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Efficacies of bacterial and fungal isolates in biocontrol of *Botrytis cinerea* and *Pseudomonas syringae* pv. *tomato* and growth promotion in tomato do not correlate

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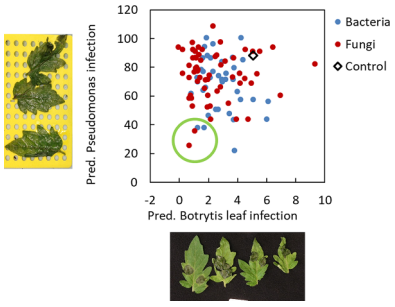
GRAPHICAL ABSTRACT



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ABSTRACT

There is a need to develop more biological control agents to fulfil the increasing demand for biological crop protection. Testing for consistent efficacy in disease control under the relevant range of environmental conditions is one of the most demanding steps during screening programs. Bioassays were conducted to target three major diseases of tomato, stem canker caused by *Botrytis cinerea*, leaf spot caused by *B. cinerea*, and bacterial spot caused by *Pseudomonas syringae* pv. *tomato*, and to assess possible growth promotion of tomato seedlings. Nine quantitative screening approaches were analyzed for a test panel of approximately 100 isolates of bacteria and fungi, all obtained from tomato, and several known antagonists as reference isolates. Even with such a limited number of isolates promising antagonists, partly not yet described as antagonists, could be selected for control of the targeted diseases when labor and resource demanding *in planta* bioassays had been applied. Also some promising isolates enhancing seedling development could be identified. Independent screening assays for the different traits were needed since no correlation between the different traits were found. Attempts to simplify screening assays to high-throughput systems failed since there were no positive correlations with *in planta* bioassays. In conclusion, the often suggested first screening rounds using *in vitro* tests for huge numbers of isolates followed by *in planta* testing of a selected group of candidates, e.g. those with high *in vitro* production of certain secondary metabolites or biosurfactants, may not exploit the entire potential of antagonists. Especially antagonists combining various modes of action may be excluded by *in vitro* screening with a bias on a specific mode of action. Therefore, independent *in planta* assays are proposed to screen against different pathogens and for growth promotion.

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1. Introduction

Microbial biocontrol agents (BCA) are sustainable tools in crop protection against damage caused by plant pathogens. In integrated pest management, the use of effective biocontrol agents is considered first before chemical pesticides are applied (Barzman et al., 2015). In conventional cropping systems, biological control agents as substitute for fungicide applications may be particularly interesting at the end of a growing season in high value crops such as fruit to avoid residue levels above the limits set by the markets.

There is a need to develop more BCAs to fulfil this increasing demand for biological crop protection. For commercial use microbial BCAs have to fulfill many different criteria regarding efficacy, safety, ecological characteristics and economical demands (Whitesides et al., 1994; Schisler & Slininger, 1997; Köhl et al., 2011). An efficient system of adequate screening assays allows the targeted selection of suitable candidate antagonists with the input of limited resources (Köhl et al., 2019a). Testing for consistent efficacy in disease control under the relevant range of environmental conditions is one of the most demanding steps during such a screening program. Efficacy of antagonists depends on their effective mode of action. In nature, antagonism is a common feature of many organisms interfering for their position in an ecological niche. A broad range of different modes of action may be involved (Köhl et al., 2019b). Most organisms may exploit different modes of action in combination or alternation in a sequence of antagonistic events (Nygren et al., 2018; Piombo et al., 2018; Köhl et al., 2019b).

For screening programs, different strategies can be followed. Antagonists can be screened independent from a specific mode of action. This allows the selection of antagonists with possibly still unknown combinations of modes of action. The final effect on disease suppression will be measured in a microcosm approach which simulate under controlled conditions the environment where applications finally will take place. This is typically carried out with potted plants in growth chambers or plant parts in moist chambers with controlled environment (temperature, humidity, light intensity and day length), artificial inoculation with the pathogen, and application of candidate antagonists to seeds, soil, leaves, flowers or fruits. When comparing different microorganisms in such assays, wet conditions may favor bacteria that need water films to swarm and exert typically rapid biocontrol activity. However, yeast and hyphal fungi may show stronger biocontrol activity under lower and variable moisture conditions. Bioassays under controlled conditions can be complemented by the evaluation of a number of candidate antagonists under field conditions in adequately simplified assays (Köhl et al., 1995a).

Advantage of bioassays using microcosms mimicking the crop's situation are that positive correlations between bioassays and field results can be expected. Furthermore, antagonists with different combinations of modes of action or even still unknown modes of action may be recognized so that new opportunities for next generations of BCAs may be utilized. An obvious disadvantage is that they are labor-intensive, depend on cultivation of plants and demand high quality climate facilities and complex planning of research activities. Further disadvantages are possibly large variations of results within and between repeated trials due to inconsistent environmental conditions, and the risk of possible losses of trials due to technical failures, e.g. of climate facilities, during assays lasting a few weeks or even longer.

In contrast, screening for a specific mode of action, e.g. for production and secretion of certain antifungal secondary metabolites such as lipopeptides (Cawoy et al., 2014) has the advantage that simplified assays such as biosurfactants production assays based on the assessment of droplet dispersal allow high-throughput screening of huge numbers of candidates at low costs per candidate in a short time (Raymaekers et al., 2020). Also, environmental factors can better be controlled resulting in reduced risks of failures. The obvious disadvantages are that (i) only a specific group of the antagonists will be discovered while the majority of antagonistic organisms with different and possibly still unknown modes of action may not be detected, and (ii) the selection of antagonists depends on the conditions of the simplified environment, e.g. in agar plates screening for production of secondary metabolites depends on the prevailing nutrient conditions of the growth medium (Knudsen et al., 1997).

BCAs are often selective for certain diseases or groups of related diseases. For commercial use it may be attractive to have a broader range of activity. Screening antagonists for efficacy against a range of different pathogens *in vitro* on dual cultures can be conducted with limited resource input (Daranas et al., 2019). However, screening *in planta* in separate tests against different pathogens is resource demanding. Knowledge on possibly positive correlations of the effects of candidate antagonists (i) against a pathogen measured by *in vitro* assays versus *in planta* assays, or (ii) against different pathogens measured in *in planta* bioassays may allow less resource-demanding screening strategies.

The objective of the present study was to compare different screening strategies for microbial antagonists. This information is essential for the design of powerful and cost-effective screening programs for the development of new BCAs. Results of seven different bioassays for testing antagonistic and plant growth stimulating properties of a set of microbial isolates were compared and possible correlations between results of the bioassays were analyzed. Three major diseases in tomato were targeted, stem canker caused by *Botrytis cinerea*, leaf rot caused by *B. cinerea*, and bacterial leaf spot caused by *Pseudomonas syringae* pv. *tomato*. Bioassays were conducted with tomato plants, detached tomato leaves, tomato stem segments and tobacco plants as alternative test plant for *P. syringae* pv. *tomato*. Additionally, *in vitro* assays without plants or plant parts were conducted. Bioassays on callus formation and rooting on tomato stem segments were included to assess characteristics that may correlate with the growth promotion potential of isolates and hampering *B. cinerea* and *P. fluorescens* pv. *tomato* infection (Finiti et al., 2013). The test panel consisted of approximately 100 isolates of bacteria and fungi, all obtained from tomato, and several known antagonists as reference isolates.

2. Materials and methods

2.1. Origin and identification of bacterial and fungal isolates

Samples of roots, stems and leaves of various tomato plants were collected in five commercial tomato crops grown in greenhouses in the Western part of the Netherlands. Surfaces of the sampled roots, stems and leaves were slightly pressed on tryptic soy agar (1/10 strength; tryptic soy broth 3 g l^{-1}, agar 15 g l^{-1}) containing 50 mg l^{-1} cycloheximide (TSA) for the isolation of epiphytic bacteria and on malt agar (MA; half strength; malt extract 5 g l^{-1}, agar 15 g l^{-1}) containing 100 mg/l streptomycine and 15 mg l^{-1} tetracycline for the isolation of epiphytic fungi including yeasts. Other samples were surface-sterilized by submerging samples for 1 min in sodium hypochloride (0.25%) and for 0.5 min in ethanol (70%) followed by three washing steps in sterile tap water. Small pieces sized approximately 2 × 2 mm were cut from the surface-sterilized samples using a sterile scalpel and placed on TSA and MA for isolation of endophytic bacteria and fungi. Plates were incubated at 20 °C and bacterial and fungal isolates were obtained from developing colonies during two weeks of incubation. Pure cultures were obtained by subculturing hyphal tips or cells. From the obtained collection of approximate 600 isolates, a sub-set of 40 bacteria and 60 hyphal fungi (including 20 yeasts or yeast-like fungi) were selected. Selected isolates represented the five different tomato crops and different compartments of the plants, so that in total 60 epiphytic and 40 endophytic colonizers of roots, stems and leaves were represented in the sub-set (Table 1). Isolates *Aureobasidium pullulans* 490 and *Clonostachys rosea* 016 (former isolate code: *Gliocladium roseum* 1813), both known as efficient antagonists against *B. cinerea* (Köhl et al., 1995b), and isolate *B. amyloliquefaciens* S499, a known producer of lipopeptides

such as surfactin (Cawoy et al., 2014), kindly provided by Marc Ongena, University of Liège, were used as reference isolates.

Cells from bacterial colonies grown on nutrient agar (NA; 28 g l^{-1}) and spores or mycelium from hyphal fungal colonies grown on oatmeal agar (OA; 20 g l^{-1} oatmeal, 15 g l^{-1} agar) and yeasts grown on potato dextrose agar (PDA; 39 g l^{-1}) were used for isolation of genomic DNA. Bacterial and fungal tissues were lyophilized and total DNA was extracted using Sbeadex mini plant kit (LGC) and KingFisher™ Flex (Thermo Scientific). Lyophilized fungal tissue was disrupted using a TissueLyser II (Qiagen) and one stainless steel bead (3.2 µm) for 30 sec with a frequency of 30 Hz. After disruption, 200 µl lysis solution with 0.5 µl RNase (2 mg ml^{-1}) were added and further DNA extraction was carried out according to the protocol supplied by the manufacturer. For bacterial isolates, 27F/1492R DNA amplification (Lane, 1991; Turner et al., 1999) was performed. For isolates of hyphal fungi and yeasts, ITS1/ITS4 DNA amplification (White et al., 1990) was performed. Quality and quantity of 27F/1492R and ITS1/ITS4- PCR products were checked by electrophoresis on 1.0% agarose gels and they were purified. The 27F/1492R PCR products were sequenced using primer 800R and ITS1/ITS4 products were sequenced by ITS1/ITS4 primers, performed by Macrogen Europe (Amsterdam, The Netherlands). The 27F/1492R and ITS1/ITS4 DNA sequences were analyzed using blastn (http://www.ncbi.nlm.nih.gov/BLAST) with the default parameters and taxonomical groups were identified at a similarity of 99 to 100%. In the few cases where highest similarity was shown for two or more different taxonomic groups, such groups were indicated together.

2.2. Growing of bacteria, fungi and plants

Bacterial isolates were grown on NA for four days at 25 °C, isolates of hyphal fungi on OA for 14 days at 20 °C, and isolates of yeast-like fungi on PDA for seven days at 20 °C. Suspensions of bacteria were prepared in a sterile buffer (0.1 M $MgSO_4 \cdot 7H_2O$) and concentrations adjusted to OD_{600nm} = 0.8 A to yield approximately 10^8 cells ml^{-1}. Spores of hyphal fungi were separated from mycelium by filtering through gauze (200 µm mesh), and concentrations were adjusted to 1×10^7 spores ml^{-1} in the buffer amended with 0.01% Tween 80. Yeast cells were suspended in the buffer amended with 0.01% Tween 80 and adjusted to 1×10^8 spores ml^{-1}. *Botrytis cinerea* 700 was grown on PDA for seven days. A conidial suspension (5×10^5 conidia ml^{-1}) was prepared in a 50 mM filter-sterilized glucose solution. *Pseudomonas syringae* pv. *tomato* DC3000 was grown on NA. Suspensions were prepared and adjusted to OD_{600nm} = 0.1 A. To obtain supernatants, microorganisms were grown in the liquid medium of the same composition mentioned above but without agar, i.e. potato dextrose broth instead of PDA for yeasts, oatmeal broth instead of OA for hyphal fungi and nutrient broth instead of NA for bacteria, in flasks on a shaker for the same period as mentioned above. Subsequently, cultures were centrifuged. Supernatants were filtered through a 0.22 µm filter and stored in 1.5 ml-microfuge tubes at −20 °C until use.

Tomato (*Solanum lycopersicum*) cv. 'Moneymaker' was grown in 3 l-pots in a peat-based substrate in a greenhouse at 20 °C and 80% relative humidity, and fertilized regularly as recommended for the crop. Tobacco (*Nicotiana tabacum*) cv. 'White burley' was cultivated under the same conditions as tomato. Plants were used for the experiments, when they were at the four fully-expanded leaf stage.

2.3. Biocontrol bioassays

2.3.1. Bioassay on tomato stem infection by Botrytis cinerea

Tomato plants cultivated till the onset of flowering were used for stem segments assays. The main stems were cut into 2.5 cm-long segments using a sharp knife, simulating the wound produced by pruning in commercial tomato production. Segments obtained from the lower and middle part of stems were used for the experiments on stem protection from *B. cinerea* invasion. Stem segments from the upper stem parts were used in experiments on the effect of candidate isolates on rooting and callus formation (described below). Each stem segment was fixed to a push pin through a screened 200 µl PVC pipette-tip tray to keep an upright position. The surface of the upper cut of fixed stem segments was then treated with a 50 µl droplet of the suspension of a candidate isolate. After 5 min, when the stem surface was dry again, the surface was inoculated with a 25 µl droplet of the conidial suspension of *B. cinerea*. A treatment without application of candidate isolates but with a conidial suspension of *B. cinerea* served as positive control in all experiments. In some experiments, additionally a negative control treatment without pathogen inoculation was included. A total of six stem pieces were fixed to the pipette tip tray and obtained the same treatment. Two of such trays were placed on a moistened filter paper in a plastic box with lid (sized 20 cm × 10 cm 10 cm). The experiment was carried out in a fully randomized design with four replicates per treatment. On average 20 candidate isolates were tested per experiment. A set of arbitrarily chosen candidate isolates was tested twice in independent experiments. Treated stem segments were incubated for seven days at 20 °C in the dark.

Stem segments were evaluated for the presence of *B. cinerea* sporulation, following the disease rating scale proposed by Dik et al. (1999) ranging from 0 to 4, where 0 = treated surface remains green, 1 = treated surface is brownish, 2, 3 and 4 = treated surface brownish with > 0–50, > 50–75 and > 75% of the stem surface covered with *B. cinerea* sporulation, respectively. From the obtained score for each stem segment, the McKinney index (McKinney, 1923) for disease severity was calculated, according to the formula below, and multiplied by 100 to convert the 0–1 range to percent.

$$\frac{\sum_{k=0}^{n} kx_k}{nT}$$

where k is the disease rating from '0' to 'n', according to the scale, x_k is the number of stem segments with the disease rating k, n is the maximum disease rating from the scale (in our case n = 4) and T the total number of stem segments considered (in our case T = 6).

2.3.2. Bioassay on tomato leaf infection by Botrytis cinerea

Leaflets from 8-week old tomato plants were excised from the middle and upper part of the plants. They were immediately disposed on autoclaved plastic trays (three leaflets per tray) in a sterile transparent plastic box with lid (sized 20 cm × 10 cm 10 cm) containing moistened filter paper. Suspensions (50 µl per tray) of each antagonist were sprayed on the detached leaflets using atomizers. Water-treated leaflets served as positive control. Leaflets were allowed to dry in a flow cabinet for approximately 20 min. Thereafter, each leaflet was inoculated with two 5 µl droplets of *B. cinerea* conidia suspension. Each treatment, consisting of three leaflets in a sterile plastic box as experimental unit, was repeated four times. A set of arbitrarily chosen isolates was tested twice in independent experiments. The boxes with the leaflets were kept in a fully randomized arrangement in the dark at 20 °C for three days. Thereafter, lesion diameters were measured using a calliper rule.

Table 1
Origin of bacteria and fungi from tomato tissues selected for screening assays.

	Number of isolates from[1]						
	Total	Roots		Stems		Leaves	
Bacteria	40	17	(7/10)	11	(6/5)	12	(11/1)
Fungi	60	26	(9/17)	14	(9/5)	20	(18/2)
Total	100	43	(16/27)	25	(15/10)	32	(29/3)

[1] Number of epiphytic isolates / endophytic isolates in brackets.

2.3.3. Bioassay on tomato leaf infection by Pseudomonas syringae pv. tomato

Sets of three whole tomato plants at the second true leaf stage were spray-inoculated with a suspension of each of the bacterial or fungal isolates with ca. 10 ml per plant. Leaf surfaces of treated plants were dried in a downflow cabinet for five minutes and then inoculated with a suspension of *P. syringae* pv. *tomato* with ca. 10 ml per plant. Treated plants were arranged in a fully randomized design and individually incubated within a wet plastic cover for 24 hrs at 20 °C to favour stomatal penetration by the pathogen. Thereafter, three leaflets from the second true leaves were excised and placed on a plastic grid on a wet paper in a plastic box with cover lid. Leaflets in the moist chambers (boxes) were incubated at 21 ± 1 °C, 14 hrs light at 200 $\mu mol.m^{-2}.s^{-1}$. Each replicate consisted of three leaflets (from the same plant) and each treatment was replicated three times (with leaflets from different plants). Leaflets were rated after six days, according to an adapted rating scale ranging from 0 to 4, where 0: no symptom, 1, 2, 3 and 4 corresponding to 1–25, 26–50, 51–75 and 76–100% leaf area with water soaking symptom, respectively. With the highly disease conducive incubation conditions, the symptoms did not evolve to a typical chlorosis but rather to water soaking followed by tissue maceration. From the obtained score for each leaflet a disease severity index (T = 3) was calculated using the same formula as for bioassays on tomato stem infection (see 2.3.1).

2.3.4. Bioassay on tobacco leaf infection by Pseudomonas syringae pv. tomato after injection of culture filtrates

Aliquots of sterile supernatants of each isolate were added to an equal volume of a suspension of *P. syringae* pv. *tomato* DC3000 at OD = $0.1A_{600}$ nm. The resulting suspensions were kept on ice and used within 5 hrs. An average 5 µl of each suspension was infiltrated into the underside of a tobacco leaf with a 5 ml syringe without needle. The individual infiltration points were separated longwise from each other by the lateral venules and from one side of the leaf blade to the other by the middle vein leading to trapezoidal fractions of the leaf. The first and last trapezes from each leaf side were not used. The number of tested strains per leaf depended on available spaces delimited by the veins. On each leaf, supernatants of up to 20 randomly chosen different isolates or control treatments were tested, 10 on each leaf side (separated by the middle vein). Each treatment was labelled at the edge of the leaf on its upper side using a marker pen. Autoclaved tap water without *P. syringae* pv. *tomato* or supernatants was infiltrated as negative control treatment in each tobacco leaf used in the experiment. A *P. syringae* pv. *tomato* suspension mixed with autoclaved tap water (1:1) was used as positive control. Each treatment was done in three replicates on different plants. Treated plants were kept in a growth cabinet with 14 hrs light, 200 $\mu mol.m^{-2}.s^{-1}$ light, and 20 °C at a relative humidity of approximately 60% for five days. The diameter of the chlorotic lesions that developed was measured using a calliper rule. The experiment was repeated twice using new batches of plants, supernatants and suspensions of *P. syringae* pv. *tomato*.

2.3.5. Biosurfactants production assays

Surfactant molecules produced by potential biocontrol candidates may cause direct inhibition of bacterial or fungal plant pathogens. *In vitro* assays were conducted to screen isolates with the potential to produce biosurfactants. Sterile broths of Tryptone soy broth (TSB; 15 g l^{-1}), normal saline (NS; 9 g NaCl l^{-1}), nutrient broth (NB; 13 g l^{-1}), potato dextrose broth (PDB; 12 g l^{-1}), and oatmeal broth (OB; 20 g l^{-1}) were prepared. Droplets (30 µl) of the broth media were pipetted on sterile square polystyrene Petri dishes (120 × 120 × 17 mm) in a regular pattern of five rows each with five droplets, using the same medium on a single plate. Bacterial isolates were grown on NA for two days, hyphal fungi on OA for seven days, and yeasts on PDA for five days at 18 °C. Growing colonies of bacterial or fungal isolates were touched with a sterile needle and subsequently an individual droplet was touched by the needle for inoculation. Sterile supernatants of the bacterial and fungal strains (prepared as described above) were added to another set of NS droplets (30 µl). For each set of droplets on a Petri dish, randomly chosen isolates were applied. Each isolate or supernatant was tested in four droplets per medium on independent plates (replicates). All isolates were tested in TSB and NS and supernatants of all isolates were tested in NS. Additionally, bacterial isolates were tested in NB, hyphal fungi in OB and isolates of yeast-like fungi in PDB. In each plate one droplet of the respective medium without addition of suspensions with isolates or supernatants but with sterile water served as negative control. Droplets with added supernatants or suspensions of *B. amyloliquefaciens* S499, *C. rosea* 016 or *A. pullulans* 490 were used as references on Petri dishes with bacteria, hyphal fungi or yeasts. Each individual droplet treated with supernatants of isolates was visually assessed 2 h after treatment for possible droplet dispersal, i.e. the ability of the microbial-produced surfactant to break the surface tension of the droplet and allow the liquid to spread on the polystyrene surface. According to the strength of such dispersal, the treatment has been visually rated using the following scale: 0 = no dispersal, 0.5 = droplet-polystyrene interface at least doubled without dispersal and 1 = full dispersal. Petri dishes with droplets treated with isolates were placed with open lids in a closed box (29 × 16 × 9 cm) on moist filter paper and were incubated for three days at 20 °C. Thereafter, each individual droplet was assessed for possible dispersal, using the same scale.

2.4. Growth promotion bioassays

2.4.1. Bioassay on growth promotion of tomato seedlings

Seeds of *Solanum lycopersicum* cv. 'Merlice' were primed in suspensions of the bacterial and fungal isolates. Ten seeds were added to 0.5 ml of the bacterial or fungal suspensions in Eppendorf tubes sized 1.5 ml and shaken on a shaker (Titertek, Flow Laboratories, at position 4) for 4.5 h at 20 °C. Treated seeds were transferred from the tubes without additional drying step in soil at 5 mm depth in plastic pots (10 cm height, 5.5 cm diameter), containing 75 ml of potting soil and a top layer (1 cm) of sowing soil. For each isolate, three replicate pots were used, each with two seeds. Seeds only treated in the buffer serving as negative control were transferred to six replicate pots. Pots with treated seeds were arranged in a block design with full randomization within blocks (replicates) in a climate cabinet with a 16-hour day length. Temperature was 22 °C during the day and 19 °C during the night; humidity was set at RH = 80%. Pots were covered during the first five days with a plastic foil. After 11 days, pots were weighted and water was added to compensate for possible evaporation. Seedling emergence was recorded after seven days. One plant was left in the pot, the second emerged plant was cut. Tomato plants were cut at soil surface after 21 days and plant length from soil line to top was measured. Plants without roots were subsequently dried overnight at 105 °C and the dry weight was measured. Each isolate was tested twice in independent experiments.

2.4.2. Bioassay on callus formation and rooting on tomato stem pieces

Experiments with stem segments of tomato cv. 'Moneymaker' were conducted in parallel to the experiments on tomato stem infection by *Botrytis cinerea* described above. Stem segments were arranged in the same way on pipette-tip trays using push pins and treated with bacterial or fungal suspensions. Water was added in the negative control. Treated stem segments were incubated in plastic boxes with lid for seven days at 20 °C in the dark. Callus formation on the upper wounded end of stem segments and rooting was assessed using a rating scale ranging from 0 to 4, where 0 = no visible callus, 1 = few calluses covering less than 25% of the stem piece circumference, 2 = several calluses covering between 25 and 50% of the stem piece circumference, 3 = calluses covering more than 50% of the stem piece circumference, and 4 = calluses not only around the circumference of the stem piece but also

covering the wounded internal stem piece tissue, and 0 = no roots, 1 = 1 root, 2 = 2–5 roots, 3 = 6–10, and 4 = > 10 roots for root formation. A callus formation index (CFI) and a rooting index (RI) were calculated using the same formula as for bioassays on tomato stem infection (T = 6; see 2.3.1).

2.5. Statistics

Quantitative results of the various bioassays were subjected to ANOVA separately for each experiment. Significant treatment effects compared to the control treatments were identified by two-sided protected LSD-tests (p = 0.05). Additional to the separate analysis of each experiment, an overall analysis of the results of each set of experiments conducted for the different bioassays was performed using a REML-model with experiment as random factor and isolate as fixed factor. This analysis resulted for each of the different bioassays in predicted means for each isolate corrected for the fact that they were tested in different experiments. This correction was based on the control treatments, that were part of all experiments and on other isolates that were tested twice. Significant predicted treatment effects in comparison with the predicted control treatments were identified by LSD-tests and isolates were ranked according to their predicted means. Predicted means obtained for the individual bacterial and fungal isolates in the various bioassays were used to estimate pairwise correlations between results of different bioassays.

3. Results

3.1. Bioassay on tomato stem infection by Botrytis cinerea

In total, 13 experiments have been conducted with tomato stem segments under controlled conditions with 3 to 38 isolates tested per experiment. The disease index (DI) for *B. cinerea* in the positive control varied between 12.5 and 95.8% (mean 56.9%) in the different experiments (Table 2A). Significant treatment effects were found in all experiments except in experiments 7 and 10. However, the LSD values (p = 0.05) varied between 8.5 and 32.2 for the different experiments. More than 50% of the tested isolates reduced DI in all experiments with DI of > 40% in the positive control. In experiments 7, 10 and 13 with DI < 40% in the positive controls, a few or none of the tested isolates significantly reduced DI. For the further data analysis, experiments 7, 10 and 13 were excluded because of the restricted symptom development *by B. cinerea.*

In total, 40 bacterial isolates and 57 fungal isolates were tested in the further analysed ten experiments of which 13 bacterial and 24 fungal isolates were tested twice in independent experiments. The REML-analysis showed that there were significant differences between treatments with different isolates (p < 0.001). The predicted disease index for the positive control was DI = 66.9. The approximate LSD (p = 0.05) for comparing treatment effects of isolates with the control treatment was LSD = 20.4 (and LSD = 14.8 for isolates that were tested twice). Thirty five bacterial isolates and 49 fungal isolates significantly reduced DI by a value of at least 20.4 compared to the control. The mean predicted efficacy in DI reduction was 71.3% for the 40 analysed bacterial isolates and 65.6% for the 57 analysed fungal isolates (Table 2B).

The ten most effective isolates were in decreasing order: *C. rosea* 016, *Chryseobacterium* sp. HTS012, *Pseudomonas* sp. HTS043, *Rhizobium* sp. / *Agrobacterium* sp. HTS123, *Lysobacter* sp. HTS263, *Pseudomonas* sp. HTS042, *Aureobasidium pullulans / A. proteae* HTS508, *A. pullulans / A. proteae* HTS551, *Pythium aphanidermatum* HTS401, and *Pseudomonas* sp. HTS143.

3.2. Bioassay on tomato leaf infection by Botrytis cinerea

Seven experiments have been conducted with detached tomato leaves under controlled conditions. The lesion diameter caused by *B. cinerea* in the positive control varied between 1.07 and 7.65 mm (mean 4.49 mm) in the different experiments (Table 3A). Significant treatment effects were found in each experiment except in experiment 2. However, the LSD values (p = 0.05) varied between 1.63 and 3.25 mm for the different experiments. The number of isolates tested was 19 to 42 per experiment. More than 80% of the tested isolates caused significant reduction of lesion development in experiments 1 and 7 with the highest levels *of B. cinerea* development. In experiments 3 to 6 with moderate *B. cinerea* development 17–36% of the tested isolates significantly reduced disease development. For the further data analysis, experiment 2 was excluded because of the very restricted symptom development by *B. cinerea* in the positive control.

In total, 102 isolates (41 bacterial isolates, 61 fungal isolates) were tested in the further analysed experiments of which 12 bacterial and 50 fungal isolates were tested twice in independent experiments. The REML-analysis showed that there were significant differences between treatments with different isolates (p < 0.001). The predicted lesion diameter for the positive control was 5.06 mm. The approximate LSD (p = 0.05) for comparing treatment effects of isolates with the control was LSD = 2.30 (and LSD = 1.73 for isolates that were tested twice). Forty one fungal isolates and 22 bacterial isolates significantly reduced lesion development by more than 2.30 mm compared to the positive control. Mean predicted efficacy for bacterial isolates was 44.8%, and for fungal isolates 53.1% (Table 3B).

The ten most effective isolates were in decreasing order: *Fusarium oxysporum* HTS519, *A. pullulans / A. proteae* HTS551, *A. pullulans* 490, *Sporobolomyces roseus* HTS354, *Botryosporium longibrachiatum* HTS402, *Coniochaeta hoffmannii* HTS460, *Candida palmioleophila* HTS589, *A. pullulans / A. proteae* HTS448, *Chryseobacterium* sp. HTS080, and *Trichoderma asperellum / T. hamatum* HTS386.

3.3. Bioassay on tomato leaf infection by Pseudomonas syringae pv. tomato

In total, eight experiments have been conducted with detached tomato leaves under controlled conditions. The disease index (DI) caused in the positive control treatment varied between 19.4 and 100.0

Table 2
Effect of bacterial and fungal isolates on disease index (DI) for *Botrytis cinerea* infection of tomato stem pieces.

A. Overview on 13 experiments conducted.

Experiment	DI (0–100) in positive control	Number of isolates		LSD value (p = 0.05)
		tested	significantly reducing DI	
1	55.6	10	10	13.0
2	43.1	8	6	16.6
3	70.8	15	13	11.5
4	66.7	6	3	19.1
5	95.8	10	10	29.8
6	69.4	7	6	20.5
7	12.5	21	0	17.0
8	95.8	21	18	25.7
9	53.5	19	19	8.5
10	29.2	3	0	9.0
11	38.9	4	1	17.2
12	79.2	30	19	32.2
13	29.2	38	3	24.9

B. Mean predicted disease index (PDI) for treatments with bacterial and fungal isolates[1].

	Number of isolates	Mean PDI[2]	Mean efficacy (%) [3]
Bacteria	40	19.2 (−3.7–75.0)	71.3
Fungi	57	23.0 (−7.3–92.9)	65.6

[1] PDI of positive control was PDI = 66.9.
[2] Range in brackets.
[3] Disease reduction (%) in comparison to positive control.

Table 3
Effect of bacterial and fungal isolates on lesion diameter caused by *Botrytis cinerea* on detached tomato leaves.

A. Overview on seven experiments conducted.

Experiment	Lesion diameter (mm) in positive control	Number of isolates		LSD value (p = 0.05)
		tested	significantly reducing lesion diameter	
1	7.07	22	21	3.07
2	1.07	27	0	2.48
3	2.94	24	4	2.23
4	2.18	42	12	1.88
5	6.31	19	7	3.75
6	4.24	29	8	2.39
7	7.65	28	24	1.99

B. Mean predicted lesion diameter for treatments with bacterial or fungal isolates[1].

	Number of isolates	Mean Predicted lesion diameter (mm) [2]	Mean efficacy (%) [3]
Bacteria	41	2.80 (0.79–6.11)	44.8
Fungi	61	2.38 (−0.07–9.30)	53.1

[1] Predicted lesion diameter of positive control was 5.06 mm.
[2] Range in brackets.
[3] Disease reduction (%) in comparison to positive control.

(mean = 79.5) in the different experiments (Table 4A). Significant treatment effects were found in each experiment except for experiments 3 and 6. LSD values (p = 0.05) varied considerably between 14.1 and 53.1 for the different experiments. The number of isolates tested was 9 to 35 per experiment. Approximately 50% of the tested isolates caused significant reduction of symptom development in experiments 1 and 2. In these experiments mainly bacterial isolates had been tested. In the remaining experiments less than 25% of the tested isolates caused a significant reduction of disease symptoms. In experiment 3, the disease development generally was low with DI < 20 for the positive control treatment. This experiment was therefore excluded from further data analysis.

In total, 104 isolates (41 bacterial isolates, 63 fungal isolates) were tested in the seven further analysed experiments of which 40 fungal and 41 bacterial isolates were tested twice in independent experiments. The REML-analysis showed that there were significant differences between treatments with different isolates (p < 0.001). The predicted disease index for the positive control was 88.1. The approximate LSD (p = 0.05) for comparing treatment effects of isolates with the positive control was LSD = 37.5 (and LSD = 26.0 for isolates that were tested twice). Five fungal isolates and seven bacterial isolates significantly reduced DI by more than 37.5 compared to the control. Mean predicted efficacy for bacterial isolates was 21.1%, and for fungal isolates 13.9% (Table 4B).

The ten most effective isolates were in decreasing order: *Herbaspirillum* sp. HTS116, *S. roseus* HTS354, *Engyodontium album* / *Torrubiella* sp. HTS331, *Serratia* sp. HTS071, *Shinella* sp. HTS262, *Citrobacter* sp. HTS218, *Bacillus simplex* / *Brevibacterium frigoritolerans* HTS064, *Verticillium* sp. / *Simplicillium lamellicola* / *Guignardia vaccinii* HTS341, *Engyodontium parvisporum* / *Lecanicillium tenuipes* HTS351, and *P. aphanidermatum* HTS401.

3.4. Bioassay on tobacco leaf infection by Pseudomonas syringae pv. tomato after injection of culture filtrates

In total, culture filtrates of 103 isolates (41 bacterial isolates, 62 fungal isolates) were tested twice in the two independent experiments. The lesion diameter caused *by P. syringae* pv. *tomato* in the positive control were 8.50 and 11.08 mm in the two experiments (Table 5A). Significant treatment effects were found in both experiments with LSD values (p = 0.05) of 2.14 in experiment 1 and 3.12 mm in experiment 2.

The REML-analysis showed that there were significant differences between treatments with different isolates (p < 0.001). The predicted lesion diameter for the positive control was 9.79 mm. The approximate LSD (p = 0.05) for comparing treatment effects of isolates with the control was LSD = 2.17 mm for all isolates tested twice. Six fungal isolates and no bacterial isolate significantly reduced lesion diameter by more than 2.17 mm compared to the positive control. Mean predicted efficacy in reducing lesion diameter was −7.20% for culture filtrates of bacterial isolates and −1.61% for culture filtrates of fungal isolates (Table 5B).

The ten most effective isolates in decreasing order were: *Aspergillus* sp. HTS458, *A. pullulans* / *A. proteae* HTS508, *S. roseus* HTS600, *C. rosea* 016, *E. parvisporum* / *L. tenuipes* HTS351, *Cladosporium* sp. HTS590, *A. pullulans* 490, *Bacillus* sp. HTS003, *A. pullulans* / *A. proteae* HTS347, and *Bacillus* sp. HTS125.

3.5. Biosurfactants production assays

From the 41 bacteria tested in droplets of three different growth media, *Chryseobacterium* sp. HTS012, *Pseudomonas* sp. HTS043, *Chryseobacterium* sp. HTS080, *Chryseobacterium* sp. HTS093, *Variovorax* sp. HTS173, *Pseudomonas* sp. HTS174, *Rhodococcus* sp. HTS191, *Variovorax* sp. HTS231, *Lysobacter* sp. HTS263 and the reference isolate *B. amyloliquefaciens* S499 caused a dispersal of NB droplets. Four out of these ten isolates also caused dispersal of NS droplets and nine isolates of TSB droplets. Additionally, isolates *Pseudomonas* sp. isolates HTS042, HTS104 and HTS143, and *Sphingomonas* sp. HTS078 caused dispersal only of TSB droplets. *Bacillus amyloliquefaciens* S499, the reference isolates known for surfactin production, caused droplet dispersal in all cases. Amongst the 41 hyphal fungi, only the reference isolate *C. rosea* 016 caused droplet dispersal (found for all three media) and *T. asperellum* / *T. hamatum* HTS386 increased the droplet-polystyrene interface without causing any dispersal. However, the following nine out of 21 tested isolates of yeast-like fungi caused dispersal of NS and PDB droplets: *A. pullulans* / *A. proteae* isolates HTS347, HTS448, HTS508, HTS541 and HTS551, *S. roseus* isolates HTS354, HTS503 and HTS600, and the reference isolate *A. pullulans* 490. Results were similar for TSB droplets, except for *S. roseus* HTS503, causing no reaction. Additionally, *C. palmioleophila* HTS584 increased the TSB droplet-polystyrene

Table 4
Effect of bacterial and fungal isolates on disease index (DI) caused by *Pseudomonas syringae* pv. *tomato* on detached tomato leaves.

A. Overview on eight experiments conducted.

Experiment	DI (0 – 100) in positive control	Number of isolates		LSD value (p = 0.05)
		tested	significantly reducing DI	
1	100.0	30	13	14.1
2	100.0	35	18	45.0
3	19.4	20	0	29.2
4	80.6	35	0	n.s. [1]
5	100.0	26	3	32.1
6	47.2	29	0	n.s.
7	100.0	21	0	n.s.
8	88.9	9	2	21.6

B. Mean predicted disease index (PDI) for treatments with bacterial or fungal isolates [2].

	Number of isolates	Mean PDI [3]	Mean efficacy (%) [4]
Bacteria	41	69.5 (22–100.7)	21.1
Fungi	62	75.1 (25.8–108.8)	14.7

[1] No significant differences.
[2] PDI of positive control was PDI = 88.1.
[3] Range in brackets.
[4] Disease reduction (%) in comparison to positive control.

Table 5
Effect of culture filtrates of bacterial and fungal isolates on the diameter of lesions caused by *Pseudomonas syringae* pv. *tomato* on attached tobacco leaves.

A. Overview on two experiments conducted.

Experiment	Lesion diameter in positive control (mm)	Number of isolates tested	significantly reducing lesion diameter	LSD value (p = 0.05)
1	8.50	103	18	2.14
2	11.08	103	6	3.12

B. Mean predicted lesion diameter for treatments with culture filtrates of bacterial and fungal isolates on the diameter of lesions caused by *Pseudomonas syringae* pv. *tomato* on attached tobacco leaves[1].

	Number of isolates	Mean predicted lesion diameter (mm) [2]	Mean efficacy (%) [3]
Bacterial isolates	41	10.55 (8.01 – 13.10)	−7.20
Fungal isolates	62	9.95 (3.87 – 14.03)	−1.61

[1] Predicted mean lesion diameter on leaves of the positive control was 9.79 mm.
[2] Range in brackets.
[3] Disease reduction (%) in comparison to positive control.

interface without causing any dispersal.

Most supernatants of bacterial isolates and of isolates of hyphal fungi generally caused no reactions of treated droplets or, in few cases weak reactions. Only supernatants of *Chryseobacterium* sp. HTS080 and the reference isolate *B. amyloliquefaciens* S499 consistently caused droplet dispersal. Amongst the yeast-like fungi, isolates HTS347, HTS448, HTS508 and HTS551 of *A. pullulans / A. proteae*, *S. roseus* HTS354 and the reference isolate *A. pullulans* 490 caused droplet dispersal.

3.6. Bioassay on growth promotion of tomato seedlings

Five experiments with 95 isolates (40 bacterial isolates, 55 fungal isolates) were conducted with tomato seedlings. Each isolate was tested

Table 6
Effect of treatments with bacterial or fungal isolates on weight and height of tomato seedlings.

A. Seedling dry weight. Overview on five experiments conducted.

Experiment	Seedling weight (mg dry weight) in negative control	Number of isolates		LSD value (p = 0.05)
		tested	significantly increasing/decreasing seedling weight	
1	255.0	40	0 / 2	129.6
2	140.0	36	0 / 0	n.s. [1]
3	158.2	40	4 / 3	93.9
4	210.0	37	0 / 6	77.8
5	103.0	37	0 / 0	n.s.

B. Seedling height. Overview on five experiments conducted.

Experiment	Seedling height (mm) in negative control	Number of isolates		LSD value (p = 0.05)
		tested	significantly increasing/decreasing seedling height	
1	77.0	40	0 / 3	1.56
2	80.0	36	0 / 0	n.s.
3	67.3	40	17 / 2	1.89
4	85.0	37	0 / 4	1.66
5	69.5	37	0 / 0	n.s.

C. Mean predicted weight of tomato seedling treated by bacterial or fungal isolates[2].

	Number of isolates	Predicted mean seedling weight (mg dry weight) [3]	Mean efficacy (%)[4]
Bacterial isolates	40	203.8 (19.65 – 280.40)	17.60
Fungal isolates	55	197.22 (59.78 – 282.48)	13.79

D. Mean predicted height of tomato seedling treated by bacterial or fungal isolates[5].

	Number of isolates	Mean predicted mean seedling height (mm) [3]	Mean efficacy (%) [4]
Bacterial isolates	40	80.48 (38.11 – 91.65)	6.20
Fungal isolates	55	80.15 (34.13 – 94.66)	5.67

[1] No significant differences.
[2] Predicted weight of tomatoes of negative control was 173.3 mg.
[3] Range in brackets.
[4] Growth increase (%) in comparison to negative control.
[5] Predicted height of tomatoes of negative control was 75.8 mm.

twice in independent experiments. The mean seedling weight in the negative control treatments varied between 103 mg and 255 mg in the different experiments (Table 6 A). A significant increase of seedling weight was found only in four cases (experiment 3). In 11 cases, seedling weight was significantly decreased by a treatment with bacterial or fungal isolates. Seedling height ranged between 69.5 mm and 85.0 mm in the control treatments of the five experiments (Table 6 B). Seventeen isolates significantly increased seedling heights whereas in nine cases height was significantly decreased by isolate treatments.

The REML-analysis showed that there were significant differences in the seedling weight between treatments with different isolates ($p < 0.001$). The predicted seedling weight for the negative control was 173.3 mg. The approximate LSD ($p = 0.05$) for comparing treatment effects of isolates with the control treatment was LSD = 67.1 mg for all isolates tested twice. Ten bacterial isolates and ten fungal isolates significantly increased seedling weight by more than 67.1 mg compared to the control. Three bacterial isolates and one fungal isolate significantly decreased seedling weight by more than 67.1 mg compared to the control. Mean efficacy in increasing seedling weight for bacterial isolates was 17.60%, and for fungal isolates 13.79% (Table 6 C). For measurements of tomato seedling height, the REML-analysis showed that there were significant differences in height between treatments with different isolates ($p < 0.001$). The predicted seedling height for the control was 75.8 mm. The approximate LSD ($p = 0.05$) for comparing treatment effects of isolates with the control treatment was LSD = 12.1 mm for all isolates tested twice. Five bacterial isolates and nine fungal isolates significantly increased seedling height by more than 12.1 mm compared to control. Three bacterial isolates and one fungal isolate significantly decreased seedling height by more than 12.1 mm compared to the control. Mean efficacy in increasing seedling height for bacterial isolates was 6.20%, and for fungal isolates 5.67% (Table 6 D).

The ten most effective isolates increasing tomato seedling weight in decreasing order were: *Alternaria* sp. HTS537, *Pseudomonas* sp. HTS043, *T. asperellum / T. hamatum* HTS386, *C. sphaerospermum* HTS505, *Pseudomonas* sp. HTS076, *Chryseobacterium* sp. HTS080, *Alternaria* sp. HTS591, *Paenibacillus* sp. HTS008, *Cladosporium* sp. HTS495, and *Frigoribacterium faeni* HTS271. The ten most effective isolates increasing tomato seedling height in decreasing order were: *Alternaria* sp. HTS537, *Agrobacterium* sp./ *Rhizobium* sp. / HTS123, *Cladosporium* sp. HTS495, *Cladosporium* sp. HTS522, *Variovorax* sp. HTS231, *Arthrobacter* sp. HTS151, *C. sphaerospermum* HTS373, *Epicoccum nigrum* HTS471, *T. asperellum / T. hamatum* HTS386, and *Paenibacillus* sp. HTS008.

3.7. Bioassay on callus formation and rooting on tomato stem pieces

Five experiments have been conducted with tomato stem segments. The callus formation index (CFI; 0–4) in the negative control varied between CFI = 0.75 and CFI = 1.47 in the different experiments (Table 7 A). CFl was significantly enhanced in 21 cases compared to the control whereas significantly reduced in nine cases. In the same experiments, root formation on the tomato stem pieces was assessed. The rooting index (RI; 0–4) for stem pieces of the control treatments ranged between 0.75 and 1.33 of the five experiments (Table 7 B). In 72 out of 121 cases, treatments with bacterial or fungal isolates significantly increased RI. In 11 cases RI was significantly reduced by such treatments.

In total, 100 isolates (60 fungal isolates, 40 bacterial isolates) were tested in five experiments of which one fungal isolate and 20 bacterial

Table 7
Effect of treatments with bacterial or fungal isolates on callus formation index (CFI) and rooting index (RI) on tomato stem segments.

A. Callus formation index. Overview on five experiments conducted.

Experiment	CFI (0–4) in negative control	Number of isolates tested	Number of isolates significantly increasing/ decreasing seedling CFI	LSD value ($p = 0.05$)
1	1.47	12	0 / 5	0.91
2	1.42	8	0 / 0	1.07
3	1.08	29	10 / 0	0.93
4	0.75	58	11 / 1	0.60
5	1.00	14	0 / 3	0.99

B. Rooting index. Overview on five experiments conducted.

Experiment	RI (0–4) in negative control	Number of isolates tested	Number of isolates significantly increasing/ decreasing seedling RI	LSD value ($p = 0.05$)
1	1.00	12	5 / 1	0.69
2	1.00	8	0 / 2	0.84
3	1.33	29	15 / 3	0.68
4	0.75	58	46 / 0	0.68
5	0.81	14	6 / 5	0.52

C. Mean predicted callus formation index (CFI) for treatments with bacterial or fungal isolates[1].

	Number of isolates	Predicted CFI[2]	Mean efficacy (%) [3]
Bacterial isolates	40	1.20 (0.02 – 2.24)	−0.83
Fungal isolates	60	1.15 (0.13 – 3.16)	−4.96

D. Mean predicted rooting index (RI) for treatments with bacterial or fungal isolates[1].

	Number of isolates	Predicted RI[2]	Mean efficacy (%) [3]
Bacterial isolates	40	1.01 (-0.68 – 2.45)	29.49
Fungal isolates	60	2.07 (0.62 – 3.25)	165.38

[1] CFI of stem pieces of negative control was PDI = 1.21.
[2] Range in brackets.
[3] Increase (%) in comparison to negative control.
[4] Predicted RI of stem pieces of negative control was RI = 0.78.

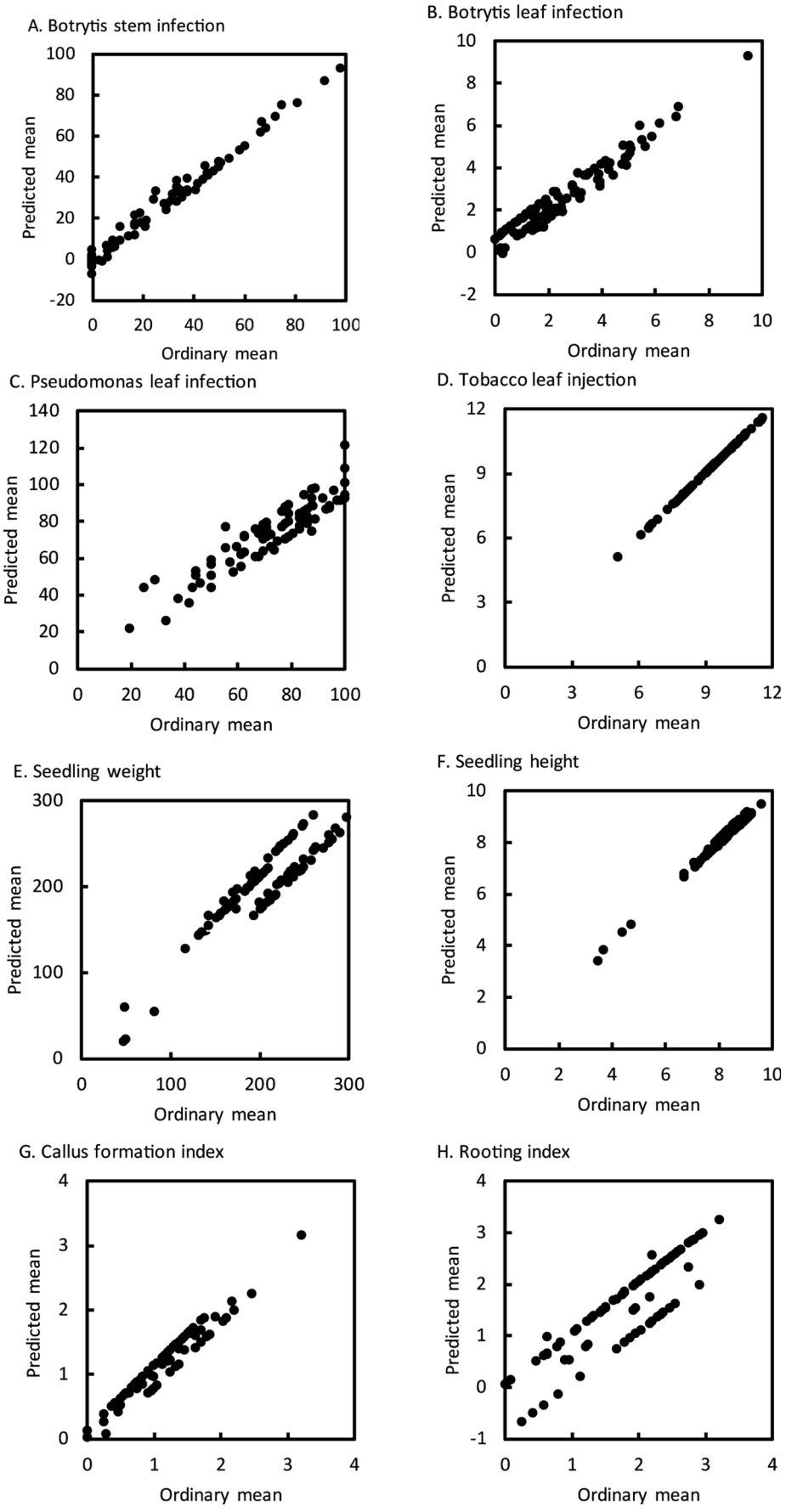

Fig. 1. Relationship between ordinary means and means predicted by REML analysis for the series of individual experiments executed for A. Bioassay on tomato stem infection by *Botrytis cinerea*; B. Bioassay on tomato leaf infection by *B. cinerea*; C. Bioassay on tomato leaf infection by *Pseudomonas syringae* pv. *tomato*; D. Bioassay on tobacco leaf infection by *Pseudomonas syringae* pv. *tomato* after injection of culture filtrates; E. Bioassay on growth promotion of tomato seedlings – Seedling weight; F. Bioassay on growth promotion of tomato seedlings – Seedling height; G. Bioassay on callus formation on tomato stem pieces; H. Bioassay on rooting on tomato stem pieces.

Table 8
Predicted means of treatments with bacterial and fungal isolates in comparison with the positive control in bioassays on tomato stem infection by *B. cinerea*, on tomato leaf infection by *B. cinerea* and *P. syringae* pv. *tomato*, on tobacco leaf infection by *P. syringae* pv. *tomato* after injection of culture filtrates of the isolates, and with the negative control in bioassays on growth promotion of tomato seedling, and callus formation and rooting in tomato stem segments.

Isolate	Predicted means[1] of variates assessed in bioassays on antagonism								Predicted means of variates assessed in bioassays on growth promotion							
	Botrytis stem infection[2]		Botrytis leaf infection[3]		Pseudomas leaf infection[4]		Tobacco leaf injection[5]		Seedling weight[6]		Seedling height[7]		Callus formation[8]		Rooting[9]	
Bacterial isolates																
Acinetobacter sp. HTS038	16.646	(50)	1.908	(45)	88.157	(83)	10.443	(58)	215.40	(36)	81.99	(48)	2.135	(3)	1.491	(60)
Arthrobacter sp. HTS151	38.257	(74)	4.211	(87)	57.602	(21)	12.737	(98)	222.56	(24)	90.77	(6)	1.575	(23)	1.031	(77)
Bacillus amyloliquefaciens S499	*		1.730	(41)	94.549	(97)	11.620	(86)	*		*		1.382	(38)	0.526	(90)
Bacillus simplex/Brevibacterium frigoritolerans HTS064	45.430	(84)	6.010	(98)	43.836	(7)	12.430	(96)	232.07	(22)	82.65	(43)	0.977	(64)	1.387	(65)
Bacillus sp. HTS003	69.648	(92)	2.292	(55)	96.491	(98)	8.013	(8)	182.07	(69)	67.99	(90)	0.852	(69)	1.346	(67)
Bacillus sp. HTS125	47.039	(85)	3.974	(83)	63.157	(28)	8.320	(10)	203.74	(50)	87.32	(16)	1.991	(4)	1.447	(63)
Bacillus sp. HTS150	0.985	(22)	2.300	(56)	77.046	(58)	13.097	(101)	217.07	(34)	83.65	(37)	1.394	(37)	1.554	(55)
Bacillus sp. HTS199	16.035	(49)	3.750	(80)	79.824	(65)	10.567	(65)	207.07	(47)	88.65	(12)	2.241	(2)	1.364	(66)
Bosea sp./*Rhizobium* sp. HTS244	33.312	(67)	1.219	(24)	72.880	(46)	9.640	(37)	189.23	(63)	80.61	(60)	*		*	
Chryseobacterium sp. HTS012	−3.655	(2)	1.604	(36)	86.769	(78)	10.740	(68)	174.23	(76)	82.61	(45)	0.825	(73)	−0.678	(100)
Chryseobacterium sp. HTS080	28.675	(61)	0.794	(9)	61.769	(27)	10.137	(49)	262.56	(6)	82.94	(41)	1.158	(53)	0.739	(84)
Chryseobacterium sp. HTS093	31.923	(64)	1.622	(37)	64.206	(30)	9.613	(35)	230.90	(23)	84.44	(31)	0.700	(83)	0.197	(92)
Citrobacter sp. HTS218	4.923	(39)	3.724	(78)	43.713	(6)	10.423	(56)	192.07	(61)	84.49	(30)	1.218	(50)	0.532	(89)
Dyella terrae HTS119	47.425	(86)	5.074	(95)	57.602	(20)	10.200	(51)	184.23	(65)	80.77	(57)	1.593	(18)	0.824	(82)
Flexivirga alba HTS120	27.289	(58)	2.648	(62)	50.681	(14)	9.837	(41)	250.90	(12)	83.94	(34)	1.616	(17)	0.947	(79)
Frigoribacterium faeni HTS162	−0.021	(19)	3.191	(72)	75.917	(56)	12.930	(100)	190.90	(62)	80.27	(61)	0.769	(78)	0.512	(91)
Frigoribacterium faeni HTS271	35.474	(72)	2.947	(69)	70.102	(37)	11.947	(92)	254.23	(10)	84.94	(26)	0.968	(65)	0.782	(83)
Herbaspirillum sp. HTS116	2.140	(36)	3.784	(81)	21.992	(1)	11.880	(90)	175.90	(74)	79.61	(66)	0.519	(90)	0.137	(93)
Kocuria palustris HTS010	33.539	(69)	2.502	(59)	50.657	(13)	8.967	(20)	242.07	(19)	86.99	(18)	1.144	(54)	1.679	(52)
Lysobacter sp. HTS263	−3.655	(5)	1.630	(38)	77.070	(59)	9.607	(34)	210.90	(42)	79.77	(65)	1.885	(6)	1.532	(57)
Microbacterium sp. HTS211	4.923	(38)	1.787	(42)	65.959	(32)	9.593	(33)	245.40	(15)	86.32	(22)	1.408	(36)	1.281	(69)
Microbacterium sp. HTS282	74.979	(94)	4.365	(89)	85.380	(74)	11.108	(76)	219.23	(29)	80.77	(58)	1.033	(63)	−0.344	(98)
Nesterenkonia sp. HTS237	11.431	(45)	2.680	(63)	74.405	(50)	8.887	(16)	217.56	(32)	84.77	(27)	0.741	(79)	1.281	(70)
Paenibacillus sp. HTS008	69.648	(93)	1.492	(34)	78.435	(62)	10.873	(70)	260.40	(8)	88.82	(10)	1.677	(13)	1.741	(50)
Paenibacillus sp. HTS277	33.845	(71)	3.437	(74)	48.003	(12)	11.887	(91)	210.90	(43)	84.11	(33)	1.116	(58)	1.989	(40)
Pseudomonas sp. HTS042	−3.152	(6)	1.254	(28)	70.102	(36)	10.100	(48)	19.56	(95)	38.11	(94)	0.019	(99)	0.054	(95)
Pseudomonas sp. HTS043	−3.655	(3)	2.898	(67)	83.991	(70)	10.470	(59)	280.40	(2)	83.65	(36)	0.852	(70)	1.846	(45)
Pseudomonas sp. HTS076	−0.021	(16)	2.122	(52)	72.880	(45)	9.657	(38)	267.07	(5)	87.82	(15)	1.227	(49)	1.554	(54)
Pseudomonas sp. HTS104	−0.021	(18)	1.994	(49)	46.491	(11)	11.773	(89)	22.06	(94)	45.11	(93)	0.019	(100)	0.054	(96)
Pseudomonas sp. HTS143	−1.183	(10)	2.899	(68)	71.491	(40)	12.297	(95)	54.23	(93)	48.27	(92)	0.075	(98)	−0.511	(99)
Pseudomonas sp. HTS174	−1.183	(12)	2.148	(54)	86.769	(79)	10.473	(60)	202.07	(54)	86.49	(21)	1.885	(5)	2.324	(25)
Rhizobium sp./*Agrobact.* sp. HTS123	−3.655	(4)	0.963	(15)	92.324	(89)	10.323	(54)	218.74	(30)	91.65	(2)	1.408	(35)	1.614	(53)
Rhodanobacter sp./*Dyella* sp. HTS254	17.652	(51)	4.229	(88)	100.657	(102)	9.250	(28)	180.90	(72)	81.44	(53)	1.866	(9)	1.239	(72)
Rhodococcus sp. HTS191	21.590	(54)	3.154	(71)	71.491	(41)	10.927	(72)	203.74	(51)	71.99	(84)	1.116	(56)	1.531	(58)
Serratia sp. HTS071	0.985	(21)	1.678	(39)	38.157	(4)	10.073	(47)	222.07	(25)	80.15	(63)	1.632	(15)	2.450	(20)
Shinella sp. HTS262	−0.374	(14)	1.250	(27)	38.157	(5)	8.820	(15)	165.90	(82)	77.27	(76)	0.783	(77)	−0.136	(97)
Solibacillus sp./*Bacillus* sp./*Caryophanon* sp. HTS023	42.126	(80)	1.840	(44)	100.657	(101)	10.147	(50)	243.74	(18)	83.82	(35)	1.491	(26)	1.406	(64)
Sphingomonas sp. HTS078	33.683	(70)	4.139	(85)	75.818	(53)	12.157	(94)	204.23	(49)	86.94	(19)	1.825	(11)	1.447	(62)
Unidentified HTS101	−0.021	(17)	6.107	(99)	56.237	(19)	10.663	(67)	217.56	(31)	83.61	(38)	0.269	(96)	0.096	(94)
Variovorax sp. HTS173	−1.183	(11)	3.659	(76)	86.929	(80)	11.670	(88)	213.74	(38)	82.65	(44)	1.491	(27)	0.864	(81)
Variovorax sp. HTS231	−0.021	(20)	3.677	(77)	71.491	(42)	8.437	(12)	244.23	(16)	90.94	(5)	1.116	(57)	1.114	(75)
Fungal isolates																
Alternaria alternata / A. tenuissima HTS536	−0.829	(13)	1.171	(23)	80.175	(66)	10.613	(66)	214.78	(37)	75.63	(79)	1.045	(62)	1.954	(44)
Alternaria infectoria / A. triticina HTS444	24.171	(56)	1.226	(25)	77.397	(60)	9.573	(32)	*		*		1.045	(60)	1.329	(68)
Alternaria infectoria / A. triticina HTS472	55.421	(89)	6.915	(101)	60.730	(25)	11.530	(83)	146.44	(87)	66.63	(91)	1.336	(42)	1.954	(43)
Alternaria sp. HTS537	*		1.012	(16)	76.928	(57)	12.920	(99)	282.48	(1)	94.66	(1)	0.628	(87)	0.621	(88)

(*continued on next page*)

Table 8 (*continued*)

Isolate	Predicted means[1] of variates assessed in bioassays on antagonism								Predicted means of variates assessed in bioassays on growth promotion							
	Botrytis stem infection[2]		Botrytis leaf infection[3]		Pseudomas leaf infection[4]		Tobacco leaf injection[5]		Seedling weight[6]		Seedling height[7]		Callus formation[8]		Rooting[9]	
Alternaria sp. HTS572	38.755	(76)	4.170	(86)	69.063	(35)	9.047	(22)	203.11	(52)	82.96	(40)	0.670	(85)	2.829	(11)
Alternaria sp. HTS591	1.179	(34)	2.448	(58)	87.604	(82)	8.700	(14)	260.82	(7)	88.82	(11)	0.128	(97)	1.121	(74)
Alternaria sp. HTS597	*		9.295	(102)	82.283	(69)	11.373	(80)	203.11	(53)	76.46	(78)	0.836	(72)	1.704	(51)
Aspergillus sp. HTS458	92.921	(97)	2.827	(65)	97.662	(100)	3.873	(1)	232.48	(21)	85.82	(23)	0.795	(75)	2.246	(29)
Aureobasidium pullulans / A. proteae HTS448	18.098	(52)	0.769	(8)	60.540	(24)	10.523	(64)	221.44	(26)	82.46	(46)	1.461	(29)	2.788	(12)
Aureobasidium pullulans / A. proteae HTS508	−2.692	(7)	1.548	(35)	92.484	(90)	6.420	(2)	208.11	(46)	80.79	(56)	1.378	(40)	2.038	(37)
Aureobasidium pullulans / A. proteae HTS541	1.179	(31)	1.409	(32)	73.040	(48)	10.067	(46)	144.78	(88)	79.46	(67)	0.378	(95)	1.496	(59)
Aureobasidium pullulans / A. proteae HTS551	−2.692	(8)	0.205	(2)	92.484	(91)	11.283	(79)	219.78	(28)	78.46	(71)	0.628	(88)	2.204	(32)
Aureobasidium pullulans 490	*		0.217	(3)	75.818	(55)	7.960	(7)	*		*		3.157	(1)	2.561	(16)
Aureobasidium pullulans/A. proteae HTS347	1.179	(23)	4.126	(84)	75.818	(54)	8.210	(9)	194.78	(58)	84.63	(29)	1.420	(31)	2.371	(22)
Botryosporium longibrachiatum HTS377	28.338	(59)	5.322	(96)	75.440	(52)	11.993	(93)	174.78	(75)	79.79	(64)	1.378	(39)	2.038	(36)
Botryosporium longibrachiatum HTS402	76.255	(95)	0.656	(5)	58.773	(23)	9.113	(24)	212.48	(40)	84.99	(25)	1.628	(16)	2.829	(9)
Botrytis cinerea / Sclerotinia sclerotiorum HTS452	86.671	(96)	5.031	(94)	91.286	(88)	9.223	(26)	*		*		0.503	(91)	0.663	(85)
Botrytis cinerea / Sclerotinia sclerotiorum HTS531	32.505	(66)	4.928	(93)	69.063	(34)	9.627	(36)	168.11	(78)	71.79	(85)	1.253	(48)	2.996	(2)
Candida palmioleophila HTS584	1.179	(32)	4.535	(91)	92.484	(92)	10.897	(71)	211.44	(41)	80.63	(59)	0.795	(76)	2.954	(7)
Candida palmioleophila HTS589	1.179	(33)	0.656	(7)	81.373	(67)	9.463	(31)	128.11	(91)	71.63	(86)	0.878	(68)	2.163	(34)
Cladosporium sp. HTS332	42.921	(81)	1.796	(43)	71.273	(39)	10.508	(63)	254.15	(11)	87.99	(14)	0.920	(67)	2.204	(30)
Cladosporium sp. HTS362	39.233	(77)	1.331	(31)	88.993	(85)	10.257	(52)	217.48	(33)	84.32	(32)	1.586	(20)	2.371	(23)
Cladosporium sp. HTS455	*		0.894	(12)	53.218	(17)	10.057	(45)	182.48	(68)	78.49	(70)	1.836	(10)	2.871	(8)
Cladosporium sp. HTS495	40.838	(78)	1.473	(33)	85.162	(73)	10.320	(53)	259.15	(9)	91.16	(3)	0.711	(81)	1.829	(48)
Cladosporium sp. HTS522	1.179	(29)	4.478	(90)	72.326	(44)	8.913	(17)	240.82	(20)	90.99	(4)	*		*	
Cladosporium sp. HTS529	45.005	(83)	1.718	(40)	65.382	(31)	9.660	(39)	192.48	(60)	81.16	(54)	1.170	(52)	2.288	(26)
Cladosporium sp. HTS590	1.255	(35)	2.313	(57)	108.773	(103)	7.560	(6)	197.48	(57)	81.99	(49)	1.420	(33)	2.038	(38)
Cladosporium sphaerospermum HTS373	45.005	(82)	2.101	(51)	53.218	(16)	11.103	(75)	244.15	(17)	90.66	(7)	1.336	(41)	2.954	(3)
Cladosporium sphaerospermum HTS505	29.265	(62)	1.144	(21)	86.551	(76)	9.835	(40)	270.82	(4)	86.82	(20)	1.336	(43)	2.413	(21)
Cladosporium sphaerospermum HTS545	22.308	(55)	2.855	(66)	81.613	(68)	8.930	(18)	246.48	(14)	81.16	(55)	0.670	(84)	1.079	(76)
Cladosporium sphaerospermum HTS576	49.171	(87)	2.805	(64)	66.286	(33)	8.933	(19)	166.44	(81)	77.29	(75)	1.128	(55)	2.079	(35)
Clonostachys rosea 016	−7.289	(1)	1.170	(22)	78.218	(61)	7.197	(4)	*		*		0.407	(92)	0.978	(78)
Coniochaeta hoffmannii HTS460	6.735	(42)	0.656	(6)	73.040	(47)	11.650	(87)	209.78	(44)	81.63	(50)	1.045	(61)	0.663	(86)
Cryptococcus laurentii HTS343	−0.084	(15)	1.957	(47)	71.651	(43)	11.587	(85)	206.44	(48)	85.79	(24)	1.045	(59)	1.788	(49)
Cryptococcus stepposus / Filobasidium globisporum HTS511	1.179	(27)	1.247	(26)	70.262	(38)	9.877	(42)	209.78	(45)	88.46	(13)	1.586	(22)	2.496	(19)
Cryptococcus victoriae HTS553	5.641	(40)	0.911	(13)	86.593	(77)	11.260	(78)	166.44	(80)	81.63	(51)	1.670	(14)	2.579	(15)
Engyodontium album/Torrubiella sp. HTS331	9.512	(44)	1.048	(17)	35.730	(3)	11.390	(81)	178.11	(73)	77.63	(74)	0.702	(82)	0.640	(87)
Engyodontium parvisporum / Lecanicillium tenuipes HTS351	61.671	(90)	4.698	(92)	44.063	(9)	7.397	(5)	173.11	(77)	78.96	(68)	1.586	(19)	3.246	(1)
Epicoccum nigrum HTS471	*		0.879	(11)	97.326	(99)	10.943	(73)	249.15	(13)	90.66	(8)	0.711	(80)	0.871	(80)
Epicoccum nigrum HTS491	11.671	(47)	6.419	(100)	94.063	(94)	10.507	(62)	*		*		1.878	(8)	1.829	(47)
Fusarium oxysporum HTS519	1.179	(28)	−0.066	(1)	94.063	(95)	9.423	(30)	184.78	(64)	74.13	(82)	*		*	
Penicillium citrinum / P. griseofulvum HTS378	33.462	(68)	3.729	(79)	86.551	(75)	9.037	(21)	165.82	(83)	80.16	(62)	1.253	(45)	2.954	(4)
Plectosphaerella cucumerina / Colletotrichum pisi HTS528	1.179	(30)	1.274	(29)	78.595	(63)	10.753	(69)	213.11	(39)	82.46	(47)	0.378	(94)	1.454	(61)
Plectosphaerella sp. HTS407	28.338	(60)	2.542	(60)	88.508	(84)	11.393	(82)	143.11	(89)	71.46	(87)	0.836	(71)	2.829	(10)
Plectosphaerella sp. HTS415	1.179	(24)	0.931	(14)	92.584	(93)	9.367	(29)	183.11	(66)	76.79	(77)	0.545	(89)	1.538	(56)
Plectosphaerella sp. HTS417	36.671	(73)	3.638	(75)	73.139	(49)	9.103	(23)	216.44	(35)	83.29	(39)	0.378	(93)	1.267	(71)
Plectosphaerella sp. HTS421	38.755	(75)	2.586	(61)	63.508	(29)	8.483	(13)	183.11	(67)	78.29	(72)	0.795	(74)	1.121	(73)
Plectosphaerella sp. HTS429	1.179	(25)	3.151	(70)	84.251	(71)	10.413	(55)	181.44	(71)	74.46	(81)	0.628	(86)	1.829	(46)
Plectosphaerella sp. HTS473	1.179	(26)	*		84.251	(72)	10.440	(57)	198.11	(56)	77.79	(73)	0.961	(66)	2.204	(31)
Pseudolachnella sp. HTS410	63.755	(91)	5.508	(97)	91.286	(87)	11.183	(77)	*		*		1.253	(46)	2.538	(17)
Pseudolachnella sp. HTS431	30.421	(63)	3.358	(73)	55.175	(18)	9.907	(43)	*		*		1.253	(47)	2.954	(6)

(*continued on next page*)

Table 8 (*continued*)

Isolate	Predicted means[1] of variates assessed in bioassays on antagonism					Predicted means of variates assessed in bioassays on growth promotion				
	Botrytis stem infection[2]		Botrytis leaf infection[3]	Pseudomas leaf infection[4]	Tobacco leaf injection[5]	Seedling weight[6]		Seedling height[7]	Callus formation[8]	Rooting[9]
Pythium aphanidermatum HTS401	−1.908	(9)	2.126 (53)	44.063 (10)	9.950 (44)	59.78	(92)	34.13 (95)	1.878 (7)	1.954 (42)
Sporobolomyces roseus HTS354	6.336	(41)	0.656 (4)	25.818 (2)	8.367 (11)	181.44	(70)	78.79 (69)	1.461 (28)	2.621 (14)
Sporobolomyces roseus HTS453	18.836	(53)	2.031 (50)	74.528 (51)	11.023 (74)	146.44	(86)	70.46 (88)	1.711 (12)	2.246 (28)
Sporobolomyces roseus HTS503	27.169	(57)	1.097 (19)	86.929 (81)	13.417 (102)	128.11	(90)	74.63 (80)	1.586 (21)	2.496 (18)
Sporobolomyces roseus HTS600	32.030	(65)	1.079 (18)	91.095 (86)	7.093 (3)	168.11	(79)	81.46 (52)	1.420 (34)	2.288 (27)
Trichoderma asperellum / *T. hamatum* HTS386	11.671	(46)	0.815 (10)	58.773 (22)	14.027 (103)	272.48	(3)	89.66 (9)	1.170 (51)	2.371 (24)
Trichoderma asperellum / *T. hamatum* HTS387	9.203	(43)	1.100 (20)	79.607 (64)	9.213 (25)	192.48	(59)	84.66 (28)	1.503 (25)	2.954 (5)
Verticillium sp. / *Simplicillium lamellicola* / *Guignardia vaccinii* HTS341	15.838	(48)	3.899 (82)	44.063 (8)	12.450 (97)	219.78	(27)	82.79 (42)	1.295 (44)	1.954 (41)
Verticillium sp. / *Simplicillium lamellicola* / *Guignardia vaccinii* HTS549	4.342	(37)	1.917 (46)	52.397 (15)	9.227 (27)	199.78	(55)	87.13 (17)	1.461 (30)	1.996 (39)
Verticillium sp. / *Simplicillium lamellicola* / *Guignardia vaccinii* HTS561	53.338	(88)	1.959 (48)	60.730 (26)	10.493 (61)	163.11	(84)	72.79 (83)	1.420 (32)	2.663 (13)
Verticillium sp. / *V. cf. aranearum* HTS565	40.838	(79)	1.292 (30)	94.063 (96)	11.537 (84)	154.78	(85)	70.46 (89)	1.545 (24)	2.163 (33)
Control	66.875		5.064	88.095	9.789	173.30		75.77	1.206	0.784

[1] Ranking of isolates for effects on disease control and growth promotion in brackets.
[2] Disease index (DI, 0–100); [3] Lesion diameter (mm); [4] Disease index (DI, 0 – 100); [5] Lesion diameter (mm); [6] Seedling weight (mg dry weight); [7] Seedling height (mm); [8] Callus formation index (CFI, 0 – 4); [9] Rooting index (RI, 0 – 4).

isolates were tested twice in independent experiments. The REML-analysis showed that there are significant differences in the CFI between treatments with different isolates ($p < 0.001$). The predicted CFI for the control treatment was CFI = 1.21. The approximate LSD ($p = 0.05$) for comparing treatment effects of isolates with the control treatment was LSD = 0.53 for isolates tested once and LSD = 0.51 for isolates that were tested twice. Four fungal isolates and seven bacterial isolates significantly increased the predicted CFI by more than 0.53 compared to the control. Fifteen fungal isolates and eight bacterial isolates significantly decreased the predicted CFI by more than 0.53 compared to the control. Mean efficacy in increasing the predicted CFI for bacterial isolates was −0.83% and for fungal isolates −4.96% (Table 7 C). For root formation, REML-analysis showed that there are significant differences in the predicted RI between treatments with different isolates ($p < 0.001$). The predicted RI for the control was RI = 0.78. The approximate LSD ($p = 0.05$) for comparing treatment effects of isolates with the control was LSD = 0.55 for isolates tested once and LSD = 0.51 for isolates that were tested twice. Fifty fungal isolates and 18 bacterial isolates significantly increased RI by more than 0.53 compared to the control. No fungal isolates and nine bacterial isolates significantly decreased the predicted RI by more than 0.53 compared to the control. Mean efficacy in increasing the RI for bacterial isolates was 29.49%, and for fungal isolates 165.38% (Table 7 D).

The ten most effective isolates increasing CFI were in decreasing order: *A. pullulans* 490, *Bacillus* sp. HTS199, *Acinetobacter* sp. HTS038, *Bacillus* sp. HTS125, *Lysobacter* sp. HTS263, *Pseudomonas* sp. HTS174, *P. aphanidermatum* HTS401, *E. nigrum* HTS491, *Rhodanobacter* sp. / *Dyella* sp. HTS254, and *Cladosporium* sp. HTS455. The ten most effective isolates increasing RI were in decreasing order: *E. parvisporum* / *L. tenuipes* HTS351, *B. cinerea* / *Sclerotinia sclerotiorum* HTS531, *C. sphaerospermum* HTS373, *Penicillium citrinum* / *P. griseofulvum* HTS378, *T. asperellum* / *T. hamatum* HTS387, *Pseudolachnella* sp. HTS431, *C. palmioleophila* HTS584, *Cladosporium* sp. HTS455, *Botryosporium longibrachiatum* HTS402, and *Plectosphaerella* sp. HTS407.

3.8. Overall analysis of isolate rankings in different bioassays

Correlations between predicted means for the various pairs of bioassays were determined. Antagonistic effects of bacterial and fungal isolates have been quantified in bioassays on tomato stem infection by *B. cinerea*, on tomato leaf infection by *B. cinerea* and on tomato leaf infection by *P. syringae* pv. *tomato*. Bioassays on tobacco leaf infection by *P. syringae* pv. *tomato* after injection of culture filtrates and potential of isolates to produce biosurfactants causing droplet dispersal were conducted to assess for characteristics that may correlate with the antagonistic potential of the isolates. Possible effects of the bacterial and fungal isolates on plant growth were quantified in the bioassay on growth promotion of tomato seedlings and in bioassays on callus formation and rooting on tomato stem segments.

For several bioassays, levels of the quantified parameter, e.g. disease levels, differed between individual experiments. REML analysis correcting for such differences were used for a combined analysis of results of the individual series of experiments for each type of bioassay (except for the bioassay on droplet dispersal). Ordinary means for treatments in the various experiment strongly correlated with the respective predicted means (Fig. 1). The predicted means for each bioassay (except for the bioassay on droplet dispersal) allowed the identification of significant treatment effects and a ranking of all tested isolates in their potential in antagonism or growth promotion (Table 8). Generally, there was no correlation between effects of isolates on the different variates (Table 9; Fig. 2). A weak significant correlation was found for fungal isolates between Botrytis stem infection and Botrytis leaf infection. Only growth promotion of tomato and callus formation and rooting on stem pieces showed a positive significant correlation for bacteria. For fungal isolates, a positive correlation between tomato growth promotion and rooting was found. However, the correlation

with callus formation was negative.

Results obtained in the various bioassays for isolates causing droplet dispersal versus isolates not causing droplet dispersal were analysed separately for bacteria, filamentous fungi and yeast-like fungi. For Botrytis stem infection and Botrytis leaf infections, the means of these groups causing droplet dispersal were lower compared to the means for the groups not causing dispersal. However, strong antagonistic effects against *B. cinerea* infection of stems or leaves were also found for isolates of bacteria, filamentous fungi and yeast-like fungi that did not cause droplet dispersal (Fig. 3). For all other bioassays, means of the groups causing dispersal and groups not causing dispersal did not differ (data not presented). The same pattern was found for tests with supernatants of the isolates (data not presented).

4. Discussion

Approximately hundred isolates of bacteria and fungi from tomato have been tested in bioassays on whole or parts of tomato plants for their efficacy in reducing disease symptoms under controlled conditions. These bioassays on biological control of Botrytis stem infection, Botrytis leaf infection and Pseudomonas leaf infection allowed to rank the candidate antagonists according to their efficacy, and sets of best performing antagonists were selected. The reference isolates with known antagonism against *B. cinerea*, *A. pullulans* 490 and *C. rosea* (syn *Gliocladium roseum* (Dik et al., 1999; Köhl et al., 1995) ranked amongst the best isolates, confirming results of earlier conducted bioassays. Series of 13, 7 and 8 individual bioassays on Botrytis stem canker, Botrytis leaf rot and Pseudomonas leaf rot were needed to test approximately hundred isolates once or in many cases twice. A considerable variation in disease levels was observed between the individual tests, e.g. the disease index (DI) for Botrytis stem infection for the positive controls ranged between 12.5 and 95.8% and LSD-values indicating statistically significant differences between treatments ranged between 8.5 and 32.2 for the 13 conducted bioassays. Results of three out of 13 bioassays were not further evaluated because DI of the positive control was considerably lower compared to the remaining

Table 9
Correlations between predicted results of the effects of treatments with bacterial and fungal isolates in various bioassays [1].

A. Bacterial isolates.

	Botrytis stem infection	Botrytis leaf infection	Pseudomonas leaf infection	Tobacco leaf injection	Seedling weight	Seedling height	Callus formation
Botrytis leaf infection	0.2191						
	40						
	0.1742						
Pseudomonas leaf infection	0.1024	−0.2160					
	40	41					
	0.5293	0.1749					
Tobacco leaf injection	−0.0805	0.2748	−0.0994				
	40	41	41				
	0.6213	0.0821	0.5365				
Seedling weight	0.2704	0.0817	0.1243	−0.1536			
	40	40	40	40			
	0.0915	0.6160	0.4446	0.3439			
Seedling height	0.1858	0.1825	0.0915	−0.1117	**0.8925**		
	40	40	40	40	40		
	0.2511	0.2597	0.5742	0.4926	< 0.001		
Callus formation	0.2324	0.0183	0.3079	−0.1489	**0.5304**	**0.6404**	
	39	40	40	40	39	39	
	0.1545	0.9108	0.0533	0.3598	< 0.001	< 0.001	
Rooting	0.1101	−0.0667	0.1440	−0.1697	**0.5256**	**0.4598**	**0.6388**
	39	40	40	40	39	39	40
	0.5045	0.6824	0.3752	0.2952	< 0.001	0.0032	< 0.001

B. Fungal isolates.

	Botrytis stem infection	Botrytis leaf infection	Pseudomonas leaf infection	Tobacco leaf injection	Seedling weight	Seedling height	Callus formation
Botrytis leaf infection	**0.3480**						
	56						
	0.0086						
Pseudomonas leaf infection	−0.0458	0.0237					
	57	61					
	0.7354	0.8560					
Tobacco leaf injection	−0.1756	0.0216	−0.1279				
	57	61	62				
	0.1914	0.8689	0.3217				
Seedling weight	0.0565	−0.0949	0.1593	0.0222			
	51	54	55	55			
	0.6936	0.4947	0.2455	0.8721			
Seedling height	0.0947	−0.1274	0.1639	−0.0435	**0.8366**		
	51	54	55	55	55		
	0.5088	0.3585	0.2318	0.7524	< 0.001		
Callus formation	0.0875	−0.0810	−0.2168	−0.0230	**−0.3792**	**−0.2819**	
	55	59	60	60	53	53	
	0.5254	0.5419	0.0961	0.8617	0.0051	0.0409	
Rooting	0.1935	0.0555	−0.1559	−0.0751	−0.2338	−0.0959	**0.5550**
	55	59	60	60	53	53	60
	0.1568	0.6764	0.2344	0.5683	0.0920	0.4947	< 0.001

[1] Detailed results for individual isolates and bioassays are shown in Table 8. Correlation coefficient, number of observations and P-value for two-sided test for difference from zero are presented.

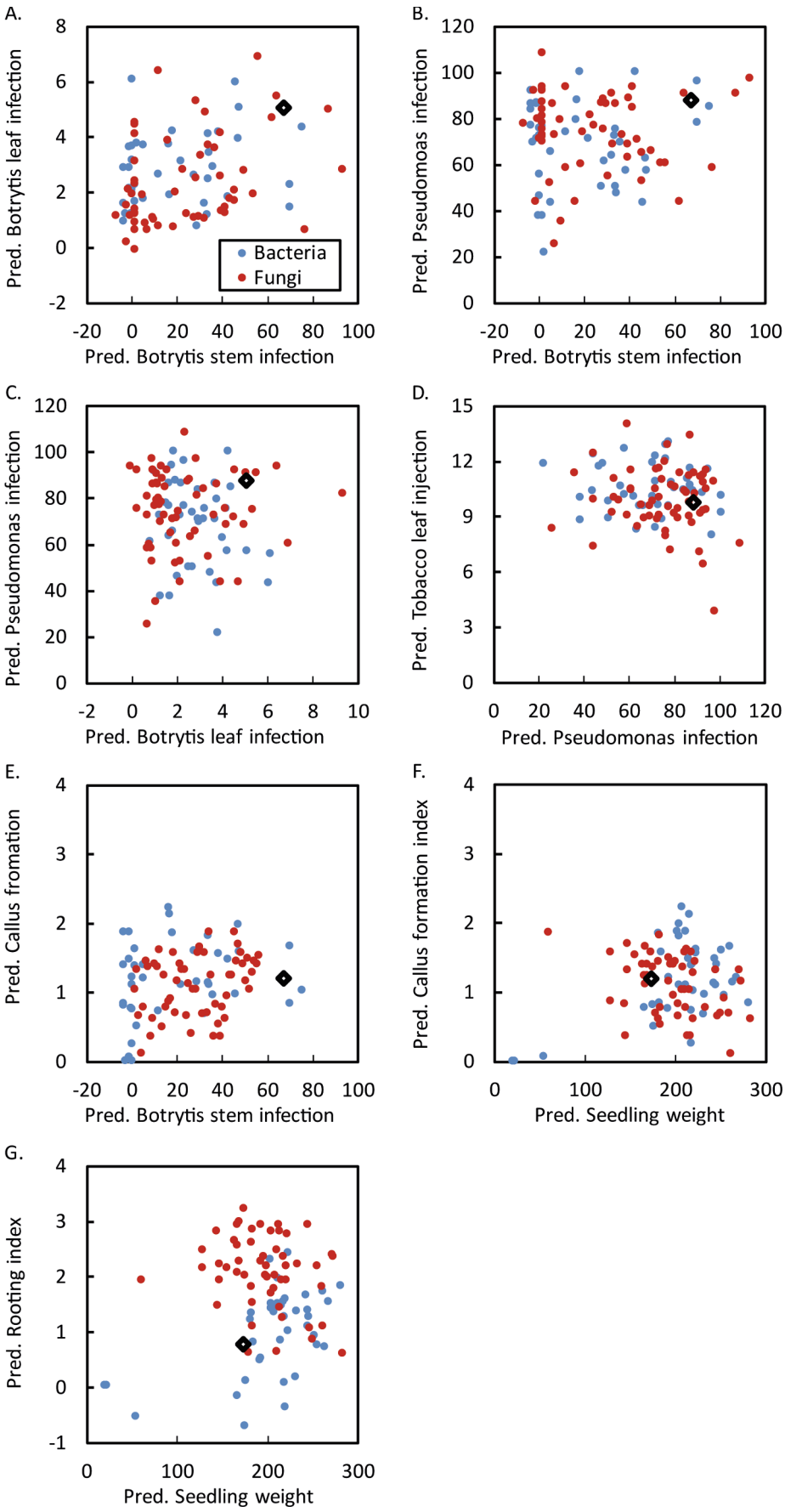

Fig. 2. Relationship between predicted means for effects by bacterial and fungal isolates in different bioassays. Values for control treatments indicated by open squares (◊). A. Bioassay on tomato stem infection by *Botrytis cinerea* and Bioassay on tomato leaf infection by *B. cinerea*; B. Bioassay on tomato stem infection by *B. cinerea* and Bioassay on tomato leaf infection by *Pseudomonas syringae* pv. *tomato*; C. Bioassay on tomato leaf infection by *B. cinerea* and Bioassay on tomato leaf infection by *P. syringae* pv. *tomato*; D. Bioassay on tomato leaf infection by *P. syringae* pv. *tomato* and Bioassay on tobacco leaf infection by *P. syringae* pv. *tomato* after injection of culture filtrates; E. Bioassay on tomato stem infection by *B. cinerea* and Bioassay on callus formation on tomato stem pieces; F. Bioassay on growth promotion of tomato seedlings – Seedling weight and Bioassay on callus formation on tomato stem pieces; and G. Bioassay on growth promotion of tomato seedlings – Seedling weight and Bioassay on rooting on tomato stem pieces.

experiments. Also for bioassays on Botrytis stem canker, variation in the level of symptoms expression was observed between individual bioassays. In two bioassays disease was too low to identify antagonistic isolates. One out of eight bioassays on Pseudomonas leaf infection had to be excluded from further data analysis for the same reason. Thus, a substantial number of individual bioassays was needed to test the set of candidate isolates for their efficacy to control the three diseases. A number of bioassays was lost due to variation in achieved disease levels. Consequently, selection strategies based on disease rating on plants or plants parts demanded substantial resources in labor and time, facilities to raise standardized plants, and climate facilities to allow repeated experiments under standardized controlled conditions. Because the assessed symptoms were the results of complex interactions between host plant, pathogen and antagonist depending on various environmental factors, variation between experiments and loss of individual experiments were common although environmental conditions were standardized as much as possible. For example, variation in host susceptibility may depend on individual plant reactions and on seasonal effects although plants were raised in greenhouse compartments with highly controlled light, temperature and humidity.

Data analysis using REML to compensate for variation between individual bioassays allowed to identify antagonists and to rank them according to their estimated efficacies against each of the three diseases. For Botrytis stem rot, candidates with high efficacy belonged to genera or species of bacteria or fungi known from literature for antagonism against plant pathogens such as *Pseudomonas* sp. (Haas & Défago, 2005), *Lysobacter* sp. (Postma et al., 2009), *Chryseobacterium* sp. (Kim et al., 2008), *Clonostachys rosea* (Sutton et al., 1997), *Rhizobium* sp. / *Agrobacterium* sp. and *Aureobasidium* sp. (Freimoser et al., 2019).

Isolates tested in this study represented bacteria, yeast and hyphal fungi from different niches of tomato crops. Such isolates had not been pre-selected according to their putative biological control traits, e.g. *Trichoderma* spp., *Bacillus* spp. or *Pseudomonas* spp. were not preferably tested amongst isolates belonging to other genera. Yet unknown antagonists of *B. cinerea* were detected besides those belonging to genera with known antagonism mentioned above even within a considerably small set of hundred isolates from a defined host crop. These finding indicates that there are plenty of unexploited opportunities for biological control to be developed. Such genera or species newly found as potential antagonists of plant pathogens have to be assessed for potential pathogenicity against humans, animals and plants to exclude potential unwanted pathogens, for example *Pythium aphanidermatum* as known pathogen of tomato (Sutton et al., 2006), was found to be antagonistic against *B. cinerea* in the bioassay. Remaining isolates are candidates for further in depth studies regarding ecology, efficacy against plant pathogens and inoculum producibility (Köhl et al., 2011).

Also for Botrytis leaf rot control, promising candidates belonged to genera or species known from literature for antagonism against plant pathogens such as *Aureobasidium* sp., *Sporobolomyces roseus* (Filonow, 1998), *Trichoderma* sp. (Harman et al., 2004) and *Chryseobacterium* sp. But again, also genera or species not described earlier as antagonists, have been selected as strong antagonists of *B. cinerea* on tomato leaves with isolates of the yeast *Candida palmioleophila* and hyphal fungi *Botryosporium longibrachiatum* and *Coniochaeta hoffmannii*. These species have been described as saprophytes and are thus, after thorough evaluation of possible pathogenicity to humans or animals, promising candidates for further screening as biocontrol agents. However, the antagonistic strain of *F. oxysporum* needs further evaluation because

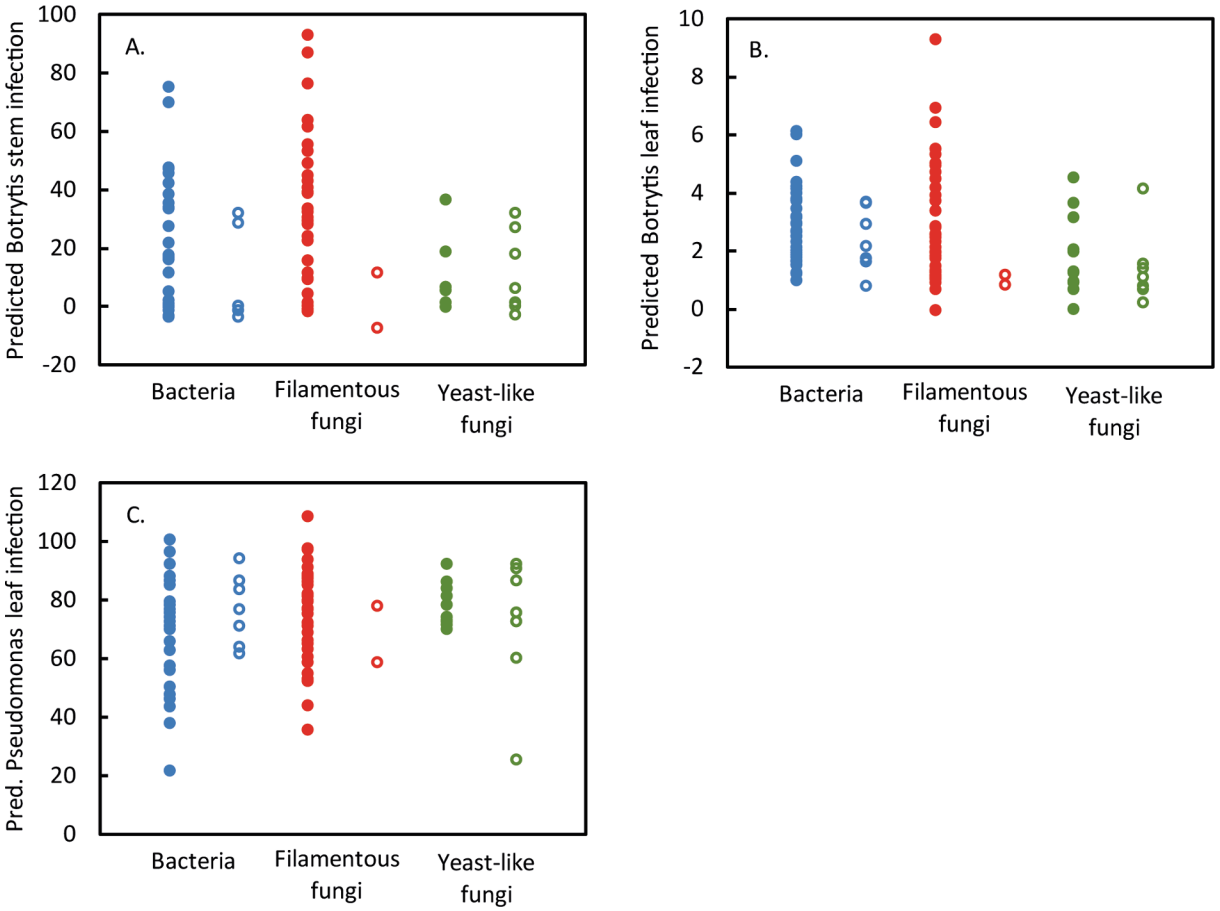

Fig. 3. Comparison of effects of strains of bacteria, filamentous fungi and yeast-like fungi causing no dispersal (solid circles) or dispersal (empty circles) of inoculated droplets and their effect on A. Predicted Botrytis stem infection (positive control: 66.88); B. Predicted Botrytis leaf infection (positive control: 5.06); and C. Predicted Pseudomonas leaf infection (positive control: 88.10).

this species includes besides saprophytic, endophytic and antagonistic strains (Aimé et al., 2013), also strains causing severe plant diseases in specific crops including tomato (Bao et al., 2002).

For biological control of Pseudomonas leaf rot, promising candidates belonged to genera or species known for antagonism against plant pathogens such as *Serratia* sp. (Soenens & Imperial, 2019), *Bacillus simplex / Brevibacterium frigoritolerans* (Miao et al., 2018) and, surprisingly, also a yeast isolate of *Sporobolomyces roseus*. An unexpected high abundance of 'new' antagonists against Pseudomonas leaf spot were found such as bacterial isolates of *Citrobacter* sp., a genus that also encompasses species with antibacterial potential (Mandal et al., 2013), and a fungal isolate of *Engyodontium album / Torrubiella* sp. Species within *Engyodontium* have been implicated in the parasitism of *Meloidogyne* eggs (Muthulakshmi et al., 2017).

Two bioassays were developed to assess the antagonistic properties of bacterial and fungal isolates in higher throughput compared to bioassays with rating of disease symptoms discussed above. A bioassay on tobacco plants was conducted using sterile supernatants of each isolate that were locally injected together with cells of *P. syringae* pv. *tomato* in tobacco leaves and local hypersensitive reaction development at the injection sites was rated. This set up allowed the testing of supernatants of approximately 100 isolates on four plants within one experiment that could be repeated on a second set of plant. Such a high-throughput screening approach encompasses modes of action related to direct inhibition of the pathogen and/or interfere on the acyl-homoserine lactone-regulated production of virulence factors (Licciardello et al., 2007). However, there was no correlation between the bioassays on Pseudomonas leaf infection and the high-throughput test based on injections of supernatant.

A main difference between the two assays is that for the *in vivo* assay living cells of the isolates were applied whereas sterile supernatants possibly containing secondary metabolites produced during culturing were tested in the high-throughput test. A positive correlation between the assays can be expected if similar effective metabolites as produced during culturing are also produced *in situ* in the *in vivo* bioassay and other modes of action are not effective in this situation. However, mixed modes of action acting in parallel or in cascades of events are common for pathogen-antagonist interactions (Köhl et al., 2019). This may explain that for a set of isolates belonging to a broad range of bacterial and fungal genera with their particular combined modes of action, a screening for a specific mode of action (as done in the high-throughput assay) cannot identify the real antagonistic potential of the isolates. For the selection of antagonists within a particular taxonomical group with antagonists expressing similar modes of action, e.g. via specific groups of secondary metabolites, such a screening may be more powerful. However, even for a screening of isolates belonging to a same bacterial genus (*Streptomyces* spp.) no correlation between *in vivo* and *in planta* tests for Fusarium root rot and Fusarium foot rot in wheat was found (Colombo et al., 2019a,b).

In a second *in vitro* assay, droplet dispersal based on the production of surfactant molecules was assessed. Such molecules have three major roles: the breakdown of plant pathogenic fungal and bacterial biofilms, direct inhibition of plant pathogens and to trigger induced resistance (Cawoy et al., 2014). Therefore, such tests may have a potential to select candidate biocontrol agents for either bacterial or fungal plant diseases. Several isolates showed *in vitro* surfactant activity and strong antagonism against *B. cinerea* or *P. fluorescens* pv. *tomato* (Fig. 3), with, surprisingly, a trend that hyphal fungi and yeast-like fungi with biosurfactant activity showed strong *in planta* activity. This trend was found for the reference isolate *C. rosea* 016, an isolate of *Trichoderma asperellum / T. hamatum* and of *Sporobolomyces roseus,* and, consistently, for isolates belonging to *Aureobasidium* sp. Biosurfactant production by *A. pullulans* has been described by Kim et al. (2015). Isolates of these fungal species that produced surfactants on the droplet dispersal test are well-described antagonists that exhibit various modes of action (Sutton et al., 1997; Filonow, 1998; Harman et al., 2004; Freimoser et al., 2019). Formation of biosurfactants may play an important role in their mix of different modes of action.

Microorganisms can promote plant growth, e.g. through hormone production or through supporting the nutrient acquisition machinery. Many commercial microbial products, particularly those designed for seed treatment or soil drench, promote plant growth along with plant protection, using beneficial organisms combining both traits or by combined use of different microorganisms with complementary traits (Lugtenberg & Kamilova, 2009; Domenech et al., 2006). Assays with approximately 100 bacterial and fungal isolates identified only few isolates that significantly increased growth of tomato seedlings (Table 6). Bacterial and fungal strains promising for plant protection against *B. cinerea* and *P. fluorescens* pv. *tomato* had minor interference on plant growth and vice-versa. Interestingly, few isolates such as *Trichoderma asperellum / T. hamatum* HTS386 and *Chryseobacterium* sp. HTS080 demonstrated combined potential in plant growth promotion and protection against *B. cinerea* in leaf infection. However, no isolate combined potential in growth promotion with control of *P. fluorescens* pv. tomato.

To enhance screening for growth promotion, callus formation and rooting by detached tomato stem pieces treated with candidate isolates were assessed as high-throughput methods. However, results of both methods did not correlate with results on growth promotion of tomato seedlings. Furthermore, the strains most promising in callus formation were mostly not promising in rooting, except for *Cladosporium* sp. HTS455. Cambium cells can deposit callus on wounds to restore the plant epidermis. When main tomato stems are cut into pieces and kept in the dark under high humidity, those callus cells differentiate into roots (Locy, 1983). However, in interaction with a pathogen, the callose deposition is blocked and the pathogen gains access to the plant tissue (Finiti et al., 2013). Induction of callus formation by applied microorganisms may thus be a component of their mode of action to prevent pathogen infection. However, there was no positive correlation for the tested isolates between reduction in symptom development by *B. cinerea* or *P. fluorescens* pv. *tomato* on tomato and the potential to induce rooting or callus formation in stem pieces. Only few strains with biocontrol potential also enhanced rooting with *Botryosporium longibrachiatum* HTS402, *Engyodontium parvisporum / Lecanicillium tenuipes* HTS351, or callus formation with *Aureobasidium pullulans* 490, *Lysobacter* sp. HTS263 and *Pythium aphanidermatum* HTS401. One of the strains, *Botrytis cinerea / Sclerotinia sclerotiorum* HTS531, promoted rooting of tomato stem segments although a pathogenic reaction was expected. Plant pathogens may colonize plants asymptomatically, eventually provide some benefit to the plant and for yet-unknown reason go rogue and infect the plant, as observed for *B. cinerea* (van Kan et al., 2014).

From the results of the seven assays conducted with a set of hundred bacterial and fungal isolates originating from tomato it can be concluded that even with such a limited number of isolates promising antagonists, partly not yet described as antagonists, could be selected for control of the targeted diseases Botrytis stem rot, Botrytis leaf rot and Pseudomonas leaf rot when labor and resource demanding *in planta* bioassays had been applied. Also some promising isolates enhancing seedling development could be identified. In the case of the targeted tomato diseases and tomato growth promotion, independent screening assays for the different traits were needed since no correlation between the different traits were found. With a sufficiently high number of isolates in a screening program with independent assays candidates with promising combinations of traits, e.g. disease control and growth promotion might be selected. Attempts to simplify screening assays to high-throughput systems failed since no positive correlations with *in planta* assays were found.

In conclusion, the often suggested first screening rounds using *in vitro* tests for huge numbers of isolates (Raymaekers et al., 2020) followed by *in planta* testing of a selected group of candidates, e.g. those with high *in vitro* production of certain secondary metabolites or

biosurfactants, may not exploit the entire potential of antagonists. Especially antagonists combining various modes of action may be excluded by *in vitro* screening with a bias on a specific mode of action. Furthermore, exceptional isolates that may combine beneficial traits in plant growth promotion and disease control have to be selected independently in specific screening assays. For the future, combining multiple genetic information for many beneficial traits of antagonists will be an alternative for screening huge number of candidates that may allow to reduce the substantial input of resources for *in planta* screening. Recent studies on transcriptomics of interactions between host plants, pathogens and antagonists are rapidly increasing insights in the modes of action of beneficial microorganisms (Nygren et al., 2018; Piombo et al., 2018). This progress may allow designing large future screening programs that combine the selection for many relevant traits and their combinations and avoid the limitations of current *in vitro* screening.

CRediT authorship contribution statement

Jürgen Köhl: Conceptualization, Formal analysis, Validation, Writing - original draft, Writing - review & editing. **Flavio H.V. Medeiros:** Conceptualization, Methodology, Validation, Writing - original draft. **Carin Lombaers-van der Plas:** Methodology, Investigation. **Lia Groenenboom-de Haas:** Methodology, Investigation. **Trudy van den Bosch:** Methodology, Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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