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Emerging multi-pathogen disease caused by *Didymella bryoniae* and pathogenic bacteria on Styrian oil pumpkin

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Abstract The Styrian oil pumpkin, *Cucurbita pepo* L. subsp. *pepo* var. *styriaca* Greb. is a crop of cultural, commercial, and medical importance. In the last decade, yield losses of pumpkins increased dramatically. The ascomycetous fungus *Didymella bryoniae* (Fuckel) Rehm was identified as main causal agent provoking gummy stem blight as well as black rot of pumpkins. We observed a remarkable phenotypic diversity of the fungal pathogen, which contrasted with a high genotypic similarity. Evidence of pathogenictiy of *D. bryoniae* on Styrian oil pumpkin was demonstrated in a newly developed greenhouse assay. Isolates representing the five observed phenotypic groups fulfilled the Koch's postulates. In the field, the fungal disease was often associated with bacterial colonization by *Pectobacterium carotovorum*, *Pseudomonas viridiflava*, *Pseudomonas syringae* and *Xanthomonas cucurbitae*. The pathogenic behaviour of bacterial isolates on pumpkin was confirmed in the greenhouse assay. The high co-incidence of fungal and bacterial disease suggests mutualistic effects in pathogenesis. With a new assay, we found that bacteria can use the mycelium of *D. bryoniae* for translocation. We argue that the rapid rise of the multi-pathogen disease of pumpkins results from combined action of versatile pathogenic bacteria and the rapid translocation on a structurally versatile mycelium of the fungal pathogen.

Keywords *Didymella bryoniae* · Black rot · Multi-pathogen disease · *Pectobacterium carotovorum* · *Pseudomonas viridiflava* · *Pseudomonas syringae* · *Xanthomonas cucurbitae* · Pumpkin · Bacterial pathogens

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During the past decade, a dramatic increase of diseases in Styrian oil pumpkin (*Cucurbita pepo* L. subsp. *pepo* var. *styriaca* Greb.) has been observed; up to 80% yield losses were reported (Huss 2007). The pumpkin cultivar 'Styrian oil pumpkin' is a phylogenetically young member of the *Cucurbitaceae* since the mutation leading to dark green seeds with stunted outer hulls arose in the 19th century

(Frühwirth and Hermetter 2007). This unique cultivar has been grown for at least 100 years in Austria but today it has much broader growing areas, including several Southern European and African countries, China, Russia, and the USA. The seed is edible and used in the bakery industry and for the production of dark-coloured seed oil. Styrian pumpkin seed oil is a regional specialty (European Union protected designation of origin) and international gourmet oil. It has an intense nutty taste and is rich in polyunsaturated fatty acids but contains also vitamins, phytosterols, minerals and polyphenols (Frühwirth and Hermetter 2007). Studies suggest usefulness of the oil in the prevention and treatment of benign prostatic hyperplasia as well as to prevent arteriosclerosis and regulate cholesterol levels (Dreikorn 2002).

As the main causal agent of black rot and gummy stem blight, *Didymella bryoniae* (Auersw.) Rehm (anamorph *Phoma cucurbitacearum*) was identified. This fungus was first reported in Europe in 1869 from *Bryonia* (wild hops) and today it is found on six continents as pathogen on a broad range of *Cucurbitaceae* (Chiu and Walker 1949; Keinath 2010). Most information about diseases caused by *D. bryoniae* focused on melons or cucumber but not yet on pumpkins. The fungus can infect any stage of plants and shows a variety of symptoms according to the crop and stage concerned; furthermore it can be seed-borne, air-borne, or soil-borne (Lee et al. 1983; van Steekelenburg 1983; Bruton 1998; Keinath 2010). Black rot is an important pre- and post-harvest fruit rot of pumpkins which seriously reduces yield and quality (Zitter and Kyle 1992; Sitterly and Keinath 1996; Babadoost and Zitter 2009). Measures to suppress pathogens in oil pumpkin cultivation include application of chemical fungicides (pyrrole derivates, phenylamide), 3-years of crop rotations and breeding strategies for resistance against *D. bryoniae*. However, due to the fact that pumpkin seeds are always harvested in the field and all residues are incorporated in soil, there is an increasing pathogen pressure in fields.

Interestingly, in our region, Styrian oil pumpkins are frequently attacked also by bacterial soft rot caused by *Pectobacterium carotovorum*. Furthermore, bacteria such as *Pseudomonas viridiflava* and *Xanthomonas cucurbitae* can affect pumpkin plants in combination with *D. bryoniae* (Huss 2011). These co-infections of *D. bryoniae* and bacterial pathogens, which colonize and destroy the whole inner pumpkin pulp, led to extremely high yield losses. Most diagnostic processes cease with the detection of the first relevant pathogen but molecular methods often show a more diverse pathogen spectrum (Crous and Gams 2000, Grube and Berg 2009). Furthermore, synergistic pathogenic effects were already reported for *D. bryoniae* (Leben 1984; Zitter and Kyle 1992). To suppress these multi-pathogen diseases, all players and their interaction have to be analyzed.

The objective of the present work was to characterize different *D. bryoniae* isolates from *C. pepo* at the phenotypic and genotypic levels. Furthermore, collected isolates were tested for their pathogenicity on oil pumpkins using a newly developed assay. In addition, also a new experimental set-up was established to study the synergistic interaction between the fungus and pathogenic bacteria.

Materials and methods

Strain collection

In 2008, *D. bryoniae* isolates were collected from diseased oil pumpkins grown in fields located in Gleisdorf (47°6′14″N, 15°42′30″E) and the district St. Peter in Graz (47°3′18″N, 15°28′30″E) located in Styria. Infected tissue was placed on water agar (water, 15 g agar l^{-1}) supplemented with chloramphenicol (100 mg l^{-1}). Presumptive colonies of *D. bryoniae* were isolated and stored at −20°C. One additional isolate of *D. bryoniae* (A-220-2b) was included (isolated from pumpkins grown in an experimental station of Bundesanstalt für alpenländische Landwirtschaft Raumberg-Gumpenstein, Stadl-Paura, 48°5′2″N, 13°51′50″E). All bacterial pathogens, *P. carotovorum* ssp. *atroseptica* 25–2, *X. cucurbitae* 6h4, *P. viridiflava* 2d1 and *P. syringae* 6g1 were originally isolated from Styrian oil pumpkins (Huss 2009; Göttinger Sammlung Phytopathogener Bakterien: GSPB, University of Göttingen, Germany). Before application, we confirmed identification by molecular analysis.

Phenotypic characterization of *D. bryoniae* isolates

Isolates of *D. bryoniae* were grown on malt agar (MA) at ambient temperature (23–25°C) in the dark for 3 days, before agar blocks from the isolates (5 mm diameter) were transferred to MA and quarter-strength potato-dextrose agar (QPDA; Roth GmbH, Karlsruhe, Germany) plates. MA plates were incubated in the dark for 7 days and the diameters of the colonies covering the agar plates were measured. Furthermore, the morphology and the colour of the colonies were assessed. Isolates on QPDA plates were grown at ambient temperature and with a photoperiod of 12 h. The isolates were characterized morphologically for three replicates of each isolate.

Genotypic characterization of *D. bryoniae* isolates

To isolate fungal DNA, all *D. bryoniae* isolates were grown on QPDA for 5 days at ambient temperature. Cultures were scraped to remove mycelium and placed in 50 ml liquid medium containing 1 g of KH_2PO_4, 0.5 g of $MgSO_4$, 6.0 g of casein hydrolysate, 100 g of sucrose, 1 mg of $FeSO_4$, 0.15 mg of $CuSO_4$, 0.10 mg of $ZnSO_4$, and 0.10 mg of $NaMoO_3$ per litre of distilled water. The medium was adjusted to pH 4.9 with 0.1 M HCl and autoclaved before inoculation. The cultures were incubated for 7 days at 23°C, at 120 rpm in the dark. Liquid cultures were then filtered through a sterile filter paper, and the mycelium was washed with sterile distilled water. After removing as much liquid as possible, mycelium was frozen and stored at −20°C. About 150 mg of the mycelia were transferred to 2 ml tubes containing glass beads (0.25–0.5 and 1.7–2.0 mm in diameter). After adding 500 µl lysing buffer (0.4 M Cetrimonium bromide (CTAB), 1 M NaCl, 7 mM Tris, 30 mM EDTA, pH 5.5), the tubes were agitated with a Fast PrepTM machine (Qbiogene BIO 101® systems, Carlsbad, CA). The tubes were incubated at 65°C for one h and mixed repeatedly. Then, 500 µl of chloroform:isoamyl alcohol (24:1) were added and vortexed briefly and centrifuged for 5 min at 13,000 × g. The supernatants were transferred to fresh tubes, and the chloroform:isoamyl alcohol step was repeated. One ml of precipitation puffer (14 mM CTAB, 40 mM NaCl) was added and incubated at room temperature for one hour. The tubes were centrifuged for 15 min at 13000 × g, supernatants were removed and pellets mixed with 350 µl 1.2 M NaCl. The treatment with chloroform:isoamyl alcohol was repeated twice. Supernatants were transferred to fresh tubes and 210 µl of isopropanol were added before samples were incubated at −20°C over night. Afterwards tubes were incubated at room temperature for 5 min and subsequently centrifuged for 20 min at 13.000 × g, DNA was washed with 70% ethanol, dried, suspended in 40 µl PCR grade water and stored at −20°C.

To obtain DNA sequences of *D. bryoniae* for analysis of genetic relationship, ITS rRNA gene region was amplified and sequenced using primers ITS 1F (5′CTT GGT CAT TTA GAG GAA GTA A 3′) and ITS 4r (5′-TCC TCC GCT TAT TGA TAT GC-3′). PCR products were purified using the peqGOLD MicroSpin Cycle Pure Kit (Peqlab, Erlangen, Germany). Purified PCR products were sequenced using the genetic analyzer AB3730 and the ABI PRISM BigDye Terminator v3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Vienna, Austria). Obtained data were subjected to BLAST search with the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov) to confirm taxonomic affiliation to *D. bryoniae (*Altschul et al. 1997). All sequences were aligned using Molecular Evolutionary Genetics Analysis (MEGA) software (Version 4.0.2). A network of relationship was constructed with the TCS program v. 1.21.

BOX-PCRs were done as described by Rademaker and De Bruijn (1997) using the BOXA1R primer 5′-CTA CGG CAA GGC GAC GCT GAC G-3′. PCR amplification was performed with a Peltier Thermal Cycler PTC-200 (Biozym Diagnostic, Hessisch Oldendorf, Germany) using an initial denaturation step at 95°C for 6 min, and subsequently 35 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min. and extension at 65°C for 8 min. followed by final extension at 65°C for 16 min A 10 µl aliquot of amplified PCR product was separated by gel electrophoresis on 1.5% agarose gels in 0.5 × TBE buffer for 6 h, stained with ethidium bromide, and photographed under UV transillumination. The reproducibility of the results was verified in at least two independent experiments.

Assay to test pathogenicty of *D. bryoniae* isolates

Oil pumpkins (*Cucurbita pepo* L. subsp. *pepo* var. *styriaca* Greb.) cultivar 'Gleisdorfer Diamant' (Saatzucht Gleisdorf, Austria) were seeded in 10 cm plastic pots filled with a soil mix containing clay (Gramoflor, Vechta, Germany). *D. bryoniae* strains SP2, SP6, SP12, GL1 and A-220-2b were grown on QPDA at ambient temperature and 12 h photoperiod until pycnidia were produced. Cultures were flooded with 0.1% sucrose-0.05% casein solution and scraped to release conidia from pycnidia. The suspension was gently mixed on a vortexer and filtered to remove mycelium of the fungus. Spore concentration was determined by counting in a haemocytometer and adjusted to 10^5 conidia per ml. Two-weeks old plants were inoculated by injecting of 50 µl spore suspension in the two first petioles. Plants inoculated with 0.1% sucrose/0.05% casein solution served as a control. The plants were covered with plastic bags for 11 days and kept at 20±2°C and about 80% humidity with a 12 h photoperiod under greenhouse conditions. Plants were assessed for development of symptoms from the 6th to 11th day after infection. Infection experiments were carried out on 12 plants per *D. bryoniae* isolate. The presence of pycnidia was checked by microscopy. The experiment was repeated independently three times. From re-isolated *D. bryoniae* strains, DNA was extracted and used as template for BOX-PCR (as described above) to compare banding patterns with those from isolates that were used for infection.

Assay to test pathogenicty of bacterial pathogens

Pathogenicity of bacterial isolates (Huss 2009) was confirmed by artificial infections of oil pumpkin plants 'GL Opal' and subsequent re-isolation of the bacterial strains: bacterial strains were grown in LB medium (Roth GmbH, Karlsruhe, Germany) at 30°C under agitation. Cells were transferred into sterile 0.15 M NaCl and counted to adjust a concentration of 1×10^8 cells ml^{-1}. Leaves were injured with a grater and suspensions as well as sterile 0.15 M NaCl (serving as a control treatment) were sprayed on the leaves. Plants were covered with plastic bags, kept at 26°C with a 12 h photoperiod and observed until 11 days after infection. Infested plant parts were homogenized with sterile NaCl (0.15 M); these suspensions were diluted and plated onto water and LB agar. Colonies from these plates were picked, streaked out and DNA was isolated from strains by cooking cells in PCR grade water for 10 min. Isolated DNAs from potential as well as applied pathogens were used for BOX-PCR (as described above). The obtained molecular fingerprints were compared with the patterns from the inoculum.

Fungal-bacterial interactions on agar medium

The system developed by Kohlmeier et al. (2005) was modified for our study on *Didymella*-bacteria interaction. One *D. bryoniae* isolate per morphogroup was grown on a disk (1.4 cm diam.) of potato dextrose agar (PDA) for 2 days. Bacterial pathogens *P. carotovorum* ssp. *atroseptica* 25–2, *X. cucurbitae* 6h4, *P. viridiflava* 2d1 and *P. syringae* 6g1 were grown O/N in Tryptic Soy Broth (TSB) (Roth GmbH, Karlsruhe, Germany) at 30°C, 120 rpm. Bacterial suspensions were centrifuged for 5 min. at 7.500 × g and resuspended in 0.15 M NaCl. Bacterial cell concentrations were determined by counting in a haemocytometer and adjusted to approximately 10^8 cells ml^{-1}. *D. bryoniae* strains grown on PDA disks were inoculated with 10 µl of bacterial suspensions and placed in 0.5–1 mm distance from a strip of QPDA (minimal medium agar; 1×3 cm) as shown in Fig. 5. The gap between the media pieces prevented swarming of motile bacteria from one agar piece to the other, whereas *D. bryoniae* could easily bridge the gap with growing hyphae. The QPDA strips colonized by the strains after 12 days of incubation were sliced and placed in 2 ml tubes. One ml of 0.15 M NaCl was added and vortexed several times to separate bacteria attached to fungal hyphae. The suspensions were plated on LB and incubated at 30°C for several days. The identity of bacterial strains was checked by their individual BOX-PCR patterns.

Statistical analysis

Analysis of Variance (ANOVA) in addition with Games-Howell post hoc test ($P<0.05$) was performed with Predictive Analysis SoftWare (PASW, Version 18.0.0) to compare diameters of *D. bryoniae* colonies.

Nucleotide sequence accession numbers

Obtained DNA sequences of *Didymella* were deposited in GenBank under accession numbers HQ684024 to HQ684032 and DNA sequences from phytopathogenic bacteria were deposited under JF906521 to JF906524.

Results

Phenotypic diversity of pumpkin-associated *D. bryoniae* isolates

Altogether nine *D. bryoniae* strains were isolated from infested pumpkins in Austria during 2008 (Table 1). Based on their morphological traits developed within 7 days in culture, these isolates could be distinguished. On MA plates, isolates SP4, SP12 and GL1 formed beige (young growth zone) and black (older zone) substrate mycelia and white hairy aerial mycelia (Fig. 1). Isolates SP1 and SP6 formed beige to reddish-brown (young growth zone) or black substrate mycelia, and the same aerial mycelia as the first group. Isolates SP2, SP9 and SP3 formed beige substrate mycelia with only a small part of black admixture; their aerial mycelia were white and fluffy-hairy. Isolate A-220-2b produced beige substrate mycelium and white aerial mycelium. On the basis of the observed differences, the isolates were grouped into four morphological groups; group I: SP4, SP12 and GL1; group II: SP1 and SP6; group III: SP2, SP9 and SP3; group IV: A-220-2b. When isolates grew more than 2 weeks, phenotypic characteristics became more similar on MA-plates. Growing on QPDA plates during a 12 h light/dark photoperiod, all isolates produced dark green to black substrate mycelia and white aerial mycelia. Pycnidia were produced in concentric rings. The daily growth had a range between 8.5 and 13.1 mm and all *D. bryoniae* isolates reached the edges of the petri dishes within 7 days.

Genotypic diversity of pumpkin-associated *D. Bryoniae* isolates

ITS rRNA gene sequence analysis of the isolates confirmed their taxonomic affiliation to *D. bryoniae*. Differences between our sequences and *D. bryoniae* sequences from the database were analyzed with TCS program which reveal their high similarity (Fig. 2): sequences were identical with the exception of isolate SP1, which differed at two nucleotide positions. Only an isolate from grapevine EU030365.1 is distinct by 12 mismatches.

Table 1 Growth and morphology of different isolates of *D. bryoniae* grown on malt agar at 22°C

Isolate	Origin	Colony diameter (mm)[a]	Fungal colony morphology	Morpho-group
SP1	St. Peter/Graz/Styria	13.1 (a)	beige to reddish brown and black substrate mycelium, white hairy aerial mycelium	II
SP2	St. Peter/Graz/Styria	11.2 (b)	beige substrate mycelium, white fluffy hairy aerial mycelium	III
SP3	St. Peter/Graz/Styria	11.1 (abc)	beige substrate mycelium, white fluffy hairy aerial mycelium	III
SP4	St. Peter/Graz/Styria	11.7 (abc)	beige to black substrate mycelium, white hairy aerial mycelium	I
SP6	St. Peter/Graz/Styria	11.8 (ab)	beige to reddish brown and black substrate mycelium, white hairy aerial mycelium	II
SP9	St. Peter/Graz/Styria	11.0 (ab)	beige substrate mycelium, white fluffy hairy aerial mycelium	III
SP12	St. Peter/Graz/Styria	8.5 (c)	beige to black substrate mycelium, white hairy aerial mycelium	I
GL1	Gleisdorf/Styria	11.0 (ab)	beige to black substrate mycelium, white hairy aerial mycelium	I
A-220-2b	Stadl Paura/Upper Austria	12.6 (ab)	beige substrate mycelium, white aerial mycelium	IV

[a] mean values of three replicates; different letters signify significant differences (ANOVA, Games-Howell $p<0.05$)

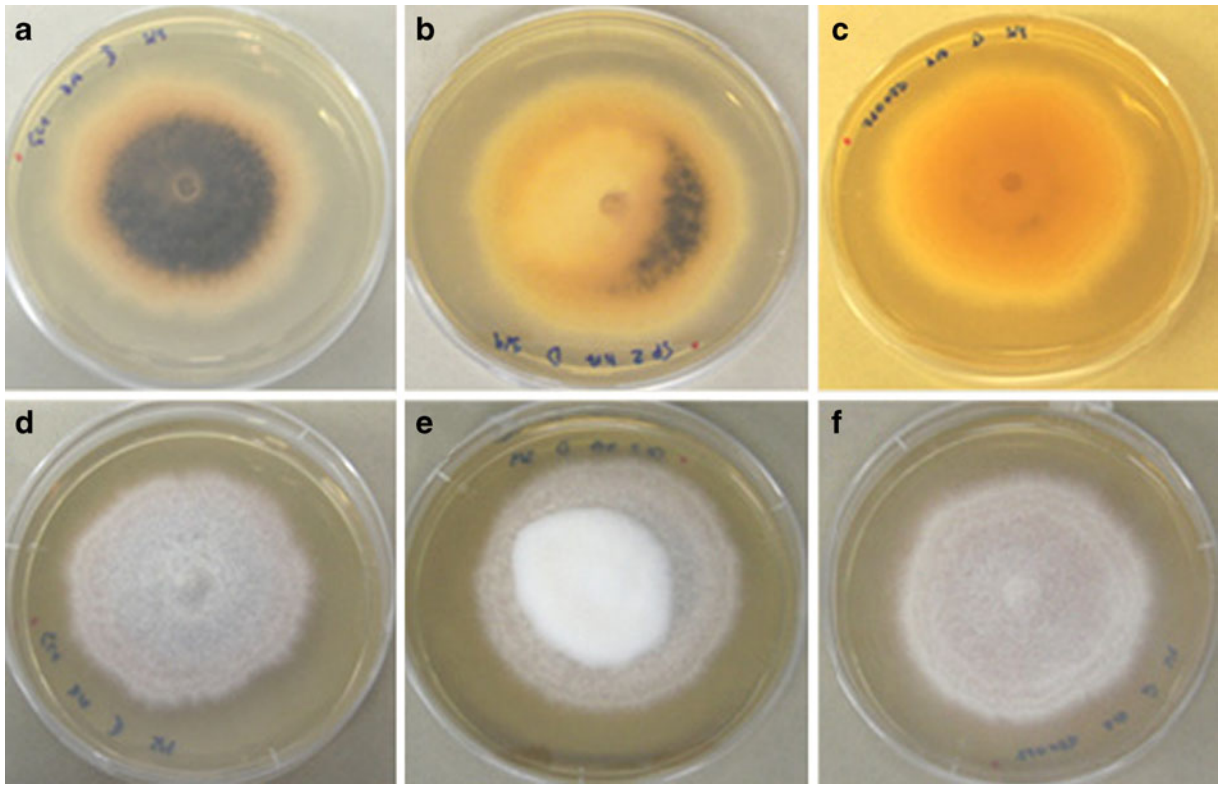

Fig. 1 Morphology of *D. bryoniae* isolates; Isolates grown on MA agar; **a**–**c**: view of cultures from below; **d**–**f**: view of cultures from top; **a** and **d**: isolate GL1 (Morphogroup I); **b** and **e**: isolate SP2 (Morphogroup II); **c** and **f**: isolate A-220-2b (Morphogroup IV)

To differentiate collected isolates at the genotypic level, all isolates were further analyzed by BOX-PCR fingerprints. The obtained BOX-PCR patterns again demonstrated a high similarity between the nine studied isolates (Fig. 3). Based on the presence/absence of an approximately 550 bp DNA fragment, they could be separated into two groups. The isolates SP1, SP2, SP3 and SP4 represent one, and SP6, SP9, SP12, GL1 and A-220-2b the other group.

Assays to test pathogenicity of *D. bryoniae* and bacterial phytopathogens on styrian oil pumpkin

Five representatives (SP2, SP6, SP12, GL1, A-220-2b), selected according to their phenotypic and genotypic diversity as well as their production of pycnidia, were used for pathogenicity tests on Styrian oil pumpkin plants. All tested isolates were found to be pathogenic; they produced typical symptoms on the first true leaves. Five days after the infection the first symptoms were visible (Fig. 4). The early symptoms found on leaf stems were water-soaked lesions. These lesions rotted and the decay extended to the whole leaf. Thereafter grey-green leaf spots were formed in irregular patterns, which later changed their colour to become dark brown. Furthermore, pycnidia on stems and leaves were noticed 11 days after infection. All tested isolates were re-isolated from infected tissues. Their identity was confirmed by morphological observation on QPDA plates and molecular BOX-fingerprint analysis.

To verify pathogenicity of bacterial isolates *P. virdiflava* 2d1, *P. carotovorum* ssp. *atrosepticum* 25–2, *P. syringae* 6g1 and *X. cucurbitae* 6h4 on oil pumpkin, infection studies were performed. In all treated plants, 4 days after infections rotten lesions on leaves were visible, which increased rapidly within

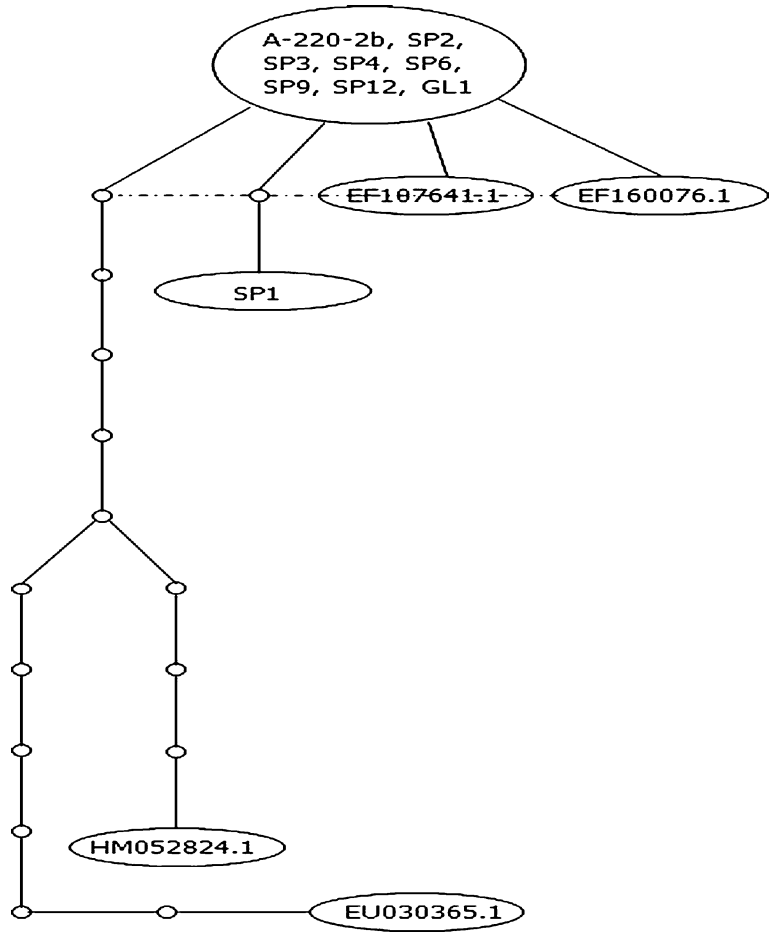

Fig. 2 TCS-analysis with obtained and reference DNA sequences of *D. bryoniae* (encoding ribosomal ITS regions, 384 bp) indicating number of non-matching bases between sequences

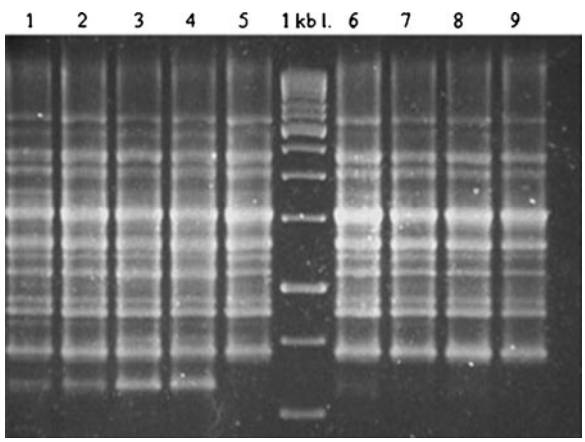

Fig. 3 BOX-PCR fingerprints of *D. bryoniae* isolates: lane 1, SP1; lane 2, SP2; lane 3, SP3; lane 4, SP4; lane 5, SP6; lane 6, SP9; lane 7, SP12; lane 8, A-220-2b; lane 9, GL1; DNA marker: 1 kb DNA-ladder; BOX-PCR products were loaded on a 1.5% (w/v) agarose gel

the next days (data not shown). From affected plant tissues tested bacteria were successfully re-isolated and their identity was confirmed by molecular fingerprints performed by BOX-PCR (data not shown).

Mycelium of *D. bryoniae* translocates pathogenic bacteria

The system used for assessing translocation of pathogenic bacteria by *D. bryoniae* is illustrated in Fig. 5. We selected four isolates of *D. bryoniae* (A-220-2b, GL1, SP2 and SP6) belonging to different morphotypes (Table 1) and bacterial phytopathogens *P. carotovorum* ssp. *atroseptica* 25–2, *X. cucurbitae* 6h4, *P. viridiflava* 2d1 and *P. syringae* 6g1. Results of the translocation experiments are summarized in Table 2. In case of SP2, SP6 and GL1, all four bacteria were detected on the surface of the QPDA gel

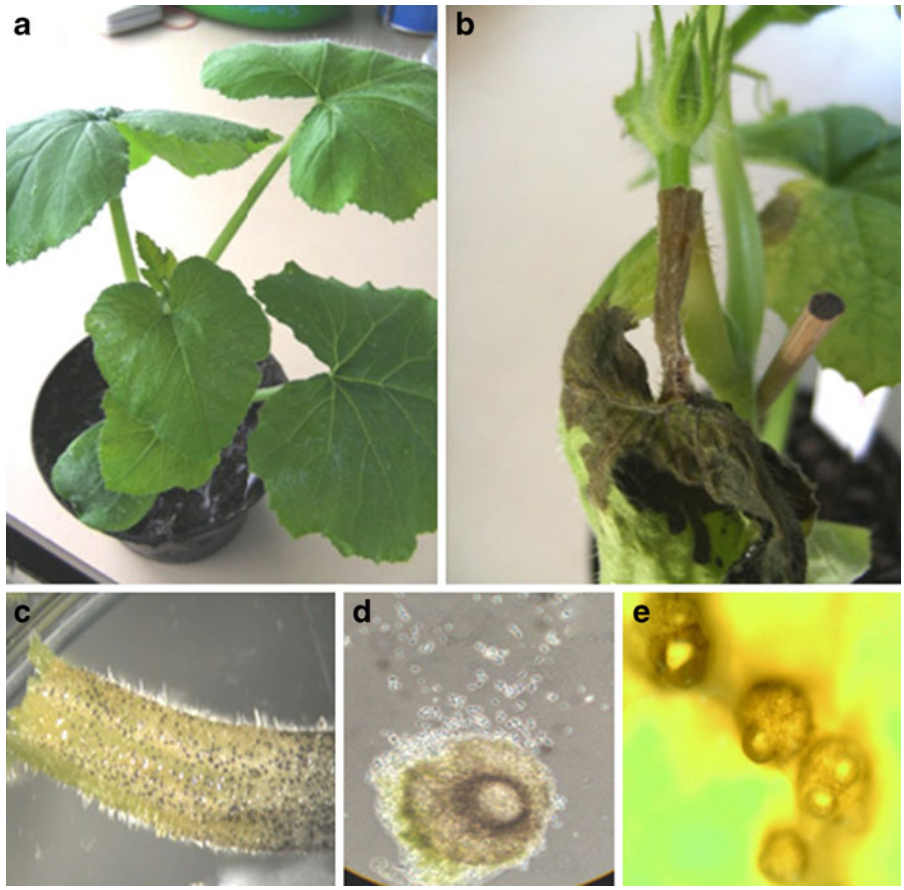

Fig. 4 Gummy stem blight of Styrian oil pumpkin caused after artificial infection with *D. bryoniae*: **a** uninfected plant; **b** infected plant with gummy stem blight lesion; **c** infected stem with pycnidia of *P. cucurbitacearum* (anamorph of *D. bryoniae*); **d** pycnidia with conidia; **e** pycnidia

slice suggesting their translocation via the fungal hyphae. In contrast, this was not observed for isolate *D. bryoniae* A-220-2b. In the negative controls, all four bacteria were only detected at the inoculated slice (PDA).

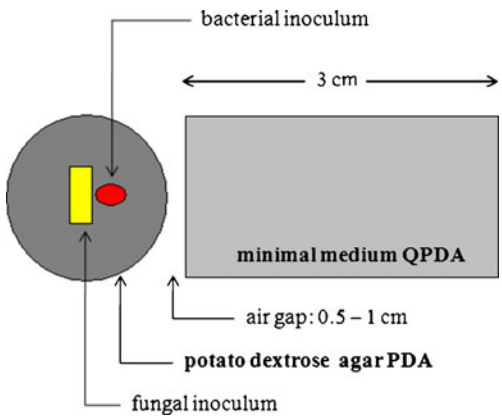

Fig. 5 Experimental design of interaction studies between *D. bryoniae* and pathogenic bacteria carried out in a sterile petri dish

Discussion

The fungus *D. bryoniae* was identified as main causal agent of high yield losses of Styrian oil pumpkin. Furthermore, *D. bryoniae* was found often associated with bacterial pathogens *P. carotovorum*, *P. viridiflava*, *P. syringae* and *X. cucurbitae,* which also contribute substantially to these yield losses. *D. bryoniae* hyphae were able to transport bacterial isolates, which was shown in vitro. These observations and the high coincidence of fungal and bacterial disease in the field suggest mutualistic effects in pathogenesis.

Based on morphological characterization in culture and by sequencing, all fungal isolates from oil pumpkins affected by black rot were classified as *D. bryoniae* (Keinath et al. 1995). *D. bryoniae* produces white aerial mycelium and olivaceous-green to black substrate mycelium. *D. bryoniae* developed considerable morphological variation. We assigned these growth variants to five groups. Because growth conditions were similar and genetic diversity of the

Table 2 Fungus-mediated mobilization of bacteria

D. bryoniae isolates	Bacterial isolates								Control
	P. carotovorum, ssp. *atrosepticum* 25-2		*P. viridiflava* 2d1		*X. cucurbitae* 6h4		*P. syringae* 6g1		without bacteria
	PDA	QPDA	PDA	QPDA	PDA	QPDA	PDA	QPDA	QPDA
A-220-2b	+	−	+	−	+	−	+	−	−
GL1	+	+	+	+	+	+	+	+	−
SP2	+	+	+	+	+	+	+	+	−
SP6	+	+	+	+	+	+	+	+	−
no fungus	+	−	+	−	+	−	+	−	

+bacteria were detected, −bacteria were not detectable

isolates was low, we suspect that epigenetic variation could be responsible for the morphological variation.

Further, we tested for the first time *D. bryoniae* isolates for their pathogenicity against oil pumpkin in a greenhouse assay. With the new assay—a modified version of the method described by Keinath et al. (1995) and Shim et al. (2006)—we showed that all tested isolates were significantly virulent to pumpkin. Five days after injection of conidial suspensions of the pathogen into leaf stems, and under conditions conducive to development of gummy stem blight, typical symptoms were observed (Lee et al. 1983), including the production of pycnidia on infected tissues. Re-isolated fungal strains from infected tissues were identified as *D. bryoniae,* which again confirmed the role in pathogenesis.

In the field, black rot was often detected together with symptoms of bacterial pathogens. We therefore suspected that some kind of interactions could take place in the development of pumpkin disease. Specific interactions of fungi with bacteria have been described in context with plant pathogenicity, in which bacteria are not independently acting as pathogens (Marpues et al. 2008). Kohlmeier et al. (2005) noticed that bacteria are mobilized by fungi and that apical growth of the fungi was not directly involved in the displacement of bacteria. Trifonova et al. (2009) reported that bacteria are able to migrate along fungal hyphae. This movement may involve bacterial growth along the hyphae or active migration on the surface of the hyphae. Continuous liquid films on the fungal surface seem to be important for translocation of bacteria (Furuno et al. 2010). Fungal hyphae mostly grow at the hyphal apices and regularly branch in regions of active growth (Wick et al. 2007). In our study, we tested the hypothesis whether a synergistic interaction between *D. bryoniae* and pathogenic bacteria *P. carotovorum* ssp. *atroseptica* 25–2, *X. cucurbitae* 6h4, *P. viridiflava* 2d1 and *P. syringae* 6g1 exist. Using our new experimental set-up to study hyphal-mediated bacterial translocation, we tested the *D. bryoniae* isolates GL1, SP6, SP2 and A-220-2b, each representing one morphological group. All tested isolates except A-220-2b could mediate the translocation of bacteria. Strain-specific differences are well known for several traits such as pathogenicity or antagonism, and these could be important in the virulence of the pumpkin diseases. This research demonstrates that *D. bryoniae* promotes the translocation of different pathogenic bacteria in a fungal strain-specific manner. We also suggest that black rot management should start with effective control of gummy stem blight in the field as reported by Babadoost and Zitter (2009) and should be synchronized with control of diseases caused by bacterial pathogens.

The joint presence of fungal and bacterial pathogens on pumpkins is representative for the emerging incidence of multi-pathogen diseases of crops. Multi-fungal diseases have been known for some time, with Esca representing the most prominent example of a grapevine disease. Esca is induced by a complex of xylem-inhabiting fungi (Crous and Gams 2000), and there is evidence for early causal agents, which allow access to saprophytic opportunists. *D. bryoniae* often occurs with different *Phoma* species on cucurbit seedlings (Somai and Keinath 2002) or with powdery mildew (Zitter and Kyle 1992). Here we show that

fungi may pave the way for rapid access for bacteria to plant tissue. The rapid rise of newly emerging plant diseases in various parts of the world could be promoted by climatic perturbations. More studies of mutiple disease-contributing microorganisms are necessary.

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