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Efficacy of four phosphate-mobilizing bacteria applied with an animal bone charcoal formulation in controlling *Pythium aphanidermatum* and *Fusarium oxysporum* f.sp. *radicis lycopersici* in tomato

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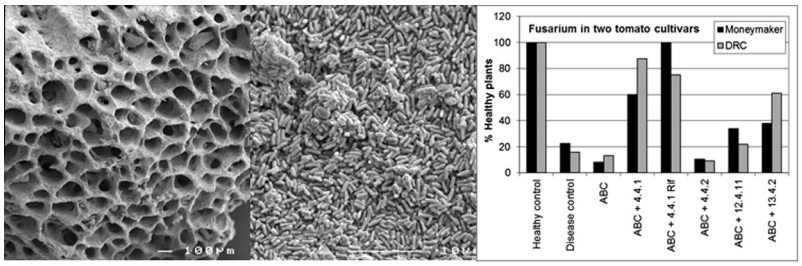
HIGHLIGHTS

- Biological control of *Pythium aphanidermatum* and *Fusarium oxysporum* in tomato.
- Bacteria with the capacity to mobilise phosphate were able to control plant diseases.
- Animal bone char can function as a novel carrier for biocontrol agents.
- *Pseudomonas chlororaphis* has high root colonizing capacity.

GRAPHICAL ABSTRACT



Animal bone charcoal as carrier - for bacterial antagonists - controlling diseases in tomato plants

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ABSTRACT

Four taxonomically different bacteria, with the ability to mobilize phosphate (P) and to colonize animal bone charcoal (ABC), were tested for their capacity to control plant pathogens. Tests were performed in the greenhouse with young tomato plants in (potting) soil and in rockwool. Plants were infested with *Pythium aphanidermatum* and *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL) causing respectively damping off and crown and root rot. ABC is a porous, phosphorous containing waste product from the food industry, and was used as carrier to introduce the bacteria into the growing media. Scanning electron microscopy (SEM) pictures showed the intensive colonization of the bacteria in the interior of ABC. Of the four tested strains, *Pseudomonas chlororaphis* 4.4.1 was most effective in controlling the diseases. It controlled *P. aphanidermatum* and FORL in tomato in each of the tests. The strain appeared to be a very good root colonizer, since 1–8% of the cultural bacterial population on the tomato roots or in rhizosphere soil consisted of the introduced strain. Population densities of *P. chlororaphis* 4.4.1 were 0.5–5 $\times$ 10^7 CFU g^{-1} root or rhizosphere soil. *Peanibacillus polymyxa* 12.4.1 and *Streptomyces pseudovenezuelae* 13.4.2 significantly controlled *P. aphanidermatum* in two tests in potting soil, whereas *Bacillus pumilus* 4.4.2 was not effective. FORL could be controlled by *B. pumilus* 4.4.2 and *S. pseudovenezuelae* 13.4.2 in only part of the tests, whereas *P. polymyxa* 12.4.1 was not effective. ABC is a novel carrier for delivery of biocontrol bacteria into soil or substrate and combines biocontrol with recycling a phosphorous-rich waste product.

1. Introduction

The continuing growth of the world population and increasing pressure on all resources used for agricultural production demand action to improve sustainability in agriculture. Chemical inputs should be replaced by other strategies to avoid harmful emissions and residues. Synthetic pesticides can be successfully reduced by the application of biological control agents. Increasing numbers of products have been developed in recent years (Glare et al., 2012). Nevertheless, the biopesticide market is still a small portion

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(3.5%) of the total agrichemical market (Glare et al., 2012). More effort and effective screening procedures must be developed to enhance the number of biological products (Glare et al., 2012; Köhl et al., 2011).

Also the use of mineral and synthetic fertilizers should to be reduced to decrease emission to the environment and to enhance nutrient use efficiency (Dungait et al., 2012). Moreover, enhanced recycling of nutrients is urgently needed (Dungait et al., 2012; Paungfoo-Lonhienne et al., 2012). An essential element for plant growth which will be depleted in the near future is phosphorus (P). Modern agriculture is using increasing amounts of P derived from phosphate rock, and the current global reserves may be depleted in 50–120 years (Cordell et al., 2009; Ott and Rechberger, 2012). It is clear that there is an urgent need to look for alternative and renewable sources of P. Animal bones are a significant source of P that can be recycled. Bones have always been regarded as a valuable manure, but have been little used in commercial agriculture since the 1950s (Warren et al., 2009). New technologies producing safe P fertilizers from animal bones will facilitate the recycling of P from food industrial waste.

Carbonization of animal bone meal, i.e. heat treatment up to 550 °C, results in a porous product which is called Animal Bone Charcoal (ABC). It mainly consists of P and calcium (Ca). Due to the high temperature, any risk of transmitting diseases is negligible. ABC is a P fertilizer of intermediate solubility between Gafsa phosphate rock and triple superphosphate fertilizer (Warren et al., 2009). P solubility can be enhanced by a variety of soil bacteria and fungi, e.g. *Burkholderia, Bacillus, Pseudomonas, Streptomyces, Aspergillus, Paenibacillus* and *Trichoderma* spp. (Hamdali et al., 2008a,c; Kim et al., 2008, 2005; Kucey et al., 1989; Mamta et al., 2010; Postma et al., 2010; Vassilev et al., 2006). Such microorganisms are naturally present in soil, but can also be introduced to improve solubility of ABC. Previous research showed that such P-solubilizing bacteria can colonize the porous ABC particles (Postma et al., 2010).

Recent research focussed on selecting such P-solubilizing bacteria to use as inoculant to improve plant growth. Co-inoculation of different bacteria with different beneficial properties, i.e. P solubilization and nitrogen fixation, showed additive positive effects (Rudresh et al., 2005; Yu et al., 2012). Another interesting strategy is to select bacteria or fungi that combine P solubilization with plant growth promoting phytohormones or antifungal activities (Cordero et al., 2012; Hamdali et al., 2008b,c; Postma et al., 2010; Vassilev et al., 2006). P-solubilizing actinomycetes strains could reduce Pythium symptoms in wheat (Hamdali et al., 2008b). However, most research did not include plant-pathogen bioassays, and as a consequence the biocontrol efficacy of such P-solubilizing bacteria in a crop is still poorly described.

The current research aims at combining recycling of P from animal bones and the application of bacteria controlling soil-borne plant diseases. The porous structure of ABC is an ideal carrier to harbor micro-organisms (Someus, 2004). ABC has a large specific surface area and microorganisms grown in such a matrix are protected against the harsh environment, when introduced into soil.

Tomato (*Lycopersicon esculentum* Mill.) is used as model crop for the validation of this new and integrated sustainable strategy. Tomato is an economically important crop in EU with a production of 22×10^6 tonnes/year (world wide: 150×10^6 tonnes/year) and an estimated value of 18×10^9 €/year (FAOSTAT 2010 http://faostat.fao.org/). Moreover, it is an intensively grown crop with high yields (on average 55 tonnes/ha in EU) and high pesticide and fertilizer inputs. Production systems use different growing media and several diseases can threaten the crop. *Pythium aphanidermatum,* a devastating oomycetes pathogen, causes pre-and post-emergence damping-off symptoms (Postma et al., 2000). No resistant varieties exist. *Fusarium oxysporum* f.sp. *radicis lycopersici* (FORL), causing crown and root rot of tomato, is an important soil-borne disease causing high yield losses in fields and commercial greenhouses (Clematis et al., 2009; Validov et al., 2007). Although resistance genes are known, it is expensive to prepare resistant varieties for all types of tomatoes. Thus, biological control of both *Pythium* and *Fusarium* would be a welcome alternative measure to reduce the use of chemical pesticides.

The challenge is to develop a new product which combines all these positive targets: re-using a valuable P-rich waste stream which delivers plant-available P, and serves as a carrier for P-solubilizing bacteria with the capacity to protect plants against pathogens. Previous research showed the possibility to grow bacteria with beneficial properties in ABC (Postma et al., 2010). In the current research, the potential to control soil-borne diseases in plant assays by antagonistic bacteria applied in ABC as carrier was tested.

Four bacterial strains from different genera with phosphate mobilizing and biological control capacities were selected. Their efficacy to control *P. aphanidermatum* and *F. oxysporum* f.sp. *radicis lycopersici* in young tomato plants, respectively in soil and rockwool, was tested in repeated bioassays in the greenhouse. Inoculum production of the four bacterial strains was performed in a two stage fermentation process, based on the addition of a cheap liquid medium with proper carbon and nitrogen contents to the solid porous P-rich ABC. Colonization of the interior of ABC by the bacterial strains was assessed with Scanning Electron Microscopy. Root colonization of the best biocontrol strain, i.e. *Pseudomonas chlororaphis*, was assessed after a rifampicin-resistant mutant was selected.

2. Materials and methods

2.1. Bacterial strains

Four bacterial strains from different genera were used to study their potential to control diseases in tomato plants. Origin, identity and other characteristics of these strains are summarized in Table 1. These strains had been selected for their capacity to mobilize phosphate and their potential to control plant pathogens (Postma et al., 2010). The strains were identified by sequencing 1500 bp of 16S rRNA followed by BLASTing the sequences with Ribosomal Database Project II release 10. Strains were cultured on R2A (Difco Laboratories, Detroit, USA) at 25 °C and stored at −80 °C in 20% glycerol.

2.2. Selection of rifampicin-resistant mutants of P. chlororaphis

Mutants of *P. chlororaphis* 4.4.1 resistant to rifampicin (Rif) were generated by exposure of the isolate to R2A amended with different Rif levels (20, 50, 100, 150 and 200 mg l^{-1}). The plates were stored in the dark for 2 days at 25 °C. Few colonies were collected from R2A with 100 mg l^{-1} Rif and transferred twice to R2A with Rif. One good growing colony was selected and tested for its growth ability in tryptic soy broth (TSB, Difco) amended with Rif 100 μg ml^{-1} and for stability of the mutant in TSB without Rif.

2.3. Inoculum production of bacteria in ABC

Animal bone charcoal (ABC) was supplied by Terra Humana Ltd. (Budapest, Hungary). It was derived from pig bone (Smits Vuren BV, the Netherlands) and prepared by heating 1 h at 550 °C. ABC is a porous material with an internal surface area of 82 m^2 g^{-1}, containing 13.4% phosphorus (P) and a pH-H_20 of 7.6 (Warren et al., 2009). The main P component is hydroxy-apatite ($Ca_5OH(PO_4)_3$) (70–75%), followed by magnesium phosphate (12–15%).