Seed bio-priming with *Serratia plymuthica* HRO-C48 for the control of *Verticillium longisporum* and *Phoma lingam* in *Brassica napus* L. spp. *oleifera*

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Abstract

Oilseed rape is one of the most important crops all over the world. It is susceptible to many fungal pathogens including soil-borne *Verticillium longisporum* (causes *Verticillium* wilt) and *Phoma lingam* (causes blackleg). Available control measures are not sufficient to control these diseases. One way to increase both quality and quantity of crop yield is by treating seeds with beneficial microorganisms before sowing.

Effect of seed bio-priming with the antagonistic bacterium, *Serratia plymuthica* HRO-C48 was investigated in oilseed rape (*Brassica napus* L. spp. *oleifera*) against wilt and black leg. Rifampicin-resistant strain of the bacterium was used in this study. The effect on wilt was assessed in cultivars Visby, Trabant, KWS 63 and KWS 136 in growth chamber experiments while the effect on black leg was observed in presence and absence of fungicides (Folicur © or Caramba ©) in Visby cultivar the field trials on-going at Birkenmoor and Hohenlienth in Germany. *V. longisporum* was artificially inoculated and *Phoma* infection in the field trail was naturally occurring.

Disease severity and plant recorded in plants with *V. longisporum* were compared with that in presence of *S. plymuthica* HRO-C48^{rif} . In field experiments, leaf infestation frequency and number of leaf pycnidia due to *Phoma* were compared between the treatments. Bacterial population of inoculated *S. plymuthica* HRO-C48^{rif} was also estimated in the rhizospheres of both growth chamber and field experiments. Summarised results showed that *S. plymuthica* HRO-C48^{rif} was able to colonise the rhizospheres of oilseed rape in both growth chamber and field trails but it was not able to reduce the infections by *V. longisporum* or *Phoma lingam* in a satisfactory way. Furthermore, the application of bacterium seemed compatible with the fungicides and the bacterial populations declined below the detection level.

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Introduction

Oilseed rape (OSR *Brassica napus* L.) is one of the most important crop all over the world, especially European Union (EU) with an acreage of 5.4 million ha in 2005 (http:/faostat.fao.org/). OSR has been cultivated in Asia for thousands of years; in India it has been grown since 3000 years from where it was introduced to China and Japan 200-500 BC (Krzymanski, 1998). OSR cropping has increased rapidly over the last 20 years, China produces 25% of the world production, Canada 20% and among the EU countries Germany, France and UK are the main producers. (Figure 1). The OSR acreage in the EU has more than doubled during the last 10 years (Harvest 2008 > 6 million ha) (www.npz.de).

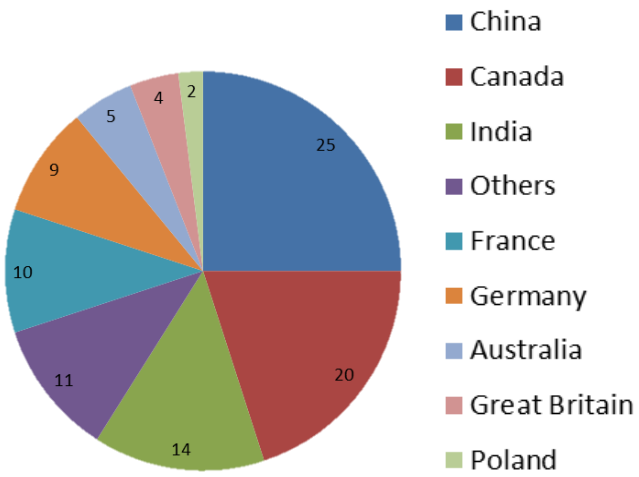

Figure 1. Countries dominating in producing oilseed rape in the world (http:/faostat.fao.org)

In Sweden, OSR cultivation is important for agriculture. In the beginning of the twentieth century its acreage was very small. The crop attracted interest during the First World War but its cultivation was soon given up. Modern OSR cultivation started in 1939. Now in Sweden about 94 887 ha were sown in 2011 (Figure 2). The main part of this acreage is located in Skåne, the southernmost province of Sweden (http://www.svenskraps.se).

The demand for rapeseed crop has increased recently due to its high-quality edible oil and also for non-food purposes as an important renewable oil resource in bio-fuel industry and as an important pre-crop to cereal crops cultivation. There are hundreds of varieties of OSR grown all over the world, depending on climate, soils, growing seasons and level of disease resistance. Among these, >50% of current OSR production for instance in Germany is derived from hybrid cultivars (Friedt *el al*. 2007).

Some oilseed rape diseases and their control

With the increased production of OSR, plant diseases causing pathogens tend to become an increasing threat. OSR is susceptible to many fungal pathogens such as those caused by *Verticillium longisporum* (earlier *V. dahliae*, causes *Verticillium* wilt), *Phoma lingam* (causes

stem canker) and *Sclerotinia sclerotiorum* (causes stem rot) (Brun, 2006). In some cases, *S. sclerotiorum* causes 95% losses if chemical fungicides are not effective (Grison *et al*. 1996).

It is common to spray fungicides against *S. sclerotiorum* at full flowering for best control. Now, new varieties have high *Phoma* resistance levels. But only low or no resistance is available against diseases caused by *S. sclerotiorum* and *V. longisporum* diseases. Estimates of losses caused by *P. lingam* 30% and *V. longisporum* are 20% (Gunnarsson 2002).

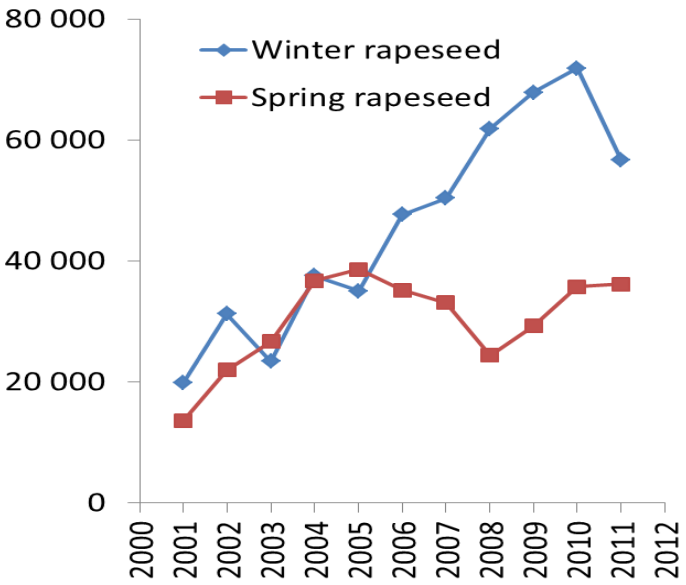

Figure 2: Oilseed rape production area in hectare in Sweden from 2001-2011 (http://www.svenskraps.se)

Verticillium longisporum

Verticillium belongs to class Deuteromycetes (Fungi imperfectii), a group which has only asexual stage. Its mycelium is hyaline, septate, multinucleate, single and branched. The nuclei are haploid. The shape of conidia is elongate and size ranges between 6.5-12 µm (Karapapa *et al.*, 1997).

Verticillium longisporum is one of the major threats of winter oilseed rape cultivation in many areas of the world (Zeise and Tiedmann 2001, 2002, Zhou *et al*. 2006). Most current OSR cultivars are susceptible to *V. longisporum* infection (Rygulla *et al*. 2008). *Verticillum* spp. is a soil-borne pathogenic fungus that causes diseases in several crops in temperate and subtropics regions. Wilt caused by *Verticillium* is especially prominent in northern Germany and Sweden, but it has also been reported from Poland, France, southern Russia and Ukraine (Heale and Karapapa 1999). The first report of Verticillium wilt in *Brassica* oil-seed crops in Sweden comes from an inventory of oilseed rape and turnip rape fields in Skåne in 1969 (Kroeker, 1970).

A few years later, Verticillium wilt severe outbreaks on *OSR* crops were reported from northern Östergötland (Björkman and Sigvald, 1980). *Verticillum* wilt causes yield and quality losses in many other crops such as alfalfa, cotton, eggplant, mint, potato, tomato and sunflower (Domsch *et al*. 1980, Dixelius *et al.*, 2005).

V. longisporum survives in the soil and plant debris as microsclerotia, which are produced in the wilting tissues of the host plant (as shown in Fig 3). Microsclerotia are subglobose, black and mostly 50-80 µm diameters. Its life cycle is monocyclic and can be divided into two phases. First phase occurs in the soil, where vascular tissues get infected during early stages and second phase occurs after germination of seed. The germination of microsclerotia is stimulated in the soil by root exudates that are secreted from the root tips and root hairs. The fungal hyphae penetrate the cortex of susceptible young roots (Schnathorst, 1981).

Phoma lingam

Phoma belongs to the class Dothideomycetes. The conidia shape of *Phoma lingam* is hyaline, cylindrical to ellipsoidal. The spores are colourless and unicellular. The pycnidia are black and depressed in the tissues of host.

Stem canker (also termed blackleg) is asexual stage and one of the most important disease is caused by *P. lingam (sexual stage Leptosphaeria maculans*) in *B. napus* (Rouxel and Balesdent 2005). This disease is economically important in the main growing areas of Australia, China, Canada and EU (West *el at* 2001) although these areas are cropped with different cultivars and different agricultural practices (West *et al*. 2001, Howlett 2004).

Symptoms of rotting and girdling at the stem basis are of uttermost economic importance as they restrict water and nutrients supply of the upper sprouts parts. Transport of water and nutrients is blocked due to rotting of plant tissues.

The fungus survives on crop debris as mycelium and releases ascospores from sexual fruiting bodies pseudotheci. The ascospores are then released in autumn during warm and humid weather. Pycnidiospores are produced in asexual pycnidia and are spread by rain splashes over short distances. Both ascospores and pycnidio-spores cause leaf infections. The spores dispersed systemically into the plant and initiate stem canker (Brazauskiene and Petraintiene 2006).

Disease management

The goal of plant disease management is to reduce economic damages caused by plant diseases (www.apsnet.org). Crop rotation is a frequently used strategy to reduce the pathogen inocula in the soil. Examples of other practices are tillage, proper drainage, irrigation or soil pH. It may also involve changing date or depth of seeding, plant spacing, pruning that allow plant to escape infection or reduce severity of disease.

Soil-borne pathogens such as *V. longisporum* (earlier *V. dahliae* var. *longisporum* Stark) cannot be controlled by chemical fungicides as is possible for *S. sclerotiorum* and *P. lingam*. Fungicides are recommended to be applied during the leaf phase of the *Phoma* disease in autumn in OSR crop. In northern Germany fungicides; Caramba ©, Folicur ©, Amistar ® etc. are generally applied to control *Phoma* infections but also to improve winter hardiness and stem stability of the crop. Their use is recommended to be optimised to achieve the economically viable response (Dixelius, 2006). Use of foliar fungicides in association with cultivars with little or no resistance has proven to be ineffective for *Phoma* stem canker control (West *et al*. 2001). Current fungicides are effective as protectants for only a short

period of time. As a result of degradation, leaf expansion and the emergence of new untreated leaves, it is important that application of fungicides is correctly timed (West *et al*. 2002).

Biological Control**:** Chemical fungicides can cause several negative effects, such as: (a) causes hazards to humans and environmental pollution, (b) development of pathogen resistance, and (c) damage to beneficial organisms, (Hungria *et al.,* 2005). It is speculated that fewer pesticides will be used in future and that greater reliance will be placed on biological technologies including the use of microorganisms as antagonists (Fridlender *et al.* 1993, Jung *et al*, 2003). One way to increase both quality and quantity of crop yield is by treating seeds or seedlings with beneficial microorganisms that are capable of inducing plant intrinsic systemic resistance (Beckers and Conrath 2007, Buensanteai *et al*. 2009). Naturally occurring beneficial microorganisms have shown to possess the potential to protect different crops against the harmful effects of their pathogens (Weller *et al*; 2002, Welbaum *et al*; 2004, Berg, 2009).

Use of beneficial microorganisms for seed treatment is considered as environment friendly because it has not shown any documented negative impact on human or environment (Jensen 2004, Mew *et al.* 2004, and Knowles *et al.* 2008). Seed priming or osmo-conditioning are terms to describe a pre-sowing hydration treatment that has been developed to improve seedling establishment (Taylor & Harman 1990). Furthermore, bio-priming has in several cases been reported to enhance and stabilize the efficiency of biocontrol agents (BCAs) (Jensen *et al*; 2004, Bennett and Whipps 2008).

It has been advised that a strain to be used for bio-priming or as BCAs must be chosen with appropriate genetic potential for effective biocontrol but also for rhizosphere competence. For commercial purpose, it is important to prepare a high biomass quality of the organism that is able to survive during storage on seeds and in the soil. A commercial bio-primer must also be environmentally safe, not deleterious to the seed (Taylor & Hermann 1990, Fravel 2005, Spadaro and Gullino 2005).

Most BCAs have a threshold level measured as live cells needed for biocontrol that is above 10^6 live cells g^{-1} seed, roots or soil (Raaijmakers *et al*; 1995, Bennett and Whipps 2008). To achieve this density, it is necessary to develop strategies to improve the performance and reliability of BCAs to be delivered and established in the potential infection zone of the host plant or in the rhizosphere and to have a consistent efficiency under varying field conditions.

Bio-priming with S. plymuthica

Serratia is ubiquitous, found in water, soil, plants and animals (including *S. marcescens in* humans). The genus *Serratia* is named after the Italian physicist Serafino Serrati and belongs to the family Enterobacteriaceae within the Gammaproteobacteria. One of its species *S. plymuthica* was first time described by Lehmann and Neumann in 1896 (Breed *et al.* 1948).

Over 5000 bacterial isolates obtained from the OSR roots were screened for antifungal properties against *V. dahliae* in a study by Kalbe et al (1996). Of the 18 *Serratia* isolates formed, 16 were identified as *S. plymuthica*. One of these (HRO-C48) selected for this study was also identified as *S. plymuthica* for its ability to control fungal pathogens, *V. dahliae, V. longisporum, S. sclerotiorum*, *Fusarium* spp, and *Rhizoctonia solani)* and leaf pathogens of

strawberry. (Kalbe *et al*. 1996, Kurze *et al*. 2001, Berg *et al*. 2001, 2002). It is the active organism in the biocontrol product Rhizostar® (e-nema GmbH, Raisdrof, Germany). *S. plymuthica* HRO-C48 has a high chitinolytic activity, produces plant growth hormone indole-3-acetic acid (IAA) and it is considered harmless to human health and environment. It has unique molecular fingerprints and has a minimum growth at temperature $\geq 4^{o}$C. (Frankowski *et al* 1998; Berg *et al*. 2000; Kalbe *et al* 1996). According to German legislation (Biostoff Verordnung: BGBI pp. 50-60), the biocontrol strain *S. plymuthica* HRO-C48 belongs to risk class one which means that the isolate is considered harmless to human and the environment (Eberl et al., 2005).

Objectives

The effect of seed bio-priming was evaluated in this study to determine the ability of *S. plymuthica* HRO-C48 to control *V. longisporum* and *P. lingam* and also indirect plant growth promotion. The aims were, therefore, to study the;

1- ability of *S. plymuthica* HRO-C48 to control *V. longisporum* in four different winter rape cultivars in growth chamber experiment.

2- population dynamics of *S. plymuthica* HRO-C48 in winter rape in *V. longisporum* infested soil.

3- effect of *S. plymuthica* HRO-C48 in comparison to effect of fungicides on naturally occurring Phoma incidence in winter rape in field condition.

4- population dynamics of *S. plymuthica* HRO-C48 in winter rape in the above field experiment with *Phoma*.

Materials and Methods

Plant material and *V. longisporum* inoculum preparation

Four hybrid cultivars of winter OSR; Visby, Trabant, KWS 63 and KWS 136 were used. Their known traits are summarised in the table 1. All the seeds used were certified.

Table 1: List of known traits of the four hybrid winter rapeseed cultivars used in this study

Traits	**Visby**	**Trabant**	**KWS 63**	**KWS 136**
Resistance to *Verticillium*	Yes	Yes	Not Known	Not Known
Resistance to *Phoma*	Moderate	Moderate	Not Known	Not Known
Yield	High	High	High	High

The pathogen *V. longisporum* isolate ELV25 used throughout in all the growth chamber experiments. Both seeds and the pathogen isolate were obtained or provided from the collection at Inst. Plant Pathology at Kiel, Germany.

Microsclerotia of *V. longisporum* were produced in Erlenmeyer flasks, containing 250ml of Czapek-Dox broth (g/litre: Sodium nitrate 2, Potassium chloride 0.5, Magnesium glycerol-phosphate 0.5, Ferrous sulphate 0.01, Potassium sulphate 0.35, Sucrose 30, Agar 12, pH 7.2) by inoculating fresh culture of the fungus and incubating on rotary shaker at 195 rpm for two weeks at 20^{o}C. The microscelerotia thus produced were used for artificial infestation.

Four litre sterilised vermiculite (Dämmstoffe Sprökhovel, Germany) was mixed with 200 ml Czapek-Dox broth and then inoculated with the fresh culture of the pathogen and incubated for four weeks. This was used as the pathogen inoculum for experiments described below.

Culturing of *S. plymuthica* and inoculum preparation

The spontaneous rifampicin-resistant mutant of the isolate *S. plymuthica* HRO-C48 was received as a stock culture stored at -80^{o}C in tryptic soybroth (TSB, 30g/litre, bioMérieux Deutschland GmbH, Nürtingen) from Prof. Ehlers Lab. University of Kiel, Germany. The mutant produced by Hammoudi (2007) was used for biopriming of all OSR seeds to enable re-isolation and identification of applied bacteria from the seed and plant rhizosphere soil (and natural) environment. The bacteria were cultured in TSB at 150 rpm and 28^{o}C for 48 hrs in dark. After 48 hrs, bacterial concentrations were determined spectrophotometrically and adjusted to 10^6 colony forming units (CFU) ml^{-1} by dilution with sterile 0.85% NaCl solution. A dilution series of *S. plymuthica* was performed on TSB supplemented with 1.6% agar and amended with 100µg ml^{-1} rifimpicin for assessment of CFU/ ml (TSA^{rif}). The CFU in the bacterial suspensions was then estimated from a calibrated growth curve.

Bio-priming and bacterial load of seeds

Two grams of seeds of each of the four OSR cultivars were primed with 2 ml bacterial suspensions by incubating for 5 h at 20^{o}C. During incubation, seeds were agitated at 150 rpm on a rotary shaker. Seeds were airdried overnight at 20^{o}C. Bacterial density on seeds was estimated before they were sown, to achieve > 10^6 CFU seed^{-1}. For the purpose of CFU estimation, ten seeds of each of the cultivars treated with *S. plymuthica* HRO-C48 by blended them for one min in 2 ml sterile 0.85% NaCl solution. The suspensions thus obtained were diluted serially and plated on TSA^{+rif} (as shown in Fig. 7). All plates were incubated at 28^{o}C in the dark for 48 h before CFUs were counted and counts transformed to CFU/seed.

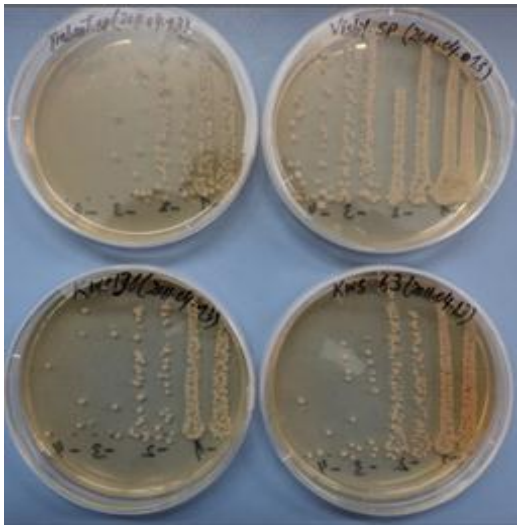

Fig. 3. CFU /seed determined by spreading appropriate dilutions on TSA^{rif}

Effect of *Serratia* on *Verticillium* wilt in growth chamber

In order to study the ability of *S. plymuthica* HRO-C48 to control *V. longisporum* in growth chamber, two different experiments were conducted, first one preparatory experiment of the pathogen inoculum and in the main experiment a high pathogen inoculum was used. The growth substrate in both experiments was the same and consisted of: a commercial soil used for greenhouse cultivation (Natural Clay, peat, compost substrate, expanded clay, lava, sand, - volume of fresh weight: 400- 600 kg /m^3, www.einheitserde.de/).

The above growth substrate was artificially infested with *Verticillium* suspension obtained above at a ratio of 20:5:1 (commercial soil: vermiculite: Verticillium in vermiculite, v/v/v) respectively in the preparatory experiment. In the main experiment, the proportions were 16:6:2 (v/v/v) respectively. Pots filled with the above substrate without *Verticillium* served as healthy controls. Four seeds per pot/cultivar were sown in pots (9cm diameter x 17 cm depth) filled with the growth substrate above. After germination, the seedlings were thinned out to two plants per pot. Different treatments that were included in the two experiments are shown in table 2.

Table 2: Treatments and replicates (in parenthesis) included in growth chamber trials with different doses of Verticillium longisporum

Preparatory trial (20:5:1)*	**Main trial** (16:6:2)*
Untreated (n=30)	Untreated (n= 19)
+ *V. longisporum* (n=60)	+*V. longisporum*, (n= 28)
+ *V. longisporum* + *S. plymuthica* (n=60)	+ *V. longisporum* + *S. plymuthica* (n= 28)

*proportion of commercial soil, vermiculite, Verticillium in vermiculite

All pots were placed in trays that were randomly placed in a growth chamber with 16/8 h light/dark regime and temperature 15^{o}C-20^{O}C respectively. Plants were watered and fertilized regularly with a commercial plant nutrient solution available for the purpose at the ratio of 1ml/L.

Verticillium wilt assessment started in the low dose experiment from 27 days after sowing and thereafter twice per week. In main experiment, *Verticillium* wilt assessment started from 32 days after sowing and thereafter twice per week. The disease assessment per pot was done according to the scale suggested by Zeise (1992) and was based on the symptoms that appeared in the leaves per pot. Table 3. Disease index (DI) was calculated according to Borges *el al.* (2003). DI= $[(n_0\times 0) + (n_1\times 1) + ...+(n_8\times 8)/ (N\times 8)] \times 100$, where n_0-n_{15} represents the number of the plants belonging to classes 0-8 receptively, and N= the total number of plants. The effect of the treatments, if any, was measured at the end of each experiment in terms of the plant height and shoot dry weight. Shoot dry weight was obtained after drying the shoots at 70^{o}C for 2 days.

Table 3: Scoring scheme suggested by Zeise (1992) for assessing disease symptoms induced by Verticillium longisporum *in oilseed rape plants*

Score	Symptom description
0	No symptoms
1	Slight symptoms on oldest leaf (yellowing, black veins)
2	Oldest leave with strong symptoms (nearly dead)
3	Loss of the oldest leaf
4	About 50% of leaves with strong symptoms
5	Loss of about 50% of leaves
6	Loss of over 50% of leaves
7	Only apical meristem still alive
8	Dead plant

Bacterial colonization of oilseed rape rhizosphere in growth chamber experiments

To estimate the bacterial colonization ability of the OSR plants rhizosphere, roots with adhering soil were aseptically sampled from 11 weeks old plants (2 plants per pot), cut into a small pieces and suspended in sterile distilled water. Care was taken to adjust the amount of water used for suspending the roots according to the weight of roots. The roots were shaken in the water at 195 rpm for 2 hrs. The rhizosphere soil suspensions thus obtained were diluted and appropriate dilutions were plated on TSA^{rif}. The CFUs were estimated after 48 h of incubation at 28^{o}C in the dark.

Effect of *S. plymuthica* on disease caused by *Phoma lingam* in field

A field trial was set up at the end of September 2010 at nine filed sites. The locations shown in Fig 4; were provided and cultivated by Germany breeding companies Schleswig-Holstein

(LKSH). Two of the field sites were in Birkenmoor (latitude 54.450) and Hohenlienth (latitude 54.266) located in Northern Germany. The two trial sites covered different climatic conditions in northern parts of Germany with intensive rapeseed cultivation.

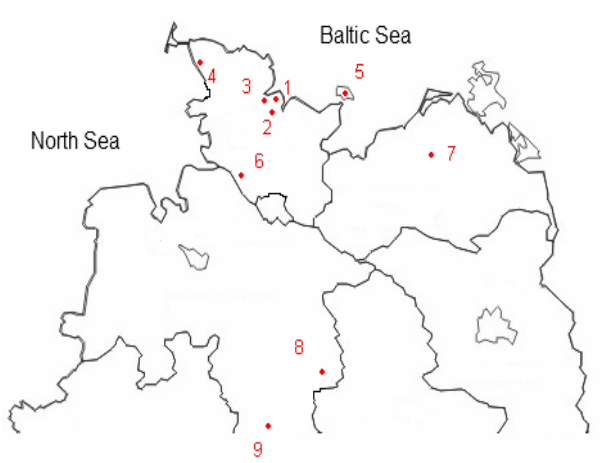

Fig 4: Location of trial sites. 1=Birkenmoor, 3=Hohenlieth

Table 4: Trial site information. AZ ("Ackerzahl", a parameter for soil quality reaching from 7 (very bad)-100 (very good)) as well as mean temperature and mean annual precipitation of the trial episode 2008-2011. Information about soils and weather data were received from project partner Schleswig-Holstein (LKSH)

No.	Site	Soil type	AZ	Mean temp (°C)	Annual precipitation
1	**Birkenmoor** (Schleswig-Holstein)	sandy loam	56	8,6	715
3	**Hohenlieth** (Schleswig-Holstein)	sandy loam	56	8,7	838

The aim was to compare the effect of *S. plymuthica* with that of fungicides (Folicur© or Caramba©) on naturally occurring infection of *P. lingam*. The soil types were sandy loam at both the sites. The growth conditions on the sites are summarised in table 4.

The cultivar Visby was used for the field trials; its seeds were bio-primed according to the procedure described above. This cultivar is moderately resistant to blackleg disease (table 5). The bio-primed seeds were sown at the rate of 45 seeds/m^2 at Birkenmoor (plot size 25 m^2) and 70 seeds/m^2 at Hohenlieth (plot size 12.7 m^2). The treatments (n=4) included in the field trial were: 1) Control (no treatment); 2) Plants sprayed with Folicur or Caramba in autumn/ spring/ and at flowering stage; 3) Seeds bio-primed with *S. plymuthica*; and 4) Seeds bio-primed with *S. plymuthica* + plants sprayed with Folicur

Recording of the leaf infestation by (natural infection of) *P. lingam* started 20 days after sowing.

Bacterial colonization of oilseed rape rhizosphere in field experiments

To estimate the bacterial colonization ability in the rhizosphere of Visby plants above, roots with adhering soils were sampled from two treatments (seeds bio-primed with *S. plymuthica*; and seeds bio-primed with *S. plymuthica* + plants sprayed with Folicur) at different time intervals; 20 October 2010, 8 Nov 2010, 18 Jan 2011, 27 April 2011 and 16 May 2011. Ten plants/ repetition in the plot were sampled. They were cut into small pieces and processed for CFU estimation TSA^{rif} according to same procedure as described above on 48 h of incubation at 28^{o}C in the dark.

Statistical analyses

All data was statistically analysed with JMP, using Tukey's HSD test to compared means after analysis of variance (ANOVA). Significant differences among treatments were computed by Tukey HSD test at $\alpha = 0.05$.The healthy controls were excluded from the statistical analysis of DI since symptoms specific to *Verticillium* wilt were absent on those plants.

Results and Discussion

Effect of *S. plymuthica* on *Verticillium longisporum* in growth chamber

S. plymuthica HRO-C48^{rif} used in this study was a spontaneous rifampicin resistant mutant of the wild type *S. plymuthica* HRO-C48. All results presented here are, therefore, based on bio-priming with *S. plymuthica* HRO-C48^{rif}. The effect of *S. plymuthica* HRO-C48^{rif} against *V. longisporum* was recorded in terms of disease index (DI) and plant height. In the trial with Phoma *lingam* the incidence of leaf infestation frequency and number of pycnidia were recorded.

One preparatory experiment and one main experiment were conducted to study the effect of bio-priming on Verticillium wilt in growth chamber. The symptoms observed in the preparatory trial (low dose of pathogen) were much weaker and sometimes unreliable than in the main trial (double dose of pathogen than in the preparatory trial). Because of this, only the results from the main trial are presented. However, the results in both the preparatory and main experiments showed similar pattern of disease occurrence.

DI did not differ significantly in sick controls of the four cultivars in the Verticillium experiment (P=0.8695)**.** As regards effect of bio-priming, there was no significant (P=0.5207) effect in any of the cultivars by the bacterium in growth chamber experiments.

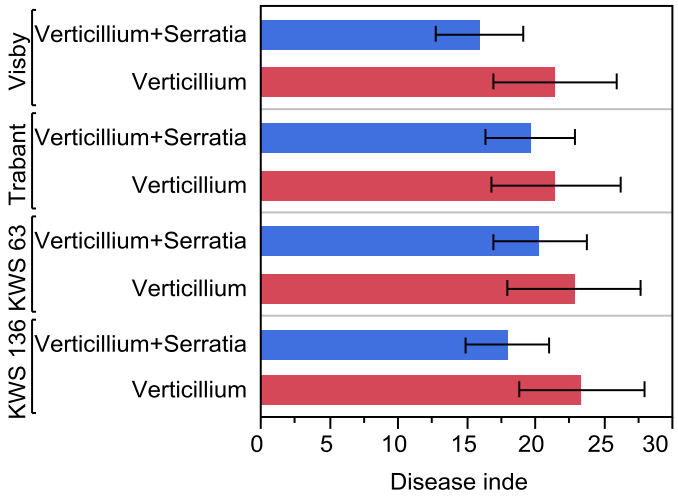

Figure 5. Disease Index in four different cultivars of winter oilseed rape inoculated with Verticillium longisporum *in presence and absence of* S. plymuthica HRO-C48^{rif} *in a growth chamber trial.* Verticillium= *sick control. Bars indicated 95 % confidence interval.*

However, the DI seemed to be lower in bacteria-treated plants of all cultivars compared to the control treatment (as shown in Fig 5), indicating some protection of bio-priming with *S. plymuthica* in oilseed rape. For instance, *S. plymuthica* HRO-C48^{rif} reduced DI in cultivars KWS 136 (P = 0.3307) from 23 to 18 and in Visby (P = 0.3308) from 21 to 16.

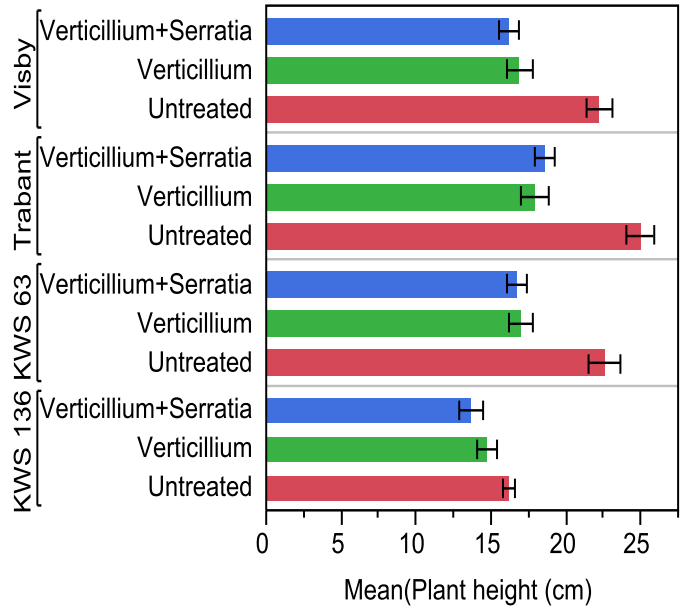

Figure 6: Effect of S. plymuthica HRO-C48^{rif} *on plant height (cm) of four OSR cultivars grown in growth substrate artificially infested of* V. longisporum. *Untreated= healthy control.* Verticillium= *sick control. Bars indicated 95 % confidence interval.*

All the results with respect to effect of different treatments on plant height are summarised in Fig. 6. The results show that the untreated (= healthy) plants grew significantly higher than those treated with *Verticillium* or *Verticillium+ Serratia* (p <0.0001*).* The negative effect of *Verticillium* inoculation was evident in terms of appearance of typical wilt symptoms as well as reduced plant height. No effect of bio-priming with *S. plymuthica* HRO-C48 *rif* against *Verticillium* infection or on plant growth was observed.

Effect of *S. plymuthica* HRO-C48*rif* on leaf infestation by *Phoma lingam* in field trial

Results on the effect of seed bio-priming with *S. plymuthica* HRO-C48 *rif* in combination with fungicides on *Phoma* are shown in Fig 7 and 8. The number of pycnidia of *Phoma* formed on leaves was higher in untreated plants in early spring at Birkenmoor site compared to that in Hohenlieth (Jan 2011). The *Serratia+fungicide* mix significantly reduced symptoms caused by *P. lingam* only in the Birkenmoore field (p<0,0141 for treatments meaned over date).

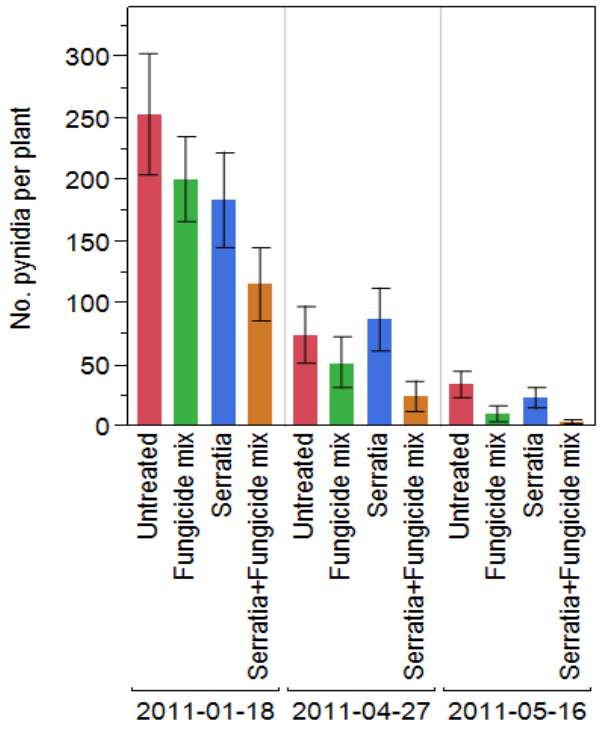

Fig 7. Effect of S. plymuthica HRO-C48rif bio-priming on blackleg disease development in cultivar Visby in a field experiment located at Birkenmoor. Bars indicated 95 % confidence interval.

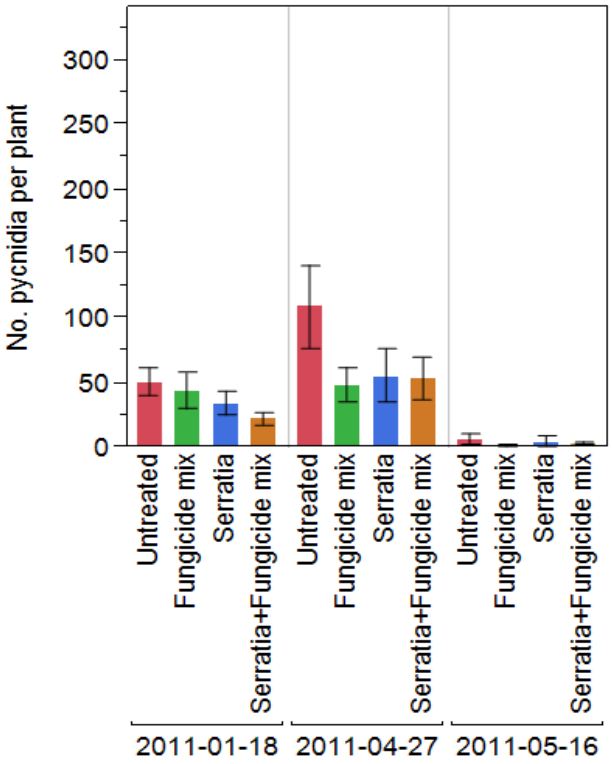

Fig 8. Effect of S. plymuthica HRO-C48*rif bio-priming on blackleg disease development in cultivar Visby in a field experiment located at Hohenlieth. Bars indicated 95 % confidence interval.*

Treatments: Red (Control), Green (Fungicides Folicur + Caramba), Blue (HRO-C48)
Orange (HRO- C48+Fungicides)

Pang *et al*. (2009) reported that *S. plymuthica* HRO-C48 was able to protect cucumber plants from damping-off caused by *Pythium aphanidermatum*. The bacterium was also shown to induce the systemic resistance in bean and tomato plants against *Botrytis cinerea*. Abuamsha *et al* (2010, 2011) also investigated the effect of *S. plymuthica* HRO-C48 on eight different cultivars of oilseed rape in terms of its ability to protect them from blackleg disease caused by *Phoma lingam*. The cultivars varied in resistance to the disease. The authors observed disease reduction in cv Trabant 71.6% after treatment with *S. plymuthica* HRO-C48. The cultivar Aragon showed the highest control compared to other cultivars which is 73.8%. The strain HRO-C48 was also found to be an effective antagonist of *Verticillium dahliae*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum* in strawberry under field conditions (Frankowski *et al*; 1998; Kurze *et al* 2001)

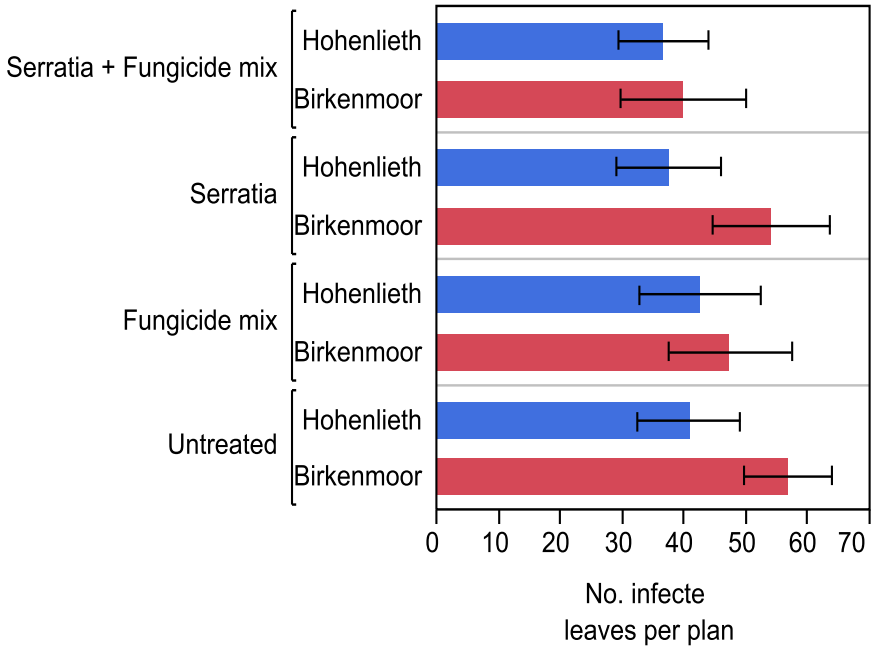

Fig 9. Effect of bio-priming of Visby seeds by S. plymuthica *HRO-C48 compared to that of fungicide mix (Caramba © and Folicur ©) on % leaf infestation by* Phoma lingam *in field experiments at Birkenmoor and Hohenlieth. Bars indicated 95 % confidence interval.*

In this study, untreated plants developed higher leaf infestation in the beginning of winter/spring but after passage of the time, old leaves had been removed and new leaves appeared. The seed bio-priming with *S. plymuthica* was unable to reduce natural infection of *Phoma lingam* in new leaves of Visby cultivar grown at both sites, Birkenmoor and Hohenlieth (Fig. 9; P= 0.0998).

Bio-priming with wild type of the bacterial strain was not included in this study. Further experimentation using the wild type of *S. plymuthica* HRO-C48 will confirm its bio-control potential against *Verticillium* or *Phoma* as it is possible that the rifampicin labelling reduced its rhizosphere competence, colonization and antagonistic ability in the soil environment. Abuamsha *et al.* (2010) claimed that the seed treatment with *S. plymuthica* HRO-C48 can minimize the incidence of disease and thus become a cost effective control measure to reduce

yield losses due to infection by blackleg pathogen, *Phoma*. According to them, the effect of microorganisms is not limited to certain cultivars and therefore they can probably be used successfully also in other OSR cultivars. A tendency of reduced disease incidence in four cultivars in my study indicates need for further studies to identify factors involved in effective control of fungal infection when treated with the rhizobacteria. A seed treatment with *Serratia plymuthica* wild type can minimize the incidence of disease and can provide protection, particularly when fungicide applications cannot be well timed to achieve maximum control (Abuamsha *et al.*, 2010)

Bacterial colonization in growth chamber

Results from estimation of bacterial colonization of roots with adhering rhizosphere soil are summarised in table 5. Only one observation of $CFUg^{-1}$ was made after sowing the bio-primed seeds, hence no statistical analysis was done. However, the results show presence of the bacterium in the four cultivars both before and after sowing. The results also indicate that the bacterium seems to have multiplied during plant growth.

Table 5: *Population size of S. plymuthica* HRO-C48^{rif} *in rhizosphere of different oilseed rape cultivars at sowing and 11 weeks after sowing.*

Cultivars	**CFU/ seed**	**CFU g^{-1} fresh root weight**
Visby	1.8×10^5	5.4×10^5
Trabant	5×10^4	6.2×10^6
KWS 63	4×10^5	6×10^6
KWS 136	2×10^5	5.9×10^6

Bacterial colonization in field

As a result of seed bio-priming, bacterial root colonization in plants from the two sites; Birkenmoor and Hohenlienth were compared between the treatments HRO-C48 and Fungicides+HRO-C48 as shown in Fig. 10. In general, *Serratia* population were higher in plants at Birkenmoor than at Hohenlienth. No differences were, however, found in both treatments at Birkenmoor (P =0.4361) or at Hohenlieth (P =0.6997). Population of *Serratia* seems to be slightly higher in presence of fungicidal treatment.

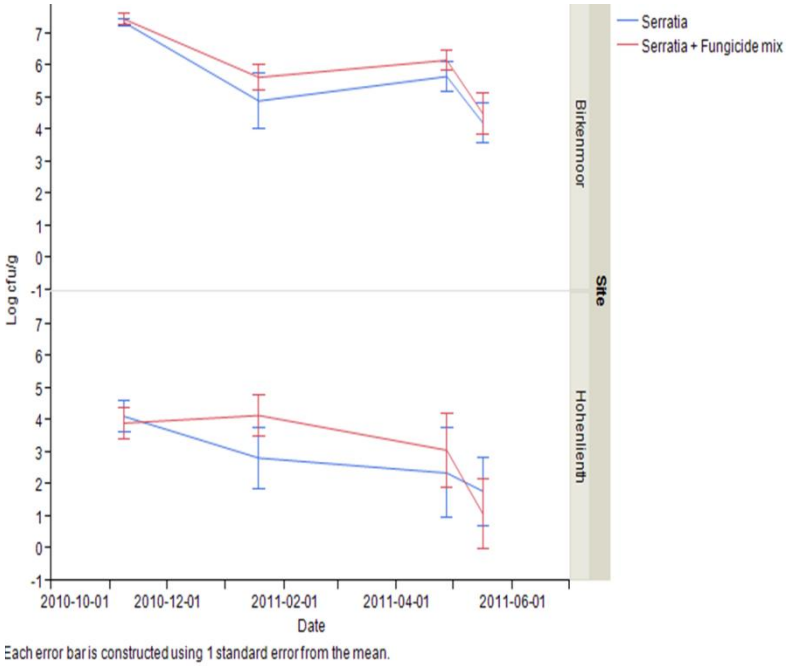

Figure 10. Bacterial populations in oilseed winter rape cv. Visby plants bio-primed with S. plymuthica *HRO-C48^{rif} with and without fungicides in field trials at Birkenmoor (P =0.4361) and Hohenlieth (P =0.6997)*

Conclusions

In conclusion, all four cultivars got infected with *V. longisporum* in growth chamber experiments. Furthermore, they showed typical wilt symptoms and the plant heights were reduced. Natural infection of *Phoma* occurred in the Visby plants at both experimental field sites. Seed bio-priming with *S. plymuthica* HRO-C48 ^{rif} gave 10^5 CFU/seed. *S. plymuthica* HRO-C48 ^{rif} was possible to re-isolate on rifampicin amended medium. More studies are needed to determine its population size in the rhizosphere as only one observation was made in growth chamber experiment and therefore results are not conclusive. Bio-priming with *S. plymuthica* HRO-C48 ^{rif} strain was unable to reduce the disease severity due to *V. longisporum* in all cultivars and natural infection by *Phoma* in the tested cultivar.

Effect of bio-priming with *S. plymuthica* HRO-C48 ^{rif} in combination with fungicides had inconsistent protective effect against *Phoma lingam.* Furthermore, *S. plymuthica* ^{rif} application seems to be compatible with fungicide application. Population of *S. plymuthica* ^{rif} differed between the two field sites included in the study and it declined over time in plots with or without fungicides in field. As regards protection ability of *S. plymuthica*, the study needs to be repeated with its wild type strain in order to draw safe conclusions about its plant beneficial ability.

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