

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 log₁₀ 3.0 to log₁₀ 6.2 CFU g⁻¹ root fresh weight in the endorhiza and from log₁₀ 2.9 to log₁₀ 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].

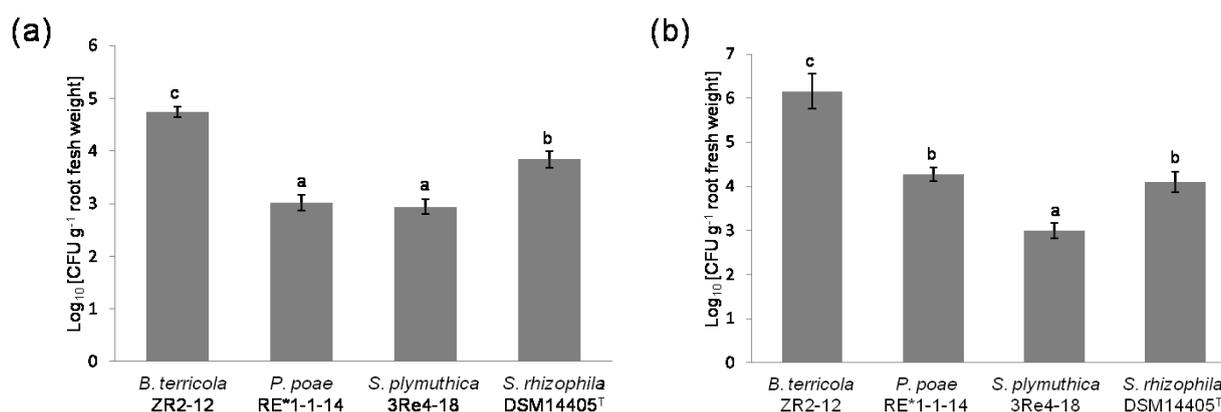
2. Results and Discussion

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.

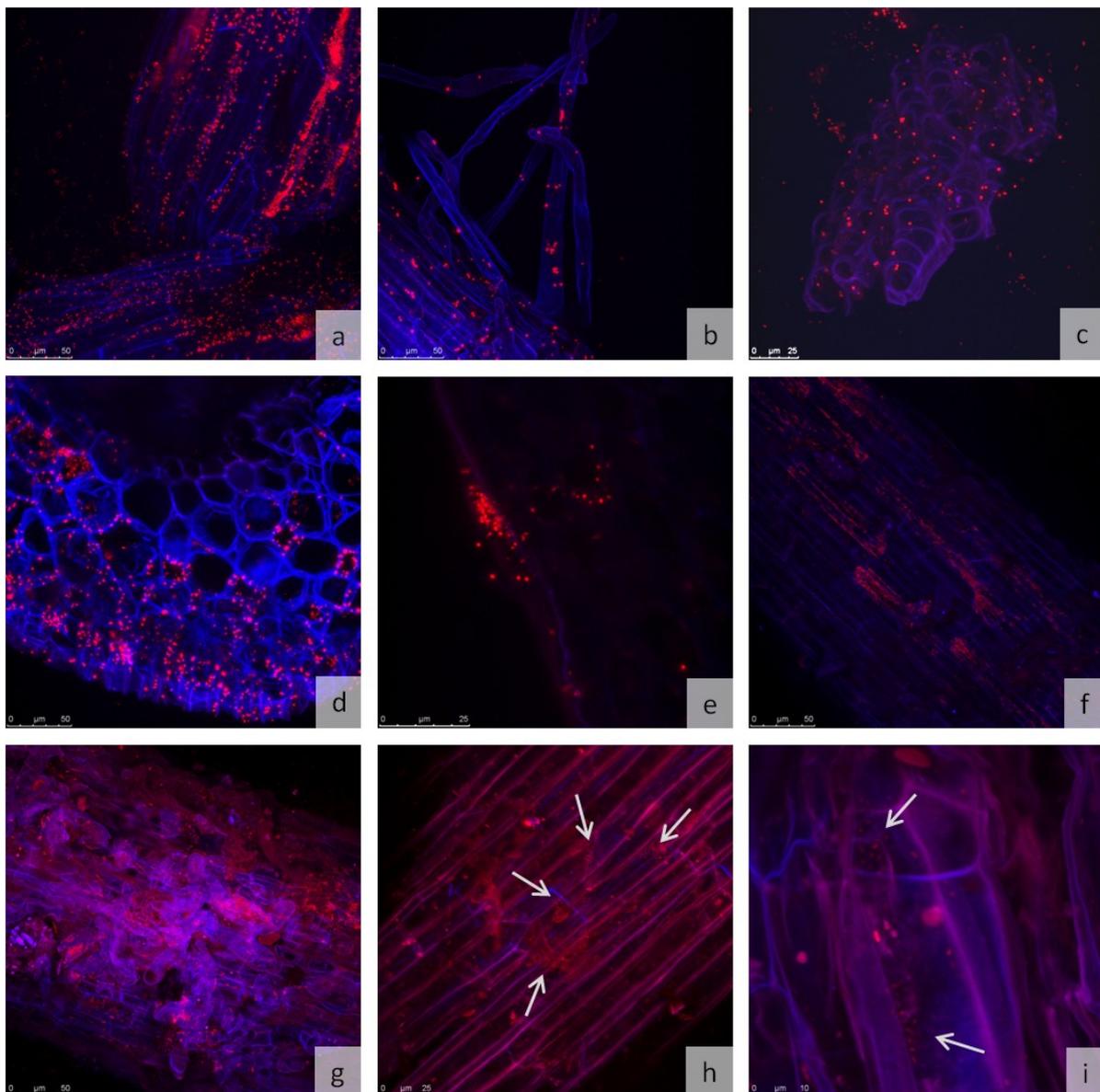
Furthermore, the potential establishments of these bacteria on seeds were analyzed. Hops seeds were inoculated and the root system cell density was determined after seven weeks. The abundances of *B. terricola* ZR2-12, *S. plymuthica* 3Re4-18, and *S. rhizophila* DSM14405^T ranged from approximately \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.

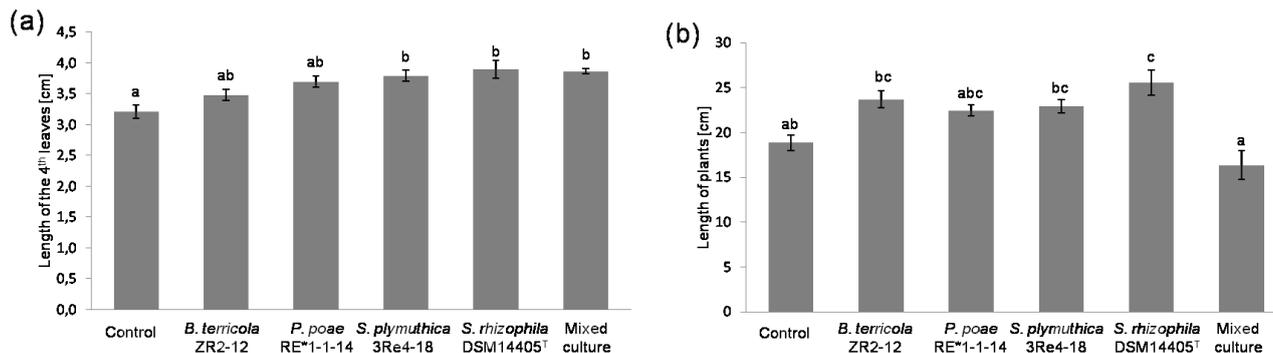
Figure 2. Colonization pattern of DsRed-transformed bacteria (red) in the root system of hops six to seven days after inoculation. *B. terricola* ZR2-12 cells showed (a) a high density on the root surface and appeared in a scattered arrangement (b) on root hairs and (c) on root tip; (d) *B. terricola* 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 10⁸ 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 rhizosphere 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 (10⁸ 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. nonalfalae* 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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