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New strategies to control Verticillium wilt in hops

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I Abstract

Verticillium wilt, caused by *Verticillium albo-atrum* and *V. dahliae*, is a devastating disease in hops, with *V. albo-atrum* being the main pathogen. Considerable economic crop losses can be the consequence. Due to hops being a perennial crop and the long persistence of *Verticillium* spp. in soil combined with the lack of effective chemical fungicides, hop wilt is very difficult to control. The only effective means are planting resistant or tolerant cultivars and carrying out phytosanitary measures. Thus, a fast and accurate detection system and new approaches for controlling the wilt is urgently required. An assay, based on DNA extraction directly from the hop bine followed by real-time polymerase chain reaction (PCR), was established. The combination of a homogenizer and a commercially available DNA isolation kit proves to be a very effective DNA extraction technique. A specific primer pair / TaqMan probe set was designed and used in a multiplex real-time PCR that enables the simultaneous detection of *V. albo-atrum* and *V. dahliae*. This detection assay is more accurate, sensitive and time-saving than the standard method based on cultivation of the fungus onto selective medium followed by PCR. The newly developed assay allows a fast and accurate detection of *Verticillium* spp. in hops and represents an important prerequisite to an effective control. Moreover, initial steps for the application of an alternative method for plant protection were performed. Biological control is an environmentally friendly strategy, based on the use of beneficial microorganisms. Therefore, four well-known beneficial bacterial strains were evaluated for their applicability in hops: *Burkholderia terricola* ZR2-12, *Pseudomonas poae* RE*1-1-14, *Serratia plymuthica* 3Re4-18, and *Stenotrophomonas rhizophila* DSM14405^{T}. The competent colonization of the root system, plant growth promoting effects, and antagonistic activity were assessed. All strains were shown to be rhizosphere and endorhiza competent. In addition, the colonization pattern and behavior of DsRed-labeled transformants was characterized and visualized by confocal laser scanning microscopy (CLSM). *B. terricola* ZR2-12, *S. plymuthica* 3Re4-18, and *S. rhizophila* DSM14405^{T} were shown to be promising candidates. Especially, *S. plymuthica* 3Re4-18 proved to be applicable with regard to all of the tested traits. Growth promoting effects on seedlings, treated with these bacteria, were assessed for *S. plymuthica* 3Re4-18 and *S. rhizophila* DSM14405^{T}. *B. terricola* ZR2-12 was characterized by a very high and stable abundance. These preliminary studies demonstrate that the biological control can be an additional means to control the hop wilt.

II Zusammenfassung

Die Verticillium Welke, die durch *Verticillium albo-atrum* und *V. dahliae* verursacht wird, ist eine schwerwiegende Krankheit bei Hopfen, wobei *V. albo-atrum* als Hauptpathogen gilt. Beträchtliche wirtschaftliche Ernteausfälle können die Folge sein. Da es sich beim Hopfen um eine Dauerkultur handelt, der Pilz lange im Boden überdauern kann und zusätzlich keine wirksamen chemischen Fungizide zur Verfügung stehen, ist die Bekämpfung besonders schwierig. Der Anbau von resistenten und toleranten Sorten und der Einsatz von phytosanitären Maßnahmen sind zurzeit die einzigen Maßnahmen. Daher ist ein schnelles und zuverlässiges Nachweisverfahren sowie neue Strategien zur Verticillium Kontrolle dringend notwendig. Ein Assay, der auf der DNA Isolierung direkt aus der Hopfenrebe gefolgt von einer real-time PCR (Echtzeit Polymerasekettenreaktion) basiert, wurde etabliert. Die Kombination von Homogenisator und kommerziell erhältlichen Kit stellt eine sehr effektive DNA Isolierungstechnik dar. Um *V. albo-atrum* und *V. dahliae* in einer real-time PCR simultan nachweisen zu können, wurde ein spezifisches Primer / TaqMan Sonden Set entwickelt. Der neu entwickelte Nachweisassay ist genauer, sensitiver und zeitsparender als die Standardmethode, die auf der Kultivierung des Pilzes auf Selektivmedium und darauffolgender PCR basiert. Des Weiteren wurde nach einer neuen und alternativen Pflanzenschutzmaßnahme gesucht. Die biologische Kontrolle erweist sich als eine umweltfreundliche Strategie. Daher wurden vier bekannte „nützliche Bakterienstämme“ auf ihre Eignung im Hopfen beurteilt: *Burkholderia terricola* ZR2-12, *Pseudomonas poae* RE*1-1-14, *Serratia plymuthica* 3Re4-18 und *Stenotrophomonas rhizophila* DSM14405^T. Die Kolonisierungskompetenz im Wurzelsystem, pflanzenfördernde Wachstumseffekte und antagonistische Aktivität wurden beurteilt. Alle Stämme besiedelten die Rhizosphäre und die Endorhiza. Zusätzlich wurde das Kolonisierungsverhalten von DsRed-markierten Transformanten mittels konfokaler Laser-Scanning-Mikroskopie (CLSM) charakterisiert. *B. terricola* ZR2-12, *S. plymuthica* 3Re4-18 und *S. rhizophila* DSM14405^T erwiesen sich als vielversprechende Kandidaten. *S. plymuthica* 3Re4-18 scheint sehr gut geeignet zu sein, da dieser Stamm alle untersuchten Eigenschaften erfüllt. *B. terricola* ZR2-12 zeichnete sich nur durch seine besonders hohe und stabile Besiedelung im Wurzelsystem aus. Pflanzenwachstumsfördernde Effekte an mit den Bakterien behandelten Samen wurden bei *S. plymuthica* 3Re4-18 und *S. rhizophila* DSM14405^T beobachtet. Diese ersten Untersuchungen zeigen, dass die biologische Kontrolle eine zusätzliche Möglichkeit zur Bekämpfung der Verticillium Welke bei Hopfen sein könnte.

III Abbreviations

ACC	1-aminocyclopropane-1-carboxylate
AHL	N-acyl-homoserine lactones
BCA	Biological control agent
BLAST	Basic Local Alignment Search Tool
CLSM	Confocal laser scanning microscope
DNA	Deoxyribonucleic acid
e.g.	For example
ELISA	Enzyme Linked Immuno Sorbent Assay
ha	Hectare
ISR	Induced systemic resistance
ITS	Internal transcribed spacer
LfL	Bavarian State Research Center for Agriculture (Bayerische Landesanstalt für Landwirtschaft)
NCBI	National Center for Biotechnology Information
PCR	Polymerase chain reaction
PGP	Plant growth promotion
PGPA	Plant growth promotion agent
PGPB	Plant growth promotion bacteria
PGPR	Plant growth promotion rhizobacteria
QS	Quorum sensing
RFLP	Restriction fragment length polymorphisms
RFW	Root fresh weight

1 Introduction

1.1 Hops – *Humulus Lupulus*

Hop, *Humulus*, belongs to the family of *Cannabaceae*. It is a genus of dioecious flowering, perennial climbing plant and grows in temperate climates. *Humulus* contains three species: *H. lupulus*, *H. japonicas*, and *H. yunnanensis*, whereof the most well-known and important species of this family is *H. lupulus* L. (common hop or in German "Echter Hopfen"), which is used for brewing beer. This species is distinguished between five botanical varieties in regard to their morphogeographic characteristics. Both sexes of *H. lupulus* contain 20 chromosomes and are normally diploid. The common hop is native to Europe, North America, and eastern Asia. The first hop cultivations can be traced back to the 5th to 7th centuries in Central Europe (Barth et al. 1994). In Germany the first documentary mention was in 736 in Geisenfeld. The main application nowadays is the production of beer. The cones of female hops are agronomically important and their ingredient resins, essential oils, and polyphenols are responsible for the flavor, the typical bitterness and preservation of beer (Mahaffee et al. 2009). Hops are also used in medicine for calmatives, cosmetics, and as an additive to liqueur and schnaps. Hops are promising for use in sugar processing, as a preservative in ethanol production and an antimicrobial additive in animal feed (Mahaffee et al. 2009).

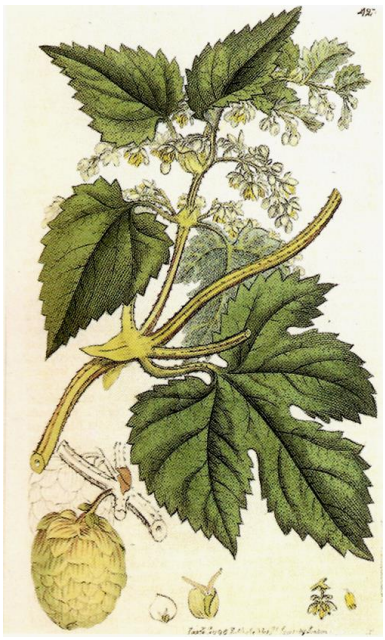

Figure 1: Cooper Engraving of *Humulus lupulus* from 1796 (Barth et al. 1994)

The growing area is limited to the 35th to the 55th degrees of latitude. Germany has the largest harvesting area with 16 849 ha, followed by USA (14 181 ha) and Czech Republic (4 339 ha) (Hopfen-Rundschau 07, data from 2013). The region Hallertau in Bavaria, Germany, is the largest, contiguous growing area worldwide. The Hop Research Center Hüll of the Bavarian State Research Center for Agriculture (Bayerische Landesanstalt für Landwirtschaft = LfL) concerns itself with every issue regarding hops, e.g. breeding, cultivation, hop quality, chemical analytics, and genome research. The institution also has an advisory service providing information on practicable issues and current research findings, in a publicly accessible form.

1.2 Diseases and Pests

Economically relevant hop diseases and pests are well-described in *Compendium of hop diseases and pests* (Barth et al. 1994), *Der Hopfen* (Kohlmann & Kastner 1974) and the information brochure *"Grünes Heft"* (LfL 2013). Downy mildew, powdery mildew, gray mold, the black root rot, and Verticillium wilt are important diseases caused by fungi and oomycetes. Downy mildew, caused by *Pseudoperonospora humuli*, is a devastating fungal disease in hops. Symptoms are stunted and chlorotic hop shoots (spikes = Bubiköpfe). In Bavaria, there exists a warning service to call for timely combat against fungicides. Powdery mildew, also named the "white mold", is clearly observable because of the powdery white colonies. The control of the pathogen *Podosphaera macularis* is based on a forecast program, which aims to prevent the initial infection. Gray mold, caused by *Botrytis cinerea*, has increasingly occurred during recent years in the Hallertau (LfL 2013). The pathogen infests the flowers and cones and therefore causes great economic losses due to poor cone quality and crop rejection (Mahaffee et al. 2009). The black root rot can have different causes. *Phytophthora citricola* is the main pathogen, but other pathogens include *Fusarium* spp. and *P. humuli*. Soil wetness, consolidation or high nitrogen supply can also cause root rot (LfL 2013). Different viruses, e.g. apple mosaic, Arabis mosaic, hop latent virus, and American hop latent virus, as well as viroids can infect hops. Diseases caused by bacteria have minor economic importance in hops. Further damages and economic failures are caused by nematodes and diverse arthropod pests, e.g. damson-hop aphid (*Phorodon humuli*), hop flea-beetle, and the two-spotted spider mite.

1.3 Verticillium Wilt

Verticillium wilt, caused by *Verticillium* spp., occurs worldwide, especially in temperate regions. The fungus can infest a wide range of host plants, including more than 200 plant species, e.g. vegetables, flowers, fruit trees, strawberries, field crops, shade and forest trees (Agrios 2005). Hop wilt is a devastating disease and causes considerable (up to 100%) economic crop losses (Down et al. 2007). The economic damage in agriculture accounts for billions of dollars (Pegg & Brady 2002). According to a new classification, *Verticillium* spp. is divided into ten species, wherein five species are newly described (Inderbitzin et al. 2011). In general, *V. dahliae* Klebahn and *V. albo-atrum* Reinke & Berthold are the most well-known and best studied species (Klosterman et al. 2009). *V. albo-atrum* and *V. dahliae* occur in hops, whereas *V. albo-atrum* is the main pathogen. *V. albo-atrum*, which infests hops, has been renamed to *V. nonalfalfae*

(Inderbitzin et al. 2011). To avoid any misinterpretation, the original name was used in this thesis. Furthermore, the new species was found in hop, potato, petunia, and spinach (Inderbitzin et al. 2011). *V. dahliae* and *V. albo-atrum* are described in detail in the reviews of Fradin and Thomma as well as Klosterman et al. (Fradin & Thomma 2006; Klosterman et al. 2009).

Verticillium spp. in hops was first reported in England in 1924 (Harris 1927). Up to now, the hop wilt has been found in Belgium, France, Germany, New Zealand, Poland, Slovenia, and USA (Neve 1991). *V. albo-atrum* is distinguished in the fluctuating (mild) and progressive (lethal) disease forms depending on the virulence, cultivar, and ecological factors (Down et al. 2007). The main difference is that the lethal form causes withering and plant death, whereas plants, which are infected with mild pathotypes, continue to grow. The lethal forms have been demonstrated only in England (Keyworth 1942), Slovenia (Radišek et al. 2003, 2006), and Germany (Seefelder et al. 2009).

1.3.1 Biology and Symptoms of *V. albo-atrum* and *V. dahliae*

Verticillium species belong to the ascomycete fungi. The two *Verticillium* species are soil-borne pathogens and can survive several years in soil by producing resting structures. *V. albo-atrum* persists via black mycelium and *V. dahliae* produces microsclerotia. Both *Verticillium* species enter the root systems by penetrating the young roots directly or through wounds before colonizing the vascular tissue. On the one hand, the fungi spread by producing spores also in higher parts of the plant and the mycelium clog up the xylem. On the other hand, *Verticillium* produces enzymes, which destroy the cell wall, and toxins (Kohlmann & Kastner 1974; Fradin & Thomma 2006). The fungi are spread by infested seeds, vegetative cuttings, and tubers, but also by wind, surface water, and soil (Agrios 2005). Verticillium spp. reproduces asexually, a sexual reproduction is not known (Pegg & Brady 2002). The morphological features are described in figures 2 and 3, taken from the studies by Inderbitzin et al. (2011). The biology and spreading of Verticillium wilt in hops is well studied (Kohlmann & Kastner 1974; Down et al. 2007).

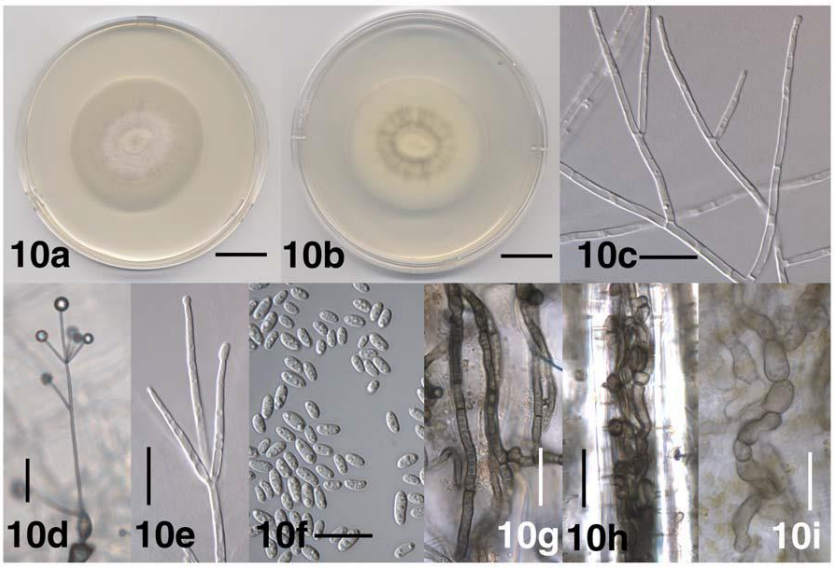

Figure 2: Morphological features of *Verticillium nonalfalfae* (formerly *V. albo-atrum*). (a-b) Colony. (c-d) Conidiophore. (e) Phialide of apical whorl. (f) Conidia. (g-i) Resting mycelium (Inderbitzin et al. 2011).

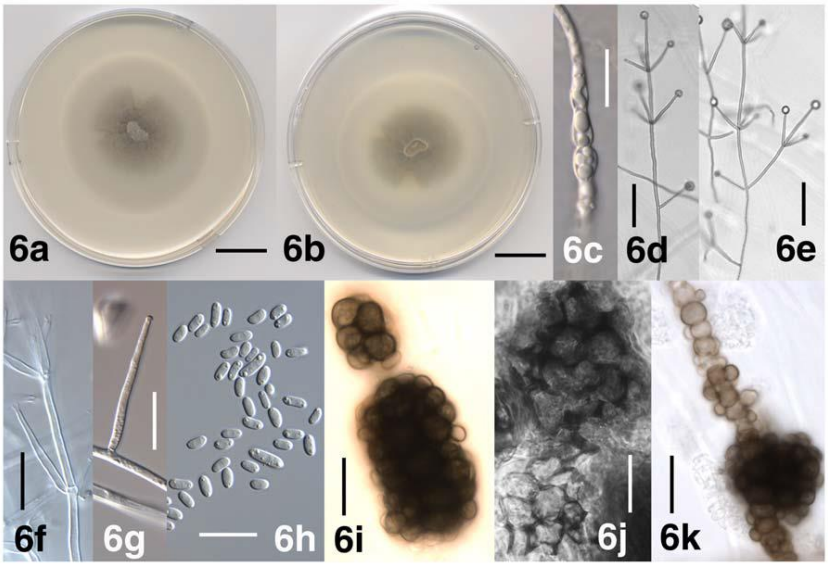

Figure 3: Morphological features of *Verticillium dahliae*. (a-b) Colony. (c) Inflated cells present in mycelium. (d-e) Conidiophore. (f) Whorl phialide. (g) Solitary phialide. (h) Conidia. (i-k) Microsclerotia (Inderbitzin et al. 2011).

Symptoms (Figure 4) of an infection are (i) yellowing and withering of the leaves (Figure 1b, d, e, f). (ii) The vascular tissue shows brown discoloration (Figure 1c) and a swollen bine can be found. (iii) The blossom cannot form and cones can show discoloration. The mild and lethal forms differ mainly in the intensity of the symptoms.

Figure 4: Symptoms of *Verticillium* in hops. (a) Healthy hop bine. (b) Hop garden with infected plants. (c) Brown discoloration of vascular tissue. (d-f) Wilting of leaves.

1.3.2 Detection of *Verticillium* in Plants

The identification and a fast detection system of *Verticillium* spp. are very important for an effective disease management. The virulence of *V. albo-atrum* isolates in hops was best identified by well-established pathogenicity tests (Sewell & Wilson 1984; Clarkson & Heale 1985). However, these bioassays are very time-consuming and laborious. In the last decades, diverse molecular genetic techniques have been applied. PCR (polymerase chain reaction) primers, based on differences in the ITS1 (internal transcribed spacer) and ITS2 region of nuclear rDNA, were developed to detect *V. albo-atrum*, *V. dahliae*, and *V. tricorpus* (Nazar et al. 1991; Robb et al. 1993; Morton et al. 1995). The standard procedure is to isolate the fungus from the hop bine on semi-selective medium according to an EPPO diagnostic guideline (Down et al. 2007). The EPPO diagnostic protocol recommends the specific primers according to Carder et

al. (1994). *V. albo-atrum* and *V. dahliae* are detected in nonalfalfae hosts by these primers and based on RFLP analysis (Carder & Barbara 1991). In addition, specific primers have been used for the detection of the mild and lethal form of *V. albo-atrum* in hops from Slovenia and Great Britain (Radišek et al. 2004). For the detection of *V. dahliae* in other crops, e.g. olive, strawberry, and artichoke, different primers are available (Mercado-Blanco et al. 2001; Mercado-Blanco et al. 2003a; Karajeh & Masoud 2006; Kuchta et al. 2008; Collado-Romero et al. 2009).

Especially, real-time PCRs are preferred in pathogen detection arrays (Lievens & Thomma 2005). This technique has several advantages compared to the standard PCR: (i) more specific, (ii) greater sensitivity, (iii) greater accuracy, (iv) faster, and (v) the detection of several pathogens or pathotypes in one PCR is enabled. For the quantification of *V. dahliae* in diverse crops, several real-time PCR assays are available (Mercado-Blanco et al. 2003b; Lievens et al. 2006; Atallah et al. 2007). Real-time PCR, based on the TaqMan technique, have been used for quantifying *V. albo-atrum* in alfalfa (Larsen et al. 2007). However, no precise detection method for routine analysis in hops is yet available.

1.3.3 Detection of *Verticillium* in Soil

Due to resting structures *Verticillium* spp. can survive several years in soil, so a detection test for soil would be desirable. Soil analysis of *V. dahliae* based on sieving and plating on agar of the microsclerotia are offered by diverse institutes in Germany and Austria (e.g. Landwirtschaftskammer Nordrhein-Westfalen, Fachhochschule Osnabrück, Österreichische Agentur für Gesundheit und Ernährungssicherheit AGES). For *V. albo-atrum* the sieving technique is not practical, because *V. albo-atrum* produces black mycelium as resting structures, which are too small for sieving. The traditional method, plating on medium, is very time-consuming and inaccurate. Several studies described molecular assays to detect *V. dahliae* in soil without sieving (Bürgmann et al. 2001; Pérez-Artés et al. 2005; Debode et al. 2011). DNA of *V. albo-atrum*, *V. dahliae*, and *V. tricorpus* were extracted directly from the soil and detected with nested PCR (Volossiouk et al. 1995; Platt & Mahuku 2000).

In this study, we tested additionally several commercially available DNA isolation kits and the method according to Bürgmann et al. (2001) and Pérez-Artés et al. (2005) to detect *V. albo-atrum* directly from the soil. The results showed that the amount of soil sample (100 to 250 mg) is not enough to detect *V. albo-atrum* with sufficient accuracy. This is supported by a study of *V. dahliae* and *V. albo-atrum* in strawberry fields (Peters 2012).

1.4 Plant Protection Measures to Control the Verticillium Wilt

Verticillium wilt is very difficult to control because of the soil habitat, the ability of the pathogen to persist for a long time due to survival structures, the infection of a wide range of hosts, and the ineffectiveness of fungicides. In general, since methyl bromide is forbidden, there are no effective chemical protection agents (Duniway 2002). In addition, hops are perennial and this complicates the control. To control Verticillium wilts, preventive and phytosanitary means are very important. Planting disease-free materials in disease-free soil is required, because *Verticillium* infested soils even in uncultivated areas have been reported (Agrios 2005; Klosterman et al. 2009). The most effective means is to remove infested plants (Fradin & Thomma 2006). Solarization, soil fumigation, and crop rotation are described to be often inefficient (Fradin & Thomma 2006).

The LfL recommends for hop farmers the following arrangements to prevent wilt (LfL 2013): (i) planting of resistant or tolerant cultivars, (ii) reduction of nitrogen fertilizer, (iii) reduction of soil cultivation to prevent injury of roots that facilitate infection, (iv) cultivation of wilt-neutral inter-tillage (e.g. cereals, corn, herbage), (v) prevention of soil consolidation, (vi) no distribution of fresh hop waste in hop gardens, and (vii) later harvest of infected hop gardens to avoid spreading of *Verticillium* spp. by machine usage.

1.5 Biological Control

An alternative strategy to conventional measurements for agriculture to control the Verticillium wilt is the biological control of soil-borne pathogens by naturally occurring antagonistic plant-associated microbes (Weller 1988; Whipps 2001). Biological control is defined as an environmentally friendly strategy to control diseases by living organisms (Lugtenberg & Kamilova 2009). In the last decades, several studies have described the promising abilities and applications of beneficial microbes. They are classified in plant growth promotion agent (PGPA) and biological control agents (BCA) (Bashan & Holguin 1998). PGPAs might act as biofertilizer, plant strengtheners, as well as phytostimulators and BCAs belong to biopesticides (Berg 2009; Lugtenberg et al. 2002). Knowledge and understanding of the role, mechanisms, and interactions of microbe, pathogen, and plant are essential for effective biological control strategies. Plant-associated microbes have to be evaluated for their ability for biological control. A successful colonization has first to be demonstrated. Confocal laser scanning microscopy (CLSM) can visualize the colonization behavior of the beneficial microbes on the plant tissue (Lugtenberg et

al. 2002; Prieto & Mercado-Blanco 2008; Zachow et al. 2010). Beneficial rhizobacteria, which promote the plant growth, are called PGPRs (Lugtenberg & Kamilova 2009). The ability to colonize the root system of PGPR is the basis for effective biocontrol approaches (Lugtenberg & Kamilova 2009). The beneficial microbes can only act locally via e.g. the effect of secondary metabolites or antibiotics. The beneficial microbes can suppress plant pathogens by different mechanisms as well as strengthen the plant and/or stimulate the plant growth. Successful application of beneficial microbes to *V. dahliae* is described in several studies. *Pseudomonas* spp. and *Serratia* spp. are effective against Verticillium wilt in several crops, such as cotton, oilseed rape, olive, and strawberry (Berg et al. 2001; Kurze et al. 2001; Müller & Berg 2008; Prieto et al. 2009; Erdogan & Benlioglu 2010). In contrast, biological control approaches against *V. albo-atrum*, respectively *V. nonalfalfae*, are rarely investigated. Naraghi et al. identified *Talaromyces flavus* as an effective BCA against *V. albo-atrum* in different crops (2010a; 2010b; 2010c).

1.5.1 The Role of Beneficial Bacteria

Microorganisms colonize different organs of plants, roots, stems, leaves, flowers, fruits, and seeds of different plants (Compant et al. 2010). The rhizosphere, which describes the roots surface and the surrounding soil, is a very attractive habitat for microorganism and also for pathogens (Weller 1988; Berg & Smalla 2009; Lugtenberg & Kamilova 2009; Raaijmakers et al. 2009). Microorganisms use and benefit from plant-produced metabolites and root exudates as nutrients. The bacterial density in the rhizosphere is 10 to 1000 times higher than in bulk soil (Compant et al. 2010). This phenomenon is called rhizosphere effect. Moreover, endophytes also show PGP effects and have a high potential for biological control (Backman & Sikora 2008; Berg et al. 2008; Hardoim et al. 2008). These beneficial bacteria are highly diverse and specific to host plants (Berg et al. 2008; Backman & Sikora 2008). The genera *Bacillus*, *Serratia*, *Pseudomonas*, *Stenotrophomonas*, and *Streptomyces* are well-investigated representatives of beneficial bacteria (Weller 1988; Berg 2009).

1.5.2 Mechanisms

Several reviews delved into the role and mechanisms of beneficial microorganisms (Weller 1988; Handelsman & Stabb 1996; Whipps 2001; Lugtenberg et al. 2002; Compant et al. 2005;

Van Loon 2007; Lugtenberg & Kamilova 2009; Berg 2009). They might promote plant growth and/or suppress plant pathogens. Diverse mechanisms and often the combinations of several mechanisms are involved. In this way, beneficial bacteria promote the plant growth directly. This means that they act in the absence of pathogens and are therefore named PGPB (plant growth promotion bacteria). PGPR are especially well discussed in many reviews (Weller 1988; Bloemberg & Lugtenberg 2001; Hardoim et al. 2008; Lugtenberg & Kamilova 2009; Raaijmakers et al. 2009; Compant et al. 2010) while other beneficial bacteria protect plants indirectly against phytopathogens and therefore act as BCAs (Lugtenberg & Kamilova 2009). The following section provides short overview.

PGPBs can act as phytostimulators, biofertilizers, and stress control agents. Further mechanisms are the induced systemic resistance (ISR) and pathogen cell signal interference.

Phytostimulator

Phytohormones enhance plant growth. They are produced by plants themselves and by their associated microbes (Berg 2009). The microbes can influence the hormonal balance. Examples for phytohormones are auxin, pyrrolquinoline quinine (PQQ), indole-3-acetic acid (IAA), ethylene, cytokinins, gibberellins, and volatiles (Berg 2009; Lugtenberg & Kamilova 2009).

Biofertilizer

Biofertilizers are bacteria, which supply plants with nutrients and which respectively increase macro- and microelements. Nitrogen (N_2)-fixing bacteria are the most important representatives. These bacteria form nodules on roots of leguminous plants, where they fix and convert atmospheric nitrogen into ammonium (NH_4). Plants need Ammonium as their nitrogen source (Bloemberg & Lugtenberg 2001; Lugtenberg & Kamilova 2009).

Stress controller

Bacteria, which produce the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, decrease the ethylene level, which is a stress hormone. They produce bioactive substances which are involved in PGP mechanisms (Lugtenberg & Kamilova 2009).

Induced systemic resistance (ISR)

Some bacteria activate the plants to defend themselves and become resistant to some pathogenic fungi, bacteria, and viruses (Van Loon 2007; Lugtenberg & Kamilova 2009).

Systemic signaling is induced by bacterial products, e.g. siderophores, salicylic acid, lipopolysaccharides, flagella, antibiotics (e.g. 2.4-DAPG, pyocyanin), volatile compounds (e.g. 2.3-butanediol), and N-acylhomoserine lactones (Bloemberg & Lugtenberg 2001; Van Loon 2007; Lugtenberg & Kamilova 2009). *Pseudomonas* and *Bacillus* strains are well-known to activate the ISR (Kloepper et al. 2004; Van Loon 2007).

Communication – Quorum sensing (QS)

Quorum sensing (QS) is the process of cell-cell communication in bacteria (Waters 2005). It occurs at high cell density and depends on bacterial behavior; in comparison these processes are inefficient by individual bacterium. QS-controlled functions are e.g. bioluminescence, biofilm formation, antibiotic secretion (rhizosphere competence), horizontal transfer of plasmids, and virulence factors (Whitehead et al. 2001; Faure et al. 2009). Small diffusible, chemical signal molecules, which are involved in QS, are called autoinducers. N-acyl-homoserine lactones (AHL) are well-described QS signal molecules. Various physiological functions and traits are controlled by AHL (Whitehead et al. 2001). The most famous representative is the control of bioluminescence by *Vibrio fischeri*. When the marine bacteria grow to high cell densities, genes for a bioluminescent signal are expressed (Whitehead et al. 2001; Waters & Bassler 2005).

BCAs, in contrast to PGPAs, obtain plant health by antagonizing pathogens. Therefore, several mechanisms play a role: competition for nutrients as well as niches, antibiotics, and parasitism (Berg 2009; Lugtenberg & Kamilova 2009).

Competition for nutrients and niches

BCAs scan the growing root for root exudate components. They suppress the pathogen in disputing nutrients and in colonizing niches in the root system (Lugtenberg & Kamilova 2009). However, great colonization competence does not necessarily imply antagonistic effects. Sometimes only specific niches are responsible for effective action of BCAs (Lugtenberg & Kamilova 2009). The competition for iron is a well investigated biocontrol mechanism. Siderophores (iron-chelating agents) repress the pathogen by binding iron and making the iron unavailable to them (Whipps 2001; Lugtenberg & Kamilova 2009).

Antibiosis

Antibiosis defines the ‘inhibition of microbial growth by diffusible antibiotics and volatile organic compounds (VOCs), toxins, and biosurfactants’ (Berg 2009). Many BCAs

produce antifungal metabolites like ammonia and hydrogen cyanide (HCN). There exists a great variety of antibiotics, e.g. phenazines, 2.4-diacetylphloroglucinol (2.4-DAPG), pyoluteorin, and pyrrolnitrin (Whipps 2001; Raaijmakers et al. 2002; Lugtenberg & Kamilova 2009).

Parasitism and extracellular enzymes

Several BCAs parasitize pathogens and thereby inhibit the plant disease (Whipps 2001; Raaijmakers et al. 2009). Beneficial bacteria and fungi can attach on hyphae of the fungal pathogen and inhibit the growth. Furthermore, extracellular lytic enzymes lyse and degrade the cell wall of pathogens, e.g. cellulases, chitinases, proteases, and glucanases.

1.5.3 Biological Control and Plant Growth Promotion in Agriculture

Biological control and PGP strategies are becoming increasingly important in sustainable, environmentally friendly agriculture. Microbial inoculants are used as biofertilizers, plant strengtheners, phytostimulators, and biopesticides (Berg 2009; Lugtenberg & Kamilova 2009). The method of screening beneficial microorganisms for the biological end-product for application lengthy and can comprise of many problems (Fravel 2005). The formulation of the PGPAs and BCAs has to be highly stable and practical for users. Various formulations are feasible: liquid-based formulations, wettable powders, water-dispersible, granules, and pellets (Berg 2009). The upscaling of biological products can be problematic because of stability and the official registration can be costly and time-consuming. Furthermore, the possible non-target effects on the micro-habitats and the potential risks to human health have to be evaluated. Berg et al. (2007) established a risk assessment for microbial antagonists. Non-target effects on the environment have to be evaluated. In tested micro-habitats no long-term effects could be observed (Berg et al. 2007). Criteria for a first evaluation include: (i) growth tests at 37°C, (ii) grouping by risk (http://www.dsmz.de), and (iii) pathogenicity assays (Berg 2009).

Several products for biological control and plant strengtheners are on the market. Many products contain *Bacillus* spp. and representatives are Serenade® (*B. subtilis* QST716; Bayer Crop Science), Companion® (*B. subtilis* GB03; Growth Products), RhizoPlus® (*B. subtilis* FZB24; ABiTEP), and RhizoVital® (*B. amyloliquefaciens* FZB42; ABiTEP). *Pseudomona*s spp. is also widely-used: Bio-Save® (*P. syringae*; JET Harvest Solutions), Cerall® and Cedomon® (*P. chlororaphis* MA 342; Intrachem Bio Deutschland), Salavida® (*P. trivialis* 3Re-27; Sourcon Padena), and BlightBan® A506 (*P. fluorescens* A506; Nufarm).

2 Objectives

The current Verticillium wilt situation in hops necessitates an effective disease management. Therefore, the purpose of this study was to develop an assay to detect *V. albo-atrum* and *V. dahliae* in hops. In addition, initial steps towards biological control of *V. albo-atrum* were investigated. Four beneficial bacteria are tested for their ability.

2.1 Molecular *In Planta* Test for the Detection of *Verticillium* Species in Hops

The standard method for detecting *V. albo-atrum* and *V. dahliae* in hops includes cultivation of the fungus by placing a piece of bine onto selective medium, DNA extraction from the fungal cultures, and PCR identification by specific primers (Down et al. 2007). This method is very time-consuming and laborious. Therefore, a detection method, which is fast, accurate, and suitable for routine use, is required. The newly developed technique is based on isolation directly from the bine and is followed by a multiplex real-time PCR assay for the simultaneous detection of *V. albo-atrum* and *V. dahliae*. The DNA extraction method by a commercially available kit is time-saving and accurate. Primer pairs and TaqMan probes were established and verified.

2.2 Initial Steps towards Biological Control – Assessment of Root Competence, PGP Effect, and Antagonistic Activity

The control of Verticillium wilt in hops is very difficult. Biological control, based on the beneficial traits of microorganisms, can be a promising additional and/or alternative plant protection strategy to the conventional disease managements. Therefore, initial steps towards a biological control were assessed. Four plant-associated bacteria were chosen because of their auspicious ability in biological control (Table 1): *Burkholderia terricola* ZR2-12 (Gasser et al. 2009), *Pseudomonas poae* RE*1-1-14 (syn. *P. trivialis*; Zachow et al. 2008), *Serratia plymuthica* 3Re4-18 (Faltin et al. 2004), and *Stenotrophomonas rhizophila* DSM14405^T (=e-p10 and =P69; Berg et al. 1996) and were evaluated for their use in hops. Many other studies already described these bacteria as applicable for biological control (Berg et al. 2005; Grosch et al. 2005; Scherwinski et al. 2008; Zachow et al. 2008, Zachow et al. 2010; Grosch et al. 2012; Schmidt et al. 2012). The competent colonization is the prerequisite towards efficient biocontrol. The objectives were: (i) to evaluate the colonization ability of these beneficial bacteria in hop root systems, (ii) to visualize the colonization patterns of DsRed-labeled transformants with confocal

laser scanning microscopy (CLSM), (iii) to investigate the potential PGP effects of treated seedlings and (iv) to assess the antagonistic activity against *V. albo-atrum* and *V. dahliae* via dual culture tests.

Table 1: Strains used in this study to assess their ability for biological control in hops, their origin, antagonistic activity, bioactive metabolites, and PGP effects

Strains	EMBL number	Origin	Antagonistic activity against[a]	Bioactive metabolites[b]	PGP	References
Burkholderia terricola ZR2-12	FN313521	Rhizosphere of sugar beet	-	ACC, Siderophores	+	(Gasser et al. 2009, 2011)
Pseudomonas poae RE*1-1-14	FN675869; CP004045	Endorhiza of sugar beet	*P. betae*, *R. solani*, *S. rolfsii*	Chitinase, Glucanase, Proteases	+	(Zachow 2008, 2009, 2010)
Serratia plymuthica 3Re4-18	FN675868	Endorhiza of potato	*B. cinerea*, *P. betae*, *R. solani*, *S. rolfsii*, *V. dahliae*	Cellulase, Chitinase, Glucanase, Pectinase, Proteases, Siderophores	+	(Faltin et al. 2004, Berg et al. 2005; Scherwinski et al. 2008; Zachow et al. 2010)
Stenotrophomonas rhizophila DSM14405^T	-	Rhizosphere of oilseed rape	*R. solani*, *S. sclerotiorum*, *V. dahliae*	Chitinase, Glucanase, Hemolysine, Proteases, Siderophores	+	(Berg et al. 1996; Minkwitz & Berg 2001; Wolf et al. 2002; Schmidt et al. 2012)

[a] *B. cinerea* = *Botrytis cinerea*; *P. betae* = *Phoma betae*; *R. solani* = *Rhizoctonia solani*; *S. rolfsii* = *Sclerotium rolfsii*; *S. sclerotiorum* = *Sclerotinia sclerotiorum*; *V. dahliae* = *Verticillium dahliae*

[b] ACC = 1-aminocyclopropane-1-carboxylate

3 Results

The experimental procedure is described in details in *Publications I* and *II*. The following sections give a short overview of the results of the studies. *Manuscript I* describes the successful application of the real-time PCR assay and summarizes the applicability of the four beneficial bacterial strains to control Verticillium wilt in hops.

3.1 Molecular *In Planta* Test for the Detection of *Verticillium* Species in Hops

The combination of the DNA extraction directly from the hop bine and the use of a commercially available DNA isolation kit is a very accurate and time-saving procedure. The working time is reduced from six to seven days in the standard procedure to only one day. For the real-time PCR to detect *V. albo-atrum* and *V. dahliae* simultaneously, primer pairs and probes were designed. The specificity was proven using BLAST analysis (NCBI) and no amplification of non-target microorganisms, which were isolated from hop bines, were observed. The standard curves and efficiency of the primer/probe sets for *V. albo-atrum*, respectively *V. dahliae*, was assessed for a single real-time PCR assay, containing additional DNA of the appropriate other species as well as healthy plant DNA. The linear coefficient R^2 was 0.998 for both species and the efficiency of *V. albo-atrum* was 92.7% and for *V. dahliae* 93.8%. The R^2 coefficient and the efficiency were not substantially impaired by the additional *Verticillium* DNA and plant DNA.

For evaluation of the real-time PCR assay, the newly developed method was compared with the standard PCR technique (Publication I). 14.6% more samples tested positive for *V. albo-atrum* with the real-time PCR assay. *V. dahliae* occurred rarely in hops, only 6.6% (five samples from 76 infected hop bines) were identified as *V. dahliae* with the newly developed method. In comparison three samples were identified as *V. dahliae* with the standard method. In general, the real-time PCR is 200 times more sensitive for *V. albo-atrum* and 533 times so for *V. dahliae* than the conventional PCR, according to Carder et al. (1994). Furthermore, *V. albo-atrum* can occur latent, without showing any symptoms, however, the newly developed assay showed only 6.2% negative results. Only one DNA extraction reported a fail, while the DNA was proven by general primer pairs for polyubiquitin in hops.

The infection is described to proceed from the bottom up (Kohlmann & Kastner 1974). The distribution was observed by the real-time PCR assay (Manuscript I). In 50% of the inspected plants *V. albo-atrum* was detected up to the top of the plant. In the other five bines *V.*

albo-atrum was found in the lower two-thirds. A continuous process was not observed. The fungus was practically observed in the side shoots and cones. The PCR of wilting leaves did not show any results. A reason for this can be inhibitions by substances, produced by wilting and browning.

3.2 Initial Steps towards Biological Control – Assessment of Root Competence, PGP Effect, and Antagonistic Activity

To evaluate the ability to control Verticillium wilt in hops, the competent colonization and PGP effects of *B. terricola* ZR2-12, *P. poae* RE*1-1-14, *S. plymuthica* 3Re4-18, and *S. rhizophila* DSM14405^T were assessed. Rifampicin-resistant mutants were re-isolated from the endorhiza and rhizosphere of hops. In general, the abundances ranged from $\log_{10}$ 3.0 to $\log_{10}$ 6.2 CFU g^{-1} root fresh weight (RFW) in the endorhiza and from $\log_{10}$ 2.9 to $\log_{10}$ 4.7 CFU g^{-1} RFW in the rhizosphere. *B. terricola* ZR2-12 showed the highest cell number ($\log_{10}$ 6.2 ± 0.4 CFU g^{-1} RFW in the endorhiza and $\log_{10}$ 4.7 ± 0.1 CFU g^{-1} RFW in the rhizosphere). This corresponds to an up to three orders of magnitude higher cell density than what was found for the other three bacteria. The colonization of *P. poae* RE*1-1-14 was $\log_{10}$ 4.3 ± 0.2 CFU g^{-1} RFW in the endorhiza and $\log_{10}$ 3.0 ± 0.2 CFU g^{-1} RFW in the rhizosphere. *S. plymuthica* 3Re4-18 showed an abundance of $\log_{10}$ 3.0 ± 0.2 CFU g^{-1} RFW in the endorhiza and $\log_{10}$ 2.9 ± 0.1 CFU g^{-1} RFW in the rhizosphere. The density of *S. rhizophila* DSM14405^T was $\log_{10}$ 4.1 ± 0.2 CFU g^{-1} RFW in the endorhiza and $\log_{10}$ 3.8 ± 0.2 CFU g^{-1} RFW in the rhizosphere. In general, the bacterial cell number was higher in the endorhiza than in the rhizosphere. Furthermore, the bacteria survive an outdoor hibernation at sub-zero temperatures. *B. terricola* ZR2-12 showed again the highest density ($\log_{10}$ 5.8 CFU g^{-1} RFW in the endorhiza and $\log_{10}$ 4.8 CFU g^{-1} RFW in the rhizosphere), whereas the colonization of the other three bacteria in the endorhiza was approximately $\log_{10}$ 3 CFU g^{-1} RFW. For a better visualization of the colonizing behavior, the patterns of DsRed-labeled transformants (*B. terricola* ZR2-12, *P. poae* RE*1-1-14, *S. plymuthica* 3Re4-18) were analyzed by CLSM. The microscopic analysis confirmed the rhizosphere competence. *B. terricola* ZR2-12 proved to be a very good root colonizer. Therefore, the endorhiza and the stem were additionally analyzed. *B. terricola* ZR2-12 showed a high cell density in the rhizosphere and scattered arrangements on root hairs and on a root tip. Also scattered arrangements were found in the endosphere. A large accumulation of colonies was found on stem surfaces. In contrast, *P. poae* RE*1-1-14 cells appeared along the epidermis cell and *S. plymuthica* 3Re4-18 showed single cells in small colonies on the surface.

The growing promotion and strengthening of the plant can support the plant health and can therefore act in biological control. Potential growth promoting effects of these bacteria were assessed by treating seeds of the cross breed of Cascade and 2007/005/504. Seven weeks after inoculation, the growth was determined by measurement of the length of the plantlets without the roots and the length of the fourth leaves including cotyledons. To assess an additive effect, plantlets treated with a mixture of *S. plymuthica* 3Re4-18 and *S. rhizophila* DSM14405^T (1:1) were analyzed. *S. plymuthica* 3Re4-18 promotes the growth in both experimental sets. A PGP effect of *S. rhizophila* DSM14405^T and the mixture was observed for the size of the fourth leaves.

The effect to antagonize pathogens is another trait of beneficial microorganisms. Therefore, dual-culture tests were arranged. Only *S. plymuthica* 3Re4-18 showed antagonistic effects against *V. albo-atrum* and *V. dahliae*, although antagonistic activity was previously described for *S. rhizophila* DSM14405^T (Minkwitz & Berg 2001).

4 Discussion

Due to the lack of effective chemical control against *Verticillium* spp. in hops, only few measures are available. Therefore, an accurate and fast detection system and new approaches for controlling the wilt are required. One of the aims of this study was to establish a molecular *in planta* test based on real-time PCR techniques. Real-time assays to detect either *V. dahliae* or *V. albo-atrum* have been successfully used in other crops (Mercado-Blanco et al. 2003b; Lievens et al. 2006; Atallah et al. 2007; Larsen et al. 2007; Duressa et al. 2012). However, a multiplexed PCR, able to detect both species simultaneously, was missing. Furthermore, an effective DNA isolation method is essential for routine analysis. The newly developed assay contains a combination of DNA extraction directly from the bine by a commercially available kit and a real-time PCR based on TaqMan technique. Pathogenicity tests and the standard culturing isolation method, which is described in the EPPO diagnostics protocol, is very time-consuming and laborious (Sewell & Wilson 1984; Clarkson & Heale 1985; Down et al. 2007). In contrast, the application of a homogenizer using big ceramic beads and granite matrix as well as the isolation kit was very suitable for hop bines, whose texture can be very ligneous. In general, the real-time PCR has many advantages against standard PCR or ELISA tests (Lievens & Thomma 2005). Real-time based methods are faster, more specific, sensitive, accurate, and are able to detect different microorganisms or species simultaneously. Compared to the standard PCR technique, the newly developed real-time PCR assay (homogenizer, DNA isolation kit, and real-time PCR) is more accurate, more effective and time-saving. This test is an application for routine use and can help to detect *Verticillium* spp. in hops early, a necessary prerequisite to an effective treatment.

To combat the Verticillium wilt, biological control offers an alternative or additional strategy to conventional disease managements. Several studies described the successful application of BCAs and PGPAs against *V. dahliae* (Berg et al. 2001; Kurze et al. 2001; Mercado-Blanco et al. 2004; Müller & Berg 2008; Erdogan & Benlioglu 2010). Biological control measures against *V. albo-atrum* are little known. The fungus *Talaromyces flavus* was described to control *V. albo-atrum* in cucumber, potato, and tomato (Naraghi et al. 2010a; 2010b; 2010c). In this study four beneficial bacteria, *B. terricola* ZR2-12, *P. poae* RE*1-1-14, *S. plymuthica* 3Re4-18, and *S. rhizophila* DSM14405^{T}, were evaluated for their use in plant protection management by biological control in hops. The colonization competence, PGP, and antagonistic effects were assessed. The ability to colonize the root system is the key to effective

control (Lugtenberg & Kamilova 2009). All of the tested spontaneous mutants resistant to Rifampicin colonized the root system of hops with general abundances of $\log_{10}$ 3.0 to $\log_{10}$ 6.2 CFU g^{-1} RFW in the endorhiza and from $\log_{10}$ 2.9 to $\log_{10}$ 4.7 CFU g^{-1} RFW in the rhizosphere. The colonization pattern of DsRed-labeled transformants (*B. terricola* ZR2-12, *P. poae* RE*1-1-14, *S. plymuthica* 3Re4-18) were visualized by CLSM. *B. terricola* ZR2-12, *S. plymuthica* 3Re4-18, and *S. plymuthica* 3Re4-18 showed to be promising candidates with regard to the tested traits. Especially *S. plymuthica* 3Re4-18 is an effective BCA. In addition to the colonization competence, plantlets, whose seeds were treated with this strain, showed PGP effects. The growth promotion was already observed for other crops (Faltin et al. 2004; Zachow et al. 2010). Furthermore, antagonistic activity against *V. albo-atrum* and *V. dahliae* was assessed via dual culture tests. *S. plymuthica* 3Re4-18 and other strains, especially *S. plymuthica* HRO-C48, are known as effective BCAs in other crops (Faltin et al. 2004; Scherwinski et al. 2006; Berg et al. 2008; Scherwinski et al. 2008; Grosch et al. 2012). Also *B. terricola* ZR2-12 has potential for the biological control due to the high and stable abundances ($\log_{10}$ 6.2 ± 0.4 CFU g^{-1} RFW in the endorhiza and $\log_{10}$ 4.7 ± 0.1 CFU g^{-1} RFW in the rhizosphere). A similar cell density was reached after an outdoor hibernation at sub-zero temperature. Hence, the colonization pattern was intensively analyzed by CLSM. A high abundance was observed in the rhizosphere and scattered arrangements were found on root hairs, root tips and in the endorhiza. Also a large accumulation of colonies was observed on stem surfaces. *B. terricola* ZR2-12 is a newly-discovered strain and the lifestyle, excellent root colonization, and PGP effect on sugar beets were described by Gasser et al. (2011). However, this strain showed neither PGP effects on hops nor antagonistic activity against *V. albo-atrum* and *V. dahliae*. *S. rhizophila* DSM14405^T can be suited to control of Verticillium wilt because of the PGP effects, which conformed to trials with other crops (Schmidt et al. 2012). *P. poae* RE*1-1-14 did not show sufficient PGP effects and antagonistic activity. This strain colonizes emerging roots of sugar beets and shows an antagonistic activity against the phytopathogens *Phoma betae*, *Rhizoctonia solani*, and *Sclerotium rolfsii* (Zachow et al. 2010). However, it seems that this strain is not suited for control of Verticillium wilt in hops.

In addition to the tested colonization behavior, PGP effects, antagonistic activities, and artificial infection tests with *V. albo-atrum* are necessary to determine the beneficial bacterial efficiency. The consistent efficacy must be verified and the control effect must be assessed under field conditions. If the promising candidates fulfill these requirements, non-target effects and the

risk for the environment have to be assessed. The preparation of a commercial plant protection agent is a very long and elaborate process.

However, this study demonstrated clues for a promising approach to control Verticillium wilt in hops. The real-time PCR assay enables a fast and accurate detection for routine use. This is the precondition for effective control. The preliminary tests of the beneficial bacteria show that the biological control can be an additional measure to the currently applied strategies and might enable establishment of an environmentally friendly, plant preserving way to control Verticillium wilt in hops.

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6 Appendix

Publication I

Real-time PCR assay to detect Verticillium albo-atrum and V. dahliae in hops: development and comparison with a standard PCR method.

Maurer KA, Radišek S, Berg G and Seefelder S (2013) Journal of Plant Diseases and Protection 120: 105-114

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Initial steps towards biocontrol in hops: successful colonization and plant growth promotion by four bacterial biocontrol agents

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Manuscript I

Untersuchungen zur Verticillium-Welke im Hopfenanbaugebiet Hallertau

Maurer KA, Berg G and Seefelder S

Gesunde Pflanzen

Additional proceeding I

Development of a rapid molecular *in planta* test for the detection of *Verticillium* pathotypes in hops and strategies for prevention of wilt.

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Publication I

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Real-time PCR assay to detect *Verticillium albo-atrum* and *V. dahliae* in hops: development and comparison with a standard PCR method

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Real-time PCR assay to detect *Verticillium albo-atrum* and *V. dahliae* in hops: development and comparison with a standard PCR method

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Abstract

Verticillium wilt, caused by *Verticillium albo-atrum* and *V. dahliae*, is a devastating disease that causes considerable economic crop losses in hop fields. The fungus can survive in soil for several years by producing resting structures, and due to the lack of effective fungicides, the disease is spreading. Thus, a fast and sensitive detection system is urgently needed. In this study we report a novel routine detection method for the identification of *V. albo-atrum* and *V. dahliae* on a molecular basis. The standard polymerase chain reaction (PCR) assay includes isolation of the fungus from affected tissue by following DNA extraction from fungal cultures and PCR identification by specific primers. In order to improve this detection assay, DNA was isolated directly from the hop bine using a commercially available DNA isolation kit. A multiplex real-time PCR assay for the simultaneous detection of *V. albo-atrum* and *V. dahliae* was established using a specific primer pairs/TaqMan probes combination. Ninety-six plants were collected from different cultivars and locations in Germany and tested by standard and real-time PCR assay. The new detection system is more accurate, sensitive and time-saving than the standard PCR method and is suitable for routine use. The test provides a valuable tool for rapid and sensitive detection of *V. albo-atrum* and *V. dahliae* in plants and gives farmers and plant protection offices the chance to react accordingly and to evaluate measures for plant disease management against *Verticillium*.

Key words: *Humulus lupulus*, *in planta*, multiplex real-time PCR, Verticillium wilt

Introduction

Hop wilt, caused by *Verticillium* spp., is a devastating disease and causes considerable economic crop failures (Down et al. 2007). In hops (*Humulus lupulus*) *V. albo-atrum* is the most widespread *Verticillium* species, whereas *V. dahliae* is rarely found (Zinkernagel 1982). Inderbitzin and coworkers (2011) recently proposed a new classification of *Verticillium* plant pathogenic species, where the currently used species name *V. albo-atrum* in hops was renamed to *V. nonalfalfae*. However, in this study, we used the original name to be more accessible for practical users. Both *Verticillium* species can survive several years in soil by producing resting structures. *V. albo-atrum* produces dark resting mycelium, whereas *V. dahliae* persists due to microsclerotia (Wilhelm 1955, Down et al. 2007). After infection of the roots, the disease progresses and the fungus colonize the vascular system resulting in yellowing and wilting of the leaves as well as brown discoloration of the vascular tissue in the bine (Engelhard 1957). Verticillium wilt on hop was first reported in England in 1924 (Harris 1927). Later the fluctuating (mild) and progressive (lethal) disease forms were distinguished, which were attributed to be virulence of the isolates (Keyworth 1942). In lethal disease form, plants show severe disease symptoms which leads to plant withering, whereas in the mild form the symptom intensity varies from year to year with low mortality rate. *Verticillium* wilt in hops has been reported in Germany, Poland, Belgium, France, Slovenia, and, outside Europe, in New Zealand and USA, caused either by *V. albo-atrum* or *V. dahliae* (Neve 1991). However, highly virulent isolates of *V. albo-atrum*, which cause the lethal disease, have been to date present only in England (Keyworth 1942), Slovenia (Radišek et al. 2003, Radišek et al. 2006) and in Germany (Seefelder et al. 2009). There are currently no effective chemical or biological treatments available to cure the infected plants. Therefore the control based primarily on planting of resistant or tolerant plants, crop rotation and phytosanitary measures (Fradin & Thomma 2006, Down et al. 2007). As an addition to all control measures, an accurate identification techniques are very important and the key for an effective disease management.

Pathogenicity tests are a well established technique to characterize virulence of *V. albo-atrum* isolates in hops (Sewell & Wilson 1984, Clarkson & Heale 1985), but they are time-consuming and laborious. PCR primers, based on the differences of the internal transcribed spacer (ITS) regions ITS1 and ITS2 of nuclear rDNA, can distinguish *V. albo-atrum*, *V. dahliae* and *V. tricorpus* (Nazar et al. 1991, Robb et al. 1993, Morton et al. 1995). Carder and coworkers (1994) developed specific primers for detection of *V. dahliae* and *V. albo-atrum* isolates from nonalfalfae hosts. These primers are based on RFLP analysis (Carder & Barbara 1991) and are now used in EPPO diagnostics protocol (Down et al. 2007). More sensitive nested PCR techniques have

been developed for detecting *V. dahliae* on olive (Mercado-Blanco et al. 2001, Mercado-Blanco et al. 2003a, Karajeh & Masoud 2006), strawberry (Kuchta et al. 2008) and artichoke (Collado-Romero et al. 2009). In hop, Radišek and coworkers (2004) developed specific primers for the detection of different *V. albo-atrum* hop pathotypes. Pathogen detection arrays, especially the use of PCR, became more and more important. In general, they are faster, more specific, sensitive, accurate and allow a detection of non-culturable microorganisms in comparison to bioassays and plating methods (reviewed in Lievens & Thomma 2005). Real-time based methods have great advantages and are preferred in pathogen detection arrays (reviewed in Lievens & Thomma 2005). The ability to detect different pathogens or pathotypes with one PCR is another great benefit. Real-time PCR is a distinguished tool to detect pathogens and is suitable for routine analysis (Mercado-Blanco et al. 2003b, Atoui et al. 2012), also in combination with TaqMan techniques (Ward et al. 2004, Van Gent-Pelzer et al. 2007, Hughes et al. 2011). In addition, several studies showed that real-time PCR enables the quantification of *V. dahliae* in other crops (Mercado-Blanco et al. 2003b, Lievens et al. 2006, Atallah et al. 2007). Larsen and coworkers (2007) developed a TaqMan assay for quantifying *V. albo-atrum* in alfalfa. Their study showed that the colonization of *Verticillium* is significantly reduced in resistant cultivars; quantitative PCR can be useful to find resistant cultivars. Despite this scientific progress, no precise detection method for *Verticillium* in hop is currently available.

The aim of this study was to develop a rapid molecular *in planta* detection test for routine use. The DNA isolation was prepared directly from the hop bine using a commercially available kit. We developed a multiplex real-time PCR assay to detect *V. albo-atrum* and *V. dahliae* simultaneously using TaqMan probes. Furthermore, we compared the developed technique with the standard PCR method.

Materials and methods

Plant material

Altogether, 96 plants were sampled in Hallertau (73 plants) and Tettnang (23 plants) in Germany in 2008 and 2009. Different hop gardens and cultivars were selected (Table 1). The bines belonged to the cultivars 'Hallertauer Mittelfrüher' (31 samples), 'Hallertauer Tradition' (29 samples), 'Perle' (18 samples), 'Herkules' (6 samples), 'Hallertauer Magnum' (5 samples), 'Hallertauer Taurus' (2 samples), 'Spalter Select' (1 sample), 'Saphir' (1 sample), 'Nugget' (1 sample), 'Brewers Gold' (1 sample) and '2005/010/709' (1 sample). The bine was sampled within the first meter above the soil. The plants were chosen because of the symptoms and the situation of the infection in the hop gardens. Seventy-six plants showed symptoms of *Verticillium*. Fourteen plants were collected from hop gardens, which were healthy from visual inspection, and six plants, which did not show symptoms, were sampled in the vicinity of infected hops. The bines were kept at −20°C until analysis.

Fungal isolates

Twenty-seven *Verticillium* isolates from hop were obtained from different locations in Europe: 14 from Slovenia, 9 from Great Britain and 4 from Germany (Table 2). Twenty-two fungi belonged to *V. albo-atrum* and 5 to *V. dahliae*. One *V. albo-atrum* isolate was from cucumber (*Cucumis sativus*) and one *V. dahliae* isolate from potato (*Solanum tuberosum*). Twenty two isolates have been previously morphological and molecular characterized by Radišek et al. 2006, whereas seven isolates (10, 15, 55, 83, Sent4, Let1 and 11043) were characterized by PCR assay during this analysis. The isolates were kept in the culture collection of Slovenia Institute for Hop Research and Brewing, Slovenia. They were stored as monospore cultures on potato dextrose agar (PDA) at 4°C or as cultures in general fungal medium in glycerol (20%) at −80°C (Weising et al. 1995). The species were identified by light microscopy.

Standard PCR method

Fungal DNA isolation using plating method. A piece of hop bine, about 6 cm in size, was surface sterilized for about 30 s with sodium hypochlorite (2.4% active chlorine). After the bine was dried, it was breamed with 70% ethanol. The interior bine was transferred onto prune lactose yeast agar containing 50 μg ml^{-1} streptomycin sulphate (Roth, Karlsruhe, Germany) and incubated at 20°C for one week (Talboys 1960). The fungus was recognized easily with the unaided eye and transferred to a new plum-agar plate. Up to 100 mg mycelia of *V. albo-atrum/V. dahliae* was scraped from cultures on prune agar containing 50 μg ml^{-1} streptomycin sulphate (Roth). For DNA extractions, the commercially available Invisorb® Spin Plant Mini kit was used according to the manufacturer´s instructions (Invitek, STRATEC Molecular, Berlin, Germany). Lysis Buffer P (400 μl) was added to the mycelia in a 2-ml vial containing about 100 mg of 0.25–0.50 mm glass beads, about 100 mg 0.75–1.00 mm glass beads and one 3 mm glass bead and homogenized at 5.5 m s^{-1} for 30 s using FastPrep 24® (MP Biomedicals, Santa Ana, CA, USA).

PCR identification of *V. albo-atrum* and *V. dahliae*. The method described in the EPPO diagnostic protocol was used for the subsequent detection of *Verticillium* species (Down et al. 2007). PCR conditions were as follows: 4 min at 94°C followed by 30 cycles consisting of 45 s at 94°C, 30 s at 54°C, 1 min at 72°C. The modified PCR reaction mixture (20 μl) contained: 1 ng of DNA, 500 nM of each primer (2/3 for *V. albo-atrum* and 19/22 for *V. dahliae*; Carder et al. 1994), 0.3 mM dNTPs (Eurogentec, Köln, Germany), 0.03 U ml^{-1} ThermoPrime *Taq* DNA polymerase (Thermo Scientific, ABgene®UK, Epsom, United Kindom), 1.5 mM $MgCl_2$, 10x Reaction Buffer IV (750 mM Tris-HCl, pH 8.8 at 25°C, 200 mM $(NH_4)_2SO_4$, 0.1% (v/v) Tween® 20). PCR was performed using Mastercycler pro or ep Gradient Thermocycler (Eppendorf, Hamburg, Germany). Every PCR reaction set included a water control (Roth, Karlsruhe, Germany) as

Table 1: List of cultivars collected from different locations in Germany, their symptoms, showing detection results for *V. albo-atrum* and *V. dahliae* using DNA kit isolation and real-time PCR and the comparison with the standard PCR method

			Newly developed method[c]		Standard PCR method[d]	
Cultivar[a]	Region	Symptoms[b]	*V. albo-atrum*	*V. dahliae*	*V. albo-atrum*	*V. dahliae*
SR	Hallertau	+	+	–	+	–
2005/010/709	Hallertau	+	+	+	–	–
HT	Hallertau	+	+	–	+	–
HT	Hallertau	+	+	–	+	–
PE	Hallertau	+	+	–	+	–
PE	Hallertau	+	+	–	+	–
HT	Hallertau	+	+	–	+	–
HT	Hallertau	+	+	–	+	–
PE	Tettnang	+	+	–	+	–
PE	Tettnang	+	+	–	+	–
PE	Tettnang	+	+	–	+	–
PE	Tettnang	+	+	–	+	–
HT	Hallertau	+	–	–	–	–
HM	Hallertau	+	+	–	–	–
PE	Hallertau	+	–	–	–	–
HA	Hallertau	+	–	–	–	–
HA	Hallertau	+	+	–	+	–
HA	Hallertau	+	+	–	–	–
HM	Hallertau	+	–	–	–	–
HA	Hallertau	–	–	–	+	–
HM	Hallertau	–	–	–	+	–
HT	Hallertau	–	+	–	+	–
HT	Hallertau	–	–	–	+	–
TU	Hallertau	–	+	–	+	–
TU	Hallertau	–	+	–	+	–
HM	Hallertau	–	–	–	–	–
HS	Hallertau	–	–	–	–	–
HA	Hallertau	–	+	–	–	–
PE	Hallertau	–	–	–	–	–
HS	Hallertau	–	–	–	–	–
HS	Hallertau	–	–	–	–	–
HM	Hallertau	–	–	–	–	–
HT	Hallertau	+	–	–	–	–
HT	Hallertau	+	–	–	–	–
HS	Hallertau	+	+	–	+	–
HS	Hallertau	+	+	–	+	–
PE	Hallertau	–	+	–	+	–
PE	Hallertau	+	+	–	+	–
HS	Hallertau	+	+	–	–	–
NU	Hallertau	+	+	–	+	–
HT*	Hallertau	+	–	–	+	–
HT	Hallertau	+	+	–	+	–
HT	Hallertau	+	+	–	+	–
HT	Hallertau	+	+	–	+	–
HT	Hallertau	+	+	–	+	–
HT	Hallertau	+	+	–	–	–
PE	Hallertau	+	+	–	+	–
HT	Hallertau	+	+	–	+	–
HT	Hallertau	+	+	–	–	–
HT	Hallertau	+	+	–	+	–

Table 1: (Continued)

Cultivar[a]	Region	Symptoms[b]	Newly developed method[c]		Standard PCR method[d]	
			V. albo-atrum	*V. dahliae*	*V. albo-atrum*	*V. dahliae*
Brewers Gold	Hallertau	+	+	–	–	–
SE	Hallertau	+	+	–	–	–
HT	Hallertau	+	+	–	+	–
HT	Hallertau	+	+	–	+	–
HA	Tettnang	+	+	–	+	–
HA	Tettnang	+	+	–	+	–
HA	Tettnang	+	+	–	–	–
HA	Tettnang	+	+	–	+	–
HA	Tettnang	–	–	–	–	–
HA	Tettnang	+	+	–	+	–
HA	Tettnang	+	+	–	+	–
HA	Tettnang	+	+	–	–	–
HA	Tettnang	+	+	–	+	–
HA	Tettnang	+	+	–	+	–
HA	Tettnang	–	+	–	+	–
HA	Tettnang	–	–	–	–	–
HA	Tettnang	–	+	–	+	–
HA	Tettnang	–	+	–	+	–
HT	Hallertau	+	–	–	–	–
HT	Hallertau	+	+	–	+	–
HT	Hallertau	+	+	–	+	–
HT	Hallertau	+	+	–	+	–
HT	Hallertau	+	+	–	+	–
HT	Hallertau	+	+	–	+	–
HT	Hallertau	+	+	–	+	–
HT	Hallertau	+	+	–	+	–
HT	Hallertau	+	+	–	+	–
HA	Hallertau	+	+	–	+	–
HA	Hallertau	+	+	–	–	–
HA	Hallertau	+	+	–	–	–
PE	Hallertau	+	+	–	+	–
PE	Tettnang	+	+	–	+	–
PE	Tettnang	+	+	–	+	–
PE	Tettnang	+	+	–	+	–
PE	Tettnang	+	–	–	+	–
PE	Tettnang	–	+	–	+	–
HA	Hallertau	+	+	+	+	–
HA	Hallertau	+	+	–	+	–
HA	Hallertau	+	–	–	+	–
HA	Hallertau	+	+	–	+	–
HA	Hallertau	+	–	+	–	+
HA	Hallertau	+	–	+	–	+
HA	Hallertau	+	–	+	–	+
HA	Hallertau	+	+	–	+	–
HA	Hallertau	+	+	–	–	–
PE	Hallertau	+	+	–	+	–

[a] SR Saphir; HT Hallertauer Tradition; PE Perle; HM Hallertauer Magnum; HA Hallertauer Mittelfrüher; TU Hallertauer Taurus; HS Herkules; SE Spalter Select; NU Nugget;

[b] Visible symptoms = +; no visible symptoms or healthy plants = –

[c] Amplification by the real-time PCR: detected = +; not detected = –

[d] Amplification by the standard PCR method: detected = +; not detected = –

* No DNA amplification for polyubiquitin

Table 2: Fungal isolates, their host and geographical origin used in this study

Isolate designation	Species	Host[a]	Geographical origin	Source[b]
10	*V. albo-atrum*	Hop	Germany	1
15	*V. albo-atrum*	Hop	Germany	1
55	*V. albo-atrum*	Hop	Germany	1
83	*V. albo-atrum*	Hop	Germany	1
Roz	*V. albo-atrum*	Hop	Slovenia	IHPS
Rec	*V. albo-atrum*	Hop	Slovenia	IHPS
Zup	*V. albo-atrum*	Hop	Slovenia	IHPS
Vran	*V. albo-atrum*	Hop	Slovenia	IHPS
T2	*V. albo-atrum*	Hop	Slovenia	IHPS
T6	*V. albo-atrum*	Hop	Slovenia	IHPS
Led	*V. albo-atrum*	Hop	Slovenia	IHPS
Urs	*V. albo-atrum*	Hop	Slovenia	IHPS
Sent4	*V. albo-atrum*	Hop	Slovenia	IHPS
Let1	*V. albo-atrum*	Hop	Slovenia	IHPS
Ciz	*V. albo-atrum*	Hop	Slovenia	IHPS
1953	*V. albo-atrum*	Hop	Great Britain	2
1985	*V. albo-atrum*	Hop	Great Britain	2
11043	*V. albo-atrum*	Hop	Great Britain	3
11055	*V. albo-atrum*	Hop	Great Britain	2
298095	*V. albo-atrum*	Hop	Great Britain	CABI
298099	*V. albo-atrum*	Hop	Great Britain	CABI
298101	*V. albo-atrum*	Hop	Great Britain	CABI
Kum	*V. albo-atrum*	Cucumber	Slovenia	IHPS
CasD	*V. dahliae*	Hop	Slovenia	IHPS
MoD	*V. dahliae*	Hop	Slovenia	IHPS
KresD	*V. dahliae*	Hop	Slovenia	IHPS
12042	*V. dahliae*	Hop	Great Britain	2
12099	*V. dahliae*	Hop	Great Britain	3
JKG8	*V. dahliae*	Potato	The Netherlands	4

[a] Hop (*Humulus lupulus*), Cucumber (*Cucumis sativus*), Potato (*Solanum tuberosum*)

[b] 1 = Dr. S. Seefelder, Bavarian State Research Center of Agriculture, Germany; IHPS = Slovenian Institute for Hop Research and Brewing, Žalec, Slovenia; 2 = Dr. G. Down, Disease Management, Horticulture Research International, West Malling, UK; CABI = Bioscience, Genetic Resources Collection, Bakeham Lane, Surrey, UK; 3 = East Malling Research, UK; 4 = Dr. JK Goud, Biological Farming Systems, Wageningen, the Netherland.

negative controls and DNA from reference isolates as positive controls. After amplification, the PCR product was visualized on 1.5% agarose gel in 1 × TBE buffer containing ethidium bromide. Samples were positive if single band of 300 bp and 580 bp were amplified for *V. albo-atrum* and *V. dahliae*, respectively. The overall time to identify *Verticillium* species following this procedure is about one week.

Novel detection assay

Fungal DNA isolation directly from plants. DNA was extracted from the bine using Invisorb® Spin Plant Mini kit according to the manufacturer´s instructions (Invitek). Prior to DNA extraction, the bark of the hop bines was removed and the xylem was cut in small pieces. For the homogenization, 2 ml tubes (Peqlab, Erlangen, Germany) containing 180 mg ± 20 mg granite matrix and two ¼ inch ceramic beads (MP Biomedicals) were used. Fresh bine (100 mg) and Lysis Buffer P were added to the lysing tubes. These plant tissues were shredded with the homogenisator FastPrep 24® (MP Biomedicals) at 5.5 m s^{-1} and 40 s. The concentration and quality of extracted DNA was determined with NanoDrop 1000 3.6.0 Spectrometer (Thermo Scientific, Wilmington, DE, USA). The samples were stored at −20°C.

Sequencing of amplicons from reference isolates. The *V. albo-atrum* amplicons were obtained using following PCR reaction mixture (60 µl): 1 µl of DNA, 200 nM of each primer 2 and 3, 0.2 mM dNTPs (Eurogentec), 0.025 U ml^{-1} Thermo-Prime *Taq* DNA polymerase (Thermo Scientific), 0.75 mM $MgCl_2$, 10x Reaction Buffer IV (750 mM Tris-HCl, pH 8.8 at 25°C, 200 mM $(NH_4)_2SO_4$, 0.1% (v/v) Tween® 20). The

PCR temperature profile was the same as was described for the current PCR method.

Ten µl of non-denaturated PCR product (the same PCR mixture as for the standard PCR were used) from *V. dahliae* reference isolates were separated on a 5% polyacrylamide gel. The amplicon of 580 bp was cut out from the gel and stored over night at 4°C in 50 µl of sterile water. After centrifugation at 13 000 rpm for 1 min, the supernatant was used for PCR reamplification following PCR protocol, described above for identification of *V. albo-atrum*, with the primer pair 19 and 22. The PCR products were purified with QIAquick PCR Purification Kit (Quiagen, Hilden, Germany) and sequencing was done by Eurofins MWG (Ebersberg, Germany). The sequences were compared with the database NCBI for verification of the correct product (National Center for Biotechnology Information, Rockville Pike, Bethesda MD, USA).

Real-time PCR primer/probe and assay design. To detect *V. albo-atrum* and *V. dahliae*, a multiplex real – time PCR assay was developed based on the sequenced amplicons. For the design of primer/probe the software Beacon Designer 7 was used (Table 3; Premier Biosoft, Palo Alto, CA, USA). The synthesis was done by Biomers (Ulm/Donau, Germany). The probe Vaa_probe was labeled at the 5' terminus with 6-Fam (flourescein posphoramidite, peak emission at 490 nm and peak excitation at 530 nm) and the 3' terminus with the black hole quencher BHQ-1 (quenching range of 500–580 nm and absorbance maximum of 534 nm), whereas Vd_probe was marked at the 5' terminus with Cy5 (indodicarbocyanine, peak emission at 670 nm and peak excitation at 650 nm) and at the 3' terminus with the quencher BHQ-2 (quenching range of 550–650 nm and absorbance maximum of 578 nm). Real-time PCR and analysis were performed in a CFX96™ Cycler with the manufacturer's software CFX Manager™ Software (BioRad, Hercules, CA, USA). To find the best annealing temperature, a gradient PCR (59°C to 65°C) was done. The optimal temperature for both primer pairs was found to be 61°C. The reaction mixture (20 µl) contained: 1 ng DNA, 10 µl SsoFast™ Probes Supermix (Biorad), 300 nM of each primer and 200 nM of the probe. The thermal cycling profile consisted of an initial step of denaturation of 1 min at 95°C, followed by 39 cycles of 5 s at 95°C and 30 s at 61°C. Each isolate was analyzed with the PCR assay twice. In all amplifications, no-template controls using sterile water (Roth) as negative controls and DNA from pure fungus as positive controls were additionally included.

To prevent false negatives, because the DNA extraction failed, the DNA isolates were tested with the primer pair 5'-ACC-ATC-ACT-TTG-GAG-GTG-GA-3' (forward primer) and 5'-GAG-ACG-GAG-GAC-AAG-GTG-AA-3' (reverse primer) for polyubiquitin (Castro et al. 2008, Oberhollenzer unpublished). The amplification proves the presence of plant DNA.

To test the specificity of primer pairs and probes, a PCR amplification of 27 unknown microorganisms (17 fungi and 10 bacteria) isolated from healthy hop bines was performed. The culturing method was used with prune agar, NA (Roth) and PDA (Roth) and the microorganisms were transferred to a new, accordant agar plate. The DNA was isolated using innuSPEED Bacteria/Fungi DNA Kit (Analytik Jena, Jena, Germany). Furthermore, references (*V. albo-atrum*: 11055, Roz, Ded, Rec, Zup, 11043, Vran, T6, Led, 298095, T2, Let1, 298101, 1953, Urs, Sent4, Kum, 298099, 1985, 10, 15, 55, 83 and *V. dahliae*: CasD, MoD, KresD, 12042, JKG8, 12099) were tested with the real-time PCR assay (Table 2).

Standard curves and efficiency of the probes. To evaluate the primers and probes, a standard curve was generated using pure *Verticillium* isolates. The same conditions as below and fivefold dilutions (ranging from 5 ng to 0.32 pg/20 µl reaction; if the concentration could not be reached, corresponding lower concentration has been inserted) of references as well as in triplicate were used. First, the efficiency of the probes was analyzed in a single real-time PCR. Further-

Table 3: Sequences of primers and TaqMan probes used for the detection of *V. albo-atrum* and *V. dahliae*

Primer combination[a]	Primer sequences	Amplification specificty	Product size (bp)
2[b]	5'-ATGGACCGAACAGCTAGGTA-3'	*V. albo-atrum*	300
3[b]	5'-TCTCAGATATATGCTGCTGC-3'	*V. albo-atrum*	
19[c]	5'-CGGTGACATAATACTGAGAG-3'	*V. dahliae*	580
22[c]	5'-GACGATGCGGATTGAACGAA-3'	*V. dahliae*	
Vaa_f	5'-GGCTTTTGCTTTCTCTTG-3'	*V. albo-atrum*	
Vaa_r	5'-GACCAAATGTAATTGTCCAG-3'	*V. albo-atrum*	150
Vaa_probe[d]	5'-CGGCTACGGCTCATGCTAAC-3'	*V. albo-atrum*	
Vd_f	5'-GGCTCAAGTTAACTACGG-3'	*V. dahliae*	
Vd_r	5'-CTGTCATGTATATAAGATACTACTG-3'	*V. dahliae*	123
Vd_probe[e]	5'-AGGTATAAGGTCCATATCCAACACGAG-3'	*V. dahliae*	

[a] f: forward primer; r: reverse primer
[b] Carder et al. 1994
[c] Carder et al. 1994
[d] 6-Fam reporter (5'); BHQ-1 quencer (3')
[e] Cy5 reporter (5'); BHQ-2 quencer (3')

more, the efficiency was validated in a multiplex real-time assay with a defined concentration of the other *Verticillium* species (1–0.5 ng) and in a single PCR with a known DNA concentration of a healthy hop bine (1 ng). The sensitivity of the assay was analyzed by a serial dilution of DNA. The threshold cycle (Ct) values and the efficiency were obtained automatically by the software Bio-Rad CFX Manager™ 2.0 with the setting Single Threshold for the Ct determination. The standard curve was plotted Ct versus logarithm of known DNA concentrations of *Verticillium* isolates.

Results

The efficiency and the quality of the results obtained with the proposed real-time PCR based identification assay was analyzed and compared with the conventional standard PCR method.

DNA extraction

The developed extraction procedure using commercial kits and isolating directly from the hop bine proved to be very efficient. Diverse kits were suited for the initial isolation (data not shown). Invisorb® Spin Plant Mini kit (Invitek, STRATEC Molecular) was chosen due to low costs. To exclude failure of the DNA isolation directly from the bine, the samples were tested with a general primer pair for polyubiquitin in hops. All DNA extractions of hop bine, except one, (Table 1; tagged with *) were successful. This isolation procedure allows a reduced time of six or seven days to one day compared to the standard procedure which is based on isolation from hop bine on prune media, the subsequent cultivation and DNA isolation using a commercial kit.

Specificity of primer and probe design

In order to have a quantitative assessment of the specificity of real-time PCR assay, the primer/probe set was tested on reference isolates (Table 2). The TaqMan assay was shown to be specific for the detection of *V. albo-atrum* and *V. dahliae*. The sequences of *V. albo-atrum* agreed with the sequence from *V. albo-atrum* (GenBank accession: NW_003315036.1) and *V. dahliae* with several isolates (GenBank accession: DQ266246.1, AF363249.1, AF363250.1, AF481987.1, AF481984.1, AF481979.1) using a BLAST analysis (NCBI). Additionally, the sequences of *V. albo-atrum* and *V. dahliae* did not show related sequences with other microorganisms. No amplification was observed for non-target and unknown microorganisms, which were isolated from hop bines.

Standard curves and efficiency of the primer/probe set for simultaneous detection of *V. albo-atrum* and *V. dahliae*

A standard curve was obtained with reference isolates for each individual primer/probe set. Standard curves show the linear correlation between the cycle threshold (Ct) value and the logarithm of the known concentration of each DNA dilution. The linear correlation coefficient (R^2) was 0,998 for *V. albo-atrum* and 0,998 for *V. dahliae* (Fig. 1). The efficiency of *V. albo-atrum* was 92.7% and for *V. dahliae* 93.8% (Fig. 1). The efficiencies and R^2 factor can be reproduced with the real-time PCR containing additional DNA of a healthy plant (*V. albo-atrum*: efficiency = 93.7%, R^2 = 0.998; *V. dahliae*: efficiency = 93.7%, R^2 = 0.996). The efficiency in a multiplex PCR assay containing DNA of the other species (1–0.5 ng) decreased to 90.3% (R^2 = 0.998) for *V. albo-atrum* and 91.0% (R^2 = 0.995) for *V. dahliae*. These factors demonstrate that plant DNA and additional *Verticillium* DNA did not substantially impair the assay.

Identification of *V. albo-atrum* and *V. dahliae* using real-time PCR technique and the standard PCR method

In total, 96 samples of hop bines were analyzed to compare the newly developed real-time assay with the current PCR identification method (Table 1). Using the developed technique, fourteen more samples (14.6%) were tested positive for *V. albo-atrum* in comparison to the current PCR method. In total, 71 hop bines were identified as infected with *V. albo-atrum*. In five samples *V. dahliae* was detected, whereas the standard PCR showed only three positive signals. Six samples, which tested positive, did not show any signals with the newly developed assay. The real-time PCR assay was confirmed as more sensitive than the conventional PCR. The detection limit of 0.05 pg *V. albo-atrum* DNA using real-time PCR clearly outperforms the limit of 0.01 ng for the conventional PCR. The final dilution of *V. dahliae* was 0.15 pg DNA for the TaqMan assay and 0.08 ng with the conventional PCR. This corresponds to 200 times and 533 times higher sensitivity for *V. albo-atrum* and *V. dahliae* more sensitive identification using real-time PCR, respectively.

Discussion

In this study, we introduced a multiplex real-time PCR assay for a fast and accurate detection and identification of *V. albo-atrum* and *V. dahliae* in hops. The first step was to develop a fast DNA isolation method. A direct isolation consisting of a homogenization, specifically optimized to the hop plant material, and isolation using a commercial kit has proven to be much more efficient and accurate than previous time-consuming and laborious cultivation techniques. Unsuccessful isolations can be easily identified and ruled out using a general primer pair for polyubiquitin. The DNA extraction directly from the bine with the kit improves the accuracy compared to the culturing isolation technique.

In a second step we developed a multiplexed real-time PCR assay for simultaneous detection of *V. albo-atrum* and *V. dahliae* in one reaction. Other studies have already described a real-time PCR assay for detection of either *V. albo-atrum* or *V. dahliae* (Mercado-Blanco et al. 2003b, Lievens et al. 2006, Atallah et al. 2007, Larsen et al. 2007).

The region of β-*tubulin 2* appears as a single copy in *V. dahliae* and was used for quantification in previous studies (Atallah et al. 2007). Larsen and coworkers (2007) developed a method based on real-time PCR for a selective quantification of *V. albo-atrum*. However, our aim was to design primers to detect both *Verticillium* species fast and reliably in one real-time PCR run. The multiplexed real-time PCR assay presented in this work is a rapid method for the simultaneous detection of *V. albo-atrum* and *V. dahliae* directly from the hop bine.

The TaqMan assay proved to be specific to these *Verticillium* species, when tested against 26 *Verticillium* reference isolates and 27 unknown microorganisms which were isolated from healthy hop bines. Furthermore, the sequences of *V. albo-atrum* and *V. dahliae* were subjected to BLAST analyses and were also species-specific and showed no homology with other fungal or bacterial DNA sequences. The new method increases the detection limit of pure *V. albo-atrum* and *V. dahliae* fungus to 200 times and about 500 times higher sensitivity than the conventional PCR. The detection limit reached for *V. dahliae* is comparable to that of other real-time PCR assays (Atallah et al. 2007, Duressa et al. 2012). The efficiency of the primer pairs is approximately 92.7% for *V. albo-atrum* and 93.8% for *V. dahliae* regardless of duplexing of both primer pairs and the presence or absence of host DNA (Fig. 1). In addition, DNA of the other *Verticillium* species did not show any significant influence on the efficiency. We were able to identify 16.7% more bines as being infected with the newly developed test than was possible with the standard PCR method. The identification success rate is also indirectly increased due to the improved isolation technique. Often when using the previous cultivation-based isolation technique, it was necessary to repeat the cultivation several times until a successful isolation of *Verticillium* species was possible. The newly developed method showed only 6.2% negative results which includes the single failed DNA extraction sample, which might be due to the absence of *Verticillium* in the part of bine used for DNA extraction. To further reduce false negatives in the future, an extraction can be done from composite samples of equal small pieces. In addition, false negatives may be a result of the inevitable methodological detection limit.

The data from the variety of sampled hops confirms the already known ubiquitous presence of *Verticillium* in hops, where *V. albo-atrum* is more common than *V. dahliae* as was already mentioned by Zinkernagel (1982). Only 6.6% of infected plants were identified as being infected with *V. dahliae*. The presented data also shows that a large amount of plants can have latent infection by *Verticillium* without showing any symptoms. In parallel, the symptoms, yellowing and wilting of leaves, can just be the effect of root damage e.g. caused by other plant diseases or pests, rather than being the symptoms of a *Verticillium* infection. Thus, the PCR allows an accurate detection of infection.

In conclusion, the combination of DNA extraction with an available commercial kit and the real-time PCR assay is a useful tool in detecting *V. albo-atrum* and *V. dahliae*. Comparing this method with a standard PCR method showed that the new developed procedure (homogenization, DNA extraction and TaqMan PCR) is not only more accurate and more sensitive but also time-saving. This technique is an application of routine analysis and can be helpful for effective plant disease management.

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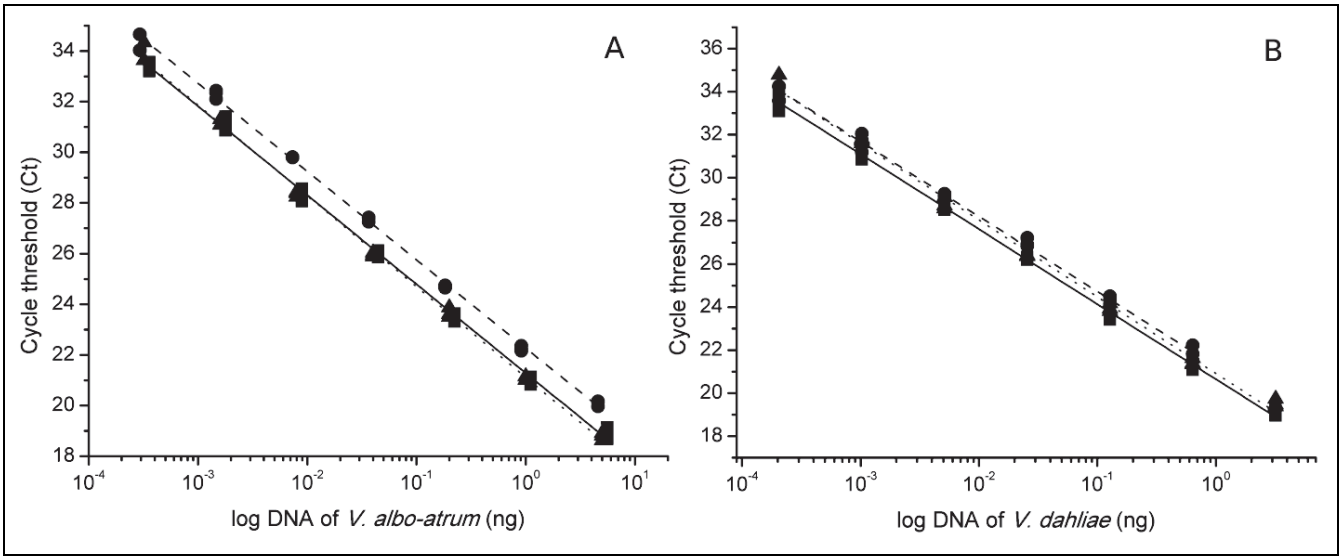

Fig. 1: Real-time PCR efficiency – seven point serial dilution of DNA from *V. albo-atrum* (A) and *V. dahliae* (B). Shown are efficiency curves for the DNA of the corresponding *Verticillium* species (■, straight line), *Verticillium* DNA with plant DNA (●, dashed line) and the corresponding *Verticillium* DNA with either *V. albo-atrum* or *V. dahliae* DNA (▲, dotted line)

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Publication II

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Initial steps towards biocontrol in hops: successful colonization and plant growth promotion by four bacterial biocontrol agents

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Contributions: Gabriele Berg and Stefan Seefelder have dominantly contributed to the conception of the work. Katja A. Maurer has designed and performed all experiments and dominantly contributed to the preparation of the manuscript. Christin Zachow has performed the statistical analysis. All coauthors contributed to scientific discussion and revision of the manuscript.

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Article

Initial Steps towards Biocontrol in Hops: Successful Colonization and Plant Growth Promotion by Four Bacterial Biocontrol Agents

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Abstract: Verticillium wilt, caused by *Verticillium nonalfalfae* and *V. dahliae*, is a devastating disease in hops that can cause considerable economic crop losses. The perennial use of hops combined with the long persistence of the pathogen in soil make it difficult to suppress the disease with conventional measures. Biological control agents (BCA) are the basis of an environmentally friendly plant protection strategy that uses plant promotion and antagonistic effects of microorganisms. We evaluated the effect of four selected beneficial bacterial strains, *Burkholderia terricola* ZR2-12, *Pseudomonas poae* RE*1-1-14, *Serratia plymuthica* 3Re4-18, and *Stenotrophomonas rhizophila* DSM14405^T for their use in hops. All strains were shown to be both rhizosphere and endorhiza competent, and their abundances ranged from $\log_{10}$ 3.0 to $\log_{10}$ 6.2 CFU g^{-1} root fresh weight in the endorhiza and from $\log_{10}$ 2.9 to $\log_{10}$ 4.7 CFU g^{-1} root fresh weight in the rhizosphere with *B. terricola* ZR2-12 showing the highest overall cell densities. Microscopic visualization of DsRed-labeled transformants with confocal laser scanning microscopy showed different colonization patterns and confirmed the rhizosphere competence. Growth promoting effects on seedlings treated with bacteria were found for

S. plymuthica 3Re4-18 and *S. rhizophila* DSM14405^T. Competent colonization and plant growth promoting effects are the most important prerequisites towards efficient biocontrol.

Keywords: *Humulus lupulus*; beneficial bacteria; root colonization; growth promotion; biological control; Verticillium wilt

1. Introduction

Verticillium albo-atrum Reinke & Berthold and *V. dahliae* Klebahn are devastating plant pathogens in many crop species [1] as Verticillium wilt is becoming a considerable threat in hops (*Humulus lupulus* L.). While first reported in England in 1924 [2], *V. albo-atrum* has since been divided and classified into two species according to Inderbitzin *et al.*: *V. albo-atrum* and the hop-infesting *V. nonalfalfae* [3]. *V. nonalfalfae* is the main pathogen of Verticillium wilt in hops that can cause devastating damage, and has been found in hop in Germany, Poland, Belgium, France, Slovenia, New Zealand, and the USA [4]. *V. nonalfalfae* is differentiated into fluctuating (mild) and progressive (lethal) pathotypes depending on the virulence of the isolates [5]. Plants infected with the lethal pathotypes, wither and often die, whereas hops infected with the mild pathotypes tolerate the disease.

Verticillium species are soil-borne pathogens, which are difficult to control with fungicides due to their ecological behavior. Resistant or tolerant hop cultivars and phytosanitary measures are the only current methods to prevent the wilt as hops are perennial crops and the fungus can survive for many years by producing resting structures. Hence, the hop infection by *Verticillium* spp. has major implications. An alternative and environmentally friendly strategy is biological control. In recent decades, many studies have proven the efficacy of biological control agents (BCAs) from the genera *Pseudomonas* and *Serratia* against *Verticillium* spp. in annual crops [6,7], but also for perennial crops such as olives [8]. In this way, the antagonistic bacteria inhibit plant pathogens via various mechanisms of disease suppression while the plant-associated microorganisms strengthen and promote plant growth [9,10]. Knowledge and understanding of these beneficial bacteria, their ability to colonize the roots, and their potential plant growth promoting (PGP) effects with pathogens interaction will help translate these properties into efficient biological control strategies [11]. Moreover, root competence was identified as a key prerequisite for successful biocontrol approaches [12].

An efficient BCA must be able to establish itself and survive in the root system. According to Weller, a root colonizer is defined as ‘a bacterium that when introduced becomes distributed along the root in natural soil, propagates, and survives several weeks in the presence of competition from the indigenous rhizosphere microflora’ [13]. Therefore, the first step towards an efficient biocontrol is to demonstrate the successful colonization in the root system of the plant. Afterwards, the bacterial treatment that could potentially strengthen the plants against diseases and/or stimulate the plant growth is analyzed. Several studies described successful biocontrol approaches towards *V. dahliae* for cotton, strawberry, olive, and oilseed rape [14–17], yet biocontrol against *V. albo-atrum* is rarely investigated. *Talaromyces flavus* was found as a fungal antagonist against *V. albo-atrum* in cucumber, potato, and tomato [18–20]. However, little is known to control Verticillium wilt in hops.

The objective of this study was to take a first step into the biological control of hops. Therefore, we (i) evaluated the root competence of four previously selected beneficial bacterial strains. Furthermore, (ii) the colonization behavior of the selected, red fluorescent protein gene (DsRed) labeled bacteria was characterized with confocal laser scanning microscopy (CLSM), and (iii) potential PGP effects were determined by bacterial treatments of seedlings. Four plant-associated bacteria were selected: *Burkholderia terricola* ZR2-12 [21], *Pseudomonas poae* RE*1-1-14 (syn. *P. trivialis*) [22], *Serratia plymuthica* 3Re4-18 [23], and *Stenotrophomonas rhizophila* DSM14405^T (= e-p10 and = P69) [24] due to their promising effects on targeted pathosystem and other plant beneficial properties [22,25–30]. For example, *B. terricola* ZR2-12 has proven to be an excellent root colonizer [21,31], and *P. poae* RE*1-1-14 was successfully applied in the sugar beet – *Rhizoctonia solani* pathosystem as reported by Zachow *et al.* [30]. The strain *S. plymuthica* 3Re4-18 was also described as a competent root colonizer and biocontrol agent in diverse crops [23,25,26,28,30,32]. The quorum-sensing-dependent antagonistic effect of *S. plymuthica* against *V. dahliae* was investigated in details [7]. Recently, the positive effect on plant growth and the strengthening of *S. rhizophila* DSM14405^T was supported [29,33] and an indirect antifungal activity against soil-borne pathogens has been demonstrated [24].

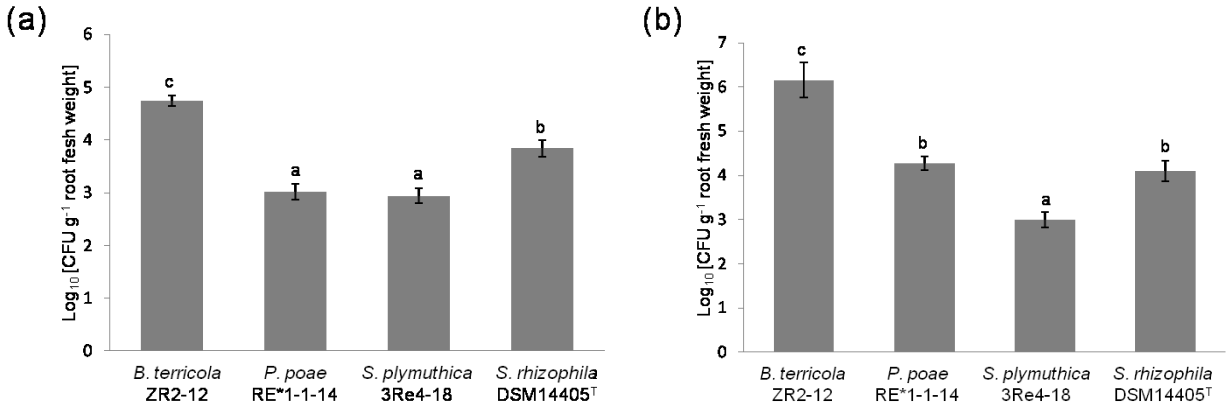
2. Results and Discussion

2.1. Rhizosphere and Endorhiza Competence by Measuring Bacterial Abundance

The competence to colonize root systems was demonstrated via the re-isolation of rifampicin-resistant mutants of *B. terricola* ZR2-12, *P. poae* RE*1-1-14, *S. plymuthica* 3Re4-18, and *S. rhizophila* DSM14405^T from the rhizosphere and endorhiza of hops. Four weeks after inoculation, bacteria from the rhizosphere and endorhiza of roots were re-isolated on nutrient agar. In general, the rhizosphere was colonized from $\log_{10}$ 2.9 to $\log_{10}$ 4.7 CFU g^{-1} root fresh weight (RFW) and the bacterial abundance ranged from $\log_{10}$ 3.0 to $\log_{10}$ 6.2 CFU g^{-1} RFW in the endorhiza (Figure 1). In this study, higher abundances were assessed for the bacteria in the endorhiza than in the rhizosphere of hop roots. *B. terricola* ZR2-12 showed the highest cell density ($\log_{10}$ 4.7 ± 0.1 CFU g^{-1} RFW in the rhizosphere and $\log_{10}$ 6.2 ± 0.4 CFU g^{-1} RFW in the endorhiza), as the abundances were up to three orders of magnitudes greater than the cell numbers of the other bacteria. *B. terricola* ZR2-12 was first selected according to its high rhizosphere competence in sugar beets roots (up to $\log_{10}$ 10 CFU g^{-1} RFW) [31], which can now also be confirmed for hops. In this study, the density of *S. plymuthica* 3Re4-18 in the hop root endorhiza was $\log_{10}$ 3.0 ± 0.2 CFU g^{-1} RFW, and the colonization of the rhizosphere was $\log_{10}$ 2.9 ± 0.1 CFU g^{-1} RFW. In *P. poae* RE*1-1-14 treated plants, the bacterial colonization was $\log_{10}$ 3.0 ± 0.2 CFU g^{-1} RFW in the rhizosphere and $\log_{10}$ 4.3 ± 0.2 CFU g^{-1} RFW in the endorhiza. Furthermore, *S. rhizophila* DSM14405^T showed similar abundances of approximately $\log_{10}$ 3.8 ± 0.2 CFU g^{-1} RFW in the rhizosphere and $\log_{10}$ 4.1 ± 0.2 CFU g^{-1} RFW in the endorhiza. In other studies, similar bacterial densities in the rhizosphere of different crops were also found. The strain *S. plymuthica* 3Re4-18 is described as an effective rhizosphere colonizer [30], and had an average abundance of $\log_{10}$ 3.6 to $\log_{10}$ 4.2 CFU g^{-1} RFW in sugar beet depending on the root section [30]. Similarly, the strain *S. plymuthica* HRO-C48 reached population densities of $\log_{10}$ 3.5 ± 1.4 CFU g^{-1} RFW in oilseed rape [34]. Lower densities of *S. plymuthica* 3Re4-18 were shown in

the root system of hops. The abundance of *P. poae* RE*1-1-14 in the rhizosphere of sugar beets ranged from $\log_{10}$ 3.7 to $\log_{10}$ 3.9 CFU g^{-1} RFW depending on root section [30], and showed superior colonization behavior in the hop rhizosphere. A large variety of life styles and interaction strategies are known for different endophytic bacteria and also rhizosphere bacteria can colonize the endorhiza [35,36]. The endophytic life style of all applied strains emphasizes their intimate plant-microbe interaction and suggests a positive role in hops.

Figure 1. Population densities of *B. terricola* ZR2-12, *P. poae* RE*1-1-14, *S. plymuthica* 3Re4-18, and *S. rhizophila* DSM14405^T in the (**a**) rhizosphere and (**b**) endorhiza of hops. Mean values followed by different letters are significantly different according to Tukey's test ($P \leq 0.05$). Error bars indicate standard error.



After outdoor hibernation, the plants sprouted under greenhouse conditions. Random samples were analyzed to see if the bacteria could survive at sub-zero temperatures. Again, *B. terricola* ZR2-12 was the best colonizer with a similar colony density as the colonization experiments above. The other three bacteria were found in the endorhiza with approximately $\log_{10}$ 3.0 CFU g^{-1} RFW. These results are important for further biological control measures and practical applications as they will help establish the bacteria in the hops roots.

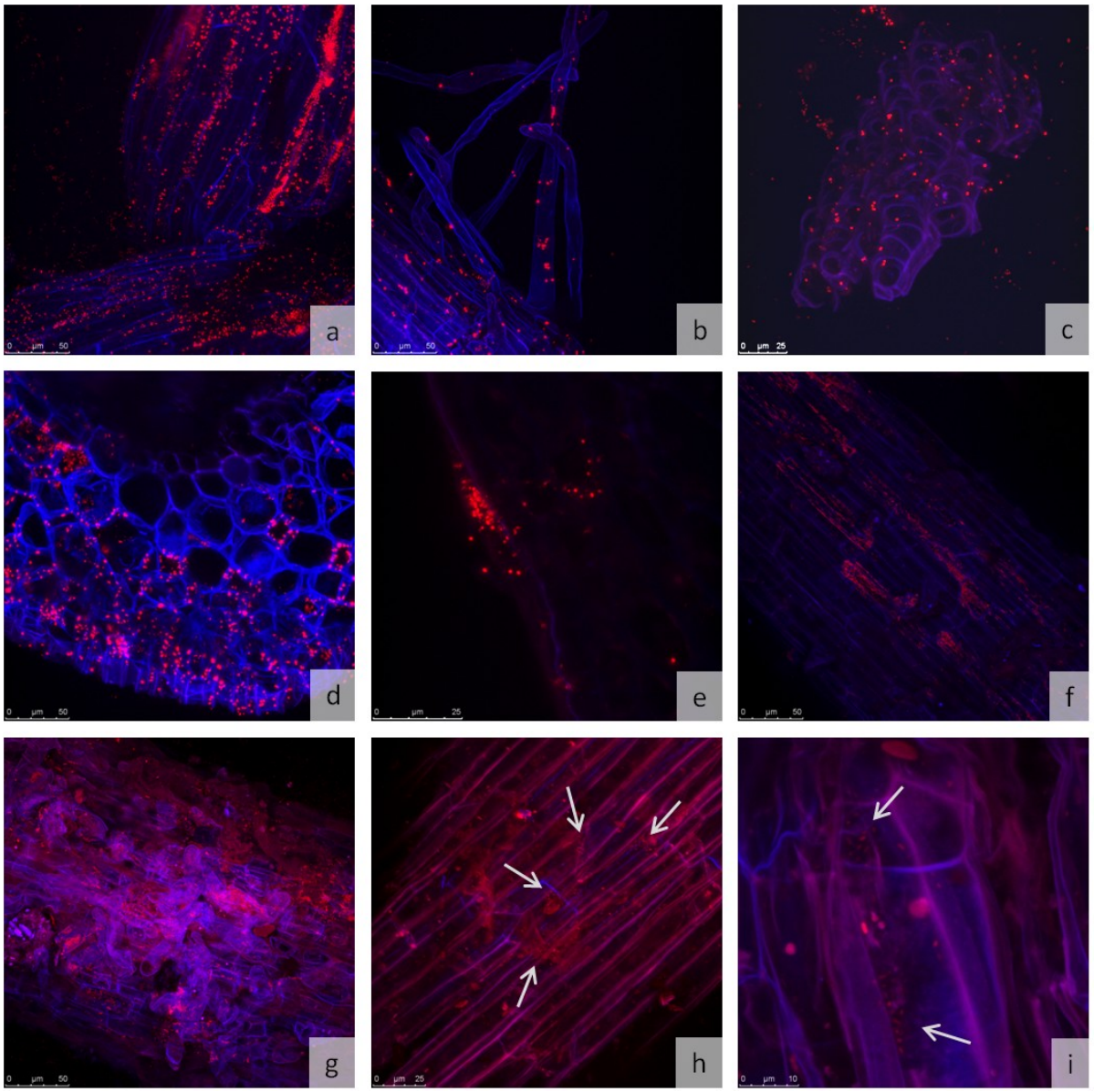
Furthermore, the potential establishments of these bacteria on seeds were analyzed. Hops seeds were inoculated and the root system cell density was determined after seven weeks. The abundances of *B. terricola* ZR2-12, *S. plymuthica* 3Re4-18, and *S. rhizophila* DSM14405^T ranged from approximately $\log_{10}$ 3.1 to $\log_{10}$ 4.1 CFU g^{-1} RFW. However, *P. poae* RE*1-1-14 showed a very low population density.

2.2. Colonization Patterns Observed by Microscopic Monitoring

Microscopic analyses were prepared using Confocal Laser Scanning Microscopy (CLSM) to visualize the colonizing behavior in the root system. Exemplarily, the colonization patterns for *B. terricola* ZR2-12, *P. poae* RE*1-1-14, and *S. plymuthica* 3Re4-18 were monitored six to seven days after inoculation. A high density of *B. terricola* ZR2-12 cells was found in the rhizosphere (Figure 2a), and scattered arrangements were found on root hairs and on the root tip (Figure 2b,c). A scattered colonization was observed in the endorhiza (Figure 2d). Moreover, *B. terricola* ZR2-12 cells showed a large accumulation of colonies on stem surfaces (Figure 2e). The endophytic *P. poae* RE*1-1-14

formed cell colonies along the epidermis cell (Figure 2f) with rod-shaped cells. In addition, *P. poae* RE*1-1-14 also showed an arrangement of single cells on the root surface with root hairs (Figure 2g) while *S. plymuthica* 3Re4-18 cells appeared as single cells in small colonies on the surface (Figure 2h,i). Zachow *et al.* reported similar rhizosphere colonization of *P. poae* RE*1-1-14 and *S. plymuthica* 3Re4-18 on roots of sugar beets [30]. *P. poae* RE*1-1-14 formed microcolonies between the epidermis cells, and *S. plymuthica* 3Re4-18 showed single cells that formed small accumulations between epidermis cells.

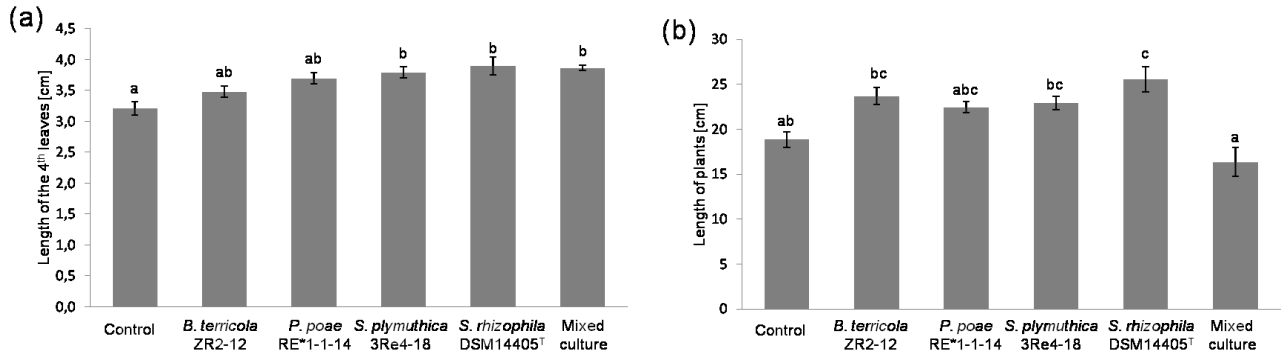
Figure 2. Colonization pattern of DsRed-transformed bacteria (red) in the root system of hops six to seven days after inoculation. *B. terricola* ZR2-12 cells showed **(a)** a high density on the root surface and appeared in a scattered arrangement **(b)** on root hairs and **(c)** on root tip; **(d)** *B. terricola* ZR2-12 cells colonized the endorhiza and **(e)** showed a large accumulation of colonies on the surface of stem; **(f)** *P. poae* RE*1-1-14 cells formed colonies along root cells and **(g)** showed a scattered arrangement on the surface of root hairs; **(h–i)** *S. plymuthica* 3Re4-18 cells formed small colonies (arrows).



2.3. Effect of Bacterial Treatment on Plant Growth

Seeds and plants were dipped in bacterial suspensions to assess the effect of *B. terricola* ZR2-12, *P. poae* RE*1-1-14, *S. plymuthica* 3Re4-18, and *S. rhizophila* DSM14405^T treatments on plant growth. Seven weeks after inoculation of the seeds, the length of the plantlets without the roots and the length of the fourth leaves were measured. Additionally, to evaluate an additive effect, seeds were treated with a mixture of *S. plymuthica* 3Re4-18 and *S. rhizophila* DSM14405^T (1:1). *S. plymuthica* 3Re4-18 showed significant PGP effects in both experimental sets (Figure 3), and *S. rhizophila* DSM14405^T and the combined strains promote plant growth as shown from the fourth leaves (Figure 3a). However, no significant PGP effects could be observed for plant length (Figure 3b), even though both strains already showed plant growth promotion in other crops [9,23,29,30]. In addition, the growth effect on taller cuttings was assessed. The weight gain four weeks after treatment was measured, but no differences in PGP could be found.

Figure 3. Growth promotion effect of *B. terricola* ZR2-12, *P. poae* RE*1-1-14, *S. plymuthica* 3Re4-18, *S. rhizophila* DSM14405^T, and mixed culture of *S. plymuthica* 3Re4-18 and *S. rhizophila* DSM14405^T on hop seedlings assessing (**a**) the length of the fourth leaves and (**b**) the length of the plant (without the root). Mean values followed by different letters are significantly different according to Scheffé test ($P \leq 0.05$). Error bars indicate standard error.



2.4. In vitro Antagonistic Activity against V. nonalfalfae and V. dahliae

The antagonistic activity of the four bacteria against *V. nonalfalfae* and *V. dahliae* was assessed using a dual culture test. The strain *S. plymuthica* 3Re4-18 showed an inhibition zone which has been confirmed with other studies for *V. dahliae* [25,30]. *P. poae* RE*1-1-14 did not have any antagonistic activity against these two *Verticillium* species in this or other studies [30]. An inhibition by *S. rhizophila* DSM14405^T and *B. terricola* ZR2-12 could also not be found, although it was described in other studies for *V. dahliae* [21,24].

3. Experimental Section

3.1. Hop Cultivars

The cultivar "Hallertauer Tradition" was used in the experiments due to its wide spread cultivation and its increased susceptibility in fields towards current pathotypes of *V. nonalfalfae*. To assess the PGP effect, seeds of the cross breed of Cascade and 2007/005/504 were treated.

3.2. Microorganisms

Four strains of bacteria, previously isolated from diverse microhabitats and crops, were used in this study. For greenhouse-experiments, spontaneous mutated isolates of *B. terricola* ZR2-12, *P. poae* RE*1-1-14, *S. plymuthica* 3Re4-18, and *S. rhizophila* DSM14405^T resistant to rifampicin (100 µg mL^{-1}; Roth, Karlsruhe, Germany) were used. No differences in growth parameters (colony morphology and growth rate) and traits (antifungal properties towards *V. nonalfalfae* and *V. dahliae*, proteolytic activity, Box PCR pattern) were found between the mutant and wild type. The strains were stored in nutrient broth (10 g of peptone, 5 g of yeast extract, Roth and 5 g of NaCl, Merck, Darmstadt, Germany in 1 L of distilled water, pH 7) containing 12.5% glycerol at −80 °C. The wildtype strains were maintained in the Strain Collection of Antagonistic Microorganisms (SCAM) at Graz University of Technology in LB medium containing 15% glycerol at −70 °C. The used *V. nonalfalfae* and *V. dahliae* strains for dual culture tests were isolated from infected hop bines. The fungi were maintained as monospore cultures on prune agar at 20 °C [37], and the species identity was verified by specific primers [38,39].

3.3. Determination of Root Colonization

Bacterial overnight culture (12 mL) in nutrient broth (100 mL containing 100 µg mL^{-1} of rifampicin, 28 °C, 120 rpm) was transferred to 500 mL of a new culture (28 °C, 120 rpm) and diluted with 0.85% NaCl solution to a final cell concentration of 10^8 CFU mL^{-1}. Roots of the cultivar "Hallertauer Tradition" were dipped in the bacterial suspension for 15 min and planted in unsterilized soil (Lorenzer potting soil, Einheitserde special, Einheitserdewerke Patzer, Sinntal-Jossa, Germany). Control plants were dipped in 0.85% NaCl solution. The experiment was done in twelve replicates for each bacterium as well as for the negative control and repeated three times under greenhouse conditions with minimum of 13 h light (artificial light between 6 am and 7 pm, if the daylight is under 40 kLux) with a minimum temperature of 22 °C at day and 19 °C at night. After four weeks, twelve plants were divided into four parts (containing three plants) for each BCA. 2.5 g of roots (soil adhering to roots) were incubated in 15 mL of 0.85% NaCl solution for 20 min and at 300 rpm to determine bacterial density in the rhizosphere. To define the colonization number in the endorhiza, 2 g of roots were cleaned, surface sterilized with sodium hypochlorite (3% active chlorine, 5 min) and washed three times with sterile water. For sterilization control, roots were dipped on a nutrient agar (nutrient broth added 15 g L^{-1} agar agar, Roth) containing rifampicin (100 µg mL^{-1}; Roth) and nystatin (20 µg mL^{-1}; Roth). The roots were then crushed with 5 mL 0.85% NaCl solution by mortar and pestle. The resulting suspensions of the rhizosphere and endorhiza were serial diluted, and 100 µL

were plated on selective nutrient agar as described above. After seven days of incubation (28 °C, in dark), the number of colonies (CFU g^{-1} fresh root weight) was determined. After the outdoor hibernation in sub-zero temperatures, six random samples of the roots, which were divided into two parts, were taken and re-isolated as described above.

3.4. Confocal Laser Scanning Microscopy (CLSM)

For microscopy analysis, DsRed-labeled strains of *B. terricola* ZR2-12, *P. poae* RE*1-1-14, and *S. plymuthica* 3Re4-18 were stored at -70 °C in nutrient broth (Luria Bertani LB, Roth) amended with 15% glycerol and subsequently employed [30,31]. The roots of seven weeks old cuttings were washed with tap water and dipped in an overnight culture of bacteria (30 °C; Luria Bertani LB supplemented with 40 µg mL^{-1} tetracycline; Roth) for *P. poae* RE*1-1-14 and *S. plymuthica* 3Re4-18, and *B. terricola* ZR2-12 with 100 µg mL^{-1} trimethoprim (Sigma Aldrich, St. Louis, MO, USA) for 30 min. They were planted in sterilized standard potting compost (Gramoflor, Profi-Substrat, Topfpikier M + Ton + Fe, Vechta, Austria) and closed with a lid. One week after incubation (14 h artificial light at 25 °C and 70% air moisture; 10 h darkness at 18 °C and 70% air moisture), the root system was observed with a TCS SPE confocal microscopy (Leica Microsystems, Germany) using the following modulations for laser lines (nm)/detection wavelengths (nm): DsRed, 532/570−620 and plant tissues (autofluorescence), 405/430−500. The rhizosphere and endorhiza of randomly selected roots were analyzed. Because of the high abundance of *B. terricola* ZR2-12 in the rhizophere and endorhiza, the stem were observed in details.

3.5. Plant Growth Promotion (PGP) in the Greenhouse

The weight of the plants before and four weeks after the bacterial treatment was measured to determine the PGP. The same dipping procedure for the determination of cell densities was used. In addition, seeds of the cross breed of Cascade and 2007/005/504 were dipped for 15 min in bacterial suspension (10^8 CFU mL^{-1}) and the control seeds were treated with sterile 0.85% NaCl solution. The seeds were planted in potting compost (Lorenzer potting soil) and grew for seven weeks. The growth was determined by the size of the plantlets without the roots and the fourth leaves (including the cotyledons). The roots were assembled and separated into two parts of 1.5 g to 2 g to control successful root colonization. The roots were then ground with 15 mL 0.85% NaCl with mortar and pestle and 100 µL plated on nutrient agar (nutrient broth added 15 g L^{-1} agar agar, Roth) containing rifampicin (100 µg mL^{-1}, Roth) and nystatin (20 µg mL^{-1}; Roth). After seven days, the colonization was determined. The growth experiments were done in twenty replicates and independently repeated two times.

3.6. Screening for Antagonistic Activity

The *in vitro* antagonism was determined in a dual culture assay. 200 µL of seven day old mycelia suspension *V. nonalfalfae* isolated from infected hops was plated onto PDA (potato extract glucose agar; Roth) and Waksman agar containing 5 g tryptone/peptone ex casein (Roth), 10 g glucose (Roth), 3 g yeast extract (Roth), 20 g agar (Roth) and filled up to 1 L with distilled water. After 30 min, the

bacteria were spotted on the plate. Antagonistic activity and the inhibition zone were assessed after seven days of incubation at 20 °C.

3.7. Statistical Analysis

Root colonization data was $\log_{10}$ transformed before statistical analysis, and the package SPSS (SPSS Inc., Chicago, IL, USA) was used for statistical data analysis. For the determination of PGP, the significance towards the control plants was analyzed using the Scheffé's test and Tukey's test ($P \leq 0.05$). The outlier test DIXON was used in the PGP experiments and the outliers were excluded from the statistical analysis. The deviation was indicated as standard error.

4. Conclusions

This study investigates the preliminary requirements of four bacteria, *B. terricola* ZR2-12, *P. poae* RE*1-1-14, *S. plymuthica* 3Re4-18, and *S. rhizophila* DSM14405^T to act as BCAs to suppress Verticillium wilt in hops. Many previous studies have already demonstrated the ability of the four bacteria for biological control in other crops. Regarding their rhizosphere competence and PGP effect, *S. plymuthica* 3Re4-18 and *S. rhizophila* DSM14405^T are promising candidates for BCA on hops, as well as *B. terricola* ZR2-12 that showed exceptionally high cell densities. Due to this rhizosphere competence even after hibernation in sub-zero temperatures, this strain can be a suitable BCA in hops. The bacterial treatment of seeds with *S. plymuthica* 3Re4-18 and *S. rhizophila* DSM14405^T was also shown to benefit plant growth. Antagonistic activity against *V. dahliae* is known for *B. terricola* ZR2-12, *S. plymuthica* 3Re4-18, and *S. rhizophila* DSM14405^T and an antagonistic effect has also been shown via dual culture test in this study for *S. plymuthica* 3Re4-18 against *V. nonalfalfae* and *V. dahliae*. Furthermore, the beneficial traits of *S. plymuthica* 3Re4-18 are well-known and contribute to its biotechnological potential in hops. Nevertheless, to objectively assess the ability of these beneficial bacteria strains towards *V. nonalfalfae* in hops, artificial infection tests and further experiments under field condition are necessary, also to assess the effects on crop yield. Furthermore, the consistent efficacy of these beneficial bacteria must be verified in the field.

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Conflicts of Interest

The authors declare no conflict of interest.

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Manuscript I

Gesunde Pflanzen

Untersuchungen zur Verticillium-Welke im Hopfenanbaugebiet Hallertau

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Zusammenfassung

Die Verticillium-Welke ist eine schwerwiegende Krankheit bei Hopfen und kann beträchtliche Ernteausfälle verursachen. Als Erreger tritt am häufigsten *Verticillium albo-atrum* auf, wohingegen *V. dahliae* nur selten vorkommt. Da der Pilz einige Jahre im Boden mit Hilfe von Überdauerungsorganen überleben kann und darüber hinaus zu seiner Bekämpfung keine Fungizide zur Verfügung stehen, ist ein schnelles Diagnosesystem erforderlich. Nur dann können rechtzeitig Maßnahmen eingeleitet werden. Ein Ziel dieser Arbeit war es daher mit einem kürzlich entwickelten molekularen Detektionssystem Stichproben von Hopfenreben aus dem Anbaugebiet Hallertau auf das Vorhandensein von *Verticillium* hin zu untersuchen. Dafür wurden 76 Hopfenpflanzen mit und ohne Welkesymptomen ausgewählt und mit einem Realtime-PCR Assay analysiert. In 57 der beprobten Pflanzen wurde *V. albo-atrum* nachgewiesen. Es zeigte sich, dass der Erreger vereinzelt auch ohne Symptomausbildung auftreten kann. Das Auftreten des Pilzes innerhalb der oberirdischen Pflanzenteile (Rebe, Seitentriebe, Blätter und Dolden) wurde analysiert. Des Weiteren wurden im Rahmen der vorliegenden Arbeit vier Bakterienstämme, *Burkholderia terricola* ZR2-12, *Pseudomonas poae* RE*1-1-14, *Serratia plymuthica* 3Re4-18 und *Stenotrophomonas rhizophila* DSM14405^T, auf ihre Eignung als biologische *Verticillium*-Gegenspieler in Hopfen untersucht. Der Schwerpunkt wurde auf die Kolonisierungskompetenz im Wurzelsystem, pflanzenfördernde Wachstumseffekte und ihre antagonistische Aktivität gelegt. Alle Bakterienstämme etablierten

sich in der Endorhiza sowie in der Rhizosphäre des Hopfens. *B. terricola* ZR2-12, *S. plymuthica* 3Re4-18 und *S. rhizophila* DSM14405^T erwiesen sich hinsichtlich ihrer Wirkung als vielversprechende Kandidaten für weitere Untersuchungen. Damit wurden erste Schritte zum präventiven Schutz von Hopfenpflanzen der Verticillium-Welke beschrieben.

Schlüsselwörter: Verticillium-Welke • *Humulus lupulus* • Realtime-PCR • Biologische Kontrolle

Research on Verticillium wilt in hop growing area Hallertau

Abstract

Verticillium wilt is a devastating disease in hops and causes considerable crop losses. *V. albo-atrum* is the most widespread species, whereas *V. dahliae* is rarely found. Due to the survival of the fungus for several years in soil by resting structures and the lack of effective chemical control, a fast detection method is needed. One aim of this study was to evaluate the occurrence of *Verticillium* spp. in the Hallertau region (Germany). Therefore, 76 hop plants, with or without wilt symptoms, were analyzed using a real time PCR (polymerase chain reaction) assay. *V. albo-atrum* was identified in 57 bine samples. The analysis proved that the pathogen can sporadically appear without showing visual symptoms. The abundance of the fungus inside the plants (bines, side shoots, leaves, and cones) was analyzed. Furthermore, four beneficial bacteria, *Burkholderia terricola* ZR2-12, *Pseudomonas poae* RE*1-1-14, *Serratia plymuthica* 3Re4-18, and *Stenotrophomonas rhizophila* DSM14405^T, were evaluated as biological control agents in hops. Therefore, the colonization competence in the root system, plant growing promotion and antagonistic effects were assessed. All strains colonized the endorhiza as well as the rhizosphere of hops. The bacterial strains *B. terricola* ZR2-12, *S. plymuthica* 3Re4-18, and *S. rhizophila* DSM14405^T are promising candidates for further biocontrol strategies. Thus, first steps towards the biological control of Verticillium wilt were presented.

Keywords: Verticillium wilt • *Humulus lupulus* • real-time PCR • biological control

Einleitung

Welkeerscheinungen bei Hopfen (*Humulus lupulus*) können von einer Vielzahl von Pilzpathogenen hervorgerufen werden. Neben den vereinzelt auftretenden Gattungen *Alternaria*

und *Phoma* (Phalip et al. 2004), ist bislang oft *Fusarium* spp. als Welkeursache bei Hopfen beschrieben worden (Neve 1991; Pichelmaier und Zinkernagel 1992; Gryndler et al. 2008). Die ökonomisch weitaus bedeutendere Krankheit stellt jedoch die Verticillium-Welke dar (Engelhard 1957), die neben hohen Ertragseinbußen auch zu einer starken Qualitätsminderung der Hopfendolden führen kann. Ist ein Hopfengarten erst einmal mit *Verticillium* befallen, kann die Ausbreitung durch das Entfernen befallener Pflanzen und Einsaat neutraler Zwischenfrüchte verhindert werden. Ein Fruchtwechsel, wie er bei einjährigen Kulturen als Bekämpfungsstrategie gegen *Verticillium* spp. beschrieben wird, lässt sich bei der Dauerkultur Hopfen in kurzen Zeitintervallen nicht durchführen. Generell stehen keine wirksamen Fungizide zur Verfügung. Daher beschränken sich die Maßnahmen auf Empfehlungen zur Prävention im Hopfenbau, die den Landwirten alljährlich z.B. von der Hopfenberatung der Bayerischen Landesanstalt für Landwirtschaft (LfL) vorgestellt werden (Anonymous 2013). Maßvolle, speziell für verschiedene Hopfensorten empfohlene Stickstoff-Düngung, Hygienemaßnahmen, Vermeidung von Bodenverdichtungen, Anbau *Verticillium*-neutraler Zwischenfrüchte usw. werden darin beschrieben.

Als Erreger bei Hopfen werden die bodenbürtigen Pilze, meist *Verticillium albo-atrum* Reinke et Berthold und seltener *V. dahliae* Klebahn beschrieben (Zinkernagel 1982). Laut einer neuen Klassifizierung der *Verticillium* Spezies wurde *V. albo-atrum* im Hopfen der neuen Spezies *V. nonalfalfae* zugeteilt (Inderbitzin et al. 2011). Jedoch wird in diesem Artikel auf Grund größeren Bekanntheit der Begriff *V. albo-atrum* synonym verwendet. Die Biologie und Verbreitung von Verticillium im Hopfen ist ausführlich beschrieben (Kohlmann und Kastner 1974; Down et al. 2007). Mit Hilfe von Überdauerungsorganen (Abbildung 1), dickwandigen schwarzen Hyphen (Dauermyzel) bei *V. albo-atrum* und Mikrosklerotien bei *V. dahliae* können die Pilze ohne Wirt fünf bis zehn Jahre im Boden überleben. Der Pilz tritt über verletzte oder junge Wurzeln in die Leitungsbahnen der Pflanze ein. Einerseits breitet sich *Verticillium* durch Sporen weiter in der Pflanze aus, anderseits verstopft das Pilzgeflecht die Leitungsbahnen und die Zellwände werden zersetzt. Die Symptome äußern sich durch von unten nach oben sich ausbreitendes Vergilben und Verwelken der Blätter (Abbildung 2a-c), welche oft nach einer leichten Berührung abfallen. Als weiteres Symptom ist häufig eine Rebenverdickung und im Querschnitt ist eine braune Verfärbung des vaskulären Gewebes auffallend (Abbildung 2d). Ein fortgeschrittener *Verticillium*-Befall verhindert meist die Blüte und Ausdoldung. Nicht abgestorbene Dolden weisen teilweise bräunliche Verfärbungen auf, die zu einer starken Qualitätsbeeinträchtigung führen können.

Die Hopfenwelke wurde 1924 erstmals in England festgestellt (Harris 1927). Von ersten Befallsauftreten von *Verticillium* in Deutschen Hopfenanbaugebieten wurde 1952 berichtet (Kohlmann und Kastner 1974). Neve (1991) beschreibt das Auftreten von *Verticillium* bei Hopfen auch in Polen, Belgien, Frankreich, Slowenien, Neuseeland und der USA. Bei Hopfen wird entsprechend der Virulenzeigenschaften zwischen milden und aggressiveren (letalen) *Verticillium*-Rassen unterschieden. Bislang wurden die aggressive Form in England (Keyworth 1942), Slowenien (Radišek et al. 2003) und seit einigen Jahren vereinzelt auch in der Hallertau (Seefelder et al. 2009), dem weltgrößten zusammenhängenden Hopfenanbaugebiet, festgestellt. Da es bei *V. albo-atrum* auch zu genetischen Rekombinationen kommen kann (Hastie 1962; Clarkson und Heale 1985a), verdeutlicht die Möglichkeit, dass aggressivere *Verticillium*-Stämme aus milderen Formen hervorgehen können und macht bei Hopfen eine generelle Untersuchung auf *Verticillium* spp. unverzichtbar. Unabhängig von ihrer Differenzierung in milde und letale Formen sind *V. albo-atrum* und *V. dahliae* als gefährliche Schadorganismen gelistet (Richtlinie 2000/29/EG) und zählen weltweit zu den „high-risk-pathogens".

Aus diesen Gründen ist ein effektives, modernes Diagnosesystem erforderlich um gezielt Maßnahmen, wie z.B. die Rodung von *Verticillium*-Nestern im Hopfengarten, zu setzen. Bisher wurde ein äußerst zeitaufwendiges Verfahren (EPPO diagnostics guideline Protokoll) angewandt, in dem der Pilz zuerst aus der Rebe auf Selektivmedien kultiviert und anschließend die Pilz-DNA isoliert wurde (Down et al. 2007). Primer, die speziell für *V. albo-atrum* und *V. dahliae* entwickelt wurden, wurden hierzu verwendet (Carder und Barbara 1991; Carder et al. 1994). Um diese Prozedur, die auch den sehr arbeitsintensiven DNS-Aufschluss über Mörsern in flüssigen Stickstoff beinhaltete, einerseits wesentlich zu vereinfachen und andererseits sensitiver zu gestalten, wurde kürzlich ein Realtime-PCR Assay entwickelt (Maurer et al. 2013a). Dabei wird die Pilz-DNS direkt aus der Hopfenrebe isoliert und anschließend mit einer Realtime-PCR (TaqMan Technik) nachgewiesen. Diese Methode erlaubt einen schnellen, effizienten und gleichzeitigen Nachweis von *V. albo-atrum* und *V. dahliae* (Abbildung 3).

Neben der Anwendung von verbesserten Diagnosesystemen, ist es von Interesse nach umweltgerechten Möglichkeiten zur Reduzierung des *Verticillium*-Pathogens auf betroffenen Hopfenflächen zu suchen. Die Anwendung nützlicher Mikroorganismen, die als natürliche Gegenspieler zur Kontrolle von bodenbürtigen Pathogenen wirken, stellt eine Möglichkeit dar (Berg et al. 2013). Erfolgreiche Ergebnisse zur biologischen Kontrolle von *Verticillium* spp. wurden bei einigen anderen wichtigen Kulturpflanzen, wie z.B. Baumwolle (Erdogan und Benlioglu 2010), Raps (Müller und Berg 2008), Oliven (Mercado-Blanco et al. 2004) oder Erdbeeren (Kurze et al. 2001) beschrieben. Diese sogenannten nützlichen Mikroorganismen sind

Vertreter der natürlichen Boden- und Pflanzen-Mikroflora und ihre Wirkmechanismen sind mittlerweile gut erforscht (Lugtenberg et al. 2002; Compant et al. 2005; Berg 2009). Sie können das Pflanzenwachstum und somit auch die Pflanzengesundheit fördern und/oder Pflanzenpathogene daran hindern die Pflanze zu befallen.

Das Ziel der vorliegenden Studie war es zum einen, die neu etablierte molekulare Diagnosemethode für Welkebefall an Hopfenpflanzen zu testen, als auch das Vorkommen des *Verticillium*-Pathogens innerhalb der Hopfenrebe zu untersuchen. In weiterer Folge sollten verschiedene Bakterienstämme *Burkholderia terricola* ZR2-12, *Pseudomonas poae* RE*1-1-14, *Serratia plymuthica* 3Re4-18 und *Stenotrophomonas rhizophila* DSM14405^T auf ihre Eignung in biologischen Pflanzenschutzmaßnamen bewertet werden. Daher wurde die Besiedelung des Wurzelsystems von Hopfenpflanzen, ihre pflanzenwachstumsfördernden Potential nach Behandlung von Hopfensamen und ihrer antagonistischen Aktivität gegen *V. albo-atrum* und *V. dahliae* mittels Dual-Kulturen Test untersucht. Dies sind wichtige Voraussetzungen für einen späteren Einsatz als Biofungizide.

Material und Methoden

Pflanzenmaterial

Zur Klärung der Welkeursachen wurden im Jahr 2012 76 zufällig ausgewählte Hopfenpflanzen aus zwölf Praxisbetrieben untersucht. Für die Analyse im Labor wurden im Feld 10 cm lange Rebenstrünke unmittelbar auf Bodenhöhe entnommen. Zur Überprüfung der Ausbreitung von *V. albo-atrum* innerhalb der Rebe wurden zusätzlich zehn zufällig ausgewählte ganze Hopfenreben mit starken makroskopisch sichtbaren Symptomen analysiert. Die Länge betrug zwischen 450 cm und 650 cm. Die Reben wurden in 50 cm große Abschnitte unterteilt. Zusätzlich wurden insgesamt 28 Seitentriebe, 27 Blätter und 18 Dolden analysiert. Das Hopfenmaterial wurde bis zur Analyse bei -20°C gelagert.

Realtime-PCR zum Befallsnachweis

Zum Nachweis von Verticillium aus den Hopfenmustern wurde ein kürzlich etablierter Realtime-PCR Assay angewandt (Maurer et al. 2013a). Die Isolierung der Gesamt-DNS erfolgte direkt aus der Hopfenrebe mit dem Invisorb® Spin Plant Mini Kit (Invitek). Zum Aufschluss der Proben wurde der Homogenisator FastPrep 24® (MP Biomedicals) mit den Aufschlussmaterialien Granitmatrix und zwei Keramikkugeln (MP Biomedicals) verwendet. In einem multiplexen Realtime-PCR-Ansatz mit Verwendung der TaqMan Technik wurde auf

beide Welkepathogene hin simultan getestet. Um falsche Negativergebnisse aufgrund möglicher DNS-Extraktionsprobleme auszuschließen, wurde für jedes zu untersuchende Muster eine spezifische PCR mit Polyubiquitin-Primern, die Hopfen-DNA amplifizieren, durchgeführt (Castro et al. 2008).

Verwendung von nützlichen Bakterien zur biologischen Kontrolle

Vier Pflanzen-assoziierte Bakterienstämme wurden auf Grund ihrer bereits beschriebenen Eigenschaften in der Anwendung zur biologischen Befallskontrolle ausgewählt: *B. terricola ZR2-12* (Gasser et al. 2009), *P. poae* RE*1-1-14 (syn. *P. trivialis*) (Zachow et al. 2008), *S. plymuthica* 3Re4-18 (Faltin et al. 2004) und *S. rhizophila* DSM14405^T (= e-p10 = P69) (Berg et al. 1996, Alavi et al. 2013) (Tabelle 2). Die Stämme stammen aus der Stammsammlung antagonistischer Mikroorganismen (Strain Collection of Antagonistic Microorganisms = SCAM) der Technischen Universität Graz, Österreich. Grundlegende Eigenschaften der Bakterienstämme, wie die Fähigkeit die Rhizosphäre und Endosphäre zu besiedeln, Effekte auf das Pflanzenwachstum bei behandelten Hopfensamen und antagonistische Aktivität gegenüber *V. albo-atrum* und *V. dahliae*, wurden beurteilt. Der Versuchsaufbau wurde detailiert beschrieben (Maurer et al. 2013b). Die Wurzeln von Hopfenstecklingen wurden mit einer Suspension bestehend aus Rifampicin-resistenten Spontanmutanten der Bakterienstämme behandelt. Nach vier Wochen wurden die Bakterien reisoliert und die Zelldichte der Rhizsophäre und Endorhiza bestimmt. Die Besiedelung der Bakterienstämme *B. terricola ZR2-12*, *P. poae* RE*1-1-14 und *S. plymuthica* 3Re4-18 an der Wurzeloberfläche wurde mittels konfokale Laser-Raster-Mikroskopie (CLSM = confocal laser scanning microscopy) veranschaulicht. Dafür wurden DsRed-markierte Transformanten der Bakterienstämme verwendet. Um mögliche pflanzenwachstumsfördernde Effekte feststellen zu können, wurden Samen mit einer Bakteriensuspension behandelt und anschließend die Größe der Pflänzchen beurteilt. Ein weitverbreiteter Dual-Kulturen Test lässt auf antagonistische Aktivitäten gegen *V. albo-atrum* und *V. dahliae* schließen.

Ergebnisse und Diskussion

Realtime-PCR Detektion der Verticillium-Welke bei Hopfen

Mit dem verwendeten Realtime-PCR Assay wurde bei 75% der untersuchten Pflanzen *V. albo-atrum* detektiert (Tabelle 1). Im Gegensatz dazu wurden bei allen 76 beprobten Rebenmustern kein *V. dahliae* festgestellt. Somit wurde bestätigt, dass *V. albo-atrum* wesentlich weiter

verbreitet ist als *V. dahliae*, wie dies schon in früheren Jahren mit verstärktem *Verticillium*-Vorkommen in der Hallertau beschrieben wurde (Zinkernagel 1982). In 73% der beprobten, visuell gesunden Hopfenpflanzen konnte kein *V. albo-atrum* nachgewiesen werden (Abbildung 4). In 27% der visuell gesunden Pflanzen konnte das Pathogen latent nachgewiesen werden, jedoch mit relativ hohen Ct-Werten von 33 bis 37, was auf einen, zumindest zum Untersuchungszeitpunkt, noch sehr geringen Erregerbefall hindeutet (Tabelle1, Abbildung 4). Der Ct-Wert (cycling threshold = Schwellenwertzyklus) beschreibt den PCR-Zyklus, bei dem das fluoreszierende Signal der TaqMan Sonde den Hintergrundlevel beziehungsweise einen definierten Schwellenwert überschreitet. Bei den drei Rebenmustern der Hopfensorte Wye Target, die leichte Welke-Erscheinungen an den Blättern aufwiesen, wurde über Realtime-PCR keine *Verticillium*-DNA nachgewiesen. Da diese Hopfensorte als *Verticillium*-tolerant bekannt ist (Talboys 1987), liegt die Vermutung nahe, dass die Symptome durch andere, eingangs beschriebene Pathogene hervorgerufen wurden.

Ausbreitung des *Verticillium* Pilzes innerhalb der Hopfenrebe

Die Verbreitung des *Verticillium*-Pathogens innerhalb fast völlig abgestorbener Reben wurde mit dem Realtime-PCR Assay verfolgt. An den zehn zufällig ausgewählten Pflanzen, die Welke-Symptome aufzeigten, konnte kein kontinuierlich von der Rebenbasis nach oben steigender Befall festgestellt werden. *V. albo-atrum* wurde bei vier Reben bis zur Spitze (bis zu 6,5 m) detektiert (Abbildung 5). Bei den restlichen sechs Reben wurde ab 3 bis 4,5 m (entspricht 54 bis 88% der Rebenlänge) kein *V. albo-atrum* mehr festgestellt (Abbildung 5). Dies bestätigt, dass der Pilz sich von unten nach oben ausbreitet. In 23 von 28 Seitentrieben sowie in 12 von 18 untersuchten Dolden wurde *V. albo-atrum* ebenso nachgewiesen. In einer früheren Studie an Hopfen konnte der *Verticillium*-Pilz über Selektivmedien in Reben, Seitentrieben und Blättern, jedoch nicht in Dolden nachgewiesen werden (Clarkson and Heale 1985b). In den Blättern der vorliegenden Untersuchung wurde trotz wiederholter DNA-Extraktion, als auch wiederholter PCR kein *V. albo-atrum* nachgewiesen. Die Feststellung, dass auch die Kontroll-PCR mit den spezifischen Polyubiquitin-Primern zu keinen positiven PCR-Produkten aus dem Hopfengenom führte, legt die Vermutung nahe, dass inhibitorische Substanzen die PCR-Reaktionen bei stark verwelkten Blättern gehemmt haben.

Der in dieser vorliegenden Arbeit angewandte Ansatz, die Kombination der Verwendung eines Homogenisators mit kommerziell erhältlichen Isolationskits und nachfolgender Realtime-PCR, hat sich zur schnellen Detektion der Verticillium-Welke bei Hopfen bewährt. Das etablierte DNS-Aufschlussverfahren über den Homogenisator und nachfolgender Verwendung eines

Isolationskits wird auch bei anderen genomanalytischen Fragestellungen an der LfL gegenwärtig angewandt. Im Vergleich zur Kulturvierungsmethode auf Pflaumenmedium wird die Arbeitszeit von einer Woche auf einen Tag reduziert. Nicht nur die DNS Isolierung direkt aus der Rebe spart Zeit, sondern auch die Verwendung der Realtime-PCR, da eine elektrophoretische Auftrennung hierbei nicht mehr erforderlich ist. Es kann auch *V. albo-atrum* und *V. dahliae* simultan detektiert werden (Abbildung 3). Die angewandte Realtime-PCR für *V. albo-atrum* ist 200 Mal und für *V. dahliae* 533 Mal sensitiver als eine herkömmliche Standard-PCR (Maurer et al. 2013a). Die Methode der Realtime-PCR wurde auch zur Detektion von *V. albo-atrum* bzw. *V. dahliae* bei anderen Fruchtarten erfolgreich angewandt (Mercado-Blanco et al. 2003; Lievens et al. 2006; Atallah et al. 2007; Larsen et al. 2007; Duressa et al. 2012). Jedoch hat dieser speziell für Hopfen etablierte Assay den Vorteil, dass *V. albo-atrum* und *V. dahliae* simultan in einem Multiplexansatz analysiert werden können. Aufgrund der erhöhten diagnostischen Sicherheit und gleichzeitiger Reduzierung des Arbeitsaufwandes hat sich die Realtime-PCR an der LfL, speziell am Institut für Pflanzenschutz, für verschiedenste Fragestellungen im Bereich der Pathogendiagnostik schon länger bestens bewährt (Bauer et al. 2006; Bauer et al. 2007; Seigner et al. 2007).

Erste Versuche als Grundlage zur biologischen Kontrollstrategie

Die Besiedelungskompetenz, mögliche positive Effekte auf das Pflanzenwachstum bei behandelten Hopfensamen und antagonistische Aktivität gegenüber *V. albo-atrum* und *V. dahliae* von ausgewählten Bakterienstämmen wurde untersucht (Tabelle 3). Durchschnittlich wurde eine Zelldichte von $\log_{10}$ 3.0 bis $\log_{10}$ 6.2 CFU (Cell Forming Unit = Anzahl der Bakterienkolonien) g^{-1} Frischgewicht der Wurzel (FGW) in der Endorhiza und von $\log_{10}$ 2.9 bis $\log_{10}$ 4.7 CFU g^{-1} FGW in der Rhizosphäre festgestellt.

Der Bakterienstamm *S. plymuthica* 3Re4-18 besiedelte sowohl die Endorhiza ($\log_{10}$ 3.0 ± 0.2 CFU g^{-1} FGW) als auch die Rhizosphäre ($\log_{10}$ 2.9 ± 0.1 CFU g^{-1} FGW). Die DsRed-markierte Bakterien weisen mittels CLSM kleine Akkumulationen auf der Wurzeloberfläche auf (Abbildung 6d). Pflanzen, deren Samen mit *S. plymuthica* 3Re4-18 behandelt wurden, zeigten ein erhöhtes Wachstum gegenüber den unbehandelten Kontrollpflanzen. Des Weiteren wurde mittels Dual-Kulturen Test eine antagonistische Aktivität gegenüber *V. albo-atrum* und *V. dahliae* festgestellt. In einigen vorausgehenden Studien wurde die erfolgreiche Verwendung dieses Stammes in der biologischen Kontrolle von Pflanzenpathogenen bei anderen Kulturarten beschrieben (Scherwinski et al. 2008; Zachow et al. 2010; Grosch et al. 2012). *B. terricola* ZR2-12 wies die höchste Zelldichte der untersuchten Bakterienstämme auf ($\log_{10}$ 6.2 ± 0.4 CFU g^{-1}

RFW in der Endorhiza und $\log_{10}$ 4.7 ± 0.1 CFU g^{-1} RFW in der Rhizosphäre). Die Kolonisierung von *B. terricola* ZR2-12 im Wurzelsystem war sehr stabil und wurde auch nach der Überwinterung, bei Temperaturen unter dem Gefrierpunkt, mit hoher Zelldichte wiedergefunden. Die CLSM Aufnahmen der Wurzeloberfläche bestätigen diese Resultate (Abbildung 6a und b). Die hohe Abundanz wurde zuvor in einer Studie bei Zuckerrüben beschrieben (Gasser et al. 2011). Jedoch konnten keine pflanzenwachstumsfördernden und antagonistischen Effekte beobachtet werden. Der dritte untersuchte Bakterienstamm *S. rhizophila* DSM14405^T zeichnete sich durch seine pflanzenwachstumsfördernde Eigenschaft aus. Die Zelldichten lagen bei $\log_{10}$ 4.1 ± 0.2 CFU g^{-1} FGW in der Endorhiza und $\log_{10}$ 3.8 ± 0.2 CFU g^{-1} FGW in der Rhizosphäre. Vergleichbare Werte ermittelten Schmidt et al. (2012) bei Baumwolle, Tomaten und Paprika. Auch *P. poae* RE*1-1-14 besiedelte sowohl die Endorhiza ($\log_{10}$ 4.3 ± 0.2 CFU g^{-1} FGW) als auch die Rhizosphäre ($\log_{10}$ 3.0 ± 0.2 CFU g^{-1} FGW). Diese Abundanz wurde auch im Wurzelsystem von Zuckerrüben erreicht (Zachow et al. 2010). DsRed-Transformanten dieses Stammes siedelten sich entlang der Wurzelzellen an (Abbildung 6c). Weder ein pflanzenwachstumsfördernder Effekt noch eine antagonistische Aktivität gegenüber *V. albo-atrum* und *V. dahliae* konnte festgestellt werden. Abschließend kann festgehalten werden, dass die untersuchten Bakterienstämme *B. terricola* ZR2-12, *S. plymuthica* 3Re4-18 und *S. rhizophila* DSM14405^T Grundvoraussetzungen wie die Besiedelungskompetenz für die weitere Verwendung als Biofungizide im Wirt-Pathogen System Hopfen-*Verticillium* erfüllen.

Schlussfolgerung

Stichprobenartige Untersuchungen von Hopfen mit und ohne Welkesymptome haben gezeigt, dass der *Verticillium*-Pilz vereinzelt auch latent in den Pflanzen auftreten kann. Die Analyse ergibt jedoch auch, dass überwiegend Verticillium-Welke die Hauptursache für die gegenwärtige Welke bei Hopfen darstellt. Das Ergebnis, dass in Mustern aus der *Verticillium*-toleranten Sorte Wye Target trotz Welke-Symptomen molekulargenetisch kein *Verticillium* spp. nachgewiesen werden konnte, zeigt, dass auch andere Ursachen vergleichbare Schadbilder hervorrufen könnten. Dies unterstreicht die Bedeutung und Notwendigkeit dieser sehr sensitiven und spezifischen molekularbiologischen Methodik. Die DNS Isolierung direkt aus der Rebe bringt arbeitstechnische und zeitliche Vorteile gegenüber der kultivierungsabhängigen Methode. In dieser Arbeit wird eine Anwendung des Realtime-PCR Assay zur schnellen Detektion von *V. albo-atrum* in Hopfen gezeigt. Dadurch lässt sich Verticillium schnell identifizieren um gegebenenfalls Maßnahmen zur Vermeidung seiner Verbreitung zu ergreifen.

Besiedelungsstudien, Untersuchungen von pflanzenwachstumsfördernden und antagonistischen Effekten zeigen, dass die Bakterienstämme *B. terricola* ZR2-12, *S. plymuthica* 3Re4-18 und *S. rhizophila* DSM14405^T als Biofungizide dienen könnten. Jedoch sind hierzu weiterführende künstliche Infektionstests mit *V. albo-atrum* und Feldversuche notwendig. Dabei bedarf es auch der Überprüfung der Etablierungsfähigkeit der Bakterienstämme im Wurzelsystem im Freiland. Zudem ist eine Risikofolgenabschätzung zur Beurteilung von Nicht-Ziel-Effekten und Umweltrisiken bei einem möglichen Einsatz in der Dauerkultur Hopfen notwendig. Mit dieser Arbeit wird ein erster Ansatz für eine mögliche Anwendung von nützlichen Bakterien zur biologischen Kontrolle der Verticillium-Welke bei Hopfen aufgezeigt.

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Tabellen und Abbildungen

Tabelle 1: Liste der Proben, Symptome, Hopfensorten, ihr Ursprung und Ct-Werte der Realtime-PCR

Probenbezeichnung	Sorte [a]	Symptome [b]	Landwirt	Ct-Wert [c]
1	SR	+	A	27,19
2	SR	-	A	-
3	SR	+	A	27,76
4	SR	+	A	28,09
5	SR	-	A	-
6	SR	+	A	36,84
7	SR	+	A	28,16
8	SR	+	A	28,88
9	SR	+	A	26,39
10	SR	+	A	27,18
11	PE	+	B	28,66
12	PE	+	B	32,47
13	PE	+	B	28,14
14	PE	+	B	28,41
15	PE	-	B	33,09
16	PE	+	C	27,46
17	PE	+	C	27,33
18	PE	+	C	26,50
19	PE	+	C	28,27
20	PE	-	C	35,04
21	SE	+	D	26,11
22	SE	+	D	27,42
23	SE	-	D	-
24	TU	-	E	-
25	TU	+	E	36,19
26	TU	+	E	26,78
27	HT	-	F	-
28	HT	+	F	28,03

29	HT	+	F	26,70
30	TA	+	A	-
31	TA	+	A	-
32	TA	+	A	-
33	PE	+	G	25,96
34	PE	-	G	-
35	PE	+	G	32,91
36	HM	-	G	-
37	HT	+	G	25,21
38	HT	-	G	37,35
39	HM	+	G	30,71
40	HM	+	G	26,39
41	HT	+	G	34,23
42	HT	+	H	28,81
43	HS	+	I	27,33
44	TU	-	I	-
45	PE	+	I	27,56
46	TU	+	I	30,40
47	HA	+	J	28,07
48	HA	-	J	-
49	HA	+	J	32,23
50	HA	+	J	27,60
51	HA	-	J	37,28
52	HA	-	J	-
53	HA	+	J	28,28
54	HA	-	J	37,25
55	HA	+	J	29,30
56	HA	+	J	29,95
57	HA	+	J	29,93
58	HA	+	J	29,34
59	HA	+	J	31,29
60	HA	+	J	27,65
61	HA	+	J	33,49
62	HA	-	J	-
63	HA	-	J	-
64	HA	-	J	-
65	HA	-	J	-
66	HA	-	J	36,61
67	HA	-	J	-
68	HT	+	K	30,44
69	HT	+	K	31,12
70	SR	+	K	27,13
71	HT	+	K	31,70
72	SE	+	K	35,02
73	SR	+	K	34,47
74	HT	+	K	28,30

75	NB	-	L	-
76	NB	+	L	22,72

[a] SR = Saphir; PE = Perle; SE = Spalter Select; TU = Hallertauer Taurus; HT = Hallertauer Tradition; TA = Wye Target; HM = Hallertauer Magnum; HS = Herkules; HA = Hallertauer Mittelfrüher; NB = Northern Brewer

[b] Sichtbare Symptome = +; keine sichtbaren Symptome = -

[c] Kein Signal bei der real-time PCR = -

Tabelle 2: Liste der Bakterienstämme und deren Ursprung

Bakterienstamm	EMBL Number	Ursprung	Referenz
Burkholderia terricola ZR2-12	FN313521	Rhizosphäre von Zuckerrübe	(Gasser et al. 2009)
Pseudomonas poae RE*1-1-14	FN675869; CP004045	Endorhiza von Zuckerrübe	(Zachow et al. 2008)
Serratia plymuthica 3Re4-18	FN675868	Endorhiza von Kartoffel	(Faltin et al. 2004)
Stenotrophomonas rhizophila DSM14405^T	-	Rhizosphäre von Raps	(Berg et al. 1996)

Tabelle 3: Eigenschaften der Bakterienstämme hinsichtlich ihrer Kolonisierungskompetenz, förderndem Pflanzenwachstum (PGP) und antagonistischer Aktivität (Maurer et al. 2013b)

	Populationsdichte [log_{10} CFU g^{-1} Frischgewicht der Wurzel]			Antagonistische Aktivität	
Bakterienstamm	Endorhiza	Rhizosphäre	PGP	*V. albo-atrum*	*V. dahliae*
B. terricola ZR2-12	6.2 ± 0.4	4.7 ± 0.1	-	-	-
P. poae RE*1-1-14	4.3 ± 0.2	3.0 ± 0.2	-	-	-
S. plymuthica 3Re4-18	3.0 ± 0.2	2.9 ± 0.1	+	+	+
S. rhizophila DSM14405^T	4.1 ± 0.2	3.8 ± 0.2	+	-	-

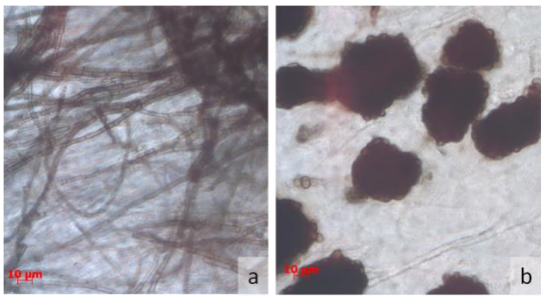

Abbildung 1: Überdauerungsorgane: (a) schwarzes Myzel von *V. albo-atrum*; (b) Mikrosklerotien von *V. dahliae.*

Abbildung 2: Symptome der Verticillium-Welke bei Hopfen. (a) – (c) Verbräunung der Blätter bis zum Absterben der gesamten Pflanze; (d) Verbräunung des vaskularen Gewebes.

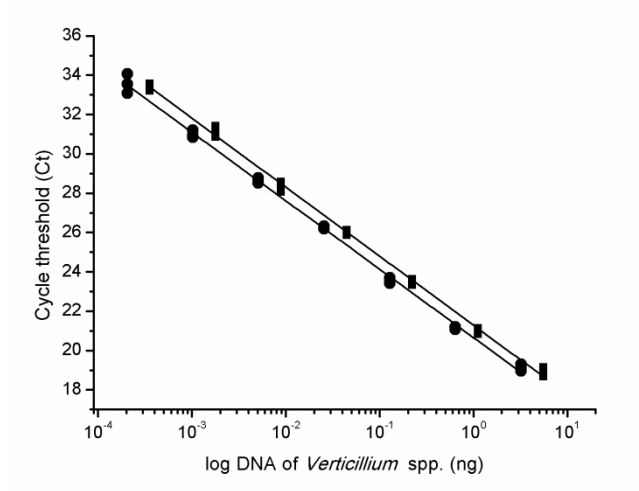

Abbildung 3: Standardgerade zur Effizienzbestimmung von *V. albo-atrum* (■) und *V. dahliae* (●): Es wurde eine sieben Punkt serielle Verdünnungsreihe mit dreifacher Bestimmung generiert. Die Effizienz von *V. albo-atrum* beträgt 92,7% (R^2 = 0,998) und von *V. dahliae* 93,8% (R^2 = 0,998).

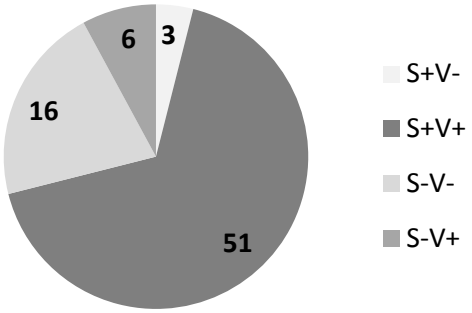

Abbildung 4: Anzahl der untersuchten Hopfenmuster hinsichtlich Symptomausbildung und molekularem *Verticillium*-Befund. S+: Welkesymtom; S-: ohne Welkesymptom; V+: *Verticillium* molekular nachgewiesen; V-: *Verticillium* molekular nicht nachweisbar.

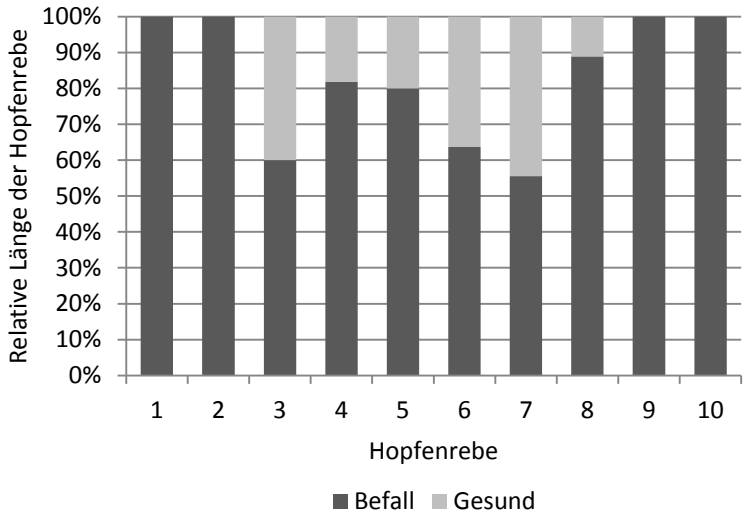

Abbildung 5: Krankheitsausbreitung von *V. albo-atrum* entlang der Hopfenrebe vom bodennahen Abschnitt beginnend.

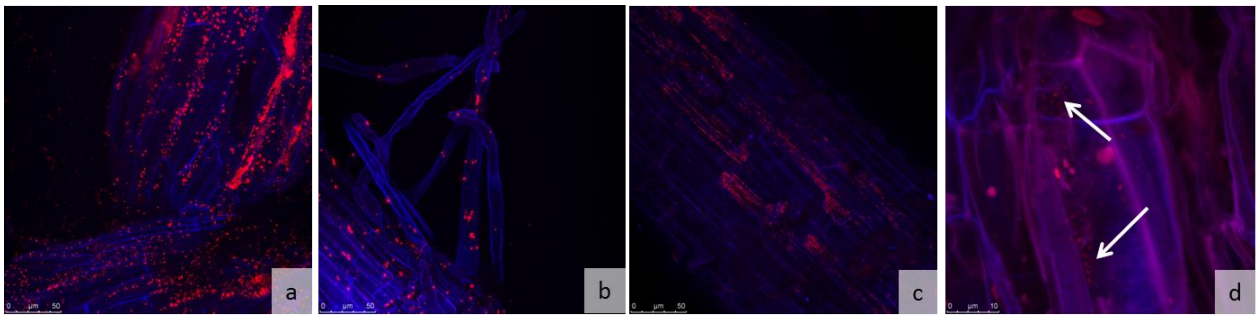

Abbildung 6: Kolonisierungsverhalten von DsRed-markierten Bakterien (rot) auf der Rhizosphäre mittels CLSM. Die Bakterien des Stammes *B. terricola ZR2-12* weisen (a) eine hohe Dichte auf der Zelloberfläche auf und (b) vereinzelt sind sie an den Wurzelhärchen zu finden. (c) Die Bakterien des Stamms *P. poae* RE*1-1-14 besiedeln die Wurzeloberfläche entlang der Pflanzenzellen. (d) An der Wurzeloberfläche sind kleine Ansammlungen des Bakterienstammes *S. plymuthica* 3Re4-18 sichtbar (Pfeile). (reproduziert mit Erlaubnis von Maurer et al. 2013b)

Additional Proceeding I

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Development of a rapid molecular *in planta* test for the detection of *Verticillium* pathotypes in hops and strategies for prevention of wilt

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Contributions: Stefan Seefelder and Katja A. Maurer have devised the concept of the presentation. Katja A. Maurer has given the oral presentation of the work. All authors contributed to scientific discussion and the preparation of the manuscript.

DEVELOPMENT OF A RAPID MOLECULAR IN-PLANTA TEST FOR THE DETECTION OF *VERTICILLIUM* PATHOTYPES IN HOPS AND STRATEGIES FOR PREVENTION OF WILT

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Abstract

Verticillium wilt is a devastating disease in hops causing high yield losses. A rapid identification would allow taking measures in time to contain and defeat the fungus. Therefore, a rapid molecular *in-planta* test on the basis of multiplex quantitative real-time polymerase chain reaction (PCR) will be developed. A rapid molecular testing of the soil can help to prevent the spreading of *Verticillium* and select fields without *Verticillium* contaminations. Biological control is a strategy for controlling and preventing of wilt. We selected five biological control agents to analyze their effects on hop plants. Preliminary experiments have proven the colonization of *Burkholderia terricola* on hop roots.

Keywords: *Verticillium*, hop, rapid molecular *in-planta* test, biological control

Introduction

Verticillium wilt, caused by *Verticillium* spp., is a dangerous disease in hop (*Humulus lupulus* L.). The fungus causes considerable economic crop failure (OEPP/EPPO 2007). *V. albo-atrum* Reinke & Berthold is the most widespread *Verticillium* species in hops, whereas *V. dahliae* Klebahn is rarely found. These species belong to the group of soilborne pathogens. *Verticillium* can survive several years in the soil by producing resting structures (OEPP/EPPO 2007). After the infection of the roots by the fungus the vascular system of the plant is colonized (Engelhard 1957).

Verticillium wilt was documented in England in 1924 for the first time by Harris (1927). Later the fluctuating (mild) and progressive (lethal) pathotypes could be differentiated (Keyworth 1942). In 1952 the mild pathogen type has been described in the Hallertau, Germany, for the first time (Kohlmann and Kastner 1974). Since 2005 *Verticillium* wilt has increased in Germany, especially in some regions of the Hallertau. The fact that former wilt resistant varieties e.g. ‘Northern Brewer’ or ‘Perle’ are showing symptoms, is a hint for the change of the virulence of the pathogen (Seefelder et al. 2009). The procedure to isolate and detect the *Verticillium* in hop bines is time-consuming and laborious. Therefore, the intention is to develop a rapid molecular *in-planta* PCR test to detect different pathotypes directly in bines. Furthermore, a quantitative real-time PCR based test should be developed to determine the concentration of *Verticillium* in soil.

Up to now, there are no effective chemical treatments available (OEPP/EPPO 2007). To prevent *Verticillium* wilt, there are only few measures, like the planting of resistant or tolerant plants and phytosanitary arrangements (Radišek et al. 2004). Biological control is an environmentally friendly way to suppress *Verticillium* wilt. The interaction between biocontrol agents, host plants, pathogen and microbial community had been described in detail by Handelsman and Stabb (1996). Berg (2009) reported about some plant-associated microorganismens which showed positive effects on plants and could prevent disease symptoms.

Methods

Rapid molecular *in-planta* PCR test. A new method to isolate the DNA directly from the bine is developed. First the interior part of the hop bine was milled with a homogenizer (MP FastPrep). Therefore different parameters such as speed, time, different bead size and bead material were tested. The DNA extraction was done by several commercial isolation kits. For the quantitative real-time PCR detection of *V. albo-atrum* and *V. dahliae* special probes were designed using Beacon Designer 7. For this step available sequences were used (Nazar et al. 1991).

Biological Control. To analyze the influence of beneficial microorganisms on hop five different bacteria and different varieties of hops were chosen. The experiments to analyze the colonization of the bacteria on roots are arranged according to Berg et al. with some modifications (2000). The roots of the plants were washed with tap water and were dipped in the bacterial suspension. Spontaneous mutants resistant to Rifampicin were used. After four weeks roots were sampled and washed in NaCl. This solution was plated on nutrient agar containing Rifampicin. After seven days the bacterial colonies were counted. The plant growth effect was analyzed. Currently, one bacterial species of bacteria *Burkholderia terricola* ZR2-12 (Grasser et al. 2011) and two cultivars of hops ‘Herkules’ and ‘Perle’ are being tested.

Results

Rapid molecular *in-planta* PCR test. The homogenization was done using specific beads. For the isolation of the DNA several tested commercial kits were effective. To detect *V. albo-atrum* and *V. dahliae* in one step a multiplex real-time PCR protocol and specific probes were developed. Results obtained by the newly developed and the original method are identical.

Biological Control. First preliminary experiments were done. *Burkholderia terricola* ZR2-12 could be re-isolated from the hop roots. The tests will be expanded using other hop varieties and beneficial bacteria and a sufficiently high number of replications.

Discussion

To develop a rapid test to detect *Verticillium albo-atrum* and *V. dahliae* is very important for the farmers. The classical method comprising the isolation and cultivation of *Verticillium* from the hop bine (Seefelder et al. 2009) and the subsequent isolation of the DNA according to a modified standard protocol (Doyle and Doyle 1990) takes four to six weeks. The new method for the DNA isolation directly from the bine to identify *V. albo-atrum* and *V. dahliae* takes only one to two days which is a significant improvement. Further work will focus on the detection of the mild and lethal pathotypes of *V. albo-atrum* using multiplex real-time PCR. Furthermore, we plan to develop a rapid molecular test to detect *Verticillium* in soil. We tested several commercial kits for the detection of DNA in the soil. Only soil samples artificially infected with *Verticillium* were tested positive. Soil samples below 1 g and inhibiting substances in the soil matrix may cause this failure to detect the pathogen by the PCR reaction. This test should be applied for preliminary investigations, whether the pathogen is already in the soil, before planting a hop garden. Similar tests already exist to determine *V. dahliae*, e.g. in strawberry fields (Neubauer and Heitmann 2011).Thereby complex tests are used, where the resting structure of *V. dahliae* (microsclerotia) are sieved and cultivated. These tests take about four weeks. The problem to detect *V. albo-atrum* in soil is that there are no resting structure can be sieved. The development of a rapid molecular test from the soil is the only way to detect *V. albo-atrum*.

Another aim of this work is to develop strategies for containment and prevention of wilt using biological control. The experiments will be done with several hop varieties and five beneficial bacteria. Successful colonization of roots with bacteria will be analyzed using confocal laser scanning microscopy. Furthermore, the interaction between the beneficial bacteria and *V. albo-atrum* should be analyzed. For this purpose the plants will be dipped into a bacterial suspension and planted in soil infected with *Verticillium* in order to test for health promoting

effects of the BCA on the plants. The objective is to find biological plant protecting agents to contain and prevent hop infection with *Verticillium*. *Serratia plymuthica* HRO-C48 and *Pseudomonas fluorescens* PICF7 represent successful applications of BCAs against *V. dahliae* (Müller et al. 2007; Prieto and Mercado-Blanco 2008). This approach will identify species of bacteria which show positive effects on hops.

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8 Curriculum Vitae

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2004 – 2007	Diploma studies of Technical Chemistry at the Technical University Graz
2010	Recipient of a scholarship of the Faculty of Technical Chemistry, Chemical Engineering and Biotechnology, Technical University Graz

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Maurer KA, Berg G and Seefelder S. Untersuchungen zur Verticillium-Welke im Hopfenangebiet Hallertau. *Gesunde Pflanzen* (in preparation)

Maurer KA, Zachow C, Seefelder S and Berg G (2013) Initial steps towards biocontrol in hops: Successful colonization and plant growth promotion by four bacterial biocontrol agents. *Agronomy* 3: 583-594

Maurer KA, Berg G, Radišek S and Seefelder S (2013) Real-time PCR assay to detect *Verticillium albo-atrum* and *V. dahliae* in hops: development and comparison with a standard PCR method. *Journal of Plant Diseases and Protection* 120: 105-114

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Oral presentations:

Development of a rapid molecular *in planta* test for the detection of *Verticillium* pathotypes in hops and strategies for prevention of wilt. Colloquium, Institute of Environmental Biotechnology, Technical University Graz, Austria, 10.08.2012

Development of a rapid molecular *in planta* test for the detection of *Verticillium* pathotypes in hops and strategies for prevention of wilt. DissertantInnen Seminar 2, Technical University Graz, Austria, 26.04.2012

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Poster presentations:

Maurer KA, Radišek S, Zachow C, Berg G and Seefelder S (2013) Molecular *in planta* test for the detection of *Verticillium* species in hops and initial steps towards biological control. 11th International Verticillium Symposium, Göttingen, Germany

Drofenigg K, Berg G, Seigner E and Seefelder S (2012) Development of a rapid molecular *in planta* test for the detection of *Verticillium* pathotypes in hops. Wissenschaftliches Kolloquium für DissertantInnen 2, University of Graz, Austria

Drofenigg K, Müller H, Berg C and Berg G (2009) Molecular analysis of endophytic communities of wild olive trees from different Mediterranean regions. 10th International Verticillium Symposium, Corfu, Greece

Drofenigg K, Aranda S, Landa BB, Berg G and Müller H (2009) Cultivar-specific *Pseudomonas* communities in the rhizosphere of olive trees. 10th International Verticillium Symposium, Corfu, Greece

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Genehmigung des Senates am 1.12.2008

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