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Effects of *Stenotrophomonas rhizophila* SPA-P69 on *Zea mays* affected by *Diabrotica virgifera*

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Affidavit

I declare that I have authored this thesis independently, that I have not used other than the declared sources/resources, and that I have explicitly indicated all material which has been quoted either literally or by content from the sources used. The text document uploaded to TUGRAZonline is identical to the present master's thesis.

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Abstract

The western corn rootworm (*Diabrotica virgifera* LeConte*)* has become a devastating pest on maize (*Zea mays L.*). Damages caused by its soil-borne larvae weaken the plant, leading to lodging and yield loss. In contrast to common pesticides, biological control of *D*. *virgifera* represents a sustainable and specific method. This method employs various organisms such as entomopathogenic nematodes, insect pathogenic fungi or beneficial bacteria. These agents can be applied individually or in combination. In this thesis the potential use of *Stenotrophomonas rhizophila* SPA-P69 as a biological control was investigated. Its ability to stimulate root growth could compensate for damages caused by larvae. In field trials, germination rate, lodging and yield were determined at three locations in Austria. The bacterial treatment reduced lodging without impairing the germination rate and increased the yield, compared to respective controls. The infection with larvae on one field site was successfully monitored with emergence traps, showing an equal larvae distribution across the field. Two different seed application techniques, priming and encapsulation, were compared. Therefore, colonization of fluorescence labelled SPA-P69 applied with those techniques, was detected using confocal laser scanning microscopy. Priming and encapsulation produced similar colonization. By development of a species-specific molecular probe, SPA-P69 was successfully quantified in the rhizosphere using quantitative real-time PCR. *Stenotrophomonas rhizophila* SPA-P69 has shown to be a promising candidate to become a beneficial component in a biocontrol application, providing advantages for economy and environment.

Zusammenfassung

Der westliche Maiswurzelbohrer (*Diabrotica virgifera* LeConte), hat sich in den letzten Jahren zu einem der verheerendsten Pflanzenschädlinge am Mais (*Zea mays*) entwickelt. Durch bodenbürtige Larven hervorgerufene Schäden führen zu verminderter Fitness der Wirtspflanze. So genanntes Lodging und Ertragseinbußen sind die Folge. Im Gegensatz zu herkömmlichen Pestiziden stellt biologische Schädlingsbekämpfung ein nachhaltiges und spezifisches Verfahren dar. In diesem Verfahren können verschiedene Organismen wie entomopathogene Nematoden, insektenpathogene Pilzen oder nützliche Bakterien einzeln oder in Kombination angewendet werden. In dieser Arbeit wurde untersucht, ob *Stenotrophomonas rhizophila* SPA-P69 einen potenziellen Kandidaten für eine solche Anwendung am Mais darstellt. Seine Fähigkeit, Wurzelwachstum zu stimulieren, könnte Fraßschäden der Käferlarven kompensieren. An drei Standorten in Österreich wurden in Feldversuchen Keimungsrate, Lodging, sowie Ertrag ermittelt. Die Behandlung mit SPA-P69 zeigte keinen negativen Einfluss auf die Keimungsrate, Lodging konnte verringert und ein höherer Ertrag erzielt werden. Die Infektion eines Feldes mit Maiswurzelbohrerlarven wurde mittels Emergenzfallen detektiert. Eine gleichmäßige Verteilung der Larven am Feld konnte festgestellt werden. Die zwei Behandlungsmethoden, Priming und Verkapselung zur Aufbringung auf den Samen, wurden verglichen. Dabei wurde fluoreszenzmarkierte SPA-P69 mit diesen Methoden appliziert und mit konfokaler Laser-Scanning Mikroskopie detektiert. Priming und Verkapselung ergaben eine ähnliche Kolonisation. Mit der Entwicklung einer spezies-spezifischen molekularen Sonde konnte SPA-P69 erfolgreich in der Rhizosphäre quantifiziert werden. Die Detektion wurde mit quantitativer Echtzeit-PCR durchgeführt. *Stenotrophomonas rhizophila* SPA-P69 stellt einen potenten Kandidaten für die Verwendung in einem Kombinationspräparat mit Vorteilen für Wirtschaft und Umwelt dar.

Table of Contents

1 Introduction

1.1 Agricultural Land Use at the Example of Maize Cultivation

According to data acquired in the timespan from 1992 to 2014 by the World Bank, the proportion of area used for agricultural purposes remains constant at 37.5 % [1]. In the same period, the global population increased from 5.5 billion in 1992 to 7.3 billion in 2014 [2]. Consequently, harvest yield improvement is needed to guarantee food and raw materials. Higher harvest yields were achieved by development and distribution of farming technologies such as genetically modified crop, synthetic fertilizers, pesticides and farm machinery [3]. These developments are also reflected individually in many cultivated crops.

For the field trials carried out in this master's thesis, the crop plant maize (*Zea mays* L.) was utilized. Worldwide maize showed a yield increase of 44.0 % between 1992 and 2014 with a total yield of 56,157 hectogram per hectare [4]. Maize has been used for many centuries as a food and energy plant for humans and food for livestock. With a production of more than 100 million tons in the year 2014, maize is the most abundant cereal crop worldwide according to Food and Agriculture Organization of the United Nations [5]. But the extensive optimization of yield and quality has, at the same time, lead to negative effects on plant fitness, including for instance weakened resistance to pathogens and insects [6]. Furthermore, reduced crop rotation, enlargement and aggregation of arable fields on a regional level are likely to increase insect outbreaks [7]. For maize, the estimated potential yield loss due to pests is 31.2 %. The highest proportion of yield loss is accounted for animal pests with about 50 %. Animal pests on maize belong to the taxonomic classes Insecta, Gastropoda, Aves, Arachnida and Mammalia. Depending on the geographical location of the area where maize was planted, animals cause different amounts of yield loss [8].

1.2 General Information and Life Cycle of the Western Corn Rootworm

Belonging to leaf beetle family (Chrysomelidae), the western corn rootworm (*Diabrotica virgifera* LeConte) is a beetle native to North America [9]. The western corn rootworm (WCR) was discovered by John Lawrence LeConte and described as a species in 1868. In 1902 it was discovered as a pest for maize plants [10, 11]. Today, it is the most devastating pest of maize in the United States. The main reasons are the strong connection in the larval and imago phase to maize and large areas planted as monoculture [12–14]. Twenty years ago, scientists pointed out that the WCR will become one of the most harmful insect species worldwide, so that humanity will face one of the biggest and most difficult entomological, agricultural, toxicological and environmental challenges [15]. It is suggested, that the WCR was introduced to Europe several times from the 1980s to the 2000s [16]. The first find in 1992 was detected in former Yugoslavia [17]. Since then, the WCR has spread over large parts of Central and Southeastern Europe. Furthermore, it has established itself as an alien invasive species [18]. In the US, losses are estimated over one billion US dollars due to control measures and harvesting losses caused by WCR. Even in Europe, economic damages are already noticeable, especially in central European countries such as Hungary, Slovenia and Austria or the Balkan peninsula [18–20]. In cultivated areas located in Southern and Eastern Europe, cultivated in monoculture, yield losses of 10-30 % occur four to five years after the first occurrence [21]. Therefore, the damage potential in Europe is estimated to be up to half a billion Euro over a long-term period [22].

The life cycle of the WCR is univoltine, meaning there is one generation per year. During diapause, stage eggs survive the winter in soil depths of about 5 to 30 cm. In this stage, no development takes place [23]. At the end of the diapause stage, the hatch from the eggs is often assumed to be spontaneous in the literature. Important parameters are in any case rising temperatures after chilling phases and the availability of water [24–27]. The hatched larvae remain immobile during the first phases, feeding on maize roots in their near surroundings. This dietary behavior of the larvae results in problems with the absorption of water and nutrients through the root, as well as an entry port for pathogens. In case of heavy infestation, the root loses the ability to keep the plant upright. So-called lodging occurs, in which the maize plants tend [13]. After passing three larval phases, the larvae form a discrete

terrestrial cell for pupation [28]. The annual time of beetle hatch differs depending on various conditions. For instance, in Croatia hatch started between the middle of June and the beginning of July in the years from 1998 to 2000 [29, 30]. On average, the pupal stadium lasts 29 days for male beetles and 32 for female beetles. The male are protandrous, they usually hatch five days before their female conspecifics [31, 32]. With this protandrous hatch it is ensured that at the time of hatching of the female beetle, enough mature males are available [33]. After the subsequent fertilization by male beetles, female beetles require protein-rich diet. In this maturation feeding phase, female beetles feed on protein-rich components of maize, such as silk and pollen. This leads to yield losses due to reduced pollination rates and a reduced number of grains on the piston [34, 35]. After a food intake of two weeks on average, the female beetles deposit 300 to 400 eggs each in the soil. There are indications that the egg-laying behavior changes and eggs are also placed not only in soil where maize is planted [36]. Adult beetles show the highest flight activity in the morning and evening hours. They feed on young leaves and ripening grains of maize and on leaves of other flowering crop plants, besides maize silk and pollen [37]. With the harvest of maize as well as the temperature drop in autumn, adult beetles die.

1.3 Measures to Control the WCR in Europe

1.3.1 Agricultural Measures

Infestation and spread of WCR are unstoppable in many areas of Europe due to active flight of beetles but also passive spread via means of transport. To slow down spread, various control measures are applied. The most effective measure to control large areas affected by WCR is crop rotation. It can suppress population development and thus reduce the natural spread of WCR [38]. Another important measure is weed control in the maize stock, which can serve as a source of food for WCR larvae [37]. Measures that strengthen the development of maize plants, can also positively contribute to preventing damage caused by the WCR. Such measures include right time of planting, targeted fertilizing and optimal preparation of the seedbed [39].

1.3.2 Chemical Pest Control

Two different application techniques for insecticides are used, seed treatment or granules to control the larvae and spray treatment control of adult beetles. Driving a maize field at the time of beetle flight to apply spray treatment is difficult because of the high stature height of

the maize plant. Not all insecticides are approved in European Union (EU) member states [40]. Insecticide application shows the highest efficiencies shortly after the introduction of WCR. Compared to crop rotation, their efficiency is estimated at 60 to 80 % [41]. Insecticides used as soil insecticides cannot prevent the rising of WCR populations, they may only prevent damage to basal roots shortly after planting, according to field trials carried out in the USA [42–44]. Furthermore, the use of neonicotinoid insecticides is not unproblematic. Residues in water, soil and parts of plants show negative effects on non-target pollination insects, such as bumblebees [45, 46]. The use of insecticides as a large-scale, nonspecific method of pest control led to a rapid resistance development of pests against these insecticides. Additional emerging issues such as the depletion of non-target insects, biodiversity loss and environmental concerns are pushing research to develop new, environmentally friendly practices [13, 47].

1.3.3 Breeding and Genetic Modification of seeding material

Plant breeding is a possible measure. New varieties should not reduce the density of beetles in the field, but on rooting by the larvae, increase the root growth. As a result, stability and nutrient exchange through the root improves. Those breeding forms are currently being investigated.

Genetic modification of maize plants to obtain WCR resistance is another measure. Individual genes from various *Bacillus thuringiensis* (Bt) strains are introduced into the plant genome. These genes encode as cry-toxins that are taken in orally by the insects. The uptake leads to pore formation in the intestine and subsequent deadly sepsis. Field trials carried out in Illinois and nine locations across the corn belt, USA, demonstrate that Bt maize hybrids show less or no damage to the maize roots caused by the WCR [48, 49]. A small amount of WCR larvae exposed to Bt toxin overlap until sexual maturity, due to possible suboptimal doses of toxin. These non-optimal doses of toxin may lead to a development of the beetle's resistance to the toxin [50, 51]. Resistance alleles in the genome of WCR are currently not explored. The only maize breed currently approved in the EU is MON810. National bans by many EU member countries, including Austria, lead to a slow distribution of these modified seeds with potential economic benefits.

1.3.4 Biological Pest Control

Another measure is the control with biological organisms. Because the WCR is an alien invasive species for Europe, it has no natural enemies in Europe as occur in North and South America. Natural enemies to the WCR are numerous pathogens, nematodes, predators, parasites, parasitoids, fungi or bacteria. Most of these natural enemies are found in soil and attack the WCR during egg or larval stage. Compared to conventional pest control measures, the use of natural antagonists has many advantages as it is considered to be less costly and more environmentally friendly than insecticidal applications [52].

Natural antagonists of WCR are entomopathogenic nematodes (EPN) of the genus *Heterorhabditis* [53]. For example, the species *Heterorhabditis bacteriophora* was discovered to significantly damp WCR. Other soil living arthropods were not affected [54]. *H. bacteriophora* does not kill the larvae, but infiltrates the WCR larva and then releases a bacterium of the genus *Photorhabdus* from the digestive system. It multiplies in the larva and releases toxins that are deadly to the larvae. Furthermore, the bacteria convert the digestive organs of the larvae into a nutritious prey for the nematodes to grow therein [55, 56]. With field trials on maize plants successfully carried out in 1996 in USA, *H. bacteriophora* treatment with 200,000 nematodes per plant reduced root damage. The potential economic loss threshold and adult hatching was reduced by 66 to 98 % [57]. From 2003 to 2007, *H. bacteriophora* was applied by different spray application methods in field trials in Hungary. All techniques showed on average a reduction of at least 50 %. Fluid application showed the highest reduction (68 %) directly during sowing of maize seed [58]. The control of WCR by these entomopathogenic nematodes represents a promising strategy. Since 2013, dianem® (E-nema, Germany), a plant protection product containing *H. bacteriophora* applied to control the larvae and pupae of the WCR, has been authorized for EU. At present, the costs and the amount of water for the application of nematodes are too high. Therefore, the application method should be further developed.

Entomopathogenic fungi (EPF) may also be of great benefit in terms of their antagonistic effects to WCR larvae [59]. The effect of EPF can be increased by applying it additionally to other control agents. Induced stress on the larvae makes them more susceptible to biocontrol agents (BCAs) [60]. Maize fields are naturally inhabited by the EPF strains, such as *Metarhizium anisopliae* (Metch.) belonging to Clavicipitaceae family from the Ascomycota

division. *M. anisopliae* showed to infect and harm the WCR [61]. The EPF *Metarhizium brunneum* (Petch.), which isolates were summarized as *Metarhizium anisopliae* var. *anisopliae* before, reduces adult WCR emergence by 31 % [62]. After infection, the mechanism of action is described by a combination of mechanical damage, lack of nutrients and intoxication caused by released toxins [63]. GranMet-P™ produced by Agrifutura in Italy is a biocontrol product containing *M. brunneum*.

1.4 New Management Strategies to Control the WCR

In the past 50 years, because of the ever-increasing use of synthetic chemical pesticides, ever higher yields could be achieved. However, broad-spectrum use has shown to have negative effects to human health and the environment [64]. Arguably due to pressure from consumers and other groups, a rethinking of Europe's approach using synthetic chemical pesticides has moved towards more sustainable methods. To reduce the use of pesticides, the European network for the durable exploitation of crop protection strategies (ENDURE) has been launched in 2007. In October 2009, European Parliament and Council adopted the directive 2009/128/EC for a more sustainable use of pesticides [65]. Article 14 of this directive deals with Integrated Pest Management (IPM). IPM combines various methods of controlling pests to minimize harm to the environment with the highest specificity against the pest. The interest on an IPM method, namely the use of BCAs, has increased since the regulation 1107/2009/EC and the directive 2009/128/EC from the European Parliament and the Council of the European Union [65, 66].

Microbes that colonize the rhizosphere can assist with plant nutrient acquisition or plant health by pathogen suppression. Seed-associated microbes may be vectors for founder microbes that establish the plant microbiome. They may subsequently contribute beneficial functions to their host plants including nutrient acquisition and promotion of plant growth [67, 68]. Important mechanisms that plant-associated microbes need are the ability to compete for niches, adhesion and colonization on host plant surface as well as the ability to form biofilms. *Stenotrophomonas rhizophila*, a gram-negative bacterium belonging to the phylum Proteobacteria, was described as a species in 2002 [69]. It is habited in different plant-associated areas such as rhizosphere, geocaulosphere and phyllosphere. *S. rhizophila* produces the osmoprotective substances trehalose and glucosylglycerol. Those stabilize enzymes and are expected to enhance the stress tolerance of the host plant [70].

Furthermore, the bacterium produces high levels of indole-3-acetic acid, the phytohormone auxin. Auxin promotes root and shoot growth [71]. The production of these substances by *S. rhizophila* have beneficial effects on host plants, such as plant growth promotion (PGP) and biocontrol against fungal pathogens [72]. This masters' thesis will focus on *S. rhizophila* strain SPA-P69 (= DSM14405^T = e-p10 = SPA-P69) with proven plant growth promotion to various host plant and biocontrol against several pathogens [73]. Additionally, there are also trials performed with *S. rhizophila* strain e-p17.

1.5 Objectives of this Study

The experiments carried out were part of the research project "DIACONT - Alternative biologische Methoden zum Schutz des Maises vor dem Maiswurzelbohrer (*Diabrotica virgifera*)" that aims to integrate *S. rhizophila* SPA-P69 as beneficial bacteria in a combination of multiple biocontrol agents. The approach combines insect pathogenic nematodes (*Heterorhabditis bacteriophora*), insect pathogenic fungi (*Metarhizium brunneum, Lecanicillium lecanii*) and *S. rhizophila* SPA-P69 as a root-growth promoter. It could be a new environmentally friendly opportunity to control the western corn rootworm, *Diabrotica virgifera*. The research project is set for a period of three years.

The aim of this study was to detect the effect of *S. rhizophila* SPA-P69 and *S. rhizophila* e-p17 applied to maize seeds under field conditions at three different locations in Austria. The treatment was combined with or without additional fungicide application on arable land naturally infected by the western corn rootworm. To ensure that the number of infestations of larvae is evenly distributed over the field, emergence traps were developed, installed and monitored. Different methods were used to evaluate and quantify the root colonization of *S. rhizophila* SPA-P69 and *S. rhizophila* e-p17.

- During the field trials in 2016, various parameter including germination rate, rates of lodging on three timespans and the amount of yield in deca tons per hectare were determined.
- Comparison of two modes of seed application by qualitative detection on seeds and by determining the colonization density of *S. rhizophila* SPA-P69 and *S. rhizophila* e-p17.
- Using confocal laser scanning microscopy (CLSM), colonization patterns of fluorescently labeled *S. rhizophila* e-p17, applied on maize seeds with two different techniques, namely priming and encapsulation, were detected.
- Development of a *S. rhizophila* SPA-P69 and *S. rhizophila* e-p17 specific TaqMan probe for the quantification of *S. rhizophila* SPA-P69 and *S. rhizophila* e-p17 in total DNA extracted from rhizosphere samples using quantitative real-time PCR.

2 Material and Methods

2.1 Culture Media

To produce the following nutrient media dH_2O was used. The components were agitated well and autoclaved at 121 °C for 20 min.

Nutrient Agar (NA)

- Nutrient Broth II (Sifin, Berlin, Germany) 15.0 g/L
    - Peptone from caseine 3.5 g/L
    - Peptone from meat 2.5 g/L
    - Peptone from gelatin 2.5 g/L
    - Yeast extract 1.5 g/L
    - Sodium chloride (NaCl) 5.0 g/L
- Agar-Agar, Kobe I (Carl Roth, Karlsruhe, Germany) 18.0 g/L

Nutrient Broth II Medium (NB II)

- Nutrient Broth II (Sifin, Berlin, Germany) 15.0 g/L
    - Peptone from caseine 3.5 g/L
    - Peptone from meat 2.5 g/L
    - Peptone from gelatin 2.5 g/L
    - Yeast extract 1.5 g/L
    - Sodium chloride (NaCl) 5.0 g/L

LB – Medium (LB)

- LB-Broth (Luria/Miller; Carl Roth, Karlsruhe, Germany) 25.0 g/L
    - Tryptone 10.0 g/L
    - Yeast extract 5.0 g/L
    - Sodium chloride (NaCl) 10.0 g/L

Selective LB-Agar with Ampicillin/IPTG/X-Gal

- LB-Broth (Luria/Miller; Carl Roth, Karlsruhe, Germany) 25.0 g/L
    - Tryptone 10.0 g/L
    - Yeast extract 5.0 g/L
    - Sodium chloride (NaCl) 10.0 g/L
- Agar-Agar, Kobe I (Carl Roth, Karlsruhe, Germany) 20.0 g/L

After autoclaving the medium was cooled to 50 °C.

50 µg/ml Ampicillin (Carl Roth, Karlsruhe, Germany) was added and afterwards the medium was poured into petri dishes.

40 µl 100 mM Isopropyl-β-D-thiogalactopyranosid (IPTG) and 40 µl 20 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) were spread over each petri dish and incubated at 37 °C for 30 min.

NA with Gentamycin/Nystatin

- Nutrient Broth II (Sifin, Berlin, Germany) 15.0 g/L
    - Peptone from caseine 3.5 g/L
    - Peptone from meat 2.5 g/L
    - Peptone from gelatin 2.5 g/L
    - Yeast extract 1.5 g/L
    - Sodium chloride (NaCl) 5.0 g/L
- Agar-Agar, Kobe I (Carl Roth, Karlsruhe, Germany) 16.0 g/L
- Nystatin (Carl Roth, Karlsruhe, Germany) 25 µg/ml
- Gentamycin (Carl Roth, Karlsruhe, Germany) 20 µg/ml

After autoclaving the medium was cooled to 50 °C.

25 µg/ml Nystatin and 20 µg/ml Gentamycin were added, after the autoclaved medium was cooled to 50 °C.

NA with Trimethoprim/Nystatin

- Nutrient Broth II (Sifin, Berlin, Germany) 15.0 g/L
    - Peptone from caseine 3.5 g/L
    - Peptone from meat 2.5 g/L
    - Peptone from gelatin 2.5 g/L
    - Yeast extract 1.5 g/L
    - Sodium chloride 5.0 g/L
- Agar-Agar, Kobe I (Carl Roth, Karlsruhe, Germany) 16.0 g/L
- Nystatin (Carl Roth, Karlsruhe, Germany) 25 µg/ml
- Trimethoprim (Sigma-Aldrich, Vienna, Austria) 50 µg/ml

25 µg/ml Nystatin and 50 µg/ml Trimethoprim were added after the autoclaved medium was cooled to 50 °C.

NBII with Trimethoprim/Nystatin

- Nutrient Broth II (Sifin, Berlin, Germany) 15.0 g/L
    - Peptone from caseine 3.5 g/L
    - Peptone from meat 2.5 g/L
    - Peptone from gelatin 2.5 g/L
    - Yeast extract 1.5 g/L
    - Sodium chloride 5.0 g/L
- Nystatin (Carl Roth, Karlsruhe, Germany) 25 µg/ml
- Trimethoprim (Sigma-Aldrich, Vienna, Austria) 50 µg/ml

25 µg/ml Nystatin and 50 µg/ml Trimethoprim were added after the autoclaved medium was cooled to 50 °C.

2.2 Solutions

To produce the solutions, liquids and components were agitated well and autoclaved at 121 °C for 20 min. To produce antibiotic solution, the antibiotics were solved in different solutions and afterwards sterile filtered with a pore size of 0.2 µm and stored at minus 20 °C.

0.85 % Natrium Chloride

- NaCl 8.5 g/L solved in ddH_2O

Ampicillin Stock 50 mg/ml

- Ampicillin 50 mg/ml solved in ddH_2O

Gentamycin Stock 10 mg/ml

- Gentamycin 50 mg/ml solved in ddH_2O

Trimethoprim Stock 50 mg/ml

- Trimethoprim 50 mg/ml dissolved in Dimethylsulfoxid (DMSO; Carl Roth, Karlsruhe, Germany)

Nystatin Stock 25 mg/ml

- Nystatin 25 mg/ml solved in ddH_2O

Isopropyl-β-D-thiogalactopyranosid (IPTG) 100mM

- IPTG 1.2 g
    - ddH_2O was added to a final volume of 50.0 ml.

The solution was sterile filtered with a pore size of 0.2 µm.

5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) 20 mg/ml

- 20 mg/ml dissolved in Dimethylformamid (DMF; Carl Roth, Karlsruhe, Germany)

Tris-Acetate-EDTA (TAE) buffer [50X]

- Tris (99.9 %) 242.0 g/L
- Glacial acetic acid 57.0 ml/L
- EDTA 0.5 M 100.0 ml/L

Tris-Borat-EDTA (TBE) buffer [5X]

- Tris (99.9 %) 54.0 g/L
- Boric acid (99.8 %) 27.5 g/L
- EDTA 0.5 M 20.0 ml/L

1% TAE[1X] Agarose Gel (final volume 400 ml)

- Tris-acetate-EDTA (TAE) buffer [50X] 8.0 ml
    - Tris (99.9 %) 242 g/L
    - Glacial acetic acid 57 ml/L
    - EDTA 0.5 M 100 ml/L
- ddH_2O 392.0 ml
- Agarose (Carl Roth, Karlsruhe, Germany) 4.0 g

All components were mixed, heated in the microwave and filled into the electrophoresis chamber.

2.3 Setup of the Field Trials

In order to determine the effects of *S. rhizophila* and, moreover, the most effective treatment method combination, field tests were carried out. Treated as well as untreated seeds of maize were planted. The field trials took place on three different locations in Austria with different soil types. One site was in Styria near Mitterdorf an der Raab (S47.175054; E15.621221), with soil consisting of light brown earth (sandy loam) on moraines and gravel. The other two sites have soil consisting of brown earth on loess and were in Upper Austria, near Reichersberg (S48.405722; E13.517692), and in Lower Austria, near Melk (S48.157633; E15.522300).

2.3.1 Overview of Maize Seed Treatment

In this section the different treatments applied on the maize seeds are explained. The seed coating contained either the fungicide MAXIM® XL (Syngenta Group Company; Basel, Switzerland) or no fungicide. Samples with fungicidal treatment were signed with an F, whereas samples with no fungicidal treatment were signed with a B. For adhesion to the seed surface, Sacrust M-621 (Kwizda Agro GmbH; Vienna, Austria) was used. The bacterial treatment was applied using two different application techniques, priming or encapsulation. *Stenotrophomonas rhizophila* SPA-P69 was applied using priming (P69F; P69B), encapsulation (P69KF; P69KB) or priming in combination with *Stenotrophomonas rhizophila* e-p17 (P6917F; P6917B) were used. In total six different samples and two negative controls (CF; CB) were used as pictured in table 1.

Table 1: Overview of the various seed treatments for the field trials

				S. rhizophila e-p17	*S. rhizophila* SPA-P69	
No.	Treatment	MAXIM® XL [mL]	Sacrust M-621 [mL]	Priming	Priming	Encapsulation [CFU/seed]
1	CF	4.2				
2	P69F	4.2			x	
3	P6917F	4.2		x	x	
4	P69KF	4.2				2.78E+10
6	CB		4.2			
7	P69B		4.2		x	
8	P6917B		4.2	x	x	
9	P69KB		4.2			2.78E+10

2.3.2 Qualitative Detection of *S. rhizophila* on the Seed surface

To examine if priming and encapsulation of maize seeds were successful, the establishment of the beneficial bacteria was determined. For this purpose, two times six seeds of the control, the seeds primed with *S. rhizophila* SPA-P69 and *S. rhizophila* e-p17, were transferred in whirl-paks® (Sigma-Aldrich, Vienna, Austria), smashed (the content of the whirlpacks with maize seeds, were transferred into a new whirlpack) with 5 ml (maize seeds) sterile 0.8 % NaCl and mixed. The suspensions were diluted: one part for the direct dilution and diluted 10^{-1} to 10^{-6}, 10 µl dropped on NA. The colonies were counted after 48 h incubation at room temperature.

The roots of the two repetitions of the control, primed with *S. rhizophila* SPA-P69 and *S. rhizophila* e-p17 in the germination pouches were harvested after one and two weeks. The roots were sliced and transferred in a whirl-pak®, smashed and mixed with 5 ml sterile 0.85 % NaCl. The suspensions were divided and prepared for cultivation as described above.

To determine if the re-isolated colonies were similar to the originally introduced bacterial strains *S. rhizophila* SPA-P69 and *S. rhizophila* e-p17, DNA was extracted from purified colonies grown *S. rhizophila* SPA-P69 on NA. BOX patterns resulting a BOX PCR of wild types were compared to the colony pattern using the software program GelComparII® (Applied Math, Kortrijk, Belgium).

2.3.2.1 DNA Extraction Protocol

Colonies were harvested under sterile conditions, suspended in 500 µl lysis-buffer (CTAB 1.4%; NaCl 1 M; Tris 7 mM; EDTA 30 mM; pH 5.5) including washed and sterile glass beads, ribolysed at 4 m s-1 for 20 s and incubated at 65°C for 1 h. 500 µl chloroform/isoamylalcohol (24:1) solution was added, vortexed and centrifuged for 5 min at 17.640 x g for phase separation. The upper phase was transferred in a new 1.5/2.0 ml tube. 500 µl chloroform was added a second time and the upper phase transferred in a new 1.5/2.0 ml tube and centrifuged (5 min at 17,640 x g). The upper phase was transferred in a new 1.5/2.0 ml tube. 1 ml of precipitation buffer (CTAB 0.5 %; NaCl 40 mM; pH 7.5) was added and the samples were incubated and stirred at room temperature for 1 h. The samples were centrifuged at 17,640 x g for 15 min. The supernatant was discarded; the DNA pellet was resuspended with 350 µl of 1.2 M NaCl solution and 500 µl chloroform were added. The samples were agitated shortly and centrifuged for 5 min at 17,640 x g. The upper phase was transferred into a new 1.5/2.0 ml tube, 210 µl of cold isopropanol were added, the samples were agitated carefully and stored

overnight into the freezer at -20 °C. The samples were defrosted for 5 min at room temperature and were centrifuged for 20 min at 17,640 x g. The supernatant was decanted carefully, the samples were put on ice, 200 µl of 80 % ethanol were added and the samples were centrifuged for 5 min at 4°C and 17,640 x g. The pellet was dried, 50 µl of sterile nuclease-free water were added, the DNA was resuspended and its concentration was measured by nanodrop 2000 (Thermo scientific, Wilmington, USA).

2.3.2.2 BOX-PCR and Gel Electrophoresis Protocol

To generate BOX patterns bacterial cultures, the PCR was performed in reactions containing 1 µl of genomic DNA from the DNA extraction protocol above in the presence of 2.5 µl BOXA1R primer 10 µM (5' CTA CGG CAA GGC GAC GCT GAC G 3') and 5 µl Taq & Go. The total reaction volume was 16 µl. The thermocycler program was composed of 35 cycles of denaturation at 94 °C for 1.0 min, primer annealing at 53 °C for 1.0 min, and extension at 65 °C for 8.0 min. 15 µl amplified PCR product with 2 µl loading dye were fractioned in 1.5 % agarose solved in 0.5 x TBE buffer (pH 8.0) for 5 h with 90 V.

2.3.3 Plot Arrangement on the Trial Fields

Each of the three trial fields consisted of 40 plots, that is, four replications per treatment method. The area of the field trials was surrounded by transition zones, consisting of four rows of maize to exclude marginal effects. A plot included four series with in each of which 50 seeds were applied. The arrangement of the different plots after randomization is shown in table 2.

Table 2: Randomized arrangement of the variant plots at the three trial fields

10	8	9	2	3
5	7	6	4	1
1	2	10	4	5
3	9	8	7	6
10	1	2	5	3
4	6	9	7	8
10	9	8	7	6
1	2	3	4	5

2.3.4 Emergence Trap Construction

To monitore the WCR density on field site Mitterdorf, ten emergence traps were installed.

2.3.4.1 Trap Design

The construction was designed similar as published by Rauch *et al*. [74]. The trap consisted of the following components.

- A plastic foil, black on one side, white on the other side, 0.07 mm thick, with a length of 100 cm and a width of 120 cm. In the middle of the foil a circle with a diameter of 25 cm was cut.
- A metal rod with a diameter of 3.0 mm and 90.0 cm length.
- Two transparent plastic mineral water bottles with a volume of 1.5 L. One was cut horizontally near the bottles half, the other was cut horizontally near the bottles floor. The bottles were interlocked with the bottle neck upwards, together stapled with staples and taped with a LUX Gewebeband Universal Silber 50 m x 50 mm (Emil Lux, Wermelskirchen, Germany)
- In the bottle opening of the lower bottle an 18.0 cm long and 5.0 cm wide plastic tube was pasted into.
- Four pieces of 20 cm long metal wire 2 mm thick.
- One cable tie with a length of 35 cm.

The assembly took place as followed. First, three stems were cut at a height of 15 cm above the soil level. The metal rod was placed in middle of the three stems. The foil was placed over the stems with the white side facing outwards, the edges digged into the soil and fixed on the four corners with the metal wires. The bottle was placed upright on the metal rod and the foil was fixed airtight on the bottle with the cable tie. In figure 1 an illustration of a ready installed trap is displayed.

Figure 1: Images of installed emergence traps at field site Mitterdorf

2.3.4.2 Monitoring of Emerging Beetles

In order to detect the exact number of hatching *D. virgifera*, from the 1st of July till the 24th of September at least every 7 days, the beetle's quantities were counted. For the counting the cable harness was opened and the beetles were taken out of the flask over the screw cap and counted.

2.3.5 Parameters to Detect Effects on Maize

2.3.5.1 Determination of Germination Rate

The germination rate in each plot was detected by counting the shoots four to seven weeks after germination of the seeds. Different weather conditions on the three field sites made it unable to sow the seeds on all three sites at the same day. At field side of Mitterdorf an der Raab, maize seeds were sown on 26th of April, at the field of Melk on 29th of April and at Reichersberg on 6th of May. Also, different weather conditions during the germination phase made it necessary to count the germination on different times, but the germination rate of one field site was always determined on the same day.

2.3.5.2 Detection of Lodging

The larvae feed on the roots leads to the so-called goose neck growth and lodging of the maize plants. The harvest of such stocks is difficult and is often only possible with losses. Therefore, lodging is a good parameter indicating the effect of the used bacteria on compactness of the root system. At the Mitterdorf field site, lodging was detected on three dates (20th of July, 5th of August and 23th of September). The sum of lodgings of all four rows of one plot was added up and mean values, based on the average growth rates, were determined from the four repeats of the plots. The determined mean values of the various points in time were then compared.

2.3.5.3 Determination and Calculation of Harvest Yield

One way to determine positive effects of the bacteria on the health of the maize plants is to detect the lodging rate, but a healthier plant should also show a higher yield. That was the reason why, during the detection of the germination rate, the number of plants in row two and three of each plot was normalized to 30 plants each. Thus, for determination of harvest yield only the rows two and three of each plot were considered. The average dry corn weight in kg of the four plots per treatment at each field site were converted in deca tons per hectare and compared.

2.4 Quantitative Determination of *S. rhizophila* SPA-P69 in Rhizosphere Samples

2.4.1 Metagenome Extraction and Processing

In order to ensure that the beneficial bacteria *S. rhizophila* SPA-P69 and *S. rhizophila* e-p17 can be successfully established in the rhizosphere of maize, 80 rhizosphere samples, two per plot from each of the three field experiments, were taken and, in addition, on every field two soil samples were taken. The sample preparation was carried out as follows.

- 5.0 g root sample was put into a plastic bag and was dissolved in 50 ml 0.8 % NaCl
- The plastic bag was mixed in a Bagmixer® for 3.0 min
- The liquid was transferred to a 50 ml tube
- After 10 min settling, two times two ml of supernatant were transferred in 1.5 ml tubes
- The samples were centrifuged for 20 min at 4 °C and 25.402 x g
- The samples were stored at -20 °C

2.4.1.1 DNA Isolation

Humic acids from soil present a problem in the analysis of metagenomes by means of Polymerase chain reactions (PCR). An over-concentration of those in the sample can lead to falsified results or non-functioning PCR. Therefore, the FastDNA® SPIN kit for Soil (MP Biomedicals, California, USA) was used to extract the DNA from soil samples. The extraction was carried out according to the recommended extraction protocol of MP Biomedicals [75] with modifications as described below.

- Add 1.0 ml to the 2.0 ml reaction vessels supplied with the kit with screw cap and ceramic beads

- Add 978 µl Sodium Phosphate Buffer and 122 µl MT Buffer to sample in Lysing Matrix E tube
- Digestion in ribolyzer at step 5.5 for 0.5 min
- Centrifuge at 14,000 x g for 10.0 min to pellet debris
- Transfer supernatant to a clean 2.0 ml microcentrifuge tube
- Add 250 µl PPS (Protein Precipitation Solution) and mix by shaking the tube by hand 10 times
- Centrifuge at 14,000 x g for 5.0 min to pellet precipitate
- Transfer supernatant to a clean 15 ml tube.
- Resuspend Binding Matrix suspension and add 1.0 ml to supernatant
- Invert by hand for 2 min to allow binding of DNA
- Place tube in a rack for 3 min to allow settling of silica matrix
- Remove and discard 500 µl of supernatant being careful to avoid settled binding matrix
- Resuspend Binding Matrix in the remaining amount of supernatant
- Transfer approximately 600 µl of the mixture to a SPIN™ Filter and centrifuge at 14,000 x g for 1 min
- Empty the catch tube and add the remaining mixture to the SPIN™ Filter and centrifuge as before
- Empty the catch tube again
- Add 500 µl prepared SEWS-M and gently resuspend the pellet using the force of the liquid from the pipet tip
- Centrifuge at 14,000 x g for 1 min
- Empty the catch tube and replace
- Without any addition of liquid, centrifuge a second time at 14,000 x g for 2 min to “dry” the matrix of residual wash solution
- Discard the catch tube and replace with a new, clean catch tube
- Air dry the SPIN® Filter for 5 min at room temperature
- Gently resuspend Binding Matrix (above the SPIN filter) in 50-100 µl of DES (DNase/ Pyrogen-Free Water)
- Centrifuge at 14,000 x g for 1 min to bring eluted DNA into the clean catch tube
- Discard the SPIN filter. DNA is now ready for PCR and other downstream applications
- Store at -20 °C for extended periods or at 4 °C for immediate use.

2.4.2 *In silico* Design of a Specific Primer

To design a specific primer for qualitative and quantitative determination of *S. rhizophila* SPA-P69 and *S. rhizophila* e-p17 in total DNA from the soil and rhizosphere samples the Linux based tool TOPSI (PCR Signature Identification) was applied [76]. The program compares large sets of prokaryotic and eukaryotic genome sequences at varying evolutionary distances, but it also provides applications to analyze genome alignments [77]. With the "align" application the genomes of *S. rhizophila* SPA-P69 and e-p17 were compared with other *Stenotrophomonas* genomes (table S1) to identify sequence regions that were unique in SPA-P69 and e-p17 as well as suitable to design specific forward and reverse primers.

The guidelines for the primer development were as described followed [78]:

- length: 18-30 bases
- GC content: 40-60 %
- not more than four bases next to one another (e.g. AAAA)
- melting point (T_m) between 55 and 80 °C
- melting points of both primers should be the same
- at 3'-end there should be one to two G or C
- the primer sequence should be specific for the desired amplification product
- the primer should not produce internal secondary structures like hair pins and they should not hybridise with one another

Of all assembled primer pairs, the ten with the highest calculated conformity were used for further testing. The sequences of the ten primer pair candidates are listed in table 3.

Table 3: Sequences of the 10 primer pairs with the highest calculated conformity

Primer number	Forward primer 5'→3'	Reverse primer 5'→3'	amplicon length
1	TACAACCAAGGGTTCGGCTA	GTGGGGACAACAGCCTTG	185
2	AGCAAACTGCTGGTTTCCAC	TGATGCGAAAATCTCGTTGA	150
5	ACAAGACTACCTGCCGTTGC	TTGAATACATGCAAATCAATGG	112
6	GGGATTGAAATGGATTACGC	TCAATGCAGGTCGAGTTGAG	167
8	CACCTGAAAGAATGTAGGAGTGG	CTCGCTCTTTTCCCTAGTGC	160
9	GGTGTTTGACGTCAGCCTTT	CATTTCGATTGGCATTGTTG	173
11	GAGAGAGGGTGGAGGTCGTT	ATCCGTATGGGAGAACAGCA	210
14	GTGCTCACAGGCTGGTATCC	AATCAAGCCAAGTGCGTCAT	123
15	TGCGGCTGCTATACATTTCA	GCGACCCTTTACATCTCTGG	190
16	TGCAGGCTATAAGAACTGCAT	CGCAACCAATGAAGGAAAT	105

2.4.3 Verification of the Developed Primers

To ensure the specificity of the primers, PCR was performed with DNA extracts from *S. rhizophila* SPA-P69 and *S. rhizophila* e-p17. In addition, different soil samples (one negative control as well as one *S. rhizophila* SPA-P69 rhizosphere sample from the field trials at Melk and Reichersberg in 2015) and different strains (*S. rhizophila* e-p14; *S. rhizophila* e-p19; *S. rhizophila* p64; *S. maltophila* e-a1; *S. maltophila* e-a22; *S. maltophila* e-p3; *S. maltophila* e-p13; *S. maltophila* e-p20) were tested. To gain the DNA extract of the various strains the `quick and dirty´ method was used, which consisted of denaturation of 500 µl ONC at 95 °C for 15 min followed by centrifugation at 14,000 x g for 20 min and cooling at 2 °C until use.

2.4.3.1 PCR and Gel Electrophoresis Protocol

The master mix for the specific PCR, with a final volume of 15 µl, was composed as follows:

- Taq&GO™ Master mix 5X 3.0 µl
- Primer (fwd/rev) 1.35 µl each (final concentration of 0.05-0.9 µM)
- DNA template 1.0 µl
- Nuclease-free water 8.3 µl

The thermocycler program included 30 cycles of denaturation at 95°C for 2.0 min, primer annealing at 55°C for 0.5 min, and extension at 72°C for 5.0 min. 1 µl amplified PCR product with 1 µl loading dye were fractioned in 1.0 % agarose solved in 1X TAE buffer for 1 h with 90 V.

2.4.3.2 Ligation and Transformation

For the transformation of the amplified DNA fragment into a pGEM™-T Vector system (Promega Corporation, Madison, USA) the PCR product was purified using Wizard® SV Gel and PCR Clean-Up System (Promega Corporation, Madison, USA). The purification was eluted with 50 µl Nuclease-free water.

The ligation of the pGEM™-T vector to the PCR product was carried out as follows:

- 2X Ligation puffer 5.5 µl
- pGEM™-T vector 1.0 µl
- Purified PCR product 2.5 µl
- T4 DNA Ligase 1.0 µl
- Nuclease-free water 1.0 µl

The ligation mix was incubated at room temperature for 2 h and stored over night at 4 °C. The pGEM™-T vector was transformed into NEB® 5-alpha competent *E. coli* (New England Biolabs, Massachusetts, USA) according to the following protocol:

- The ligation mix was added to 100 µl competent cells on ice, mixed gently and stored for 15 min on ice
- Heat shock: Put on 42 °C for 1.6 min and move carefully
- 5-7 min incubation on ice
- 1 ml LB-Medium was added and 2 h at 37° incubated
- 50 or 100 µl of the transformation mix were plated on selective LB-Agar with Ampicillin/IPTG/X-Gal plates and incubated over night at 37 °C.

2.4.3.3 Sequencing of Transformed *E. coli* Clones

By means of Sanger sequencing it was checked whether the clones carried the targeted insert. For this purpose, colony PCR was carried out. Colony forming units (CFUs) were picked with a sterile wooden toothpick, put in 50 µl nuclease-free water and incubated at 98 °C for 5 min. The master mix, with a total volume of 20 µl, was composed as follows:

- Taq&GO™ Master mix 5X 4 µl
- Primer (rsp/usp) 1 µl each
- DNA template 1 µl
- Nuclease-free water 13 µl

Primers used for insert detection and colony PCR are displayed in table 4.

Table 4: Sequence of rsp and usp primer used for colony PCR

Primer	*Sequence 5' → 3'*
usp	*GTA AAA CGA CGG CCA GT*
rsp	*CAG GAA ACA GCT ATG ACC*

The thermocycler program included 35 cycles of denaturation at 95 °C for 0.5 min, primer annealing at 55 °C for 0.5 min, and extension at 72 °C for 0.66 min. After 35 cycles, a final extension at 72 °C for 5.0 min was accomplished. 1 µl amplified PCR product with 1 µl loading dye were fractioned in 1.0 % agarose solved in 1X TAE buffer for 1 h with 90 V. The PCR samples were sent to an external laboratory (LGC Genomics GmbH, Berlin, Germany) for sequencing.

2.4.4 Quantitative Determination of DNA in Rhizosphere Samples

With the primers developed *in silico*, the abundances of *S. rhizophila* SPA-P69 and *S. rhizophila* e-p17 were determined qualitatively. To quantify DNA in soil samples, more sensitive real-time quantitative PCR (qPCR) with TaqMan® (Applied Biosystems, Massachusetts, USA) probes was applied. TaqMan™ uses a fluorogenic probe for detection of PCR product amplified during the PCR. The mechanism of action extends as follows [79]:

- Fluorogenic-labelled probes use the 5'-3' exonuclease activity of Taq-polymerase
- The probe is composed of a fluorescent reporter dye on the 5' and a quencher on the 3' end, by the relative proximity of the two (if the probe remains intact), the fluorescence is not detectable.
- The probe anneals between primer sites of the target sequence and is cleaved because of the 5'exonuclease activity of Taq-polymerase during extension.
- This cleavage separates the reporter dye from the quencher; because of that the fluorescence signal increases and removes the probe from the target strand.
- With each cycle additional reporter dye molecules are cleaved from the target probes and as a result the fluorescence intensity increases proportional to the amount of amplicon produced.

2.4.4.1 TaqMan® Probe Development

During the process of designing of specific primer candidates, specific fluorescence probes were designed additionally. Because of the high cost of the fluorescence probes, only two probes of the most promising primer candidates were tested. The guidelines for probe development were as described followed [78]:

- length: 20-30 bases
- GC content: 30-80 %
- not more than three bases next to one another (e.g. AAA)
- at 3'-end there should be no G
- melting point (T_m) between 68 and 70 °C; T_m should be 5 to 10 °C higher than the primer T_m
- The 5'end of the probe should be localized near the 3'end of the forward primer
- No complementarity between probe and primers
- the probe should not produce internal secondary structures at the target sequence.

The probes were developed, using the amplicons of the developed primer candidates with the highest conformity, according to the criteria above, are listed in table 5.

Table 5: Probe sequences developed for the qPCR

Sample number	Sequence 5'→3'
1	CGTGGACTCGGCGACTGCTG
2	ATCGGCCAGCGAAGGTGGTG*
5	CATTGCCAAGAGCAGCGCCA
6	TTCCCGGTCAATCGCCCCTT
8	CAGGGAAGCAAGCGCACCGT*
9	ATGTAGATCCTGCGCGCCGC
11	TACAGGCCCACGCTCCGTCC
14	TGTCCGCAGGAGGTTCACGG
15	CCGACGAGATGGATCGCCGT
16	AGGCGGAGTGGGGACGGAAA

*: remaining candidates for quantification

The following approach was used to perform the qPCR (total volume 10 µl):

- TaqMan® Environmental Master Mix 2.0 5 µl
- Primer (fwd/rev) 0.05 µl each
- Probe 0.2 µl
- Sample 0.5 µl (in different concentrations)
- Nuclease-free water 4.2 µl

The components were pipetted into vials and subsequently DNA content was quantified using Corbett RotorGene 6000 (Corbett Life Sciences, NSW, Australia). The temperature program for the qPCR included denaturation at 95 °C for 10.0 min, 45 cycles of annealing at 95 °C for 0.4 min and extension at 60 °C for 1.0 min.

2.4.4.2 Relative Quantification with Standard Curve Development

An internal control is required for relative quantification. This control is accomplished by a calibration line. The calibration curve is obtained using pure culture DNA extracts from *S. rhizophila* SPA-P69 in various concentrations. Because contaminants, such as humic acids, can be present in the samples extracted in 2.4.1, which can influence the qPCR results, DNA free soil extract was added to the pure DNA extracts, in the ratio 1:1. DNA was removed from these soil samples using DNase I (Sigma-Aldrich®, St. Louis, USA), as described below [80]:

- Add 8 µl extracted soil sample to 1 µl 10x Reaction Buffer and 1 µl DNase I
- Mix gently and incubate for 15 min at room temperature
- Add 1 µl Stop Solution
- Heat at 70 °C for 10 min
- Chill on ice

2.5 *Ad planta* Detection of Fluorescence Labeled *S. rhizophila* e-p17

To study the ability of *S. rhizophila* e-p17 to colonize the surface of maize roots, *ad planta* detection was performed. Furthermore, differences of colonization when *S. rhizophila* e-p17 was applied using priming or encapsulation were detected. Seeds were primed with two different *S. rhizophila* e-p17 mutants. They were labeled with green fluorescents protein (GFP) or *Discosoma* sp. red fluorescent protein (DsRed). For the labeling of *S. rhizophila* e-p17, the strain was transformed with vector pIN29 carrying the GFP gene or the vector pBAH8 with the DsRed gene. The transformed mutants were used from the cryo stocks. GFP labelled strain were plated on NA with gentamycin, DsRed labelled strains were plated on NA with trimethoprim and incubated at 30 °C. Five days after plating, the expression of fluorescence was detected using The ChemiDoc™ MP (Bio-Rad Laboratories, California, USA).

2.5.1 CLSM Sample Preparation

Maize seeds (breed: LG32.58) were primed for 4 h under constant movement. The 1:50 diluted ONC (30°C, agitation) consisted of NBII with gentamycin or trimethoprim and the GFP/DsRed labelled strains. The amount of liquid was chosen so that the seeds were covered with liquid. During the movement, they partially protruded from the liquid. Afterwards, the seeds were dried and put into germination pouches, filled with 20 ml tab water. The pouches were incubated in a clean and transparent box with a bottle filled with 200 ml sterile dH_20 without lid for humidity maintenance (16/8 h day/night cycle, 500 $mmolm^2s^{-1}$, 60/80 % relative humidity). Besides priming, the two mutants were also applied to the seeds by means of encapsulation. In addition, both treatment methods were applied on seeds. Roots of maize were examined four, six and eight days after incubation on a Leica TCS SPE confocal scanning microscope (Leica Microsystems GmbH, Wetzler, Germany) and the results of both treatment methods were compared.

3 Results

3.1 Field Trials

3.1.1 Re-isolation of *S. rhizophila* From Seeds

To verify that maize seeds were successfully inoculated for the field trial, *S. rhizophila* was re-isolated from seeds. All of the 15 re-isolated colonies showed similarity to the morphological appearance of the original strain. Based on the Pearson correlation of their BOX patterns, ten out of fifteen re-isolated colonies showed a likeness of more than 90 %, six out of these colonies showed a match higher than 96 % and one showed an equality of 98 % to control samples of *S. rhizophila* SPA-P69.The dendrogram of BOX patterns of reisolated colonies is displayed in figure 2.

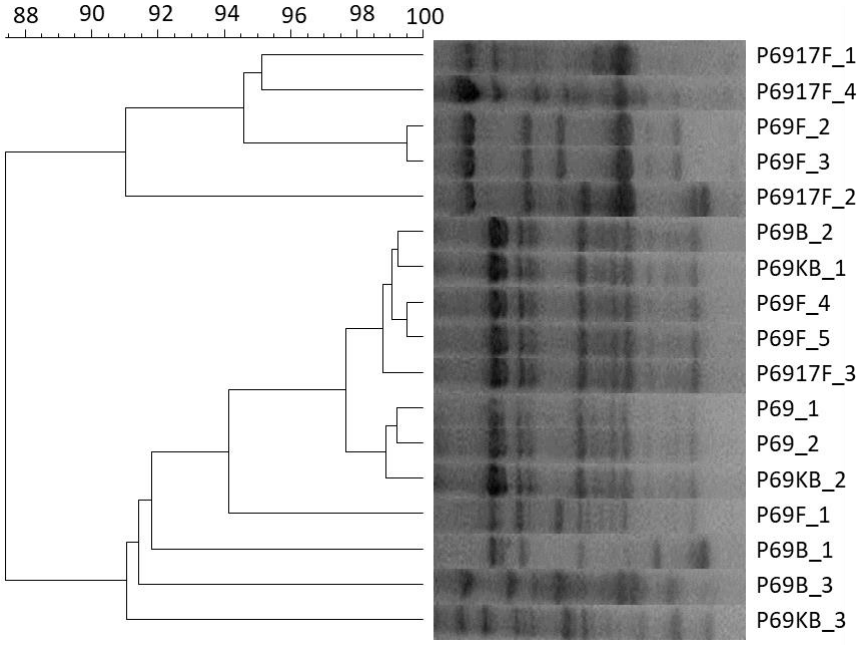


Figure 2: Unweighted pair group method with arithmetic mean (UPGMA) dendrogram of BOX patterns of re-isolated colonies using Pearson correlation similarity coefficient in percent. Abbreviations: P69F: *S. rhizophila* SPA-P69 applied by priming plus MAXIM® treatment; P6917F: *S. rhizophila* SPA-P69 and e-p17 applied by priming plus MAXIM® treatment; P69B: *S. rhizophila* SPA-P69 applied by priming; P69KB: *S. rhizophila* SPA-P69 applied by encapsulation; P69: Control sample *S. rhizophila* SPA-P69; Number 1 to 5 are sample numbers

It could be stated that only six of the fifteen samples are *S. rhizophila* SPA-P69 or *S. rhizophila* e-p17 with high probability (higher than 95 % similarity). Unfortunately, it was not possible to re-isolate colonies from all treatments, as some microorganisms originated in the seed microbiota overgrow, *S. rhizophila* SPA-P69 or *S. rhizophila* e-p17, which grows in comparison relatively slowly. In general, *S. rhizophila* SPA-P69 and *S. rhizophila* e-p17 were successfully detected for both treatment methods. Priming and encapsulation are valuable application techniques and field trials with the treated seeds were able to be carried out.

3.1.2 Monitoring of Emerging Beetles

With the emergence traps, emerging WCR imagos were successfully caught. With emerging beetles there must be larvae in the soil, so that it can be assumed that the grub on maize roots takes place in the field of Mitterdorf. The total amount of WCR imago caught in each trap is displayed in figure 3.

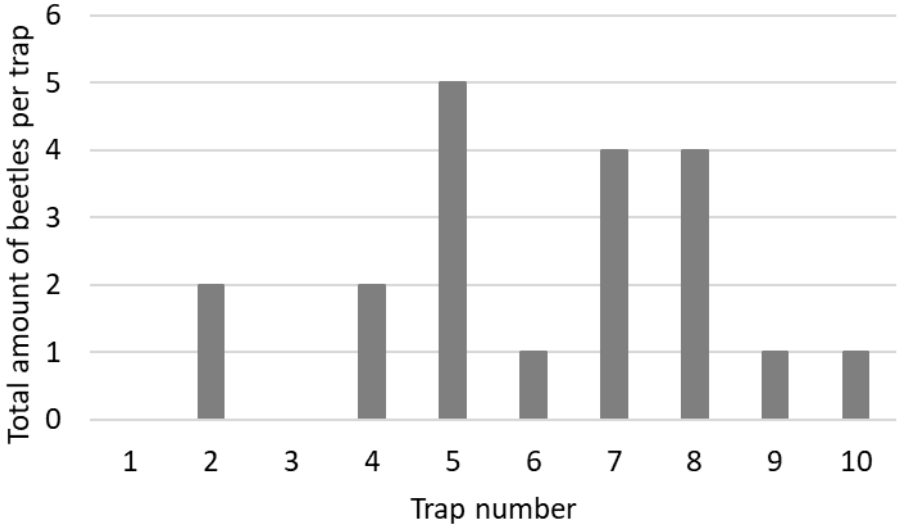
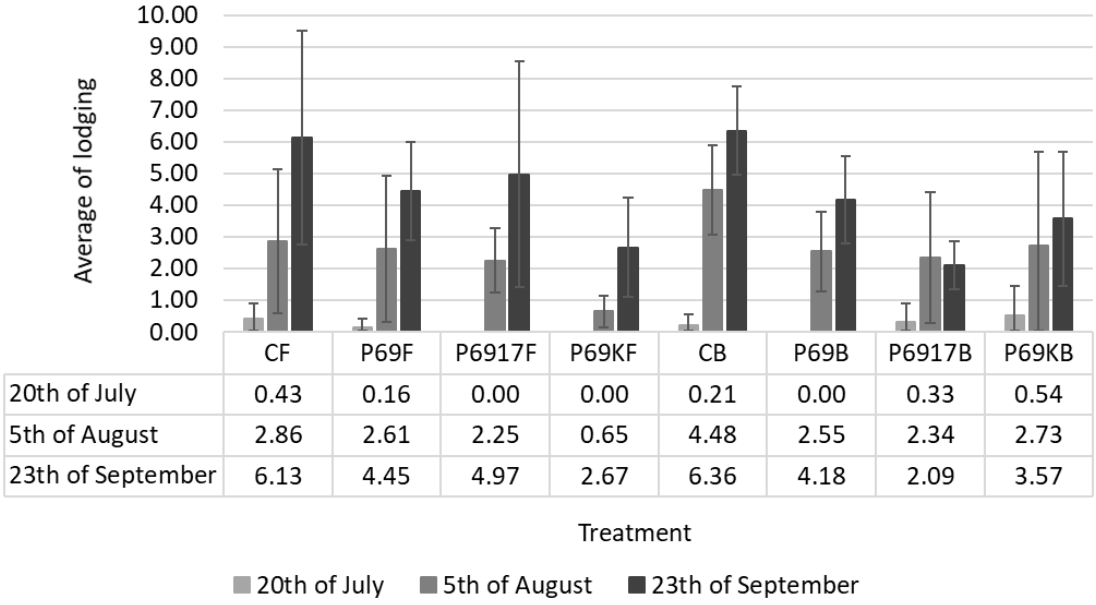


Figure 3: Total number of beetles per trap for traps number 1 to 10 from field site of Mitterdorf

Total amount of trapped beetles differed between zero and five beetles per trap. In trap numbers 1 and 3 the lowest amount, zero of *D. virgifera* beetles were caught. In trap number 5 the highest amount with five beetles was caught. In total, density of caught beetles was very low, compared to densities determined by the Agency for Health and Food Security GmbH(AGES), caught with pheromone traps at the same time span in 2016 [81]. The beetle distribution over the whole field was even. Because the number of beetles was so low, no meaningful statistical analysis could be carried out.

3.1.3 Monitoring of Lodging on Field Site Mitterdorf

The lodging of plants was determined at three times at field site of Mitterdorf, the determined mean values are illustrated in figure 4. It was successfully detected that lodging takes place on the field site.



	CF	P69F	P6917F	P69KF	CB	P69B	P6917B	P69KB
20th of July	0.43	0.16	0.00	0.00	0.21	0.00	0.33	0.54
5th of August	2.86	2.61	2.25	0.65	4.48	2.55	2.34	2.73
23th of September	6.13	4.45	4.97	2.67	6.36	4.18	2.09	3.57

Figure 4: Average number of lodged maize plants at 20th of July, 5th of August and 23th of September. Seeds were treated with *S. rhizophila* SPA-P69, e-p17 and/or MAXIM® or not at all. Abbreviations: CF: Control plus MAXIM® treatment; P69F: *S. rhizophila* SPA-P69 applied by priming plus MAXIM® treatment; P6917F: *S. rhizophila* SPA-P69 and e-p17 applied by priming plus MAXIM® treatment; P69KF: *S. rhizophila* SPA-P69 applied by encapsulation plus MAXIM® treatment; CB: Untreated control; P69B: *S. rhizophila* SPA-P69 applied by priming; P6917B: *S. rhizophila* SPA-P69 and e-p17 applied by priming; P69KB: *S. rhizophila* SPA-P69 applied by encapsulation

The average of lodging rose over the timespan from 20th of July to 23th of September. The control treated with MAXIM® showed to have no effect on lodging compared to the control with no fungicide application. Comparing the mean values of the treated seeds to the control, tendencies of less lodging of plants with seeds treated with *S. rhizophila* SPA-P69 drenching or *S. rhizophila* e-p17 drenching, as well as with encapsulated could be shown. However, no statistically significant differences could be observed.

3.1.4 Germination Rate of Field Sites Mitterdorf, Melk and Reichersberg

The average of germination of 200 seeds planted in each plot at the field sites Reichersberg, Melk and Mitterdorf are displayed in figure 5. Comparingthe variants to the respective controls, only minimal differences in germination rate were detected. It can be concluded that both *S. rhizophila* SPA-P69 and *S. rhizophila* e-p17 show no positive effect regarding an increase in the germination rate. However, negative effects like a decrease in the germination rate were not detected. Moreover, it cannot be concluded that the bacterial treatment is unable to compensate the antifungal impact achieved by the treatment with MAXIM®. Variants with no antifungal treatment showed a lower germination rate than variants treated with MAXIM®.

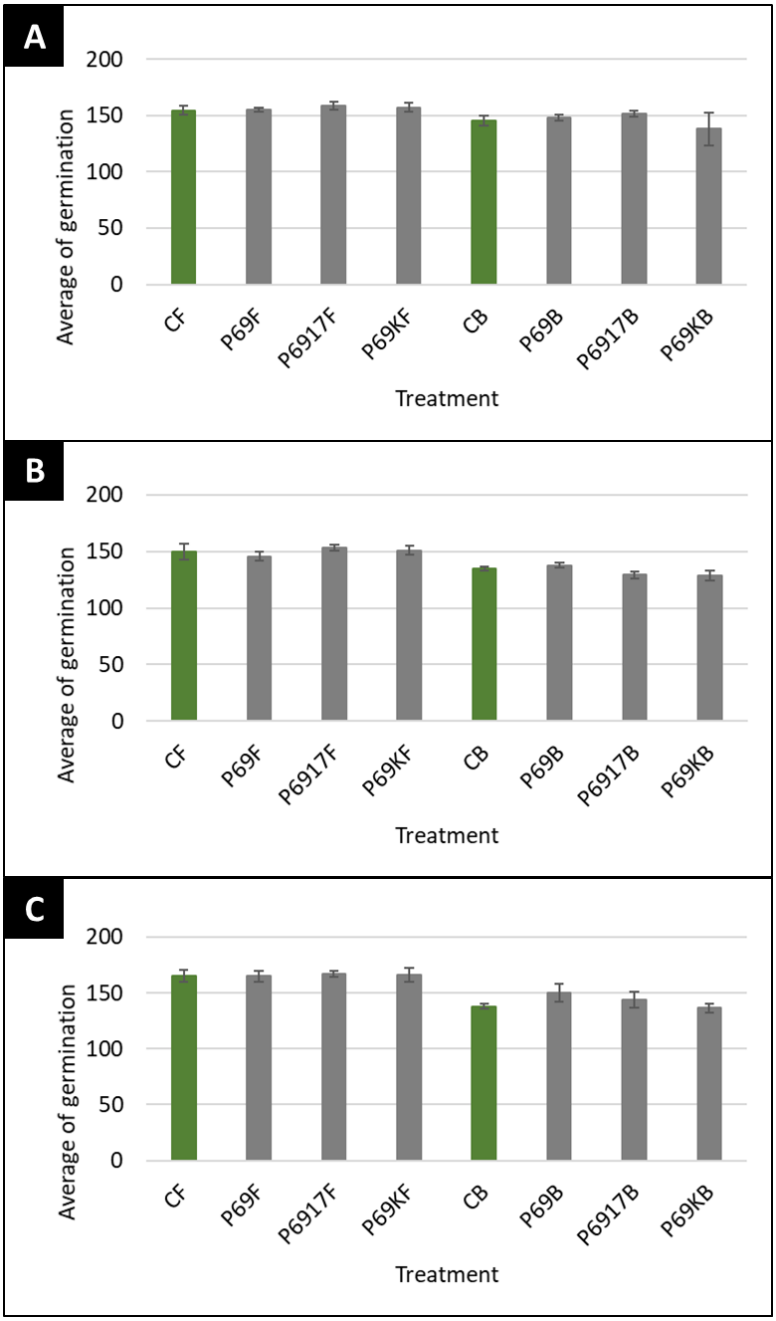
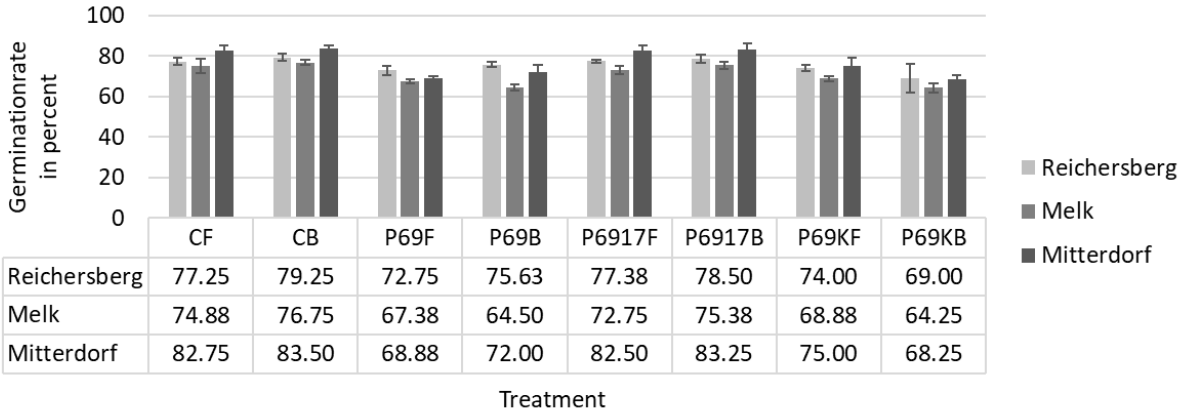


Figure 5: Average germination numbers of maize plants per plot. Per each plot 200 seeds were sown. Seeds were treated with *S. rhizophila* SPA-P69, e-p17 and/or MAXIM® or not at all. A: Field site Reichersberg; B: Field site Melk; C: Field site Mitterdorf. Abbreviations: CF: Control plus MAXIM® treatment; P69F: *S. rhizophila* SPA-P69 applied by priming plus MAXIM® treatment; P6917F: *S. rhizophila* SPA-P69 and e-p17 applied by priming plus MAXIM® treatment; P69KF: *S. rhizophila* SPA-P69 applied by encapsulation plus MAXIM® treatment; CB: Untreated control; P69B: *S. rhizophila* SPA-P69 applied by priming; P6917B: *S. rhizophila* SPA-P69 and e-p17 applied by priming; P69KB: *S. rhizophila* SPA-P69 applied by encapsulation

Germination rates in percent are displayed in figure 6. Both the controls and the variants of the respective fields showed a higher germination rate when treated with MAXIM® compared to those treated with no fungicidal treatment. The greatest differences were detected at the field site Mitterdorf, with a difference of the variants from 7.5 % (comparison of variant P69F to P69B) to 15.00 % (comparison of variant P69KF to P69KB). Additionally, the respective controls differed around 13.88 % (comparison of variant CF to CB). It can therefore be assumed that the application of no fungicide lowers the germination to a small extent. However, the decrease in germination is not significant.



	CF	CB	P69F	P69B	P6917F	P6917B	P69KF	P69KB
Reichersberg	77.25	79.25	72.75	75.63	77.38	78.50	74.00	69.00
Melk	74.88	76.75	67.38	64.50	72.75	75.38	68.88	64.25
Mitterdorf	82.75	83.50	68.88	72.00	82.50	83.25	75.00	68.25

Figure 6: Comparison of germination rate in percent from field sites Reichersberg, Melk and Mitterdorf. Seeds were treated with *S. rhizophila* SPA-P69, e-p17 and/or MAXIM® or not at all. Abbreviations: CF: Control plus MAXIM® treatment; P69F: *S. rhizophila* SPA-P69 applied by priming plus MAXIM® treatment; P6917F: *S. rhizophila* SPA-P69 and e-p17 applied by priming plus MAXIM® treatment; P69KF: *S. rhizophila* SPA-P69 applied by encapsulation plus MAXIM® treatment; CB: Untreated control; P69B: *S. rhizophila* SPA-P69 applied by priming; P6917B: *S. rhizophila* SPA-P69 and e-p17 applied by priming; P69KB: *S. rhizophila* SPA-P69 applied by encapsulation

3.1.5 Yield Comparison of Field Sites Mitterdorf, Melk and Reichersberg

The mean value of yield in deca tonnes per hectare from the field sites Reichersberg, Melk and Mitterdorf are displayed in figure 7. The lowest yield amount compared to the other two field sites was detected on field site of Reichersberg with 99.6 and 95.0 deca tonnes per hectare. Plots on field sites of Melk and Mitterdorf showed similar yield, when treated with MAXIM®. When treated with no fungicide the mean value of yield was higher on field site of Mitterdorf with 132.3 dt/ha, than in Melk with 124.1 dt/ha. The additional treatment with *S. rhizophila* SPA-P69, had no effect on amount of yield, but the yield amount was higher compared to the respective controls. Seeds primed with a mixture consisting of *S. rhizophila* SPA-P69 and *S. rhizophila* e-p17 or *S. rhizophila* SPA-P69 encapsulation had a higher yield on

field sites of Melk and Mitterdorf when not treated with fungicide. On the field site Reichersberg the bacterial treatments with MAXIM® showed a higher yield. In summary, it can be assumed that bacterial treatment with *S. rhizophila* SPA-P69 or *S. rhizophila* e-p17 with no additional treatment enable a higher yield compared to additional treatment with MAXIM®.

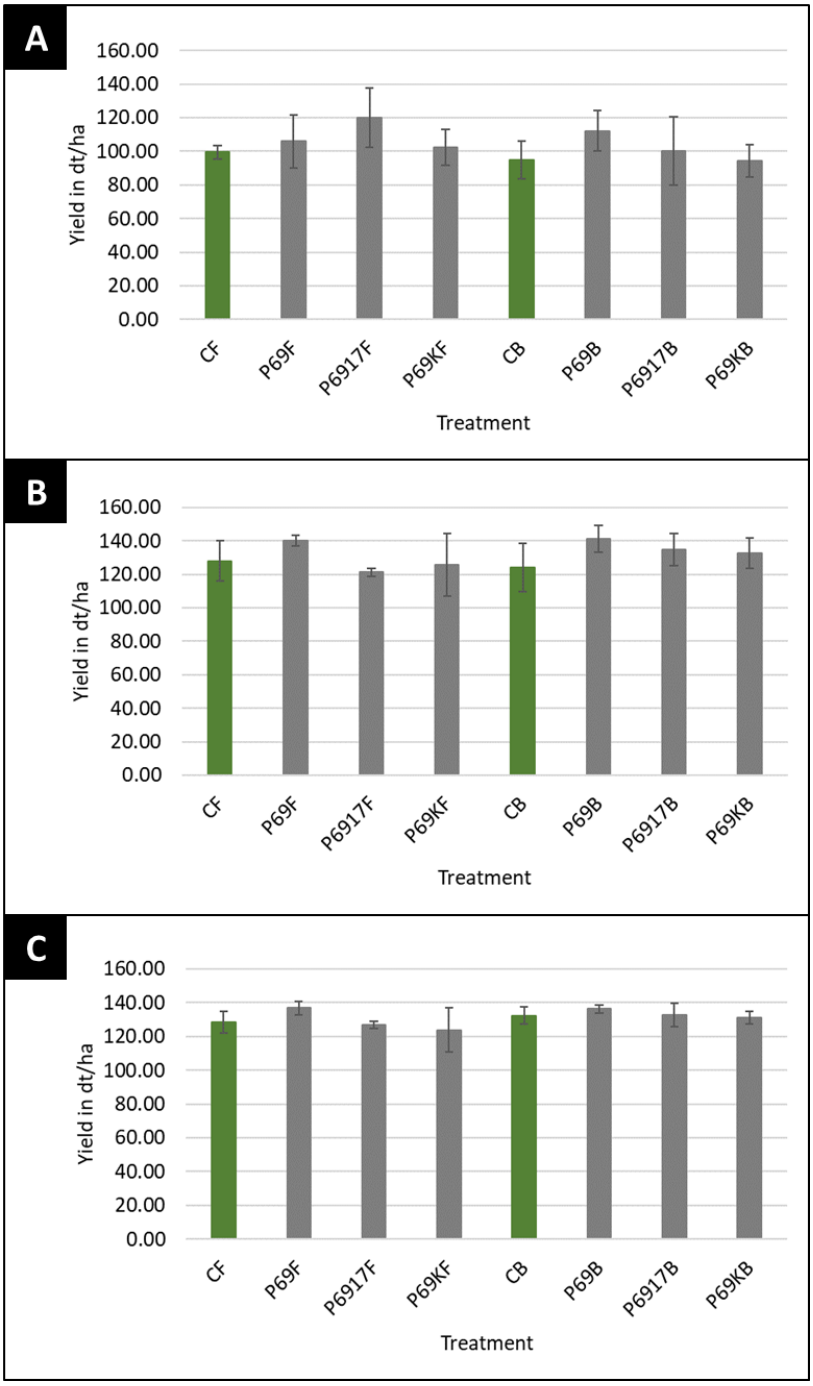


Figure 7: Mean value of yield in deca tonnes per hectare from field sites carried out in 2016. Seeds were treated with *S. rhizophila* SPA-P69, e-p17 and/or MAXIM® or not at all. A: Field site Reichersberg; B: Field site Melk; C: Field site Mitterdorf. Abbreviations: CF: Control plus MAXIM® treatment; P69F: *S. rhizophila* SPA-P69 applied by priming plus MAXIM® treatment; P6917F: *S. rhizophila* SPA-P69 and e-p17 applied by priming plus MAXIM® treatment; P69KF: *S. rhizophila* SPA-P69 applied by encapsulation plus MAXIM® treatment; CB: Untreated control; P69B: *S. rhizophila* SPA-P69 applied by priming; P6917B: *S. rhizophila* SPA-P69 and e-p17 applied by priming; P69KB: *S. rhizophila* SPA-P69 applied by encapsulation

To compare mean values of yield in deca tonnes per hectare, the results of plots of each field site with no bacterial treatment was set to 100 percent, so that harvest increase or decrease could be detected. In figure 8, yield in percent to the control (CF) of the samples from Reichersberg, Melk and Mitterdorf, of seeds treated with MAXIM®, are displayed. Seeds primed with a *S. rhizophila* SPA-P69 and *S. rhizophila* e-p17 mixture planted in Reichersberg allowed for the highest yield, with a 20.71 % higher yield compared to the respective control. In Melk and Mitterdorf, however, the yield of seeds primed with a *S. rhizophila* SPA-P69 and *S. rhizophila* e-p17 mixture was lower compared to the respective controls. Seeds primed with *S. rhizophila* SPA-P69 were the only ones with a higher yield in all fields, with a yield plus between 6.41 % and 9.41 % on the fields of Reichersberg, Melk and Mitterdorf, compared to the respective controls. All treatment methods showed a higher yield compared to the control at field of Reichersberg, with a yield plus between 2.80 % and 20.71 %. Although higher yields compared to the untreated control were measured with individual methods of treatment, no statistically significant (significance level $\alpha = 0.05$; p-value: 0.442 (Reichersberg), 0.489 (Melk), 0.371 (Mitterdorf)) differences were found.

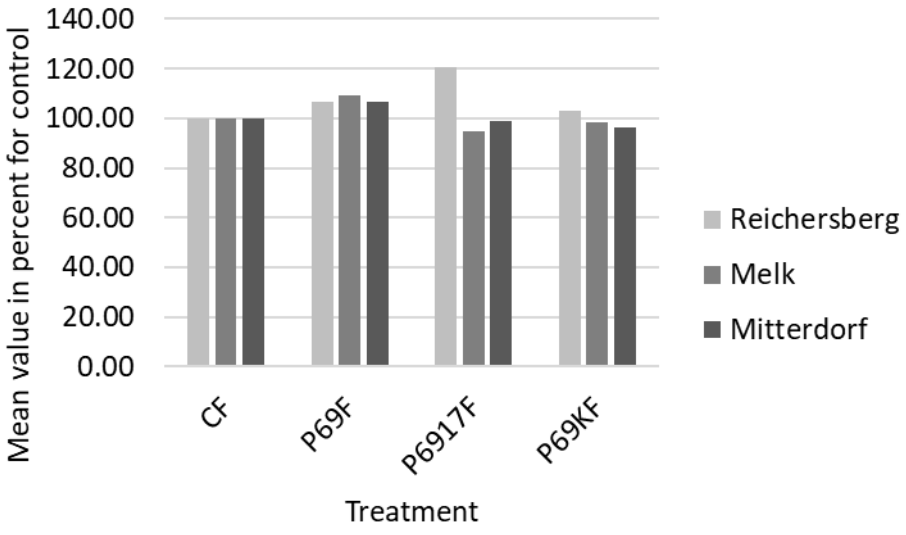


Figure 8: Comparison of the averages of crop yields (measured in deca tonnes per hectare) in percent for the control from Reichersberg, Melk and Mitterdorf in 2016. All seeds were treated with MAXIM®, the control (CF) was accepted as 100 percent. Seeds were treated with *S. rhizophila* SPA-P69, e-p17 or not at all. Abbreviations: CF: Control plus MAXIM® treatment; P69F: *S. rhizophila* SPA-P69 applied by priming plus MAXIM® treatment; P6917F: *S. rhizophila* SPA-P69 and e-p17 applied by priming plus MAXIM® treatment; P69KF: *S. rhizophila* SPA-P69 applied by encapsulation plus MAXIM® treatment

In figure 9, yield in percent to the control (CB) of the samples from Reichersberg, Melk and Mitterdorf, with no additional fungicide treatment are displayed. Seeds primed with *S. rhizophila* SPA-P69 showed a higher yield in comparison to seeds with a *S. rhizophila* SPA-P69 and *S. rhizophila* e-p17 priming or *S. rhizophila* SPA-P69 encapsulation, at all three field sites. The amount of yield was 3.1 to 18.1 percent higher, compared to the respective controls. Only on the field site of Melk all three treatment methods showed a higher yield than the respective controls with a yield plus between 6.9 to 13.8 %.

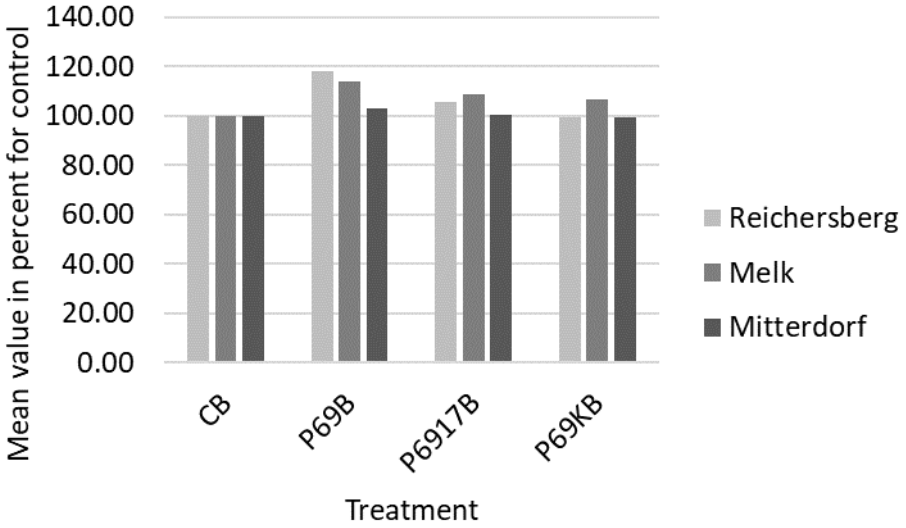


Figure 9: Comparison of the averages of crop yields (measured in deca tonnes per hectare) in percent for the control from Reichersberg, Melk and Mitterdorf in 2016. Seeds were treated with *S. rhizophila* SPA-P69, e-p17 or not at all. The control (CB) was accepted as 100 percent. Abbreviations: CB: Untreated control; P69B: *S. rhizophila* SPA-P69 applied by priming; P6917B: *S. rhizophila* SPA-P69 and e-p17 applied by priming; P69KB: *S. rhizophila* SPA-P69 applied by encapsulation

3.2 Quantitative Determination of *S. rhizophila* SPA-P69 and e-p17

3.2.1 Verification of the Primers Developed *in silico*

Testing on equality of the primer sequences of the primer candidates to sequences of various *Stenotrophomonas* genomes, which are listed in table S1, showed no compliance.

In a gel electrophoresis, amplified DNA sequences from the primer candidates, listed in table 3, were tested. The primer candidates where tested in terms of their functionality, to amplificate the desired DNA sequence. Some of the candidates amplified the wanted DNA sequence. Seven of the primer candidates, number 1, 2, 5, 8, 14, 15 and 16 were picked for further testing.

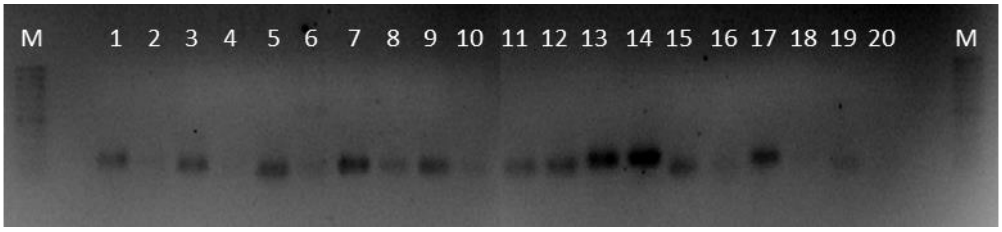


Figure 10: Gel electrophoresis, testing of the primer candidates, in terms of their functionality to amplify the wanted DNA sequence, with DNA extracts from *S. rhizophila* SPA-P69 (1,3,5,7,9,11,13,15,17,19) and *S. rhizophila* e-p17(2,4,6,8,10,12,14,16,18,20). Primer candidate 1(1,2), 2(3,4), 5(5,6), 6(7,8), 8(9,10), 9(11,12), 11(13,14), 14(15,16), 15(17,18), 16(19,20), Gene Ruler™ 500 bp DNA Ladder Plus(M)

The results of testing of the seven remaining primer candidates in terms of sensitivity to DNA from samples of different *S. rhizophila* and *S. maltophila* strains are displayed in table 6.

Table 6: Overview of testing of different *S. rhizophila* and *S. maltophila* strains to check the sensitivity of the developed primers

	S. rhizophila				*S. maltophila*			
Primer No.	e-p14	e-p19	P64	e-p20	e-p3	e-p13	e-a1	e-a22
1	x	w						
2	x							
5	x	w	w	w	w			
8	x							
14	x	w	x	w				
15	x							
16	x							

PCR product with a length between 100 and 200 bp (x), PCR product longer than 200 bp (w)

All remaining primer candidates amplified a PCR product between 100 and 200 bp with DNA extracts from *S. rhizophila* e-p14, primer 14 additionally amplified a PCR between 100 and 200 bp with DNA extracts from *S. rhizophila* P64. Because PCR products with an unwanted base pair size can affect qPCR results, primer candidates number 5 and 14, which amplified PCR products, in more than on sample, longer 200 bp, were not chosen further testing.

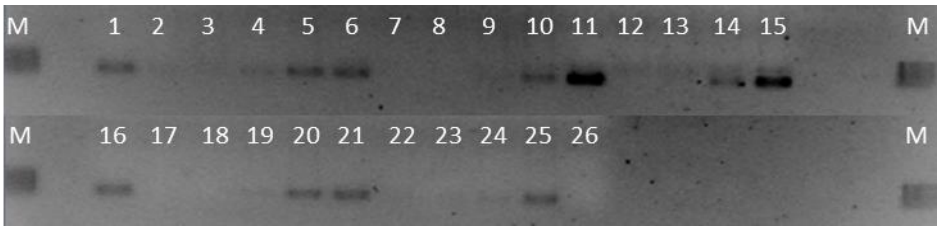


Figure 11: Gel electrophoresis, PCR products amplified of primer 1(1-5), 2(6-10), 8(11-15), 15(16-20), 16(21-25), from rhizosphere samples from Reichersberg (2,7,12,17,22) or Melk (3,8,13,18,23), rhizosphere samples of maize seeds primed with *S. rhizophila* SPA-P69 from Reichersberg (4,9,14,19,24) or Melk (5,10,15,20,25), from a negative control with nuclease-free water(26), Gene Ruler™ 500 bp DNA Ladder Plus(M)

With remaining five primer candidates number 1, 2, 8, 15 and 16, it was tested if it is possible to detect *S. rhizophila* SPA-P69 in rhizosphere samples taken in 2015 in Reichersberg and Melk. Illustrated in figure 11, all five primer candidates amplified the DNA sequence from rhizosphere of maize seeds primed with *S. rhizophila* SPA-P69 of Melk. Also, no PCR product was amplified by the five primer candidates with rhizosphere samples of maize seed with no bacterial treatment of Melk as well as Reichersberg. Only two of the five primer candidates, number 1 and 8, amplified PCR products with matching base pair length in rhizosphere samples of maize seeds primed with *S. rhizophila* SPA-P69 of Reichersberg. Finally, primer candidate number 8 was chosen to be taken for quantification of *S. rhizophila* SPA-P69 and *S. rhizophila* e-p17 using qPCR.

To be completely sure that primer number 8 amplified the wanted DNA sequence, the DNA fragment was transformed into a pGEM™-T Vector fragment which was afterwards transformed into NEB® 5-alpha competent *E. coli*. The amplicon sequence, which primer number 8 amplifies is written down in table 7.

Table 7: Amplicon, amplified by primer number 8

Primer number	Amplicon sequence 5'→3'
8	CACCTGAAAGAATGTAGGAGTGGTAAGCACCGATGCGATCCA GCGCAGAATCCGTAGGCAATTCCCCTTCCGGGCGCGCTTTTG TCGCTTCCCAGGGAAGCAAGCGCACCGTCCGCTTTTCACTCA GCATGTAGAGGTAGGCACTAGGGAAAAGAGCGAG

The DNA insert transformed into *E. coli* was sequenced, using colony PCR products from the rsp/usp primers displayed in table 4. Four CFUs were sequenced, the sequencing results are displayed in table 8. Primer 8 successfully amplifies the wanted DNA sequence.

Table 8: Sequencing results of four colony PCR products

Sample	Sequence 5'→3'
B2-33740	GACGTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCGA TTCACCTGAAAGAATGTAGGAGTGGTAAGCACCGATGCGATCCA GCGCAGAATCCGTAGGCAATTCCCCTTCCGGGCGCGCTTTTGTC GCTTCCCAGGGAAGCAAGCGCACCGTCCGCTTTTCACTCAGCAT GTAGAGGTAGGCACTAGGGAAAAGAGCAGAATCACTAGTGAATT CGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCG TTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCT TGGCGTAATCATGGTCATACTGGTTTTCCT
B2-33741	GAAACCAGCTATGACCATGATTACGCCAAGCTATTTAGGTGACA CTATAGAATACTCAAGCTATGCATCCAACGCGTTGGGAGCTCTC CCATATGGTCGACCTGCAGGCGGCCGCGAATTCACTAGTGATTC ACCTGAAAGAATGTAGGAGTGGTAAGCACCGATGCGATCCAGCG CAGAATCCGTAGGCAATTCCCCTTCCGGGCGCGCTTTTGTCGCT TCCCAGGGAAGCAAGCGCACCGTCCGCTTTTCACTCAGCATGTA GAGGTAGGCACTAGGGAAAAGAGCGAGAATCGAATTCCCGCGGC CGCCATGGCGGCCGGGAGCATGCGACGTC
B2-33742	TGACCATGATTACGCCAAGCTATTTAGGTGACACTATAGAATAC TCAAGCTATGCATCCAACGCGTTGGGAGCTCTCCCATATGGTCG ACCTGCAGGCGGCCGCGAATTCACTAGTGATTCACCTGAAAGAA TGTAGGAGTGGTAAGCACCGATGCGATCCAGCGCAGAATCCGTA GGCAATTCCCCTTCCGGGCGCGCTTTTGTCGCTTCCCAGGGAAG CAAGCGCACCGTCCGCTTTTCACTCAGCATGTAGAGGTAGGCAC TAGGGAAAAGAGCGAGAATCGAATTCCCGCGGCCGCCATGGCGG CCGGGAGCATGCGACGTC
B2-33743	TGACCATGATTACGCCAAGCTATTTAGGTGACACTATAGAATAC TCAAGCTATGCATCCAACGCGTTGGGAGCTCTCCCATATGGTCG ACCTGCAGGCGGCCGCGAATTCACTAGTGATTCACCTGAAAGAA TGTAGGAGTGGTAAGCACCGATGCGATCCAGCGCAGAATCCGTA GGCAATTCCCCTTCCGGGCGCGCTTTTGTCGCTTCCCAGGGAAG CAAGCGCACCGTCCGCTTTTCACTCAGCATGTAGAGGTAGGCAC TAGGGAAAAGAGCGAGAATCGAATTCCCGCGGCCGCCATGGCGG CCGGGAGCATGCGA

Highlighted in yellow: amplicon amplified by primer number 8

3.2.2 Quantitative Determination of DNA in Soil Samples with qPCR

To distinguish to quantity DNA fragments per ten nanogram DNA in one microliter sample, the molecular weight of the DNA insert amplified by primer 8 plus rsp/usp primer was calculated (table 9).

Table 9 Molecular weight of DNA insert amplified by primer 8 plus rsp/usp primer

DNA insert + rsp/usp [g/mol]
120534.81

DNA was prepared in different concentrations to create the calibration line. The DNA concentrations are displayed in table 10.

Table 10 Overview of the DNA quantities used to create the calibration line

DNA concentration [ng]	Log_{10} of DNA concentration
$2*10^{-1}$	-0.69
$2*10^{-2}$	-1.69
$2*10^{-3}$	-2.69
$2*10^{-4}$	-3.69
$2*10^{-5}$	-4.69
$2*10^{-6}$	-5.69
$2*10^{-7}$	-6.69

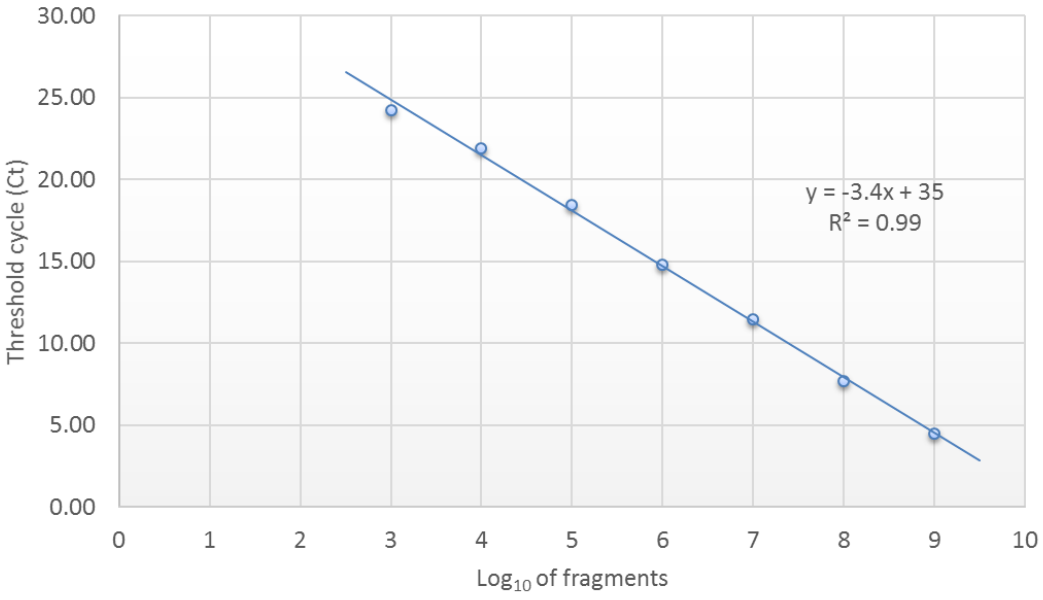


Figure 12: Correlation between threshold cycle and Logarithm of fragments

For the linear range of the correlation between threshold cycle and logarithm of fragments, illustrated in figure 12, an equation is generated:

$$Log_{10}\, fragments = (Ct - 35)/(-3.4)$$

With the primer number 8 and the developed probe, *S. rhizophila* SPA-P69 was successfully detected in soil probes from 2015. Quantities of *S. rhizophila* SPA-P69, illustrated in figure 13, from rhizosphere samples of field trials in 2015 in Reichersberg and Melk, were detected using qPCR. Log_{10} values of copies/reaction were consistently higher in samples from Melk in comparison to Reichersberg. The results show that in the rhizosphere samples of seeds treated with *S. rhizophila* SPA-P69 quantities between 3.0-3.5 (samples from Reichersberg) and between 5.0-5.6 (samples from Melk) Log_{10} copies/reaction were detected. The values are therefore in the linear range of the correlation in figure 12. All negative control samples, the seeds with no bacterial treatment as well as the soil samples from Reichersberg and Melk showed values lower 2.5 Log_{10} copies/reaction, except the samples of negative controls of seeds without fungicide treatment from Reichersberg. The majority of the values of the different control samples were not in the linear range of the correlation and thus below the detection limit of less than 3.0 Log_{10} copies/reaction.

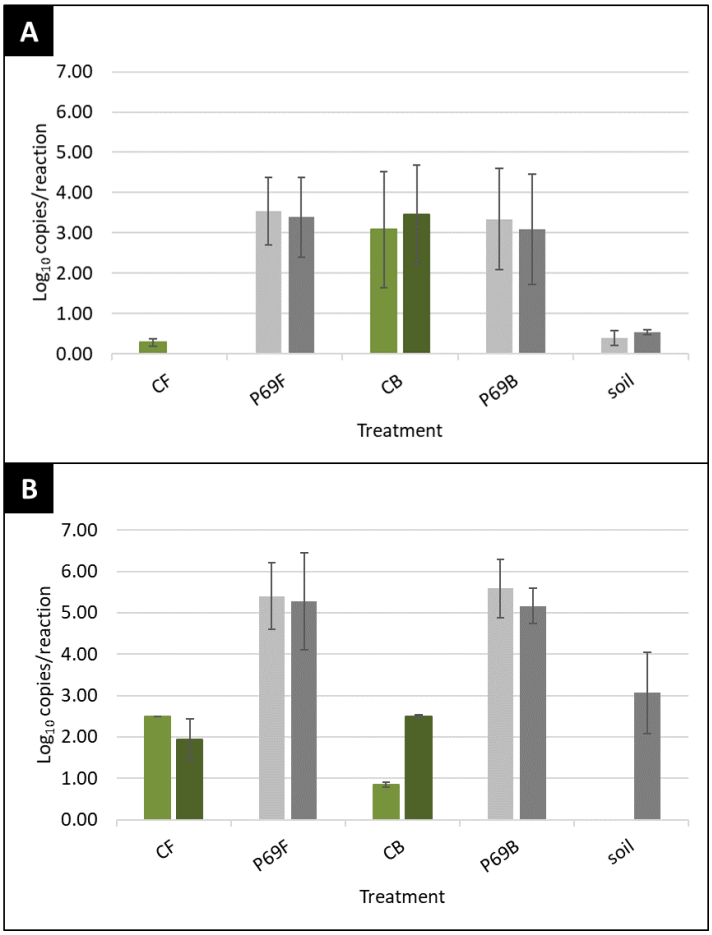


Figure 13: QPCR results in Log_{10} copies/reaction of samples from field sites Reichersberg (A) and Melk (B) in 2015. Abbreviations see table 1. Additional soil samples (soil) were taken. Seeds were treated with *S. rhizophila* SPA-P69 and/or MAXIM® or not at all. Left bar: Measurement 1; right bar: Measurement 2. Abbreviations: CF: Control plus MAXIM® treatment; P69F: *S. rhizophila* SPA-P69 applied by priming plus MAXIM® treatment; CB: Untreated control; P69B: *S. rhizophila* SPA-P69 applied by priming

With the primer number 8 and the developed probe, *S. rhizophila* SPA-P69 was successfully detected in soil probes from 2016. Quantities of *S. rhizophila* SPA-P69 and *S. rhizophila* e-p17, illustrated in figure 14, from rhizosphere samples of field trials in 2016 in Mitterdorf and Melk, were detected using qPCR. The Log_{10} values of rhizosphere samples from seeds with no bacterial treatment showed similar tends, with lower 2.5 log_{10} copies/reaction, like the different control samples from 2015. The values of *S. rhizophila* SPA-P69 samples from Mitterdorf were between 2.10-2.30 log_{10} copies/reaction and from Mitterdorf between 1.5-2.0 log_{10} copies/reaction. The values of *S. rhizophila* e-p17 from Mitterdorf and Melk were more similar with between 2.0-2.5 log_{10} copies/reaction, the values from the samples treated with encapsulated *S. rhizophila* e-p17 were very uneasy with values between 1.3-3.0 log_{10} copies/reaction. There are tendencies visible, that the copies/reaction of samples with bacterial treatment are a bit higher, but the values are below the limit of detection, therefore only qualitative and no quantitative statements can be made of the results.

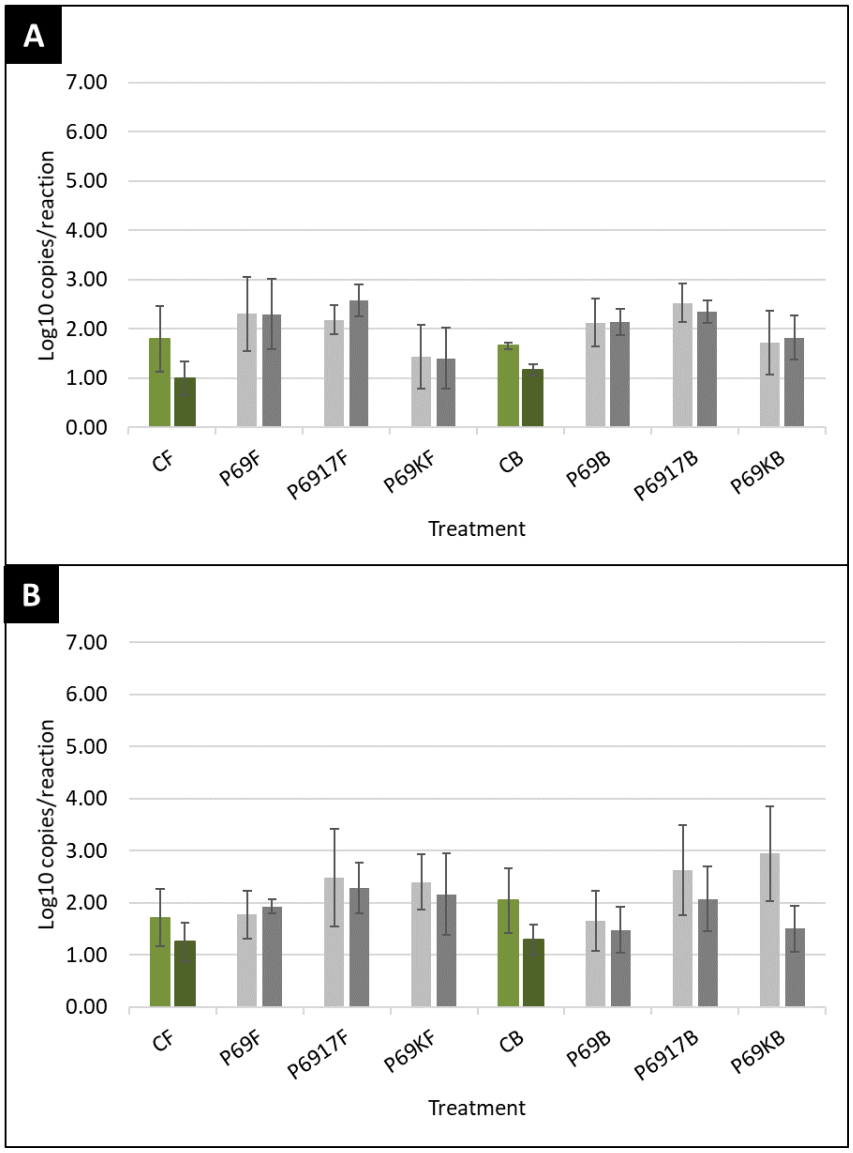


Figure 14: QPCR results in Log_{10} copies/reaction of samples from field sites Mitterdorf (A) and Melk (B) in 2016. Abbreviations see table 1. Left bar: Measurement 1; right bar: Measurement 2. Seeds were treated with *S. rhizophila* SPA-P69, e-p17 and/or MAXIM® or not at all. Abbreviations: CF: Control plus MAXIM® treatment; P69F: *S. rhizophila* SPA-P69 applied by priming plus MAXIM® treatment; P6917F: *S. rhizophila* SPA-P69 and e-p17 applied by priming plus MAXIM® treatment; P69KF: *S. rhizophila* SPA-P69 applied by encapsulation plus MAXIM® treatment; CB: Untreated control; P69B: *S. rhizophila* SPA-P69 applied by priming; P6917B: *S. rhizophila* SPA-P69 and e-p17 applied by priming; P69KB: *S. rhizophila* SPA-P69 applied by encapsulation

3.3 *Ad planta* Detection of *S. rhizophila* e-p17

3.3.1 Detection of Fluorescence Expression

The expression of fluorescence protein was detected using filters with different emission wavelengths. The *S. rhizophila* e-p17 mutant labelled with GFP emits fluorescence upon excitation at a wavelength of 530 nm. When labelled with DsRed, fluorescence is emitted upon excitation at a wavelength of 605 nm. The fluorescence image of the two mutants is shown in figure 15.

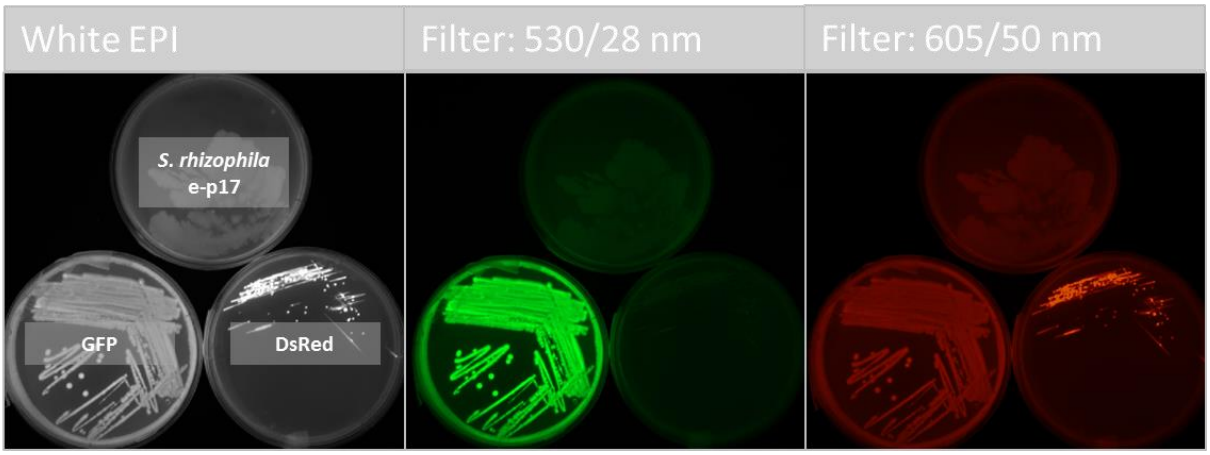


Figure 15: Detection of fluorescence expression of *S. rhizophila* e-p17 (White EPI), labelled with GFP (Filter: 530/28 nm) or DsRed (Filter: 605/50 nm) at different wavelengths

3.3.2 Detection of Colonization on Three Points in Time

To detect the colonization, three medium-sized roots of treated maize seeds per treatment were scanned using CLSM. It was detected if fluorescent bacteria are present, the results are displayed in table 11 and table 12.

Table 11: Detection of colonization of treated maize roots four, six and eight days after incubation. Fluorescence labelled (GFP or DsRed) *S. rhizophila* e-p17 was applied using the techniques priming or encapsulation. GFP and DsRed labelled e-p17 were applied solitary or in combination.

				Applied together	
	Sample	GFP priming	DsRed encapsulation	GFP priming	DsRed encapsulation
Day 4	1	-	x	-	x
	2	-	-	-	-
	3	-	-	-	-
Day 6	1	x	x	x	x
	2	-	x	x	-
	3	-	-	-	-
Day 8	1	-	x	x	x
	2	-	x	-	x
	3	-	-	-	x

visible colonization (x); no visible colonization (-)

In table 11 and table 12 the number of GFP mutant detections, regardless whether applied by encapsulation or dip bath, were lower than the number of DsRed mutants. Also, DsRed labelled *S. rhizophila* e-p17 could be detected on average for a longer timespan than the ones labelled with GFP. It was found that GFP labelled *S. rhizophila* e-p17 could be detected more frequently when encapsulated than applied by dip bath. For DsRed mutants, there was no difference in the application methods.

Table 12: Detection of colonization of treated maize roots four, six and eight days after incubation. Fluorescence labelled (GFP or DsRed) *S. rhizophila* e-p17 was applied using the techniques priming or encapsulation. DsRed and GFP labelled e-p17 were applied solitary or in combination.

				Applied together	
	Sample	DsRed priming	GFP encapsulation	DsRed priming	GFP encapsulation
Day 4	1	x	x	x	x
	2	x	-	x	-
	3	-	-	-	-
Day 6	1	x	x	x	x
	2	x	-	x	x
	3	-	-	-	-
Day 8	1	x	x	x	x
	2	x	-	x	x
	3	-	-	x	-

visible colonization (x); no visible colonization (-)

4 Discussion

The usage of microorganisms in agriculture as a biocontrol agent against host pathogens, as a plant growth promoter or as a stress protecting agent for the host plant in the context of integrated pest management is becoming increasingly important. Current hurdles in Europe are high costs in both application and registration. However, these hurdles should be taken to achieve a more sustainable and environmentally friendly agriculture [82, 83]. Under greenhouse conditions it has already been shown that *S. rhizophila* SPA-P69 promotes plant growth [73].

4.1 Beneficial Effects Achieved in the Field Trials

Field trials are indispensable in verifying the effects of beneficial bacteria which should later find use on arable land. It is important to accurately determine various parameters using controls under the same conditions to obtain comparative values. Therefore, larval intensity in field was successfully monitored. Due to great geographical distance of the fields to each other, it was only possible to measure larvae density by means of emergence traps and number of lodging in field site Mitterdorf. Emergence traps developed by Rauch et al. are the only ones which use the negative geotropic effect of the hatching beetles as a collection tool. High efficiency, low cost of material, low construction time and ease of handling are further advantages compared to other types of traps [74]. The number of detected beetles in all traps was very low, compared to the number of beetles, which were detected by AGES using pheromone traps at the nearby field site of Mitterdorf at St. Ruprecht a. d. Raab. For this reason and due to the very low number of hatched beetles, it can be assumed that the field in Mitterdorf is infected but there is no infestation yet [81]. The distribution across the field is uniform, so it can be assumed that all plots have an equally high incident pressure.

The main goal of this thesis was to determine effects of the beneficial bacteria in field trials, based on germination rate, lodging rate and yield. With the determination of the germination rate it could be excluded that the treatment with *S. rhizophila* SPA-P69 or *S. rhizophila* e-p17 leads to a reduction in the germination rate compared to the controls. However, use of MAXIM® or no fungicidal treatment showed a high but not significant effect on the germination rate. With no additional fungicidal use, a 7.5 to 15.0 percent lower germination was measured compared to the respective samples which were treated with MAXIM®. Because of these results, it can also be assumed that *S. rhizophila* SPA-P69 or

S. rhizophila e- 17 are not capable of compensating germination losses when no fungicidal treatment is applied.

The percentage of lodging correlates positively with the amount of *D. virgifera* eggs in soil, according to Sutter et al. [84]. The fact that the number of lodging increases over time, as displayed in figure 4, is due to other influences favoring lodging. Those include heavy rain or wind and the greater the attack surface presented by higher maize plants in this respect. *S. rhizophila* SPA-P69 and *S. rhizophila* e-p17 treatment showed a lower lodging level compared to the respective controls, the lowest lodging rates were observed in plants with *S. rhizophila* e-p17 encapsulation. Nevertheless, the amount of lodging was not significantly lower in plots with beneficial bacteria treatment when compared to the respective controls.

Yield comparison was not primarily about the amount of harvest income, but to recognize direct effects of the variants to the respective controls. In comparison to other variants and respective controls, *S. rhizophila* SPA-P69 treatment always increased yield regardless in which field site cultivated or whether treated with or without MAXIM®. The yield of the samples with *S. rhizophila* SPA-P69 treatment were between 3.11 and 18.05 percent higher compared to respective controls. Although no significant differences of treatment with *S. rhizophila* SPA-P69 to controls could be determined, potential positive effects which are of agricultural benefit could be shown.

4.2 Qualitative and Quantitative Determination of *S. rhizophila* SPA-P69 and e-p17

Re-isolation of *S. rhizophila* SPA-P69 and *S. rhizophila* e-p17 from seeds or roots of maize to evaluate the ability of establishment in the seed microbiome turns out to be an unpromising method for qualitative determination. On the one hand, it was not possible to unequivocally distinguish morphologically between the target strains and microorganisms of the seed microbiome. This fact makes a visual detection tricky, every single colony forming unit would need to be verified by DNA comparison. On the other hand, the growth rate of the target strains was slower compared to microorganisms of the seed microbiome, resulting in the fact that CFUs of target strains were not detected. Nevertheless, a successful establishment of some of the variants (P69B, P69F, P6917F, P69KB) could be demonstrated by calculated pearson correlation of BOX-patterns.

Detection and quantification of the bacteria directly in the soil of field by sampling rhizosphere samples turned out to be a valuable method. For this purpose, a specific primer and probe was developed *in silico* successfully. With this probe it was possible to quantify the intensity of amplicon development during quantitative real-time PCR, as it is proportional to the fluorescence intensity. Since primer number 8 showed the best performance, this primer and the additionally developed specific probe was used for the quantification. Quantitative real-time PCR provides a very sensitive method for the qualitative and quantitative detection of DNA from target organisms from soil samples. It is important to this method to reduce the influence of interfering factors in the sample, such as humic acids which reduce the efficiency of qPCR. The soil samples were diluted to reduce the influence of disturbance factors and standards were mixed in equal parts with DNA free soil samples. To isolate the DNA from the soil samples, fastDNA® SPIN Kit for Soil was used. In this isolation method, DNA losses must be expected, so that the obtained values are comparable only with each other and are below the actual values. Some measurements also showed very high values although the sample to be measured should not contain any target DNA. This is the case, for example, with the second measurement of the Melk soil sample in 2015. Such measurements can either be attributed to contamination or to "background no-template control" described in the literature in the testing of other microorganisms [86]. Despite all this, the qPCR analysis was sensitive enough to monitor the target DNA in the soil samples.

The final goal was the *in vivo* detection of fluorochrome labeled bacteria on the host root, as well as the comparison of the two treatment methods, priming and encapsulation, using confocal fluorescence microscopy. *S. rhizophila* e-p17 labelled with fluorochromes DsRed and GFP using PIN29 or pBAH8 vector emit fluorescence upon excitation with the respective wavelength. To detect the colonization of vegetative cells and their interaction with roots of the host, CLSM is a valuable tool. It enabled detection of colonization of the bacteria both by priming and encapsulation treatment, whereby no specific colonization patterns or root areas with increased colonization density could be identified. Especially at the first colonization check four days after treatment, it was very difficult to distinguish whether the bacteria adhere to the root surface or are stopped only by liquid turbulence at the root. At later times, lower bacterial density was found in the solution, which facilitated the detection of colonization. In general, the cultivation method of seeds in germination pouch should be reconsidered and possibly cultivation in a soil similar substance should be developed, because it eliminates the problem of bacteria just simply flowing around the roots and is closer to the natural conditions of field trials.

5 Directories

5.1 Index of Abbreviations

°C	degree Celsius
µl	microliter
AGES	austrian agency for health and food safety
BCAs	biocontrol agents
Bt	*Bacillus thuringiensis* Berliner
CFU	colony forming unit
CLSM	confocal laser scanning microscopy
cm	centimeter
ddH_2O	double distilled water
DNA	deoxyribonucleic acid
DsRed	*Discosoma* sp. red fluorescent protein
dt	deca tones
EDTA	Ethylenediaminetetraacetic acid
EPF	entomopathogenic fungi
EPN	entomopathogenic nematodes
EU	European Union
FAO	food and agriculture organization of the united nations
GFP	green fluorescent protein
h	hour
ha	hectare
IPM	integrated pest management
IPTG	Isopropyl-β-D-thiogalactopyranosid
IPTG	Isopropyl-β-D-thiogalactopyranosid
min	minute
min	minute
PCR	polymerase chain reaction

qPCR	quantitative polymerase chain reaction
TAE	Tris-acetate-EDTA
TBE	Tris-Borat-EDTA
WCR	western corn root worm (*Diabrotica virgifera* LeConte)
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

5.2 Index of Figures

5.3 Index of Tables

5.4 List of References

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6 Supplemental Information

A listing of the various *Stenotrophomonas* species, to which the primer sequences of the primer candidates were tested, is listed in table s1.

Table S1 *Stenotrophomonas* genomes screened for compliance with the primer sequences of the primer candidates

Species name	Strain	GenBank assembly accession	Origin
Stenotrophomonas acidaminiphila	DSM 13117		industrial waste water
Stenotrophomonas acidaminiphila	ZAC14D2_NAIMI4_2	GCA_001314305.1	sediments of a polluted river
Stenotrophomonas acidaminiphila	JCM 13310	GCA_001431595.1	sludge
Stenotrophomonas acidaminiphila	CIP 104854		cerebrospinal fluid
Stenotrophomonas chelatiphaga	DSM 21508	GCA_001431535.1	sewage sludge
Stenotrophomonas daejeonensis	JCM 16244	GCA_001431505.1	sewage
Stenotrophomonas ginsengisoli	DSM 24757	GCA_001431485.1	soil of ginseng field
Stenotrophomonas humi	DSM 18929	GCA_001431415.1	soil
Stenotrophomonas koreensis	DSM 17805	GCA_001431525.1	compost
Stenotrophomonas maltophilia	K279a	GCA_000072485.1	human bloodstream
Stenotrophomonas maltophilia	JV3	GCA_000223885.1	plants
Stenotrophomonas maltophilia	D457	GCA_000284595.1	clinical isolate
Stenotrophomonas maltophilia	5BA-I-2	GCA_000543365.1	soil
Stenotrophomonas maltophilia	R551-3	GCA_000020665.1	*Populus trichocarpa* x deltoides
Stenotrophomonas maltophilia	13637	GCA_000742995.1	oropharyngeal region of patient with mouth cancer
Stenotrophomonas maltophilia	ISMMS2R	GCA_001274675.1	human
Stenotrophomonas maltophilia	e-p3	AJ293464.1	rhizosphere of rape
Stenotrophomonas maltophilia	ISMMS3	GCA_001274595.1	human
Stenotrophomonas maltophilia	EPM1	GCA_000344215.1	human
Stenotrophomonas maltophilia	ISMMS2	GCA_001274655.1	human

Stenotrophomonas maltophilia	NBRC 14161	NZ_BCUI01000026.1	
Stenotrophomonas maltophilia	F2	GCA_001619675.1	slim Hot Spring
Stenotrophomonas maltophilia	LMG 978	GCA_001431665.1	piper Betel (plant)
Stenotrophomonas maltophilia	Sm32COP	GCA_001676295.1	horse manure (animal dumpling)
Stenotrophomonas maltophilia	SmF3	GCA_001676375.1	cattle manure (animal dumpling)
Stenotrophomonas maltophilia	SmSOFb1	GCA_001676445.1	*Bactrocera oleae* (fly)
Stenotrophomonas maltophilia	BR12	GCA_000972335.1	mice (animal)
Stenotrophomonas maltophilia	CBF10-1	GCA_001595975.1	soil
Stenotrophomonas maltophilia	SKK35	GCA_000355745.1	
Stenotrophomonas maltophilia	SAM8	GCA_001020915.1	water
Stenotrophomonas maltophilia	B418	GCA_000788095.1	barley rhizosphere
Stenotrophomonas maltophilia	ATCC 19867	GCA_000382065.1	plant
Stenotrophomonas maltophilia	RR-10	GCA_000237025.2	rice, root
Stenotrophomonas maltophilia	MF89	GCA_000455685.1	*Crassostrea virginica* (Oyster)
Stenotrophomonas maltophilia	ZBG7B	GCA_000834105.1	soil
Stenotrophomonas maltophilia	As1	GCA_001051925.1	*Anopheles stephensi*
Stenotrophomonas maltophilia	JCM9942	GCA_001431585.1	soil
Stenotrophomonas nitritireducens	2001	GCA_001700965.1	soil
Stenotrophomonas nitritireducens	DSM 12575	GCA_001431425.1	laboratory scale biofilter
Stenotrophomonas panacihumi	JCM 16536	GCA_001431645.1	soil of ginseng field
Stenotrophomonas pavanii	DSM 25135	GCA_001431565.1	stems of sugar cane
Stenotrophomonas rhizophila	QL-P4	GCA_001704155.1	fallen leaves
Stenotrophomonas rhizophila	e-p14	AJ293461.1	rhizosphere of potato
Stenotrophomonas rhizophila	Simmons01	GCA_001542955.1	tree bark
Stenotrophomonas terrae	DSM 18941	GCA_001431465.1	soil
Stenotrophomonas sp.	YM1	GCA_001562215.1	