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Promotion of growth, health and stress tolerance of Styrian oil pumpkins by bacterial endophytes

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Abstract Substantial yield losses of Styrian oil pumpkin caused by the fungus *Didymella bryoniae* and bacterial pathogens were recently reported. Here we applied bacterial endophytes with a broad antagonistic activity to pumpkin plants by seed priming. Effects of the bacterial inoculants with and without chemical seed treatments on plant growth and health were evaluated during three different field trials in two consecutive years (2010 and 2011). Biological seed treatments strongly supported the germination of pumpkin seeds. In 2010, the germination of the biologically treated seeds was comparable to the rate following a chemical treatment; whilst in 2011 effects of biological seed treatments were more obvious, including an increased emergence rate up to 109 % by *Serratia plymuthica* S13. Furthermore, tolerance against desiccation stress was observed for *Serratia* as well as for *Lysobacter gummosus* L101 treatment. The biological treatment showed different effects against fungal diseases: no effect on fruit rot was observed, whereas powdery mildew could be significantly suppressed by *Paenibacillus polymyxa* PB71 and *L. gummosus* L101 in 2010. In addition, both strains led to reproducible increases in harvest yields. In this study, we found bacterial endophytes suitable as inoculants for plant growth promotion, biocontrol, as well as for enhancing stress tolerance in Styrian oil pumpkins.

Keywords Antagonist · Biocontrol · *Didymella bryoniae* · Field trial · Fruit rot · Pumpkin diseases

Abbreviations
cw calendar week

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Introduction

During the last years, Styrian oil pumpkin (*Cucurbita pepo* L. subsp. *pepo* var. *styriaca* Greb.; Cucurbitaceae) has become an important oil crop world-wide. The cultivar originated from a natural mutation, which

was first recognized in the 19th century in the Austrian region of Styria (Teppner 2000). Owing to the absence of woody outer hulls, seeds are usable for the bakery industry and especially valued for the production of a unique, dark-green seed oil. Pumpkin seed oil is of traditional use locally but has become popular in gourmet cuisines world-wide. The oil is also known to prevent from atherosclerosis, lower urinary tract symptoms, benign prostatic hyperplasia, and to positively regulate the cholesterol level (Dreikorn 2002; Fruehwirth and Hermetter 2007). These effects have been attributed to the content of bioactive ingredients such as unsaturated fatty acids, vitamins, phytosterols, minerals and polyphenols.

For a long time, cultivation of oil pumpkins was only of regional importance in Styria but now there is an increasing production of this crop in several Southern European and African countries, China, Russia, and the USA. During the last years, substantial yield losses were reported in Styria due to fruit rot caused by the fungus *Didymella bryoniae* and the bacterial pathogen *Pectobacterium carotovorum* (Huss et al. 2007; Huss et al. 2009). Vegetative plant parts are affected by the Zucchini Yellow Mosaic Virus (ZYMV), *Pseudomonas* spp., *Xanthomonas cucurbitae* and *D. bryoniae* as well (Huss 2007; Huss and Mavridis 2007; Huss and Winkler 2009; Huss 2011). Powdery mildew can lead to significant damages on leaves especially when oil pumpkin plants are affected in an early developmental stage (personal communication, H. Huss); and desiccation stress that affected crop plants negatively in Central Europe could play an even more important role in future due to the effects of climate change (Trnka et al. 2010). Conventional methods, e.g. use of partly resistant cultivars (e.g. cv. Gl. Diamant against black rot), pesticides like Metalaxyl-M or Fludioxonil, and crop rotation exist for diminishing the degree of pathogen infestation for oil pumpkin. In addition to the conventional agricultural practice, there is demand for environmentally friendly and efficient solutions to improve health of oil pumpkin in Austria. Furthermore, this request will be underlined by the proposed complete ban of copper-based fungicides in the European Union for organic farming.

Biological control agents (BCAs) and plant growth promoting bacteria (PGPB) offer the possibility to enhance plant growth and health. PGPB serve plants directly by supplying nutrients, e.g. via the fixation of atmospheric nitrogen (N_2), phosphorous (P) solubilization, phytohormone synthesis (e.g. indole-3-acetic acid, IAA), and by lowering the host ethylene level due to ACC (1-aminocyclopropane-1-carboxylate) deaminase activity (Lugtenberg and Kamilova 2009). BCAs increase plant health by antagonizing pathogens via different modes of action: the production of antibiotics and extracellular enzymes, pathogen cell signal interference, predation and parasitism, and competition for nutrients (especially for iron) and niches. Furthermore, they can support the host immune response by induced systemic resistance (Compant et al. 2005; Berg 2009; Lugtenberg and Kamilova 2009). Bacterial endophytes undergo an intimate and effective interaction with the host plant and should be considered as beneficial inoculants for plants (Sessitsch et al. 2005; Berg and Hallmann 2006). However, only a few products on endophytic strains such as *Pseudomonas trivialis* (Salavida©) are already on the market (Mei and Flinn 2010). In a previous study, we have analyzed endophytic microbial communities from seeds (spermosphere), roots (endorhiza), flowers (anthosphere), and fruits (carposphere), of three different pumpkin cultivars. In all microenvironments potential bacterial antagonists were present (Fürnkranz et al. 2012). Six broad-spectrum antagonists were selected according to their in vitro antagonism against the oil pumpkin pathogens *D. bryoniae*, *P. carotovorum*, *P. viridiflava* and *X. cucurbitae*, and their genotypic dissimilarity, whereas five of them were active under greenhouse conditions against gummy stem blight (Fürnkranz et al. 2012).

The objective of this study was to evaluate the most promising oil pumpkin derived biocontrol agents selected in our previous work, namely *Lysobacter gummosus* L101 (endorhiza), *Paenibacillus polymyxa* PB71 (spermosphere), *Pseudomonas chlororaphis* P34 (endorhiza), and *Serratia plymuthica* S13 (anthosphere), regarding their effect on plant growth, health and stress tolerance under field conditions. Oil pumpkin plants were inoculated with bacterial strains by seed priming with and without the addition of chemical seed treatment. The effect of the different treatments on germination rate, desiccation stress tolerance, harvest yield, and 100-corn weight was assessed during a field study in the vegetation period 2010 and two field trials in 2011. As fruit rot was a serious problem for the cultivation of oil pumpkin in Styria in recent years and a high degree of infestation by powdery mildew was noticed in 2010 and 2011, the effect of bacterial treatments against pumpkin rot and

the leaf disease was additionally monitored. The overall performance of tested bacteria *ad planta* was evaluated and finally three strains were selected and suggested as biological inoculants.

Materials and methods

Bacterial broad-spectrum antagonists

The bacterial broad-spectrum antagonists investigated in this study were: *Pseudomonas chlororaphis* P34 (HQ163911), *Serratia plymuthica* S13 (HQ163914), *Paenibacillus polymyxa* PB71 (HQ163909) and *Lysobacter gummosus* L101 (HQ163910). They were isolated from different microhabitats of Styrian oil pumpkin: *P. chlororaphis* P34 and *L. gummosus* L101 from the root endosphere, *S. plymuthica* S13 from the female flower and *P. polymyxa* PB71 was found seed-borne. These strains have the capacity to suppress growth of the oil pumpkin pathogens *Didymella bryoniae* A-220-2b, and at least two of the bacterial strains *Pectobacterium carotovorum* subsp. *atrosepticum* 25-2, *Pseudomonas viridiflava* 2 d1, and *Xanthomonas cucurbitae* 6 h4 in vitro (Fürnkranz et al. 2012). The strains are stored in the Culture Collection of Antagonistic Microorganisms (SCAM) at Graz University of Technology in LB medium containing 15 % glycerol at -70°.

Seed priming

Cells were grown in LB medium at 30 °C under agitation for 3–9 h (until a favoured cell density of each strain was reached), and centrifuged for 15 min at 8.850×g before pellets were resuspended in 0.85 % NaCl. For field trial I, the concentrations of cells were adjusted to 4×10^8 colony forming units (CFU) ml^{-1} and seeds were primed in these suspensions for 8 h under agitation. For the control treatment, seeds were primed with sterile 0.85 % NaCl. One part of the seeds primed with the four bacterial suspensions was additionally treated with the chemical stripper Maxim® XL [2.4 % (w/v) Fludioxonil, 1.0 % Metalaxyl-M]. Per 1 kg seeds, 56 ml Sacrust® SK 76 including 2.2 ml Maxim® XL was used. Treated seeds were immediately sown. For field trials II and III, seeds were immersed for 9.5 h in bacterial suspensions containing 2.6×10^8 CFU ml^{-1}. The control treatment was performed as described above. In contrast to the procedure for field trial I, seeds were dried after priming in a climate chamber (Binder, KWBF 720) at 25 °C until a residual moisture content of about 8 % was reached. For field trials II and III, seeds treated with *L. gummosus* 101, *P. chlororaphis* P34, and *P. polymyxa* PB71 were sown only in addition with the chemical stripper (as described above) in contrast to *S. plymuthica* S13 that was applied without Maxim XL as well. This strategy was developed according to the results obtained from the field trial I in 2010: a high seedling emergence by *S. plymuthica* S13 was observed that could compensate the chemical treatment. An overview of the different treatments is given in Table 1.

Endophytic life-style of *S. plymuthica* S13

In contrast to the other tested broad-spectrum antagonists, *S. plymuthica* S13 was not isolated from the endosphere originally. Therefore, its capability for an endophytic life-style was analyzed. Oil pumpkin seeds (GL Opal) were inoculated with the strain as described above for field trial I and resultant plants were grown until the two-leaf stage under greenhouse conditions (Fürnkranz et al. 2012). Subsequently, roots, shoots, and cotyledons were sampled. Surface-sterilized roots were washed with tap water and placed in 5 % NaOCl for two min with stirring (shoots and cotyledons were treated with NaOCl without washing with tap water). The plant materials were then washed three times with sterile water, dipped into 70 % ethanol and flamed. Sterility checks of the superficial tissues were accomplished on agar plates with Luria-Bertani (LB) medium (Roth, Karlsruhe, Germany) that were incubated for 24 h at 30 °C overnight. Subsequently, treated plant parts were homogenized with mortar and pestle in 0.85 % NaCl. Out of resulting suspensions, dilution series were prepared and plated onto LB plates that were incubated at 30 °C for 24 h. Molecular fingerprints of the colonies were compared with the fingerprint of the inoculum by BOX-PCR (Rademaker and de Bruijn 1997) (Figure ESM4).

Field designs

Field trial I in Gleisdorf (2010)

In Gleisdorf (47°7′1″ N, 15°42′2″ E), the soil can be described as gleyed loose brown earth, loamy silt, and

Table 1 Overview of the evaluated parameters for different broad-spectrum antagonist treatments (including control treatments) in the course of oil pumpkin field trials at different field sites and vegetation periods

Treatments	Evaluated parameters tested at different field trials (site and vegetation period) with (+) and without (−) the addition of the chemical stripper Maxim XL					
	Field trial I (Gleisdorf 2010)		Field trial II (Flöcking 2011)		Field trial III (Gabersdorf 2011)	
	+	−	+	−	+	−
Control	1–6	1–6	1–6	1–6	1–4	n.e.p.
S. plymuthica S13	1–6	1–6	1–6	1–6	1–4	n.e.p.
P. polymyxa PB71	1–6	1–6	1–6	n.e.p.	1–4	n.e.p.
L. gummosus L101	1–6	1–6	1–6	n.e.p.	1–4	n.e.p.
P. chlororaphis P34	1–6	1–6	1–6	n.e.p.	n.e.p.	n.e.p.

1 germination rate, *2* harvest yield, *3* 100-corn weight, *4* fruit rot, *5* desiccation stress tolerance, *6* mildew infestation, *n.e.p* no evaluated parameter

cover loams on quaternary terrace, which were neutral to slightly acidic and deficient in lime. According to the records, 116 kg ha^{-1} mineral fertilizer (DC Rot) including nitrogen (10 %), phosphorus (8 %), and potassium (20 %) were applied on the test field in spring 2010 (before sowing). Seeds were seeded in three replicate plots per treatment (organized in a completely randomized plot design) in always two rows per plot. Eight seeds per row were seeded on 4th of May [calendar week (cw) 18] and the distance between two plants was 40 cm (240 seeds were sown per field trial I). In the 1st rows chemically treated seeds were placed, whereas in the 2nd rows seeds without chemical stripper were seeded. A few hours after the sowing, the field was treated with herbicides (Dual Gold, Centium, Flexidor). Plants were grown without artificial irrigation for 154 days.

Field trial II in Flöcking and field trial III in Gabersdorf (2011)

In Flöcking (47°5′3″ N, 15°40′2″ E), the soil was the same type as described above for field trial I. In autumn of 2010 the field was treated with cow dung (40.000 kg ha^{-1}) and 116 kg ha^{-1} mineral fertilizer (as described for field trial I). The seed was sown on 6th of May (cw 18). In Gabersdorf (46°47′22″ N, 15°35′ 37″ E), the soil was profound loose sediment brown earth. Organic and mineral fertilizers were not applied here, neither in 2010 nor 2011. The sowing was made on 27th of April (cw 17). Immediately and 5 days after the seed a herbicide treatment with the same substances used for field trial I was performed for field trials II and III respectively. Seeds were seeded in three replicate plots per treatment (organized in a completely randomized plot design). In each replicate plot seeds were seeded in five rows, and each row comprised 30 seeds (in a distance of 20 cm between seeds). Overall, 3.150 seeds were sown per field trial II and III respectively. After assessment of germination rates the number of plants was reduced to 15 per row (40 cm of distance between plants). Chemically treated and chemically untreated variants were sown in separate plots (in contrast to field trial I). Plants were grown without artificial irrigation for 125 days in Flöcking and 139 days in Gabersdorf.

Parameters assessed during field trials

The following criteria were evaluated for field trials I - III: germination rate (cw 20, 21, and 22 at field trials I, II, and III respectively), degree of fruit rot and dry weight of harvested seeds (cw 36, 37, and 40 at field trials II, III, and I respectively), and 100-corn dry weight of harvested seeds [always for each treatment, replicate plot (and plant row for field trial I)]. Due to a heavy crow grub (Figure ESM2) at field trial III, especially on the *P. chlororaphis* P34 variant (plus chemical stripper) and the non-chemical *S. plymuthica* S13 respectively control treatment paired with a low germination of these variants *per se*, they were excluded from observation in Gabersdorf. For evaluation of degrees of fruit rot and harvest yields for field trials II and III, only pumpkins from rows 2–4

(described above) were included. The number of rotten pumpkins was assessed and set in relation to the total number of pumpkins. For determination of harvest yields per treatment and replicate plot, seeds were mechanically extracted from pumpkins, dried and weighted. For field trials I and II desiccation stress resistance (in cw 27 at both field sites) and degree of infestation of leaves by powdery mildew (*Sphaerotheca fuliginea*; cw 27 and 30 at field trials II and I respectively) was observed as well. Level of infestation by powdery mildew and extent of desiccation stress (percentage of leaf area representing less turgor) was estimated always for each plant row for field trial I and for each replicate plot for field trial II (field designs are described above) by observing the respective leaf areas from the top of a ladder (Figure ESM3). The degree of mildew infestation was described either as the percentage of leaf area covered by the pathogen (for field trial I) or by scoring the infestation severity by conferring the grades 0 (until 10 % of leaf are infested), 1 (10–20 % of leaf are infested), 2 (20–30 % of leaf area infested), or 3 (> 30 % of leaf area infested) (for field trial II). An overview of the different evaluated parameters for the different treatments is presented in Table 1.

Climate data

Data for precipitation and temperature (maximal day temperatures) were registered for each day during the oil pumpkin vegetation periods in 2010 and 2011 in Gleisdorf (47°7′1″ N, 15°42′ 23″ E) and provided by ZAMG (Zentralanstalt für Meteorologie und Geodynamik, Vienna; http://www.zamg.ac.at/). The obtained recordings are representative for the conducted field trials I and II and are shown in Figure ESM1.

Statistics

Analysis of Variance (ANOVA) in addition with Games-Howell and Duncan's post-hoc tests ($P<0.1$, 0.05, 0.01) was performed with Statistical Product and Service Solutions for Windows, Rel. 11.5.1 (SPSS Inc.) to compare mean values of evaluated parameters indicating enhancement of plant growth and health.

Results

Endophytic life-style of *S. plymuthica* S13

BOX-PCR fingerprint patterns from bacteria, isolated from surface sterilized roots, shoots, and cotyledons that developed from oil pumpkin seeds that were primed with *S. plymuthica* S13, were identical to the band pattern of the inoculum (Figure ESM4). This demonstrates the capability of *S. plymuthica* S13 to live as an endophyte in oil pumpkin. The other test strains used in this study were originally isolated from the endosphere of oil pumpkin.

Parameters monitored during field trials

Germination rate

In order to assess plant growth promoting effects on Styrian oil pumpkin by selected broad-spectrum antagonists, germination rates of seeds treated with bacterial suspensions with and without addition of chemicals were monitored, compared to control treatments, and are summarized in Fig. 1 (and Table ESM1). In field trial I, bacterial inoculation (without chemical treatment) increased germination rate by 27 to 47 % compared to the non-inoculated control and could compensate the effect of the chemical stripper as it led to an equal or even better emergence of seedlings. Application of both bacteria and fungicides showed minor cumulative effects. The highest increase of germination rates were recognized for seeds inoculated with *S. plymuthica* S13 and *P. chlororaphis* P34 (always 20 %). In comparison to field trial I, inoculation with *S. plymuthica S13* (without chemical treatment) led to a considerable increase in germination (109 %) compared to the non-inoculated control in field trial II. Results from combined applications of bacteria and chemicals were similar to the results from the control, treated solely with the chemical stripper. In field trial III, the effect of *S. plymuthica* S13 applied without chemical stripper, as well as chemically treated seeds inoculated with *P. chlororaphis* P34, could not be monitored as crows fed on those seeds. In combination with chemical coatings, the highest germination rates were observed for *S. plymuthica* S13 (75.6 %) followed by *L. gummosus* L101 (72.0 %) and *P.*

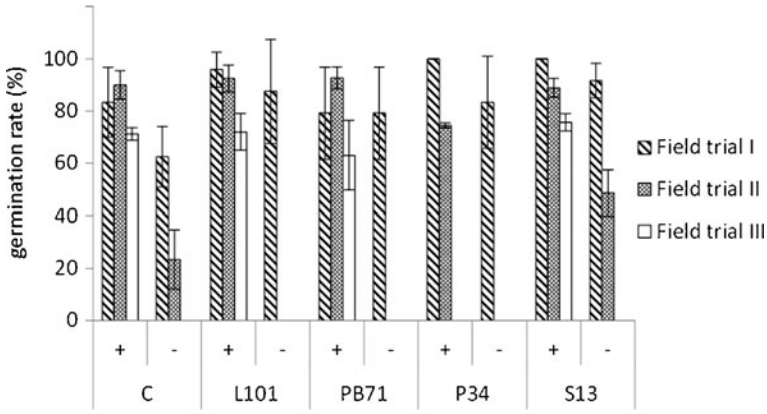

Fig. 1 Mean values of germination rates (%) of Styrian oil pumpkin after seed priming with selected broad-spectrum antagonists *L. gummosus* L101, *P. chlororaphis* P34, *P. polymyxa* PB71, and *S. plymuthica* S13 (C=control treatments) with (+) and without (−) addition of a chemical stripper at different field sites. Error bars indicate 95 % confidence intervals

polymyxa PB71 (63.0 %), and were comparable with the respective non-bacterized control.

Dry weight of harvested seeds (harvest yield)

Harvest yield, the economically most important parameter, was monitored as follows. Mean values per treatment and replicate plot were evaluated (Fig. 2). Compared to the controls, combined treatments with bacteria and chemicals resulted in similar or higher harvest yields throughout the field trials. For plants inoculated with *L. gummosus* L101, increases of harvest yields by 26 and 20 % (in comparison to the chemically treated controls) were observed in field trial II and III, respectively. The increment was statistically significant for field trial III (Duncan's multiple range test, $P<0.1$). Inoculation with *P. polymyxa* PB71 resulted in higher yields in field trials I, II and III (+ 12 %, 20 %, and 15 % respectively, compared to the chemically treated controls). In case of the chemically untreated variants, the greatest increase in harvest yield (in comparison to the respective control) was demonstrated for *S. plymuthica* S13 by 40.3 % in field trial II; the effect was statistically significant ($P<0.1$, Games-Howell test).

100-corn weight

The 1000-corn weight is of particular importance for extraction of oil from pumpkin seeds. For our field trials, not the 1000-corn weights but the weights of 100 seeds per treatment and replicate plot were compared. In field trial I, the chemical and non-chemical variants of the *P. polymyxa* PB71 treatment revealed highest increases by 8.7 % (21.68 g) and 5.0 % (20.30 g) respectively, compared to the corresponding controls. The remaining chemical variants at this field site led to 19.32 g (*L. gummosus* L101), 19.43 g

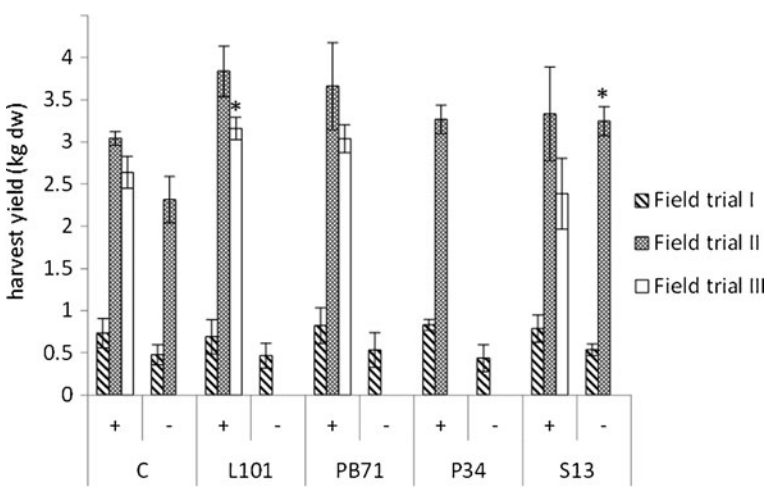

Fig. 2 Mean values for harvest yields (kg dry weight) after seed treatments with selected broad-spectrum antagonists *L. gummosus* L101, *P. polymyxa* PB71, *S. plymuthica* S13, and *P. chlororaphis* P34 (C=control treatments) with (+) and without (−) the addition of a chemical stripper at different field sites. Error bars signify 95 % confidence intervals. *significant differences compared to respective control treatments (Games-Howell and Duncan's post-hoc tests, $P<0.1$)

(*S. plymuthica* S13), and 19.97 g (*P. chlororaphis* P34), whereas the non-chemical treatments exhibited 19.72 g (*S. plymuthica* S13), 20.00 g (*P. chlororaphis* P34), and 20.27 g (*L. gummosus* L101). No considerable increases (> 5 %) in 100-corn weights were observed for inoculants at field trials II (Table 2) and III (data not shown). Values from chemical variants at field trial II were: 17.27 g for the control, 17.35 g for *L. gummosus* L101, 17.37 g for *P. chlororaphis* P34, 17.51 g for *P. polymyxa* PB71, and 17.69 g for *S. plymuthica* S13. The chemically untreated *S. plymuthica* S13 and control variant resulted in 17.68 and 18.12 g respectively. At field trial III, treatment with *L. gummosus* L101 showed the highest 100-corn weight (22.52 g), followed by *P. polymyxa* PB71 (21.78 g), the control (21.67 g), and *S. plymuthica* S13 (20.97 g).

Biocontrol of fruit rot

The causal agents of the observed fruit rot were black rot (*D. bryoniae*), soft rot (*P. carotovorum*), and rotting due to *Sclerotinia sclerotiorum* especially in field trial II. Across the different field trials, no statistical effect of bacterial treatments on pumpkin fruit health could be investigated. In field trial I, the proportions of rotten pumpkins throughout the chemically treated variants were 5.0 % for *S. plymuthica* S13, 5.4 % for the control, 6.1 % for *P. chlororaphis* P34, 10.0 % for *P. polymyxa* PB71, and 12.2 % for *L. gummosus* L101. The degrees of rotten fruits within the chemically untreated alternatives in field trial I were: 2.7 % for *S. plymuthica* S13, 3.0 % for *P. chlororaphis* P34, 6.7 % for *P. polymyxa* PB71, 12.9 % for the control, and 15.2 % for *L. gummosus* L101. In field trial II, amounts of rotten pumpkins from chemically treated variants were 2.9 % (*L. gummosus* L101), 4.8 % (control), 7.8 % (*P. chlororaphis* P34), 8.7 % (*P. polymyxa* PB71), and 13.2 % (*S. plymuthica* S13). The chemically untreated variants of *S. plymuthica* S13 and the control exhibited 3.3 % and 4.4 % respectively. For field trial III, the lowest degree of fruit rot after chemical seed treatment was found for the control (1.2 %), followed by *P. polymyxa* PB71 (1.3 %), *S. plymuthica* S13 (1.9 %), and *L. gummosus* L101 (2.3 %).

Biocontrol of powdery mildew

Leaf infestation with powdery mildew occurred in both vegetation periods. In 2010 (field trial I) it was even more pronounced due to a very intense rain event (511 mm) in cw 29 (Figure ESM1). In field trial I, the chemically treated variants exhibited a lower degree of

Table 2 Summary of the performance of selected broad-spectrum antagonists regarding promotion of growth and health of Styrian oil pumpkin after seed priming in the course of field trials I and II

Field trial	Bacterial strain	Increase in plant growth/health compared to the control treatment					Sum of weighted[a] plus
		Germination rate	Harvest yield	100-corn weight	Reduction of infestation by mildew	Increase in desiccation stress tolerance	
I	*S. plymuthica* S13	+	+	-	+	+	15
		++	+	-	+	-	
II		-	+	-	-	++	
		+++	++*	-	-	+++*	
I	*P. chlororaphis* P34	+	+	-	-	-	5
		++	-	-	-	-	
II		-	+	-	-	++	
I	*P. polymyxa* PB71	-	+	+	++***	-	6
		+	+	+	++	+	
II		-	+	-	-	-	
I	*L. gummosus* L101	+	-	-	++*	-	6
		++	-	-	+	-	
II		-	+	-	-	+++**	

White and grey fields indicate treatments with and without addition of the chemical stripper Maxim XL respectively

*, **, *** significant differences between respective mean values and means of corresponding control treatments (Duncan`s and Games-Howell post-hoc tests, $P<0.1$, 0.05 and 0.01 respectively)

-increase in plant growth/health less than 5 %; +, ++, +++increase in plant growth/health from: 5–30 % (+), 30–60 % (++) and >60 % (+++) respectively; [a] sum of plus from field trial I were halved due to the lower amount of plants in comparison to field trial II

mildew infestation. Lowest incidence of the pathogen on leaves was recorded for chemically treated variants of *P. polymyxa* PB71 (26.7 % of infested leaf area) and *L. gummosus* L101 (53.3 % of infested leaf area) that was statistically different to the respective control treatment (Duncan's multiple range test, $P<0.01$ and 0.1 respectively) (Fig. 3 and Figure ESM5). For field trial II, no effects on reduction of mildew by tested broad-spectrum antagonists were observed (Table 2 and Figure ESM6).

Desiccation stress tolerance

During both vegetation periods, the oil pumpkin plants showed signs for desiccation stress (Figure ESM3) due to relative dry periods (small amount of rainfall) in cw 26 and 27 in 2010 and 2011 (Figure ESM1), therefore the occurrence of desiccation stress symptoms on oil pumpkin plants was estimated (summarized in Table 2). Statistically significant effects were observed in field trial II by the chemical variant of the *L. gummosus* L101 treatment (Duncan's multiple range test, $P<0.05$), leading to a 100 % desiccation stress reduction compared to the respective control (0.0 % in contrast to 6.7 % of leaf area showed desiccation stress symptoms), and by the chemically untreated variant of *S. plymuthica* S13 (Duncan's multiple range test, $P<0.1$), conferring a 75 % reduction of desiccation stress in comparison to the respective control treatment by the representation of an affected leaf area of 1.7 %. Treatments *S. plymuthica* S13 and *P. chlororaphis* P34 (both chemically treated) led to 3.3 % of leaf area with less turgor, whereas 6.7 % of leaf area lacking a healthy turgor was observed for the *P. polymyxa* PB71 treatment. In field trial I, the lowest affected leaf area (23.3 %) was observed for chemically stained *S. plymuthica* S13, followed chemically stained *P. chlororaphis* P34, *P. polymyxa* PB71, and control treatment (always 26.7 %), followed by the chemically unstained *P. polymyxa* PB71 treatment (30.0 %), followed by chemically unstained *S. plymuthica* S13, *P. chlororaphis* P34 and control treatment (always 33.3 %). 36.7 % and 40.0 % of turgor-less leaf areas were found for the chemically unstained and stained *L. gummosus* L101 treatment respectively.

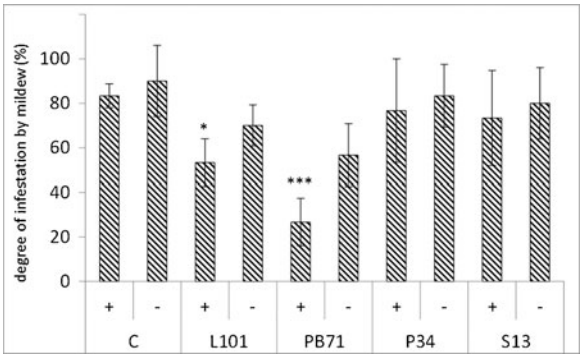

Fig. 3 Mean values for leaf area of Styrian oil pumpkin infested with powdery mildew (in %) after seed priming with selected broad-spectrum antagonists *L. gummosus* L101, *P. chlororaphis* P34, *P. polymyxa* PB71, and *S. plymuthica* S13 (C=control treatments) with (+) and without (−) the addition of a chemical stripper at field trial I. Error bars indicate 95 % confidence intervals. *, ***significant differences compared to the respective control treatment (Duncan's multiple range test, $P<0.1$ and 0.01 respectively)

Overall performance of broad-spectrum antagonists ad planta

In order to select most promising antagonists for the development of a biological product for Styrian oil pumpkin, performances *ad planta* of the four test strains were compared. According to our evaluation scheme, *S. plymuthica* S13 exhibited the best overall performance (Table 2). *P. polymyxa* PB71 and *L. gummosus* L101 showed reproducible increases in harvest yields throughout different field trials II and III (Fig. 2) and reached the second positions in the evaluation scheme (see Table 2), whereas *P. chlororaphis* P34 had the least positive effect.

Observation of climate data

Amount of precipitation and temperature fluctuations were registered for field trials I and II in 2010 and 2011 (Figure ESM1) in order to explain differences in monitored parameters among the different vegetation periods. The maximum day temperatures in the vegetation period 2010 ranged from 11.5 °C to 34.0 °C with an average of 22.5 °C, whereas in vegetation period 2011 the temperature range was from 15.5–33.4 °C (25.0 °C in average). In 2010, an intense rain event (511 mm) correlated with a high infestation of oil pumpkin plants with powdery mildew (as described above). In 2011, the highest amount of precipitation was measured in cw 23 (460 mm). In cw 26 and 27, relative small amounts of rainfall were noticed in

both vegetation periods, leading to the occurrence of desiccation stress symptoms on oil pumpkin plants (as described above).

Discussion

The selection of antagonists by in vitro assays is usually a straightforward procedure. To find BCAs that succeed under practical conditions in agriculture is actually much more complex and represents one of the main hurdles in the commercialization process (Berg 2009). Discrepancies between the antagonistic effects under in vitro conditions and the corresponding *in situ* efficacy are frequently reported (Weller and Cook 1983; Reddy et al. 1993). We revealed that three out of four bacteria, selected in a comprehensive, ecology-based study including greenhouse trials (Fürnkranz et al. 2012), were active under field conditions. Interestingly, we found that the strains showed differential activities: while *Serratia plymuthica* S13 appeared as a PGPB, and was particularly suitable to promote the germination of pumpkin seeds, *Paenibacillus polymyxa* PB71 acted as a BCA against powdery mildew infection. Furthermore, with *S. plymuthica* S13 and *Lysobacter gummosus* L101 rarely reported agents against abiotic stress were observed. However, considering all three performed field trials, differences in the efficacies of tested bacteria were found.

The highest variations were observed with respect to germination of pumpkin seeds. In field trial I, the effect of the chemical treatment on germination could be compensated by three of the four tested bacteria, whereas results from field trial II showed that the emergence of seedlings of the chemically non-treated variants was much lower compared to fungicide treated seeds. These discrepancies can be explained by the type of seed batch used for the priming procedure for field trials in 2011. The immersion bath negatively affected the cuticles due to the low seed quality, leading to a minor germination capacity. The different environmental conditions in vegetation periods 2010 and 2011 had probably a minor influence on observed germination rates. However, when the negative effect of priming on oil pumpkin seeds was subtracted from the beneficial effect on seedling emergence by the tested bacteria, oil pumpkin seed germination was increased up to 109 % (by *S. plymuthica* S13 at field trial II). We therefore suggest the exploration of other application methods than seed priming for *S. plymuthica* S13 (Müller and Berg 2008) in order to develop a biological alternative for conventional fungicides as well as copper-based formulations, essential in organic farming. For *P. polymyxa* PB71 spore-based formulations can be developed; our preliminary results indicated a high shelf-life of encapsulated spores (no decrease of the inoculum after 5 months of storage at 4 °C).

The effect of the selected bacterial strains against *D. bryoniae*, the causal agent of fruit rot, was actually of interest. Although all selected broad-spectrum antagonists showed in vitro inhibition of *D. bryoniae* (Fürnkranz et al. 2012), no biocontrol effect was observed in the field experiments. The degree of black rot depends mainly on the climatic conditions, the oil pumpkin cultivar, and field management (Huss et al. 2007; Babadoost and Zitter 2009; Huss 2011). In our study, the amount of rotten pumpkins was relatively low in comparison to other vegetation periods (Huss et al. 2007). The occurrence of affected fruits on the fields was irregular, indicating a high influence of soil characteristics, especially drainage, on black rot. These circumstances did not allow a representative evaluation of selected bacteria on the suppression of oil pumpkin rot. However, a preventative biocontrol strategy to diminish the risk for black rot and gummy stem blight, which is a world-wide problem for other crops belonging to the Cucurbitaceae (Lee et al. 1984; Sitterly and Keinath 1996; Keinath 2011), is urgently needed. In contrast to the low degree of fruit rot, powdery mildew on leaves was significant. The symptoms ranged from partly affected to fully covered leaves by the fungus. In field trial I, a statistically significant reduction of disease symptoms was observed for treatments with *L. gummosus* L101 and especially *P. polymyxa* PB71. In field trial II, the degree of mildew infestation was low due to climatic conditions, and therefore disease suppression by the bacteria was hardly assessable.

Positive effects also on the physiological status of field grown oil pumpkin plants were observed for tested bacteria. Especially *L. gummosus* L101 and *S. plymuthica* S13 were able to suppress symptoms of desiccation of pumpkin plants. Although several mechanisms of biocontrol and plant growth promotion have already been reported, e.g. for *S. plymuthica*

(Koo and Cho 2009; Müller et al. 2009), nothing is known about the relation of plant-associated bacteria and desiccation stress of plants. Due to challenges of climate change, bacterial inoculants can serve here as a promising solution. The better physiological constitution of oil pumpkin plants after bacterial inoculation was accompanied with increased harvest yields. Consistently enhanced crop yields among different field sites were obtained by *L. gummosus* L101 and *P. polymyxa* PB71 in 2011. Another parameter, which is correlated with harvest yield, is the 100-corn weight. Especially for oil extraction from pumpkin seeds, a high seed size is desirable. Here, also strain *P. polymyxa* PB71 offers potential but this effect was not observed in all field trails and should be investigated further.

In this study we found bacterial strains suitable as inoculants for plant growth promotion, biocontrol as well as for stress tolerance in Styrian oil pumpkins. Only one of the four tested strains, *P. chlororaphis* P34, showed no clear effects under field conditions. Further investigations are necessary to develop a formulation with a high shelf-life for the three promising bacteria. Although all strains belong to risk groups 1 (without risk for humans and environments), further risk assessment is important (Berg et al. 2009).

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