Aeromonas media in compost amendments contributes to suppression of *Pythium ultimum* in cress

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Abstract

   Soil-borne diseases such as damping-off caused by *Pythium* sp. are responsible for high yield losses in organic vegetable production and are difficult to control. Compost amendments have been shown to improve in many cases survival and growth of plants in soils infested with soil-borne diseases. Yet, not all composts are disease suppressive and suppressiveness of composts can be pathogen-specific. Cress (*Lepidium sativum* L.) was grown in a standard peat substrate amended either with coco fiber (conducive control) or with composts differing in their suppressiveness towards *Pythium ultimum*. Bacteria were isolated from the rhizoplane and the most abundant species identified by MALDI-TOF MS. The bacterial composition in the rhizoplane of plants grown in non-suppressive substrates and in the suppressive compost was essentially different. *Aeromonas media* was the main species isolated from the rhizoplane of plants grown in a suppressive compost, whereas *Enterobacter cloacae* was the dominating species in the less suppressive compost and in the conducive control. *A. media* was then added to all substrates to evaluate its role in disease suppression. Addition of *A. media* improved suppressiveness against *P. ultimum* in all substrates. The effect was most pronounced in the substrate amended with the compost with low suppressiveness, resulting in levels of suppressiveness comparable to the highly suppressive compost. We conclude that presence of *A. media* in composts can contribute to disease suppression and might provide a useful marker for qualitative analysis of composts.

Keywords: suppressive composts, cress, *Pythium ultimum*, MALDI-TOF MS, *Aeromonas media*, *Enterobacter cloacae*

INTRODUCTION

   Soil-borne diseases such as damping-off caused by *Pythium* sp. are responsible for high yield losses in crop production and are difficult to control (Raaijmakers et al., 2009). Compost amendments in peat or soil substrates have the potential to reduce soil-borne diseases. This suppressive effect is mainly due to living microorganisms in the composts whereas abiotic factors are less important (Bonanomi et al., 2010; Mehta et al., 2014; Noble and Coventry, 2005). Yet, not all composts are disease suppressive and suppressiveness of composts can be pathogen-specific (Termorshuizen et al., 2006). One of the most challenging issues in compost research is the difficulty to repeat experiments, since the microbial composition of a compost changes over time (van Rijn et al., 2007). This is also a major constraint for the application of composts in the practice, rendering its beneficial effect unpredictable. The suppressiveness of composts has been shown to depend on several parameters, including the composting process, the maturation stage and storage conditions. The main fungal and bacterial families occurring during different phases of composting processes have been identified by different methods such as high-throughput sequencing (Neher et al., 2013), and their occurrence correlated to disease suppression (Bonanomi et

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al., 2010; Mehta et al., 2014; Yu et al., 2015). Yet, on a species or isolate level, only little is known about the occurrence and abundance of specific microbes and their role in disease suppression. Furthermore, only few studies investigated microbial populations at the site of action such as on seeds after sowing and roots (Chen and Nelson, 2008; Chen et al., 2012).

In the present work, the most abundant bacterial species were isolated from the rhizoplane of cress plants grown in substrates containing composts with different levels of suppressiveness against *Pythium ultimum*, and identified by MALDI-TOF MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry). Suppressiveness of the most abundant bacterium isolated from the suppressive compost was then evaluated in a cress-*Pythium ultimum* bioassay.

MATERIALS AND METHODS

Substrates

The substrates used for disease suppression bioassays were a mixture of 70% of standard peat substrate (Einheitserde/Torf, Type 0 from Gebr. Patzer GmbH & Co. KG, Sinntal, Germany) with (i) 30%, of compost (composition: 55% wood from branches, 30% foliage, 10% yard and household wastes, and 5% digestate (wet basis); sieved to 10 mm, Leureko AG, Leibstadt, Switzerland) or (ii) 30% of coco fibers (Coco Ter, Ökohum, Herrendorf, Switzerland). The compost lost its diseases suppressive effect over time, most likely because of unsuitable storage conditions (Compost 1). Therefore, fresh compost (Compost 2) from the same producer, with the same characteristics, was used for further experiments. The substrates were prepared at least 3 days before sowing of cress and stored in boxes at room temperature. In some experiments, *Aeromonas media* (isolated from the rhizoplane of cress grown in substrate amended with compost 2) was grown in liquid medium M1 (Fuchs et al., 2000) at 150 rpm for 16 h at 28°C, washed with tap water (centrifugation at 3000 g for 20 min) and added to the substrate at a final concentration of 10^9 colony forming units (cfu) L^{-1} of substrate 3 days before sowing of cress.

Disease suppression bioassay

Disease suppressiveness of substrates was assessed using the model system *Pythium ultimum* (Pu) - cress (*Lepidium sativum* L., Bigler, CH) in standard peat substrate as described before in detail (Thuerig et al., 2009). Shortly, the pathogen was grown on a millet medium (24 g of millet seeds (Bio Goldhirse, Coop, Basel, Switzerland) and 20 mL of demineralized water) in a 250 mL Schott bottle. After 7-10 d incubation at room temperature, Pu colonized millet was hashed with an onion chopper and mixed with sand (Vogelsand Vitakraft Sandy, Coop, Basel, Switzerland) in order to ensure a more homogenous distribution of Pu within the substrate. To each substrate, 5 g L^{-1} of this sand/Pu/millet-seed mixture was added to obtain a final Pu/millet concentration of 0, 0.25, 1, 2, or 4 g L^{-1} of substrate, respectively. Six replicates per Pu inoculum concentration and substrate were tested in pots of 100 mL volume. Pots were filled with substrate and then 0.6 g seeds (approximately 60 seeds) were sown at the surface. After sowing, pots were watered by placing them in a basin with tap water until saturation of the substrate. Pots were then covered with a plastic foil for the first two days to ensure high humidity conditions for germination. Plants were grown in a climate chamber (15 m^2 utility space, equipped with 72 Philips TLD-reflex 85 W/light color 640 performing at 100%) with a day/night length of 16 h at 23°C and of 8 h at 18°C, respectively. The effect of the treatment on the development of the plants was assessed by harvesting shoot fresh weight (g) pot^{-1}.

Substrates were compared using a two way ANOVA with substrate and Pu concentration as factors, followed by a Tukey-B test. Treatments at each Pu concentration were compared using a one way ANOVA followed by a Tukey-B test.

Quantitative and qualitative assessment of bacterial root colonization

For assessment of bacterial colonization of the rhizosphere and rhizoplane, roots of two randomly selected pots of the treatments Coco, Compost 1 and Compost 2

(concentrations of 0 and 0.25 g L^{-1} Pu), respectively, were harvested under tap water and approximately 1 g of root segments were transferred into sterile 100 ml Erlenmeyer flasks containing 50 mL of 0.1% w/w proteose peptone in deionized water. Flasks were then shaken on a rotary shaker for 30 s. The resulting suspensions were filtrated through glass wool into 50 mL falcon tubes, centrifuged for 20 min at 3000 g, and the pellet was re-suspended in 1 mL 0.1 w/w proteose peptone. These rhizosphere samples were immediately processed for dilution plating on King medium B agar (KBA, (King et al., 1954) and R2A (Carl Roth, Karlsruhe, Germany). To isolate microorganisms from the rhizoplane, resulting root segments from the previous step were transferred with sterilized forceps into new, sterile 100 mL erlenmeyer flasks containing 20 mL of 0.1% w/w proteose peptone and 7.5 g glass beads (3 mm diameter). To bring rhizoplane microorganisms into suspension, the Erlenmeyer flasks were vigorously shaken at 250 rpm for 15 min. The resulting suspensions were filtrated through glass wool into 50 mL Falcon tubes, centrifuged for 20 min at 3000 g, and the pellet was re-suspended as described above in Eppendorf tubes and immediately processed for analysis with dilution plating on KBA and R2A. KBA dishes were incubated for 20 h at 28°C and R2A dishes for 4 d at 18°C. Colonies were counted at an optimal dilution resulting in 10-50 colonies dish^{-1}.

Qualitative assessment of bacterial root colonization (MALDI-TOF)

For information about the qualitative composition of the rhizoplane community, MALDI TOF analysis (Benagli et al., 2012) of the previously isolated bacteria (see above) was performed. Bacterial colonies were isolated from KBA plates. From each treatment one dilution plate was chosen, which exhibited a sector containing well separated 40 bacterial colonies. Each of these 40 colonies was streaked on a new KBA plate and after incubation for 3 d at 18°C, one single colony was transferred with a sterilized toothpick into an Eppendorf tube containing 0.5 mL liquid King medium B (King et al., 1954). The tubes were incubated for 20 h at 28°C. From each tube, 0.1 mL liquid was transferred to a new tube, centrifuged at 20,000 g for 1 min, the supernatant removed and the pellet was suspended in 0.4 mL EtOH 70%. These samples were sent to the laboratory Mabritec (Riehen, Switzerland) for MALDI-TOF MS analysis (Benagli et al., 2012). The remaining 0.4 mL of each liquid bacterial culture was mixed with 0.4 mL glycerol (87%), and stored at -80°C as a backup for further analysis. MALDI-TOF MS was calibrated for the bacterial genera *Aeromonas* spp. and *Pseudomonas* spp. with housekeeping genes on species level for *Aeromonas* spp. (Benagli et al., 2012) or species group level for *Pseudomonas* spp. (Mulet et al., 2012).

RESULTS AND DISCUSSION

Compost amended to standard peat substrate resulted in a higher resilience of cress towards damping off caused by *P. ultimum* compared to coco fiber amended substrate (Figure 1) (p<0.001, Tukey-B). However, Compost 1 and Compost 2, which originated from the same kind of raw materials and were obtained from the same production site, differed significantly in disease suppression (p<0.001, Tukey-B). Compost 1, which had initially been disease suppressive as well (data not shown), had lost its suppressive properties over time, most likely due to high temperatures at the storage location which was not sufficiently protected from heat during summer. This is in line with many publications reporting that heating and unappropriated storage are causes for the loss of disease suppressiveness (Noble and Coventry, 2005).

Compost 1 and Compost 2 significantly differed in the number of cfu isolated from the rhizoplane samples plated on KBA (Figure 2), with significantly lower numbers of cfu in the rhizoplane of the more suppressive Compost 2 compared to Compost 1 (two way ANOVA, p<0.05). Thus, the higher suppressiveness cannot be explained by higher quantities of root-associated microorganisms. The same pattern was observed when rhizoplane extracts were plated on the medium R2A instead of KBA, but differences were not significant (two way ANOVA, data not shown). While KBA favors fast growers, such as some species groups of the genus *Pseudomonas*, R2A medium was included to assess also slow growing bacteria.
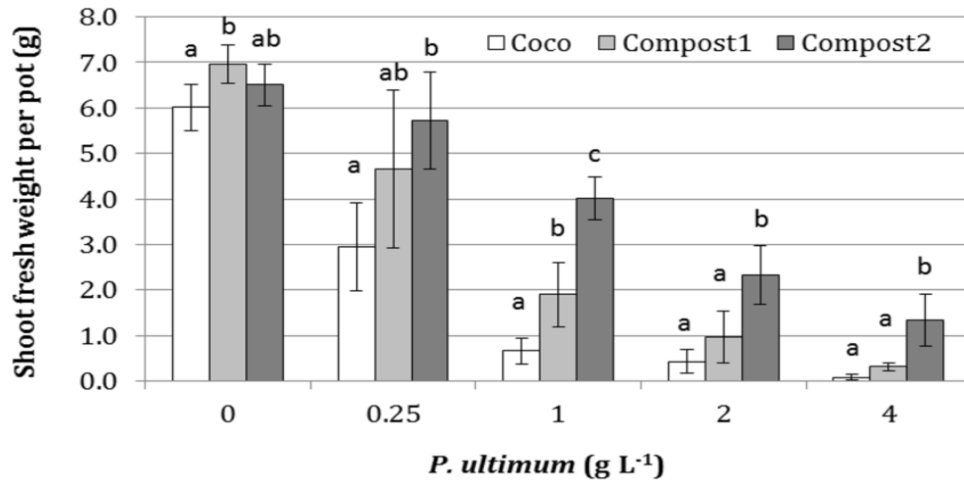
Figure 1. Suppressiveness of three different substrates (Coco, Compost 1, Compost 2) in the system cress-*P. ultimum*. The y-axis shows fresh weight of cress after one week in soils amended with different concentrations of *P. ultimum* (0, 0.25, 1, 2 and 4 g L^{-1}). The figure shows means±SD (n=6). Different letters indicate significant differences between substrates at a certain concentration of *P. ultimum* (Tukey-B, p<0.05).
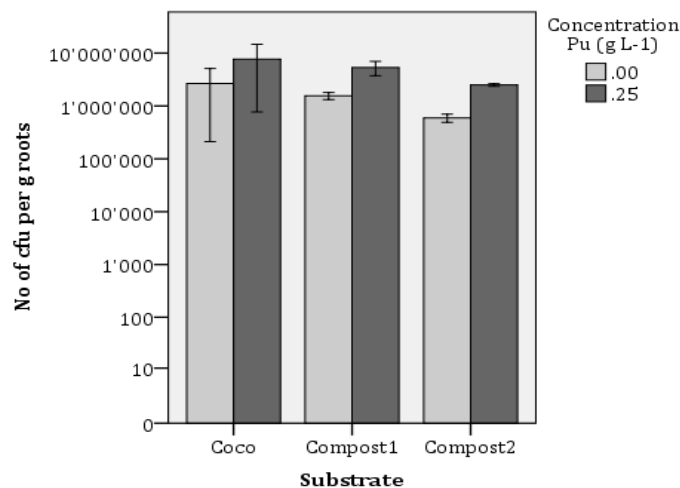

Figure 2. Total number of colony forming units (cfu) on King's medium B agar plates isolated from the rhizoplane of cress plants grown in the substrates Coco, Compost 1 or Compost 2 non-amended (0 g Pu) or amended with 0.25 g *P. ultimum* L^{-1} substrate. The figure shows means±SD from two randomly selected individual pots per treatment.

The qualitative MALDI-TOF MS analysis of the most abundant rhizoplane bacterial isolates revealed completely different species to be predominantly present in substrates with low suppressiveness (Coco, Compost 1) and in the suppressive substrate (Compost 2) (Figure 3). *Aeromonas media* was the dominant bacterial species in the rhizoplane of plants grown in the substrate compost 2, whereas *Enterobacter cloacae* was dominant in the rhizoplane of plants grown in the substrates Compost 1 and Coco. The difference in suppressiveness of the two composts could therefore be the result of this categorical difference.
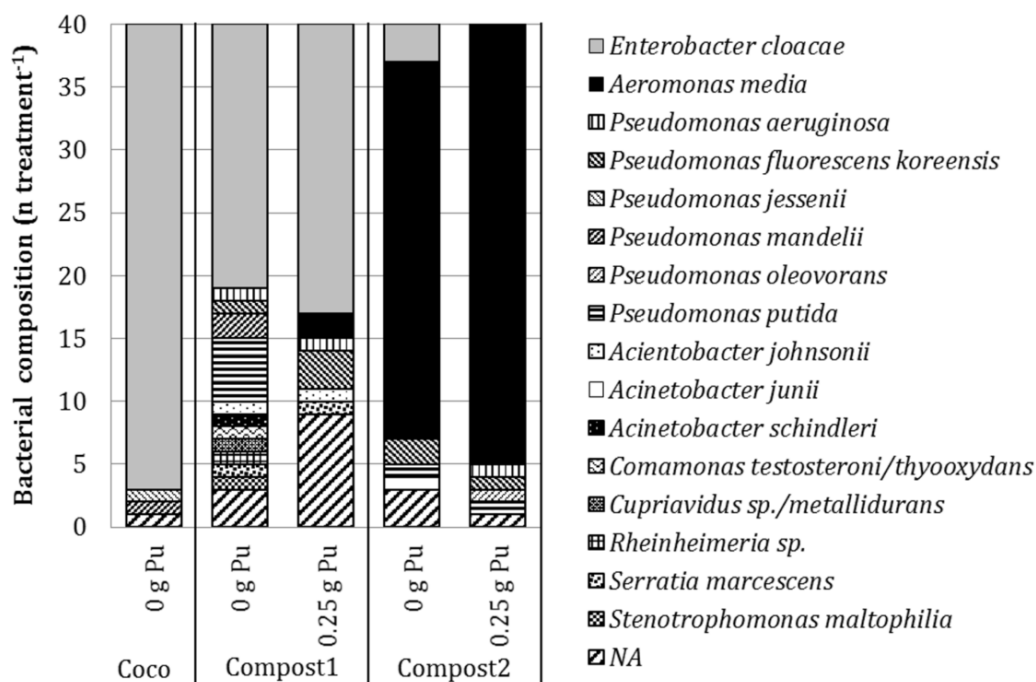
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Figure 3. Bacterial composition of the rhizoplane of cress grown in the substrates Coco, Compost 1 or Compost 2 non-amended (0 g Pu) or amended with 0.25 g L^{-1} substrate *P. ultimum* (0.25 g Pu). For each treatment, a sector of a King's B agar plate containing 40 bacterial colonies was marked and all of these 40 colonies were sub-cultured and finally analyzed by MALDI-TOF MS.

To test the hypothesis that *A. media* is responsible for disease suppressiveness of Compost 2, *A. media* was added to substrates amended with Coco fiber, Compost 1 or Compost 2 (Figure 4). In all substrates, the amendment of *A. media* improved plant growth. Best responses were obtained in substrate amended with Compost 1 (Figure 4B).

Until now, *Aeromonas* sp. were identified just in few cases as being part of the microbial community of composts (Lim et al., 2014; MacCready et al., 2013; Mehta et al., 2014), and little is known about *Aeromonas* spp. as potential biocontrol organisms against pathogens. Strains of *Aeromonas hydrophila* and *A. caviae* are reported to produce 2,4-diacetylphloroglucinol (Strunz et al., 1978) and chitinase (Inbar and Chet, 1991), respectively, and were effective against soil-borne fungal pathogens of the genera *Pythium*, *Rhizoctonia* and *Fusarium*. In aquatic systems *Aeromonas media* is reported as a biocontrol organism against the fish pathogenic oomycete *Saprolegnia parasitica* (Lategan et al., 2006). In another context, isolates of *A. media* or closely related *Aeromonas* sp. were shown to have potential as PGPR (plant growth promoting rhizobacteria) by solubilizing inorganic phosphorous and/or producing IAA (indole-3-acetic acid) (Aarab et al., 2015; Arraktham et al., 2016). Hence, *Aeromonas* sp. merit further studies to elucidate their potential as biocontrol or biofertiliser organisms.
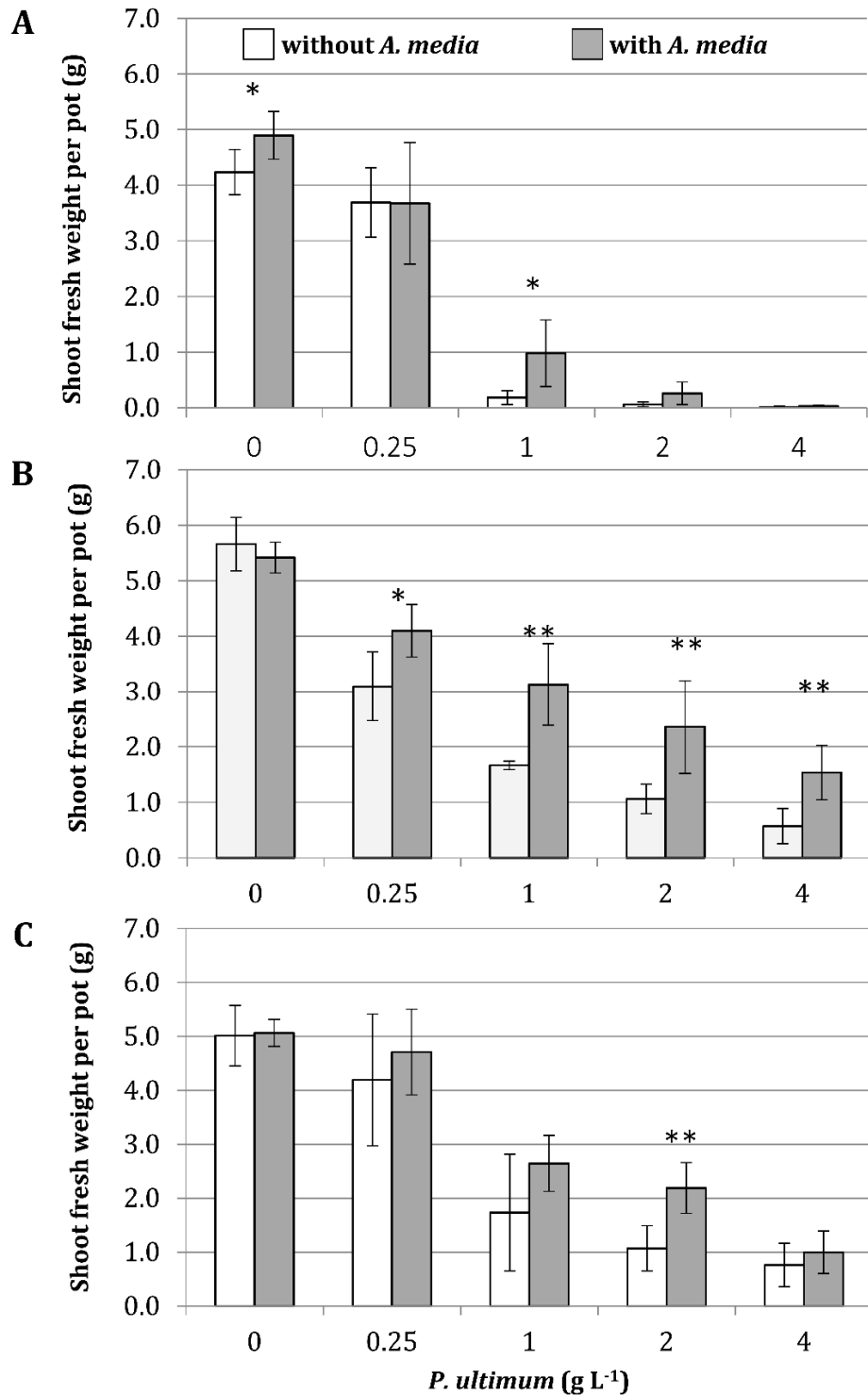
Figure 4. Suppressiveness of the substrates Coco (A), Compost 1 (B) or Compost 2 (C) not amended or amended with *A. media* in the system cress-*P. ultimum*. The y-axis shows fresh weight of cress after one week in soils amended with different amounts of *P. ultimum* (0, 0.25, 1, 2 and 4 g L^{-1}). The figure shows means±SD (*n*=6). Stars indicate significant differences between substrates at a certain concentration of *P. ultimum* (t-test, * $0.01<p<0.05$; ** $p<0.01$).

CONCLUSIONS

- Compost amendments have the potential to protect plants from soil-borne diseases, but the potential beneficial effect can get lost due to unfavorable environmental conditions during storage, due to changes in microbial composition.
- *Aeromonas media* abundantly present on the rhizoplane of cress plants was positively correlated with higher suppressiveness of substrates, and might thus be a marker for suppressiveness of composts.
- The use of a set of microbial markers could be a fast and reliable tool to assess suppressiveness of composts.

ACKNOWLEDGEMENTS

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