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# Assessing populations of a disease suppressive microorganism and a plant pathogen using DNA arrays

Bart Lievens [a,b], Loes Claes [b], Matthew S. Krause [b], Alfons C.R.C. Vanachter [b], Bruno P.A. Cammue [a], Bart P.H.J. Thomma [c,*]

[a] *Centre of Microbial and Plant Genetics (CMPG), Katholieke Universiteit Leuven, Kasteelpark Arenberg 20, B-3001 Heverlee-Leuven, Belgium*
[b] *Scientia Terrae Research Institute, Fortsesteenweg 30A, B-2860 Sint-Katelijne-Waver, Belgium*
[c] *Laboratory of Phytopathology, Wageningen University, Binnenhaven 5, 6709 PD Wageningen, The Netherlands*

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**Abstract**

Understanding the relationships between disease suppressive microbial populations and plant pathogens is essential to develop procedures for effective and consistent disease control. Currently, DNA array technology is the most suitable technique to simultaneously detect multiple microorganisms. Although this technology has been successfully applied for diagnostic purposes, its utility to assess different microbial populations, as a basis for further study of population dynamics and their potential interactions, has not yet been investigated. In this study, a DNA macroarray with multiple levels of phylogenetic specificity was developed to measure population densities of a specific disease suppressive microorganism, *Trichoderma hamatum* isolate 382, and the plant pathogen *Rhizoctonia solani*. Amongst others, the DNA array contained genus-, species- and isolate-specific detector oligonucleotides and was optimized for sensitive detection and reliable quantification of the target organisms in potting mix samples. Furthermore, this DNA array was used to quantify disease severity as well as incidence of severe disease based on pathogen population densities in the growing medium. Taking into account the unlimited expanding possibilities of DNA arrays to include detector oligonucleotides for other and more microorganisms, this technique has the potential for studying the population dynamics and ecology of several target populations in a single assay.
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## 1. Introduction

Largely driven by concerns about the detrimental effects of the use of synthetic chemicals on the environment and on public health, integrated pest management (IPM) has become the preferred strategy for managing plant diseases over the last few decades [1]. However, IPM has been severely limited by the lack of fast, accurate, and reliable means by which plant pathogens can be detected, identified, and accurately quantified in a timely fashion. Accurate quantification is particularly important since it serves as the basis for establishing population thresholds whereby a pathogen causes disease and at which point measures may be employed to effectively limit or prevent losses.

Beneficial microorganisms are used in IPM programs to increase plant health and yield by limiting or minimizing disease severity and incidence. These disease suppressive microorganisms (DSMs) may be indigenous to the soil or, alternatively, introduced into the rhizosphere. In general, DSM-mediated disease suppression can only be achieved when the pathogen is present under a certain threshold level, and the DSM is active and above a certain threshold level [2]. Therefore, similar as for plant pathogens accurate assessment of beneficial microorganisms is important when DSMs are being considered in disease management programs.

The inability to rapidly identify microorganisms using culture-based, morphological techniques has prompted the development of culture-independent molecular diagnostic methods. Most of these assays are designed for the detection of individual or small numbers of target organisms [3]. In addition, population levels of both pathogens and DSMs have rarely been measured in the same study. Currently, DNA array

* Corresponding author. Tel.: +31 317 484536; fax: +31 317 483412.
*E-mail address:* bart.thomma@wur.nl (B.P.H.J. Thomma).

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technology is the most suitable technique to detect several target organisms simultaneously [4–6]. Typically, a detection array consists of many specific detector oligonucleotides that are immobilized on a solid support such as a nylon membrane and that are complementary to the DNA sequences of interest. Generally, for signal amplification, the target DNA is amplified with a primer pair that targets a genomic region containing the specific sequences, and is simultaneously labeled. Subsequently, labeled amplicons are hybridized to the array under stringent conditions [7–9]. This can be conducted in such a manner that reliable detection and quantification of multiple microorganisms is possible in one assay [10,11]. This technology has been successfully applied in diagnostics of human, animal, and plant pathogens [4]. However, the possibilities of using DNA arrays for simultaneously assessing the populations of both pathogens and specific DSMs and relating such data to disease severity and incidence have not been reported to date.

In this manuscript, we describe the use of DNA arrays to simultaneously measure population densities of a specific biocontrol agent and a fungal pathogen, as well as to relate their presence to disease development. Overall, this work describes a technical advance with potential applications for studying the population dynamics and ecology of target populations in complex media such as potting mixes or soils. As a model, the well-established interaction between the biocontrol agent *Trichoderma hamatum* (Bon.) Bain isolate 382 ($T_{382}$) and the pathogen *Rhizoctonia solani* Kühn was used in a damping-off of radish bioassay [19].

## 2. Materials and methods

### 2.1. Oligonucleotide selection

A number of oligonucleotides used in this study (Table 1) were designed previously, including the *R. solani* detector (Rso1) and the control oligonucleotides (Fun1, Sce1, Dig1, and Con1) [10]. In addition, oligonucleotides to detect the genus *Trichoderma* (Tgn1) and the isolate $T_{382}$ (Tha382) were designed as described previously [9]. Whereas the first oligonucleotide is based on an ITS sequence, the latter is derived from the random amplified polymorphic DNA (RAPD) marker SCE16 [12]. Specificity of the oligonucleotides selected was checked by BLAST analysis and cross-hybridization testing with over 225 related and non-related fungal strains of which the most relevant strains are listed in Table 2. The quantitative character of the oligonucleotides was verified as described previously [10]. To conduct these tests, DNA extraction from reference cultures, PCR amplification, labeling, and hybridization were performed as described previously [9,10,13].

Table 1
Detector oligonucleotides used for DNA array analysis

| Code | Specificity | Sequence (5′–3′) | Target |
|---|---|---|---|
| Rsol[a] | *Rhizoctonia solani* | GCCTGTTTGAGTATCATGAAAT | ITS II |
| Tgn1[b] | *Trichoderma* sp. | GTCATTTCAACCCTCGAACCC | ITS II |
| Tha382[b] | *T. hamatum* isolate 382 | ATTCACGACATATGATCTAATC | SCE16[c] |
| Fun1[a] | Fungi | GCTGCGTTCTTCATCGATGC | 5.8S rDNA |
| Sce1[a] | *Saccharomyces cerevisiae* | GTGTTTTGGATGGTGGTAAGAA | *erg11* gene |
| Dig1[a,d] | None | GTCCAGACAGGATCAGGATTG | – |
| Con1[a] | None | GTCCAGACAGGATCAGGATTG | – |

[a] Ref. [10].
[b] This study.
[c] Ref. [12].
[d] 3′-End digoxigenin-labeled.

### 2.2. DNA array production

All oligonucleotides were synthesized with a 5′ $NH_2$ group and a C6 linker. DNA arrays were produced as described previously [9,10,13]. Briefly, 8.0 fmol of the detector oligonucleotides was spotted in duplicate on Immunodyne ABC membrane strips (PALL Europe Limited, Portsmouth, UK) using a pin replicator (V & P Scientific, Inc., San Diego, CA, USA). The calibration oligonucleotide Dig1 was spotted at 2.0 fmol. To accurately quantify total fungal DNA Fun1 was spotted at different amounts including 8.0, 0.2, and 0.1 fmol [10]. Printed membranes were air dried overnight and subsequently blocked for 30 min at room temperature. Finally, the membranes were air dried and stored at room temperature until use.

Table 2
*Rhizoctonia* and *Trichoderma* isolates used in this study

| Species | Isolate[a] |
|---|---|
| *Rhizoctonia oryzae* | CBS 273.38, CBS 474.82 |
| *R. oryzae-sativae* | CBS 235.91 |
| *R. solani* | 19 (AG-4), CBS 101590 (AG-4), CBS 101761, CBS 323.84, MUCL 9418, ST 44.02, ST 50.03 |
| *Trichoderma aggressivum* f. *aggressivum* | CPK 361, CPK 365 |
| *T. aggressivum* f. *europeum* | CPK 366, CPK 375 |
| *T. asperellum* | CPK 247, CPK 358, CPK 654, CPK 655, MUCL 41923, MUCL 41924, MUCL 41925, MUCL 41926, MUCL 41927, MUCL 41928 |
| *T. atroviride* | CPK 369 |
| *T. hamatum* | $T_{382}$, CPK 253, CPK 301, CPK 308, CPK 309, CPK 310, CPK 311, CPK 313, CPK 314, CPK 316, CPK 328, CPK 357 |
| *T. harzianum* | CPK 51, CPK 206, CPK 211, CPK 217, CPK 221, CPK 261, CPK 271, CPK 274, MUCL 19412, MUCL 28446 |
| *T. inhamatum* | CPK 239 |
| *T. longibrachiatum* | CPK 41D, CPK 47D, CPK 57D, CPK 59D |
| *T. pubescens* | CPK 489 |
| *T. virens* | CPK 389, CPK 396, CPK 400, CPK 432, CPK 521 |
| *T. viride* | CPK 421, CPK 525 |

[a] CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CPK: collection of C.P. Kubicek, Vienna University of Technology, Vienna, Austria; MUCL: Mycothèque de l'Université Catholique de Louvain, Louvain-la-Neuve, Belgium; ST: Scientia Terrae Research Institute, Sint-Katelijne-Waver, Belgium.

### 2.3. Potting mixes and Trichoderma fortification

Two types of potting mixes that should differ in disease suppressive potential were used. The first type, "dark Sphagnum peat" (DSP) mix, is a substrate that, because of its highly decomposed organic matter and subsequent low microbial carrying capacity, typically provides little or no support to DSMs that induce suppression to damping-off diseases [14–17]. This potting mix was formulated by blending dark Sphagnum peat ($H_3$–$H_4$ on the von Post peat decomposition scale [18]; Bas van Buuren, Maasland, The Netherlands) with medium horticultural grade perlite (7:3 v/v), then adding 1.1 g superphosphate and 1.0 g $K_2SO_4$ per liter of mix. The second type of potting mix used was a "composted pine bark" (CPB) mix that, due to its low but stable decomposition level and consequent higher microbial carrying capacity, is able to support the disease suppressive activities of DSMs such as $T_{382}$ against Rhizoctonia damping-off diseases [17]. This mix was prepared with the same batch of dark peat used in the previous mix, composted pine bark (DCM Corp., Grobbendonk, Belgium), medium horticultural grade vermiculite, and medium horticultural grade perlite at a ratio of 45:30:15:10 (v/v). Agricultural-grade $CaCO_3$ and $Ca(NO_3)_2$ (<100 mesh) were added at a ratio of 4:1 (w/w) to adjust potting mixes to pH 5.8–6.0. In addition, tap water was added to potting mixes to adjust moisture content to 35–40% of the water-holding capacity of each mix.

Portions of each potting mix were subjected to either a 25 or a 60 °C ("heated") initial incubation treatment for 5 days to promote the colonization by or diminish levels of mesophilic, naturally present microorganism in the mixes, respectively [19]. $T_{382}$-fortified potting mix treatments were prepared by thoroughly blending a dry microgranular preparation of $T_{382}$ (Sylvan Bioproducts, Inc., Cabot, PA, USA) with these potting mixes to achieve an initial density of $2 \times 10^7$ CFU per liter potting mix (corresponding to $4 \times 10^4$ CFU $g^{-1}$ fresh weight), which is a recommended rate to achieve disease suppression [17]. Initial density was determined by dilution plate enumeration on *Trichoderma* selective medium [20] as described further. Portions of each potting mix not blended with $T_{382}$ served as non-fortified ("natural") control treatments. Thereafter, all potting mixes were incubated at 25 °C for 7 days to allow $T_{382}$ to colonize fortified mixes before adding fertilizer and pathogen inoculum. The following day, just before seeding, slow release fertilizer (8–5–7, 1:1 Ecomix 1-Ecomix 4 blend, DCM Corp., Grobbendonk, Belgium) was incorporated into all mixes at a rate of 2.5 g/l.

### 2.4. Rhizoctonia damping-off of radish bioassay

Experiments were conducted using the Rhizoctonia damping-off of radish (*Raphanus sativus* L. cv. 'Early Scarlet Globe') bioassay optimized by Krause et al. [17] to (i) identify the relationships between hybridization signal intensity and Rhizoctonia damping-off severity as well as incidence of severe disease, and (ii) evaluate the application of the DNA array for measuring the population densities of a DSM and a pathogen in a disease suppressive system.

Potato soil inoculum of *R. solani* isolate 19 (AG-4) was prepared as described by Ko and Hora [21]. Air-dried inoculum was ground using a mortar and pestle and was sieved to retain 1–2-mm-sized particles [17,19,22]. In the first experiment, this inoculum was incorporated into potting mix at rates of 0.125, 0.25, 0.375, 0.5, and 0.625 g/l potting mix when fertilizer was incorporated just before seeding. In the second experiment, potting mixes were inoculated with 0.5 g of this inoculum per liter mix [17] at fertilizer incorporation before seeding. In both experiments, non-infested control mixes did not receive *R. solani* inoculum. Using a vacuum seeder, 32 radish seeds (85% germination, Shaffer Seeds, Akron, OH, USA) were deposited evenly across the surface of 10-cm-diameter polystyrene foam pots containing approximately 400 ml of potting mix. Seeds were covered with approximately 1 cm of potting mix, then pots were irrigated until mix saturation. As a fungicide control treatment, tolclofos-methyl (0.03 mg a.i. $ml^{-1}$ water) was applied as a drench to pathogen-infested non-fortified potting mixes (125 ml of solution per pot). Pots were incubated in a growth chamber at 24 °C under continuous illumination (225 μEinstein $m^{-2}$ $s^{-1}$) and were irrigated as needed. All bioassays were configured according to a randomized complete block design with five pots (replicates) per treatment and were conducted twice with similar results.

Damping-off severity was determined 7 days after incubation based on a damping-off severity rating scale in which: 1 = symptomless; 2 = small root or stem lesion; 3 = large root or stem lesion; 4 = post-emergence damping-off; 5 = pre-emergence damping-off [17,22]. Incidence of severely diseased seedlings (i.e. proportion of plants with a disease severity rating >2) was also evaluated using this data as described by Krause et al. [17]. Immediately after rating, for each of the five replicate pots in each treatment, five samples of mix were taken by inserting a 1.5-cm-diameter cork borer completely through the depth of the pot. Subsequently, samples were combined, homogenized and used for DNA extraction and plating on semi-selective medium. Additionally, the causal agent of the disease was verified by recovery of the pathogen from surface disinfected seedlings on a *Rhizoctonia* semi-selective medium (1.5% water agar amended with 250 ppm chloramphenicol and 250 ppm metalaxyl) followed by microscopic examination.

### 2.5. DNA extraction, amplification, labeling and hybridization

Genomic DNA was extracted from 0.5 g potting mix using the UltraClean Soil DNA Isolation Kit according to the manufacturer's specifications (Mo Bio Laboratories, Inc., Solana Beach, CA, USA). DNA extracts were subsequently diluted 10-fold to avoid inhibitory concentrations of potential PCR inhibitors and stored at −20 °C until further analysis.

Fungal ribosomal DNA (rDNA) spanning the two internal transcribed spacers (ITS) and the 5.8S rDNA region was amplified using the primer set ITS1-F and ITS4 [23] and was simultaneously labeled with alkaline-labile digoxigenin (Roche Diagnostics GmbH, Mannheim, Germany). To specifically detect $T_{382}$, amplification and labeling was performed using

primers OPE16-F and OPE16-R [12]. Samples were amplified in 20 μl reaction volumes containing 0.15 mM digoxigenin-11-dUTP mix (Roche Diagnostics GmbH, Mannheim, Germany), 0.5 μM of each primer, 1 unit Titanium Taq DNA polymerase (Clontech Laboratories, Inc., Palo Alto, CA, USA), and 1 μl genomic DNA. Thermal cycling conditions comprised an initial denaturation at 94 °C for 2 min, followed by a PCR cycling protocol of denaturation at 94 °C for 45 s, annealing at 59 °C for 45 s and elongation at 72 °C for 45 s, and a final elongation step at 72 °C for 10 min. PCR amplification was performed using 30 cycles to ensure accurate end-point quantification [10]. In order to check for differences in PCR efficiency, a separate PCR reaction was run for each sample in which 100 pg of exogenous control DNA from *Saccharomyces cerevisiae* was added to 1 μl target DNA [10]. Amplification and simultaneous labeling was done using primers $P450_1$ and $P450_2$ [24] according to the same thermal program described above. Labeled amplicons were subsequently combined and used for DNA array hybridization as previously described [9,10,13]. All assays were conducted at least twice.

### 2.6. Plating and real-time PCR as reference techniques

In order to validate DNA array analysis, both culture-dependent classical plating and culture-independent real-time PCR methods were used. For classical enumeration of *Trichoderma* propagules, a series of 10-fold dilutions of 10 g potting mix was made, followed by spreading 100 μl aliquots of each dilution in triplicate on a *Trichoderma* selective medium [20]. *Trichoderma* colonies were counted after 5 days of incubation at 25 °C in darkness. Since dilution plating cannot be used to adequately recover and quantify discrete propagules of *R. solani*, 50 randomly picked clumps (between 10 and 15 $mm^3$) of the potting mix were directly plated on *Rhizoctonia* selective medium [21,25]. Plates were subsequently incubated at 25 °C in darkness and were checked daily for *R. solani* growth.

In parallel, real-time PCR amplifications were performed in a total volume of 25 μl using the intercalating dye SYBR® Green I on a SmartCyclerII® instrument (Cepheid, Sunnyvale, CA, USA). Each reaction mixture contained 2 μl of the target DNA extract, 12.5 μl of the QuantiTect™ SYBR® Green PCR Master Mix (Qiagen, Inc., Valencia, CA, USA), 0.625 μl of each primer (20 μM), and 9.25 μl sterile distilled water. The forward primers ST-RS1 [10] and ST-Tgn1 (5′-TTCAACCCTCGAACCCCTC) were combined with the universal reverse primer ITS4 [26] to detect and quantify rDNA from *R. solani* and *Trichoderma* spp., respectively. Thermal cycling conditions consisted of 10 min at 95 °C followed by 45 amplification cycles of 15 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C with a final 2-min elongation step at 72 °C. Fluorescence was detected at the end of the elongation phase of each cycle. To evaluate amplification specificity, melt curve analysis was performed at the end of the PCR run. A melt curve profile was obtained by slowly heating the mixture from 60 to 95 °C at 0.2 °C $s^{-1}$ with continuous measurement of fluorescence. Standard curves were generated by plotting the threshold cycle ($C_t$) of a 10-fold dilution series of standard DNA against the logarithm of the concentration. The regression line was used to calculate the DNA concentration of *R. solani* and *Trichoderma* in the analyzed samples via their $C_t$-values [27,28].

### 2.7. Statistical analyses

Analysis of variance (ANOVA, Minitab (Release 13, Minitab Inc., State College, PA, USA)) was used to determine the effects of potting mix treatment on damping-off severity and on the hybridization signals obtained. Two aspects of damping-off severity were analyzed as response variables, including mean disease severity and mean incidence of severe disease [17]. The first response variable, disease severity rating for each replication ($R$), was transformed to $R^* = (R^{1.5} - 1)/1.5$ to obtain a linear scale and an approximately normal distribution with constant variance. The second response variable, defined as the proportion of seedlings in each replication with damping-off severity rating $>2$ ($y$), was transformed to $y^* = \text{arcsine}(\sqrt{y})$ to obtain a constant variance [17]. To determine the effects of potting mix treatment on the hybridization signals obtained, data were not transformed. Hybridization strength was reported relative to the average integrated optical density of the digoxigenin-labeled reference control (Dig1). Least significant differences (LSD) at $P = 0.05$ were calculated to compare means.

## 3. Results

### 3.1. Quantification of DNA dilutions using a DNA array

Prerequisite to any study related to assessing microbial populations and their dynamics are accurate detection, identification, and reliable quantification of the microorganisms of interest. For detection of *R. solani*, the previously selected ITS-based oligonucleotide Rso1 [10] was used. *Trichoderma* species are present in virtually all soils and may act as indigenous buffers against a wide range of plant diseases. Therefore, Tgn1, a genus-specific oligonucleotide, was developed for this genus, covering many species that have been reported to suppress various plant diseases. However, since disease suppression ability may differ among communities, species and isolates of *Trichoderma*, specific detection of $T_{382}$, a well-known and documented DSM, was pursued. Discrimination of $T_{382}$ from other *T. hamatum* isolates was not possible based on ITS sequences. Therefore, another genomic region, namely the $T_{382}$-specific RAPD marker SCE16 [12], was utilized to develop an isolate-specific oligonucleotide (Tha382). To investigate the quantitative properties of these detector oligonucleotides, a 10-fold dilution series of genomic DNA from *R. solani* isolate 19 and $T_{382}$, ranging from 2.5 ng to 0.25 pg, was quantified after 30 cycles of PCR amplification. Hybridization signals revealed that from 0.25 to 25 pg a linear logarithmic relationship between the signal intensity and template DNA concentration occurred for the oligonucleotides Tgn1 ($R^2 = 0.98$) and Tha382 ($R^2 = 0.98$) (Fig. 1). The curves

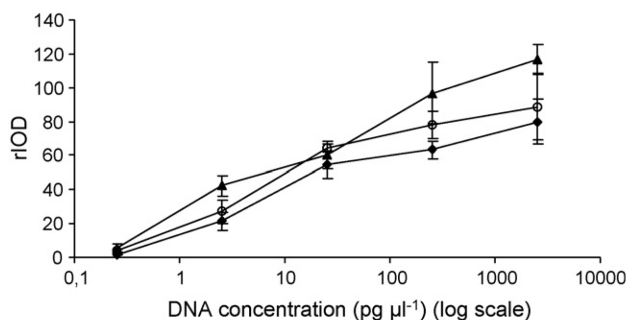


Fig. 1. Quantification of a dilution series of *Rhizoctonia solani* isolate 19 and *Trichoderma hamatum* isolate 382 ($T_{382}$) genomic DNA after PCR using a DNA array containing detector sequences Tgn1 (◆), Tha382 (○), and Rso1 (▲) to detect the genus *Trichoderma*, $T_{382}$ and the pathogen *R. solani*, respectively. Hybridization signal strength is reported relative to the average integrated optical density of the digoxigenin-labeled reference control (rIOD) and plotted against the logarithmic DNA concentration. Data represent means from two hybridization runs using amplicons from a single PCR reaction ($n = 4$). Error bars indicate standard errors.

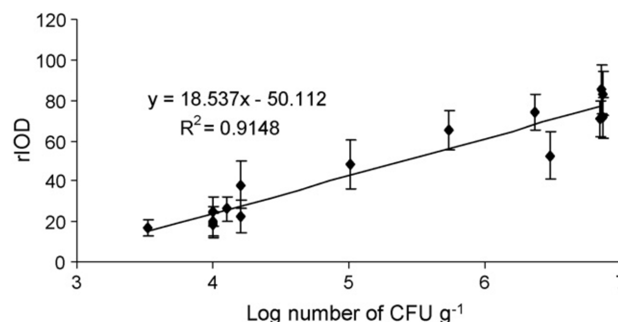


Fig. 2. Quantitative assessment of *T. hamatum* isolate 382 ($T_{382}$) in fortified potting mixes using a DNA array. Hybridization signal strength is reported relative to the average integrated optical density of the digoxigenin-labeled reference control (rIOD) and plotted against the number of $T_{382}$ propagules present in the sample (determined by classical enumeration; log CFU $g^{-1}$ fresh weight). Data represent means of hybridization signals generated by Tha382 from two hybridization runs using amplicons from a single PCR reaction ($n = 4$). Error bars indicate standard errors.

deviated from linearity when amplifying and hybridizing over 25 pg genomic DNA as the signals obtained for these oligonucleotides reached their saturation level. For Rso1, the relation was nearly linear over the complete concentration range tested ($R^2 = 0.99$). As was also observed in previous studies [10,28–30], adding 2.5 ng of DNA extracted from plant or potting mixes to all samples of the dilution series did not influence template quantification, demonstrating that amplification and hybridization efficiency is not affected by non-target DNA (data not shown). In addition to this, PCR efficiencies evaluated by addition of 100 pg exogenous *S. cerevisiae* control DNA to each sample, followed by PCR amplification and hybridization, were highly comparable among samples and were consistent with other experiments.

### 3.2. Quantification of fungal biomass of $T_{382}$ using a DNA array

Since the population density of $T_{382}$ is a crucial factor for effective suppression of Rhizoctonia damping-off and crown and root rot diseases [17,19,20], accurate quantification of $T_{382}$ using the DNA array was pursued. At the end of the bioassays, $T_{382}$ population densities were determined by the classical plating technique as well as by the DNA array. In Fig. 2, the logarithmic relationship between the hybridization signals obtained with detector Tha382 and the number of CFU $g^{-1}$ fresh weight is presented for multiple fortified potting mixes, demonstrating that accurate quantification of the biocontrol agent is possible between $10^3$ and $10^7$ CFU $g^{-1}$ fresh weight ($R^2 = 0.91$).

### 3.3. Relationship between hybridization signal intensity and disease severity and incidence

Establishing a relationship between the pathogen inoculum density in the substrate and the corresponding plant disease severity is essential for understanding and predicting potential outcomes of the disease, as well as for taking appropriate control measures. While the density of the pathogen propagules generally is related to the potential for disease development, it is more efficient to directly link hybridization signal strength to disease severity or incidence. Radish plants grown in potting mixes infested with different densities of *R. solani* inoculum were rated for damping-off severity after 7 days of incubation when each of the different damping-off severity ratings were observed. At that time, DNA was extracted from the potting mixes for DNA array hybridization (Fig. 3). Rso1 hybridization signal intensity correlated positively with both mean damping-off severity ($R^2 = 0.80$; Fig. 3A) and mean incidence of severely diseased seedlings ($R^2 = 0.80$; Fig. 3B), demonstrating the feasibility of the technique to estimate substrate infestation and potential impact on plant health.

### 3.4. Assessing populations of $T_{382}$ and R. solani in potting mixes with a DNA array

Effects of the different potting mixes on severity of Rhizoctonia damping-off, incidence of severely diseased seedlings, and hybridization signal intensities obtained using the array are summarized in Table 3. Transformed mean damping-off severity, as well as transformed mean incidence of severely diseased seedlings in the infested natural non-heated treatment, was significantly ($P \leq 0.05$) lower with the CPB mix than with the DSP mix. These observations were corroborated by DNA array data that displayed significantly ($P \leq 0.05$) weaker hybridization signals for *R. solani* in the CPB mix than in the corresponding DSP treatment. Heating these mixes eliminated or decreased this suppressive effect, as both the transformed mean disease severity and incidence of severely diseased seedlings increased greatly (Table 3). In parallel, for both mixes hybridization signal strength more than doubled, reaching a rIOD of 110.51 and 76.76 for the naturally infested DSP and CPB mix, respectively. Heating the mixes followed by fortification with $T_{382}$ significantly decreased transformed mean damping-off severity and incidence of severe symptoms again. Likewise, the hybridization signals for *R. solani*

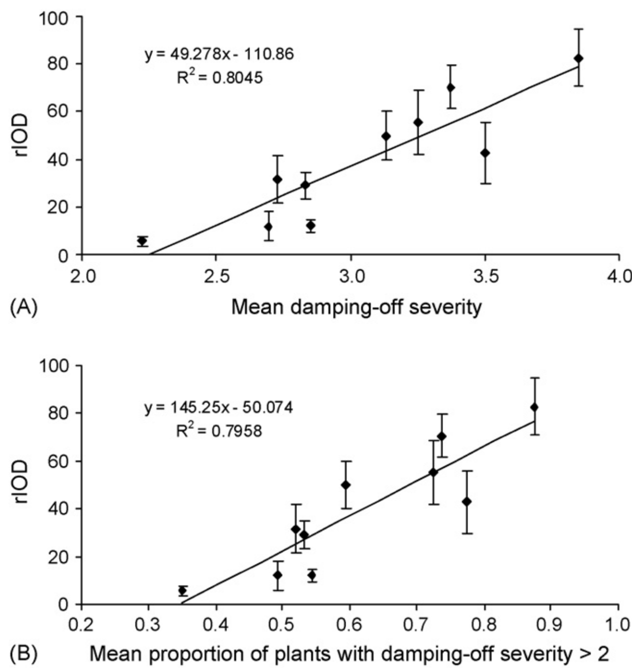


Fig. 3. Quantitative assessment of *R. solani* in artificially infested potting mixes using a DNA array. Regression lines for DNA array analysis refer to (A) mean Rhizoctonia damping-off severity and (B) mean incidence of severely diseased plants (proportion of plants with a disease severity rating >2). Damping-off severity and incidence of severely diseased seedlings was determined 7 days after planting. Rating was based on five pots of 32 plants each and a damping-off severity scale in which 1 = symptomless; 2 = small root or stem lesion; 3 = large root or stem lesion but not damped-off; 4 = post-emergence damping-off; 5 = pre-emergence damping-off. Hybridization signal strength is reported relative to the average integrated optical density of the digoxigenin-labeled reference control (rIOD). Data represent means of hybridization signals generated by Rso1 from two hybridization runs using amplicons from a single PCR reaction ($n = 4$). Error bars indicate standard errors.

decreased from a rIOD of 110.51 in the natural heated DSP mix to a rIOD of 68.02 in the $T_{382}$-fortified heated DSP mix. However, this decrease was not observed among the same treatments of the CPB mix. For both fortified mixes similar, strong hybridization signals were observed for the *Trichoderma* and $T_{382}$ oligonucleotides (Table 3). When these mixes were not preheated, inoculation with $T_{382}$ did not significantly affect transformed mean disease severity and incidence of heavily diseased seedlings, either for the DSP mix or the CPB mix (Table 3). In general, no relationship could be established between *Trichoderma* quantification and disease severity. In contrast, hybridization signal strength for *R. solani* corresponded well to disease severity and incidence of severe disease, which is consistent with the data presented in Fig. 3. However, this was not observed when the fungicide was applied to the infested potting mixes. In the case of the fungicide-drenched heated treatment plants were not diseased, yet hybridization intensities for the pathogen were as strong as with treatments for which significant disease was observed, suggesting that DNA from the killed pathogen was detected. A non-discriminative detector oligonucleotide (Fun1; Table 1) was added to the array in order to measure the total amount of fungal DNA. Since oligonucleotides spotted at 8.0 fmol get saturated by high target DNA concentrations, this oligonucleotide was also printed at lower amounts, including 0.2 and 0.1 fmol, to enable accurate discrimination between relatively high DNA concentrations [10]. Relatively weak signals were obtained for Fun1 when mixes were heated, non-fortified, and non-infested, thus confirming the initial pasteurizing effect of heating potting mixes. In contrast, relatively strong Fun1 hybridization signals were obtained for the non-heated, non-fortified, and non-infested mixes, indicating the high endogenous fungal presence in these mixes (Table 3). In addition to the hybridization assays, real-time PCR analyses were performed and parallel sets of the samples were plated on semi-selective medium to validate detection and quantification of the pathogen and the biocontrol agent. With regard to $T_{382}$, results from the DNA array were corroborated by these two reference methods (Figs. 2 and 4). Because of the lack of a good selective medium for *R. solani*, it was impossible to accurately distinguish and thus quantify the pathogen in the samples by classical plating. However, in all instances where hybridization signals were obtained for *R. solani*, except for fungicide treatments, the presence of the pathogen was confirmed by plating. Nevertheless, a very high degree of correlation ($R^2 = 0.90$) was found between hybridization strength and the calculated DNA concentration obtained by real-time PCR analysis (Fig. 4), demonstrating the reliability of the quantitative results obtained with the DNA array.

## 4. Discussion

Soils, soil-less growing media, and other substrates may naturally contain a multitude of potential plant pathogens and beneficial microorganisms, including those that naturally suppress plant diseases. Alternatively, controlled inoculation with specific DSMs frequently helps to assure that the proper microorganisms are in place to suppress various diseases. *T. hamatum* isolate 382, one such DSM, has been shown to suppress a broad spectrum of plant diseases caused by different soilborne [17,19,31,32] and foliar pathogens [33–35] when it is incorporated into suitable growing media. However, effective use of $T_{382}$ and other DSMs for disease suppression is based on numerous factors, including physical, chemical and organic matter qualities of the substrate or niche, pathogen infestation level, and DSM activity and density [2].

Microbial detection and identification methods continue to evolve, each with its own strengths and limitations. DNA array technology is currently the most suitable technique to qualitatively and quantitatively detect several target organisms simultaneously [4–6,10,11], making DNA arrays highly attractive for measuring microbial populations and studying their interactions. Currently, rDNA is one of the most common DNA targets in fungal diagnostics. Spacer regions within the rDNA have been shown to possess features that enable discrimination at the genus and species level [3–5]. In addition, universal primers flanking this region are available [26], which makes this gene highly attractive for DNA array-based microorganism detection [4–6]. Using this approach a multitude of organisms can be differentiated using a single PCR, even if they differ in only a single to a few bases in the gene that

Table 3
Effects of different potting mixes on suppression of Rhizoctonia damping-off of radish and on populations of *T. hamatum* isolate 382 ($T_{382}$) and *R. solani* measured with a DNA array

| Potting mix[a] | $T_{inc}$[b] | *R. solani* control method[c] | *R. solani*[d] | Rhizoctonia damping-off[e] | | | | Hybridization signal strength[f] | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Mean disease severity ($R$)[g] | Transformed mean disease severity ($R^*$)[h] | Mean incidence of severe disease ($y$)[i] | Transformed mean incidence of severe disease ($y^*$)[j] | Tgn1 (8.0 fmol) | Tha382 (8.0 fmol) | Rso1 (8.0 fmol) | Sce1 (8.0 fmol) | Fun1 (8.0 fmol) | Fun1 (0.2 fmol) | Fun1 (0.1 fmol) |
| DSP | 25 | – | – | 1.28 | 0.30 | 0.07 | 0.24 | 0.00 | 0.00 | 0.00 | 90.85 | 99.55 | 44.11 | 12.32 |
| | 60 | – | – | 1.18 | 0.20 | 0.04 | 0.18 | 0.00 | 0.00 | 0.00 | 91.02 | 50.95 | 0.04 | 0.00 |
| | 25 | $T_{382}$ | – | 1.20 | 0.21 | 0.05 | 0.20 | 25.08 | 35.84 | 0.00 | 94.83 | 101.16 | 47.09 | 17.06 |
| | 60 | $T_{382}$ | – | 1.23 | 0.24 | 0.06 | 0.21 | 86.07 | 127.61 | 0.00 | 95.15 | 97.27 | 72.29 | 25.12 |
| | 25 | – | + | 3.19 | 3.15 | 0.67 | 0.96 | 0.00 | 0.00 | 46.21 | 93.88 | 102.34 | 50.25 | 26.69 |
| | 60 | – | + | 4.34 | 5.37 | 0.90 | 1.27 | 0.00 | 0.00 | 110.51 | 97.87 | 100.68 | 37.71 | 8.79 |
| | 25 | $T_{382}$ | + | 3.66 | 4.02 | 0.80 | 1.11 | 35.28 | 16.46 | 63.27 | 91.85 | 102.48 | 42.88 | 29.42 |
| | 60 | $T_{382}$ | + | 3.38 | 3.49 | 0.71 | 1.00 | 61.90 | 110.39 | 68.02 | 94.98 | 102.83 | 43.60 | 5.00 |
| | 25 | Fungicide | + | 1.65 | 0.78 | 0.16 | 0.4 | 0.00 | 0.00 | 1.22 | 89.69 | 103.45 | 38.93 | 17.70 |
| | 60 | Fungicide | + | 1.09 | 0.09 | 0.03 | 0.14 | 0.00 | 0.00 | 70.79 | 93.41 | 116.62 | 16.74 | 1.75 |
| CPB | 25 | – | – | 1.23 | 0.24 | 0.06 | 0.23 | 0.00 | 0.00 | 0.00 | 97.55 | 101.77 | 41.32 | 13.44 |
| | 60 | – | – | 1.33 | 0.36 | 0.08 | 0.25 | 0.00 | 0.00 | 0.00 | 94.08 | 55.99 | 0.35 | 0.00 |
| | 25 | $T_{382}$ | – | 1.31 | 0.33 | 0.08 | 0.27 | 17.05 | 18.12 | 0.00 | 90.45 | 123.87 | 75.84 | 26.34 |
| | 60 | $T_{382}$ | – | 1.23 | 0.26 | 0.06 | 0.18 | 82.06 | 86.75 | 0.00 | 96.20 | 89.99 | 53.89 | 30.01 |
| | 25 | – | + | 2.57 | 2.08 | 0.48 | 0.76 | 0.00 | 0.00 | 21.25 | 90.11 | 106.99 | 62.18 | 33.18 |
| | 60 | – | + | 4.19 | 5.06 | 0.94 | 1.38 | 0.00 | 0.00 | 76.76 | 92.81 | 83.54 | 10.72 | 0.96 |
| | 25 | $T_{382}$ | + | 2.24 | 1.60 | 0.38 | 0.65 | 18.44 | 44.35 | 35.83 | 96.40 | 107.64 | 75.76 | 47.08 |
| | 60 | $T_{382}$ | + | 3.29 | 3.32 | 0.68 | 0.97 | 71.07 | 100.32 | 87.03 | 90.22 | 107.67 | 50.92 | 12.13 |
| | 25 | Fungicide | + | 1.25 | 0.27 | 0.06 | 0.22 | 7.80 | 0.00 | 0.00 | 94.83 | 104.02 | 58.15 | 34.68 |
| | 60 | Fungicide | + | 1.30 | 0.33 | 0.08 | 0.27 | 0.00 | 0.00 | 37.75 | 90.12 | 83.05 | 6.03 | 0.00 |
| $LSD_{0.05}$[k] | | | | – | 0.56 | – | 0.16 | 16.80 | 9.83 | 24.25 | 9.86 | 17.49 | 23.30 | 16.39 |

[a] DSP = dark Sphagnum peat mix; CPB = composted pine bark mix.

[b] Incubation temperature (°C). Potting mixes were incubated at 25 °C or heated at 60 °C for 5 days prior to fortification or incubation.

[c] Potting mixes were fortified with $T_{382}$ to achieve an initial density of $2 \times 10^7$ CFU per liter potting mix ($T_{382}$), not fortified (–), or drenched with tolclofos-methyl (fungicide; 0.03 mg a.i. $ml^{-1}$ water; 125 ml of solution per pot).

[d] +: potting mixes infested with 0.5 g of *R. solani* isolate 19 inoculum per liter of mix; −: not infested.

[e] Damping-off severity and incidence of severely diseased seedlings was determined 7 days after planting. Rating was based on five pots of 32 plants each and a damping-off severity scale in which 1 = symptomless; 2 = small root or stem lesion; 3 = large root or stem lesion but not damped-off; 4 = post-emergence damping-off; 5 = pre-emergence damping-off. Values are means ± S.E. ($n = 5$).

[f] Hybridization signal strength is relative to the average integrated optical density of the digoxigenin-labeled reference control (rIOD). Values are means from two hybridization runs using amplicons from a single PCR reaction ($n = 4$). Oligonucleotides Tgn1, Tha382, Rso1 and Sce1 (all spotted at 8.0 fmol) were selected to detect the genus *Trichoderma*, $T_{382}$, *R. solani* and the exogenous control, *S. cerevisiae*, respectively. Oligonucleotide Fun1 was spotted at different amounts, including 8.0, 0.2, and 0.1 fmol to reflect total fungal biomass.

[g] Mean damping-off severity ($R$).

[h] Disease rating for each replication ($R$) was transformed to $R^* = (R^{1.5} - 1)/1.5$ to obtain a linear scale and an approximately normally distributed variable with constant variance.

[i] Mean proportion of plants in each replication with a disease severity rating >2 ($y$).

[j] Mean proportion of plants in each replication with a disease severity rating >2 ($y$) was transformed to $y^* = \text{arcsine}(\sqrt{y})$ to obtain a constant variance.

[k] Differences in $R^*$, $y^*$, and rIOD larger than the LSD calculated are significantly different ($P = 0.05$).

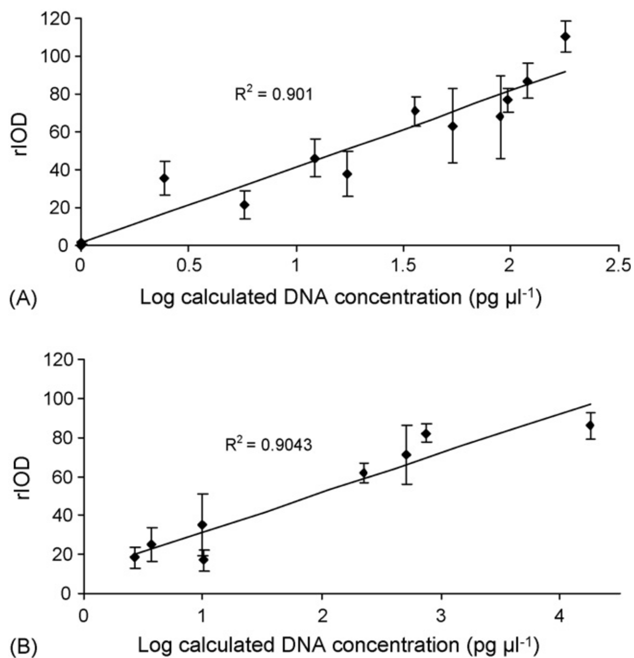


Fig. 4. Quantitative assessment of (A) *R. solani* and (B) *T. hamatum* isolate 382 ($T_{382}$) in all artificially infested and $T_{382}$-fortified potting mixes from a single bioassay, respectively. Hybridization signal strength is reported relative to the average integrated optical density of the digoxigenin-labeled reference control (rIOD) and plotted against the logarithmic calculated DNA concentration using real-time PCR. Data represent means of hybridization signals generated with Rso1 and Tgn1 from two hybridization runs using amplicons from a single PCR reaction ($n = 4$). Error bars indicate standard errors.

is targeted [13]. In this study, ITS-based detector oligonucleotides were selected to detect and identify members of the *R. solani* species complex (Rso1) and the genus *Trichoderma* (Tgn1), encompassing several pathogenic groups [36] and disease suppressive species [37], respectively. Nevertheless, ribosomal sequences do not always provide the desired degree of selectivity, especially below species level [9,38,39]. As a result, other diagnostic regions of the genome should be assessed, preferably directly linked to virulence for pathogens or disease suppressive activity for DSMs. This can be achieved by several techniques, including RAPD analysis to generate randomly amplified fragments from the genome [40]. Using this strategy, Abbasi et al. [12] developed a series of PCR primers to specifically detect $T_{382}$. However, under this protocol that was developed to ensure reliable detection, three individual PCR reactions are required to rule out the presence of other isolates [12], rendering the assay unattractive to conduct, and unsuitable for direct quantification. In this study, a detector sequence derived from one of these markers (Tha382) was added to the DNA array containing the ITS-based oligonucleotides. Whereas the primers OPE16-F and OPE16-R generated amplicons of the expected length for more than half of the *Trichoderma* isolates tested, cross-hybridization to Tha382 was limited to three *Trichoderma* isolates, including *T. harzianum* CPK 51, *T. inhamatum* CPK 239, and *T. longibrachiatum* 57D (data not shown). It is currently unknown whether these isolates manifest any levels of suppressiveness to diseases caused by *R. solani*.

In practice, plant disease symptoms often result from infection by multiple pathogens rather than by a single pathogen, complicating classical diagnosis. In addition, the disease-causing potential of potting mixes is currently evaluated in bioassays in which scoring of symptoms is often subjective and sometimes even problematic (due to a lack of robust and reliable scoring methods). As a result, other methods than just visually observing plants are needed to measure plant and substrate health. In addition, in order to take preventive measures (e.g. before crops are grown), it is desired to predict the risk of disease based on pathogen population densities in the growing medium. In this study, the power of DNA arrays to estimate disease severity by analyzing the growing medium (Fig. 3) was shown, which corroborate previous results [10]. A reasonable correlation ($R^2 \geq 0.79$) was found between Rso1 hybridization signal and disease severity as well as incidence of severe disease. The lack of a stronger correlation may be explained by the biological variation that impacts symptom development, but also by the way the severity of disease is scored. More robust and reliable disease scoring methods need to be developed to support this hypothesis.

We further demonstrated the utility of the DNA array approach to measure different microbial populations in a single assay (Table 3), offering perspectives for studying population dynamics and interactions of target populations in complex media. The array was shown to perform well in both systems studied. These encompassed a simplified system, in which the presence of endogenous microorganisms was diminished, and a natural system, in which the endogenous microbial population was abundantly present (confirmed by Fun1 hybridization signals (Table 3)). Mixes were inoculated with the pathogen at an inoculum density of the pathogen that consistently causes seedling infection and with the biocontrol agent at the recommended rate for disease suppression [17].

Sustained biological suppression of *R. solani* requires the presence of specific DSMs that eradicate pathogen propagules by predation or suppress pathogen growth by production of biostatic agents. The most important microorganisms that are suppressive to diseases caused by *R. solani* are members of the genus *Trichoderma* [41–44] and *Penicillium* [45]. These organisms often interact with a number of bacterial species that enhance their suppressiveness [19,46]. When monitoring a mycopredatory interaction, one would expect a decline in the pathogen level concomitant with an increase in the predator population. However, in this study, we only observed a significant decline in the hybridization signals for *R. solani* (decrease in rIOD from 110.51 to 68.02) after adding $T_{382}$ to the mix for the heated DSP mix (Table 3), while this was not observed for the other treatments. This suggests that factors in addition to eradication by $T_{382}$ contributed to suppression of Rhizoctonia damping-off, which are most probably biotic and abiotic environmental factors that are inherent to the mixes themselves. As high levels of endogenous fungi were measured in the natural mixes using Fun1, a non-discriminative measure of the total amount of fungal DNA (Table 3), these factors may include the undetected presence of naturally occurring DSMs. The microbial carrying capacity of the potting medium, which

is determined by the decomposition level of the organic matter fraction [16], is an important factor in suppression of Rhizoctonia damping-off [17]. Assessment of the microbial activity by the rate of hydrolysis of fluorescein diacetate (FDA) [47] revealed higher microbial activity in the CPB mixes than in the DSP mixes as the average rates of FDA hydrolysis for the CPB and DSP mixes were 4.6 and 3.6 μg FDA hydrolyzed $g^{-1}$ dry weight $min^{-1}$, respectively. Though it is rare for potting mixes to be naturally suppressive to Rhizoctonia damping-off without allowing several months for these materials to be colonized by naturally occurring DSMs [17,43], it may be possible that the source of composted pine bark used in our experiments already contained such suppressive microorganisms. Hence, the higher microbial carrying capacity of the CPB mixes, coupled to the natural presence of suppressive microorganisms in the compost, may possibly explain why natural suppression of Rhizoctonia damping-off was observed in the infested natural CPB mix and why fortifying this mix with $T_{382}$ did not further reduce disease severity and incidence of severe disease.

A potential limitation of DNA-based techniques is the possibility of detecting DNA from inactive or dead organisms. Indeed, in this study, *R. solani* was still detected in heated mixes that were drenched with a fungicide specifically labeled for protection against *R. solani* diseases (Table 3). Based on the hybridization signals generated by Fun1, the non-discriminative fungal detector oligonucleotide, we concluded that the presence and, consequently, the activity of fungi were rather low in these mixes. As DNA degradation is dependent on microbial activity [48,49], this may potentially explain why *R. solani* was still detected in these heated, fungicide-drenched mixes using the DNA array. When these mixes were reanalyzed 3 weeks after planting, general fungal presence was higher and the pathogen was no longer detectable (data not shown), thus confirming this hypothesis.

In this study, a relation was established between the pathogen level and the degree of disease (Fig. 3). However, although it would be very valuable to be able to predict the suppressive potential of a potting mix by quantifying the DSM population, no relation could be observed between the $T_{382}$ population and disease development (Table 3), again demonstrating that disease suppression is dependent on multiple factors. One of the most important advantages of DNA arrays is the possibility to extend the array to detect an unlimited number of microorganisms, including pathogenic and beneficial organisms. By expanding the DNA array to include detector oligonucleotides for other DSMs it may, however, be possible to predict the suppressive potential of a potting mix. Additionally, specific functional genes that have been identified as important to biological disease suppression or pathogenicity may be included in such arrays as well, further elevating the appeal of using DNA arrays in ecological studies.

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