the observed control of the pathogen only to its direct effect on *P. cubensis.* Therefore, the results of investigations about the effect on the photosynthetic apparatus including elevated chlorophyll contents (water 8.2 mg/ g DW, licorice 10.2 mg/ g DW) in extract treated plants, point to two modes of action of the licorice extract: direct effect on the pathogen and induced resistance.

Besides the effect on the chlorophyll fluorescence Schmitt et al. (2005) found an elevated amount of H_2O_2 in leaf tissues of Milsana® treated plants as well as an up-regulation of pathogenesis related proteins (PR proteins). Both observations are indicators for induced resistance.

To give evidence to effects of licorice extract the status of H_2O_2 and PR-1 protein, which is part of the induced resistance mechanism, the systemic acquired resistance (key substance is salicylic acid) (Raskin 1992, Yalpani et al. 1993), in licorice treated cucumber leaves was investigated. By DAB staining a burst of H_2O_2 could be detected in leaf discs treated with licorice extract. This burst seemed to be even higher in fraction

F6 treated leaf discs (darker coloration). The level was highest after 6 to 7 hours of treatment. Also the up-regulation of PR-1 mRNA in licorice treated plants could be shown. These results were important for the explanation of the observed inhibition of *P. cubensis* zoospore germination (germ tube length: water = 23.5 µm, licorice = 2.8 µm). This might have been due to the direct effect of licorice extract. But PR-1 is known for its antifungal properties. Therefore it seems to be more likely that the inhibition of germination was due to the up-regulation of PR-1. Also, the concentration of another potential antibacterial and antifungal substance was elevated in licorice extract treated plants. The amount of anthocyanin of *G. glabra* treated plants was approximately 7 units higher than that of water treated plants. These results imply that the crude extract and also fraction F6 induce resistance.

7.2. Conclusion

7.2.1. Evidences

The ethanolic leaf extract of *Glycyrrhiza glabra* (licorice) is a highly potent alternative control agent against cucumber downy mildew, which is caused by the Oomycete *Pseudoperonospora cubensis*. Protective licorice extract application results in stable high efficacies even under semi-commercial conditions.

In addition to the direct effect against the pathogen, treatment with *G. glabra* affects the plant itself in a positive way. The antenna complex is somehow stimulated by the extract and the plant becomes prepared against infections via induced resistance. It is certain that the detected compounds of the licorice extract, pinocembrin, licoflavanon and glabranin play a role in the mode of action. The same is true for the up regulated PR-1 in licorice treated plants.

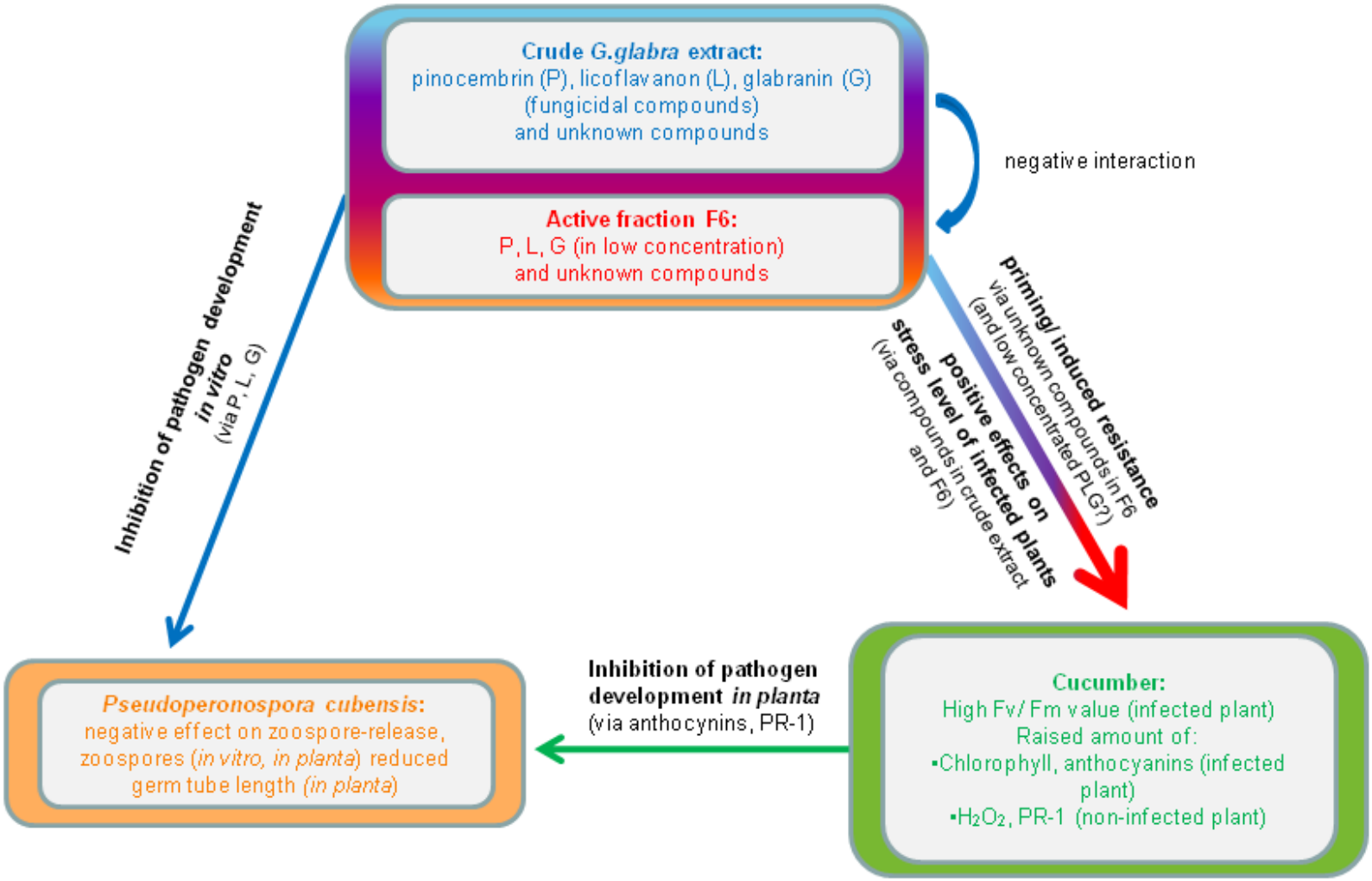

Figure.1 Diagram about the interactions between the licorice extract, *P.cubensis* and the cucumber

7.2.2. Issues that remains undiscovered

In this study the main issues about the interaction between *G. glabra* extract, *P. cubensis* and the host plant, *Cucumis sativus*, could be resolved. However, some details are not yet clear. It remains unanswered whether low concentrations of pinocembrin, licoflavanon and glabranin, which seem to cause the direct effect of the crude extract, play a role in induced resistance. Further, compounds involved in resistance inducing are conjecturable but still undiscovered. Another question is, whether the elevated anthocyanin content in licorice treated plants can be appraised as an indicator for the resistance inducing effect of the leaf extract or not. Also, the pathway of this resistance induction needs to be elucidated. Investigations on downy mildew infested *Arabidopsis* mutants indicate that the mode of action of inducing resistance of the licorice extract is neither the salicylic acid pathway, jasmonic acid pathway, nor ABA pathway (Poidevin 2010).

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8. SUMMARY

In ancient times, right after they started to domesticate plants, humans had to become plant protectors. The first protection strategies were mainly against herbivorous animals of any kind, arthropods as well as vertebrates. Thousands of years later humans discovered microorganisms like bacteria and fungi and soon correlated them with the disease they, their livestock and their plants had to suffer. The control agents they used against plant pests were mainly inorganic substances such as sulfur, arsenic or mercury. Their own diseases, in contrast, were fought with medicinal plants for thousands of years, more or less successfully.

After problems caused by chemical pesticides, like DDT, became obvious (enrichment in the food chain and with that in the human tissues as well), the search for alternatives started.

In more recent times more and more scientific studies investigate the potential of medicinal plants as plant protection agents.

The aim of this thesis was to investigate the potential of ethanolic extract of leaves of the medicinal plant *Glycyrrhiza glabra* (licorice) as a control agent against one of the main pests in cucumbers, the Oomycete *Pseudoperonospora cubensis* (downy mildew).

The first question addressed was:

Has the licorice extract a potential as alternative control agent, not only in the laboratory, but also under commercial conditions in greenhouses?

The second question addressed was:

If it shows a potential, what is the active ingredient?

And the final question addressed was:

What is the mode of action?

Under semi-commercial conditions the licorice extract has a high potential as control agent against cucumber downy mildew (efficacy up to 83.0%, application interval 7 -11 days, 3% extract concentration). Besides this, in semi-commercial trials a dark green color of licorice extract treated plants was observed.

The crude licorice extract was fractionated by shake-out procedure in 6 fractions. The active ingredient was found to be part of extract fraction F6, which contains acidic substances. This fraction reached efficacies up to 97.6% in bioassays on treated cumber plants. Three flavanoids were detected in sub-fractions of fraction F6 and

identified as glabranin, licoflavanon and pinocembrin. All three are known for their antimicrobial effect and were effective against another Oomycete, *Phytophthora infestans* in *in vitro* assays. Also, it is known that *G. glabra* extract kills zoospores of *P. cubenis in vitro* (Schuster et al. 2010) which may be due to the detected flavanoids. However, the effect of fraction F6 is not explainable by the action of the antimicrobials glabranin, licoflavanon and pinocembrin alone. Sub-fractions not containing those three substances showed efficacies up to 89.2%.

This together with the observed dark green color of treated plants in semi-commercial trials led to the assumption that licorice leaf extract has not only a direct effect on the pathogen but influences the plant itself. This assumption was supported by the finding of elevated chlorophyll and anthocyanin content in treated plants and a positive effect on the stress indicator chlorophyll fluorescence. Even in highly infected fraction F6 treated plants (disease severity 92.5%) the Fv/Fm value (0.80) was stable during the whole measuring period, whereas the Fv/Fm value of also highly infected water treated plants decreased (disease severity 100.0%) from 0.80 to 0.69 in the same time.

Furthermore, an elevated H_2O_2 level in the leaf tissues of licorice extract treated uninfected cucumber plants and an up-regulation of PR-1 mRNA could be found. The level of PR-1 mRNA was approximately 4 times higher than in water treated plants. Both, an elevated H_2O_2 level and an up-regulation of Pr-1mRNA expression are strong indicators for induced resistance. Since PR-1 has antifungal activity, this high amount of PR-1 is a possible explanation for inhibition of germ tube growth of *P. cubensis* zoospores on licorice treated leaf discs found in this thesis. The germ tube length on water treated leaf discs was 17.3-26.6 µm, whereas the germ tube length on licorice treated leaf disc was only 1.6-6.4 µm.

Based on these results it was concluded that the ethanolic leaf extract of *G. glabra* is a highly potent control agent against cucumber downy mildew (*P. cubensis*) and its mode of action is a combination of direct effects on the pathogen development and induced resistance.

ZUSAMMENFASSUNG

Schon in historischer Zeit, als sie begannen Pflanzen zu domestizieren, wurden die Menschen zu Pflanzenschützern. Die ersten Strategien dienten vor allem dem Schutz gegen jegliche herbivore Tiere, sowohl Arthropoden als auch Vertebraten. Tausende Jahre später wurden Bakterien und Pilze erstmals mit den Krankheiten der Haustiere und Pflanzen in Zusammenhang gebracht. Die nun eingesetzten Schutzmittel waren vor allem anorganische Substanzen wie Schwefel, Arsen und Quecksilber. Die humanen Krankheiten jedoch wurden, mehr oder weniger erfolgreich, über tausende Jahre mit Medizinalpflanzen kuriert.

Nachdem die Probleme, die durch chemische Pestizide, wie DDT, hervorgerufen sichtbar wurden

(Anreicherung in der Nahrungskette und auch in menschlichem Gewebe) begann die Suche nach Alternativen.
In jüngerer Zeit wurden nun mehr und mehr Studien durchgeführt, die das Potential von Medizinalpflanzen als Pflanzenschutzmittel bewerteten.
Das Ziel dieser Arbeit war es dieses Potential eines ethanolischen Blattextraktes der Medizinalpflanze *Glycyrrhiza glabra* (Süßholz) gegen den Erreger des Falschen Mehltaus an der Gurke, den Oomyceten *Pseudoperonospora cubensis,* zu erforschen.

Die erste Frage war:
Zeigt der Süßholzextrakt ein Potential als alternatives Pflanzenschutzmittel nicht nur im Labor sondern auch unter kommerziellen Bedingungen im Gewächshaus?

Die zweite Frage war:
Wenn der Extrakt dieses Potential zeigt, welche Substanzen sind an der Wirkung beteiligt?

Und die abschließende Frage war:
Welcher Wirkmechanismus liegt vor?

Unter semi-kommerziellen Bedingungen wurde das große Potential des Süßholzblattextraktes als alternatives Schutzmittel deutlich (Wirkungsgrade bis zu 83,0%, Applikationsintervall 7-11 Tage, 3%ige Extraktkonzentration). Zudem wurde in diesen semi-kommerziellen Versuchen eine dunklere Grünfärbung der Pflanzen nach der Behandlung mit Süßholzextrakt beobachtet.
Die Fraktionierung des Extraktes erfolgte durch ein Ausschüttelverfahren im Scheidetrichter. Der Rohextrakt konnte in 6 Fraktionen unterteilt werden. Die Wirksubstanzen wurden in der sauren Fraktion F6 gefunden. Mit dieser Fraktion konnten in Biotests, an behandelten Gurkenpflanzen, Wirkungsgrade von bis zu 97,6% erzielt werden. Aus Unterfraktionen der Fraktion F6 konnten die drei Flavonoide, Pinocembrin, Licoflavanon und Glabranin, bestimmt werden. Alle drei Substanzen sind bekannt für ihre antimikrobielle Wirkung. Zudem zeigten sie in *in vitro* Tests eine Wirkung gegen den Oomyceten, *Phytophthora infestans*. Auch die von Schuster et al. (2010*) in vitro* beobachtete Mortalität von *P. cubensis* Zoosporen nach Kontakt mit *G. glabra* Extrakt scheint auf die drei Flavonoide zurückführbar zu sein.
Dennoch kann die Wirkung von Fraktion F6 nicht alleine mit der Wirkung von Pinocembrin, Licoflavanon und Glabranin erklärt werden. Unterfraktionen, welche nicht die Flavonoide enthielten zeigten in Biotests Wirkungsgrade bis zu 89,2%.
Diese Resultate, zusammen mit der beobachteten starken Grünfärbung der Pflanzen in den semi-kommerziellen Versuchen, legten die Vermutung nahe, dass der Extrakt neben dem direkten Effekt gegen das Pathogen auch die Pflanze selbst beeinflusst.
Untermauert wurde diese Vermutung durch den Nachweis eines erhöhten Chlorophyll- und Anthocyan-Gehaltes in den Süßholz- behandelten Pflanzen. Zudem hatte die Behandlung der Gurken einen positiven Effekt auf den Stressindikator Chlorophyllfluoreszenz. In stark befallenen Fraktion F6 behandelten Pflanzen (Befall 92.5%) lag der Fv/Fm Wert bei 0,80 über die gesamte Versuchszeit. Der Fv/Fm Wert von ebenfalls stark befallenen Wasserkontrollpflanzen (Befall 100%) fiel in derselben Zeit von 0.80 auf 0,69.
Auch konnte ein Anstieg von H_2O_2 und PR-1 mRNA im Gewebe von Süßholz-behandelten Pflanzen nachgewiesen werden. Der Gehalt von PR-1 mRNA war circa vierfach erhöht gegenüber den Wasserkontrollpflanzen. Sowohl ein Anstieg von H_2O_2 als auch die Überexpression von PR-1 mRNA kann als starker Indikator für eine Resistenzinduktion durch den Extrakt gewertet werden. Da PR-1 eine

bekannte pilzhemmende Wirkung hat, ist dieser hohe Gehalt auch eine mögliche Erklärung für die zu beobachtende geringere Keimschlauchlänge von *P. cubensis* Zoosporen auf Süßholz-behandelten Blattscheiben. Die Keimschlauchlänge auf Wasser-behandelten Blattscheiben betrug 17.3-26.6 µm, während die Keimschläuche auf Süßholz-behandelten Blattscheiben lediglich eine Länge von 1.6-6.4 µm aufwiesen.

Basierend auf all den genannten Ergebnissen ist festzuhalten, dass der Blattextrakt aus *G. glabra* ein hohes Potential als alternatives Schutzmittel gegen den Falschen Mehltau an der Gurke (*P. cubensis)* aufweist. Der Wirkmechanismus ist eine Kombination aus direkten Effekten des Extraktes auf die Pathogene als auch auf Induzierter Resistenz in den Gurkenblättern.

Andrea Scherf (geb. Nowak)

Berufserfahrung

09/07- heute	**Doktorandin am Julius-Kühn-Institut, Darmstadt** Promotionsthema: „ Licorice, cucumber, downy mildew – tracing the secret“	Deutschland
09/07-12/10	**Wissenschaftliche Angestellte im JKI Institut für Biologischen Pflanzenschutz, Darmstadt**	Deutschland
11/05-02/06	**Wissenschaftliche Hilfskraft im Prüfungsamt der Biologische Fakultät der Universität Leipzig**	Deutschland

Ausbildung

10/02 - 09/05	**Universität Leipzig, Leipzig** Diplomthema:“ Craniometrische und genetische Analyse an Chromosomenrassen der Waldspitzmaus (*Sorex araneus* L.) in Sachsen“	Deutschland
10/99 - 03/02	**Grundstudium an der Johann-Wolfgang Goethe Universität, Frankfurt am Main**	Deutschland
05/99	**Abitur an der Marienschule der Ursulinen, Offenbach am Main**	Deutschland

Projektmitarbeit

10/03 - 07/04	**Universität Leipzig, Leipzig** Geographische Unterschiede in Schädelmessstrecken der Waldspitzmaus (Sorex araneus L.)	Deutschland
04/03 – 09/03	**Universität Leipzig, Leipzig** Projekt zur Populationsdichte und Bruterfolg der Rauchschewalbe im Kohrener Land	Deutschland
04/02-07/02	**Field Assistant an der University of Miami, Coral Gables (Fl)** Langzeitstudie zur Populationsdynamik von Kleinsäugern im Everglades Nationalpark Kartierungsstudie zu der Säugetier Fauna in den Florida Keys	USA

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