Scientia Horticulturae 173 (2014) 106–113

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Monitoring rhizosphere microbial communities in healthy and *Pythium ultimum* inoculated tomato plants in soilless growing systems

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ARTICLE INFO

Article history:
Received 20 September 2013
Received in revised form 28 April 2014
Accepted 29 April 2014
Available online 24 May 2014

Keywords:
CLPP
PCR–DGGE
Root microorganisms
Oomycete
Hydroponic systems

ABSTRACT

Closed hydroponic growing systems are commonly used for greenhouse production of vegetables. One of the main problems associated with these systems is the potential spread of plant root pathogens. The purpose of this study was to investigate whether Community Level Physiological Profiling (CLPP) can be used as a method to monitor changes in the rhizosphere microbial communities inflicted by a pathogen. We studied the microbial communities of the roots from three different physiological stages of *Pythium ultimum* inoculated and non-inoculated tomato plants, with culture-dependent (CLPP and viable counts) and culture-independent methods (PCR–DGGE). The results showed that the presence of *P. ultimum* changed the utilization of carbon sources by the root microbiota, and significant differences were found between inoculated and non-inoculated plants. However, the differences in utilization patterns were larger between plant physiological stages than between treatments. Also with the results from PCR–DGGE it was confirmed that plant age was a stronger driver of the community structure than the introduction of a pathogen. CLPP is hence a good method for examining changes in microbial communities related to plant development, but regarding changes caused by the presence of a pathogen the method shows less potential.

1. Introduction

Hydroponic greenhouse production of tomato in open cropping systems, where excess nutrient solution is drained away in a run-to-waste system, contributes significantly to eutrophication of streams and lakes. As much as 40% of the nitrogen supplied to the plants is lost with the drainage water (Dorais & Dubé, 2011; Hansson, 2003). European legislation demands that the release of nutrients be minimized (EU Water Framework Directive., 2000), and therefore growers are encouraged to change from open to closed irrigation systems where the nutrient solution, after pH and nutrient adjustment, is recirculated. One of the main problems associated with closed irrigation systems is the potential spread of plant pathogens. Pathogens can be introduced into the system with the irrigation water, inoculated seeds or transplants, by insects, and by workers in the greenhouse (Postma et al., 2008). In particular, oomycetous pathogens such as *Pythium* and *Phytophthora* can easily spread and propagate explosively under favorable conditions, causing serious damage (MacDonald et al., 1994; Stanghellini & Rasmussen, 1994).

There are many factors involved in disease development in plant cultivation systems. For instance the infectious dose needed to cause disease, the threshold, is different for each pathogen. With *Pythium aphanidermatum*, Postma et al. (2001) showed that as few as 0.1–0.4 colony forming units (CFU) per mL irrigation water were enough to result in *Pythium* root rot of cucumber crops, whereas higher concentrations were needed for other pathogens in the same cultivation system. Although this specific threshold has been set, there is an endless combination of pathogens, crops, and growing media that have not been investigated for their threshold values. There are commercial methods in use for disinfection of the recirculating nutrient solution, such as slow filtration, UV-irradiation, or heat treatments (Ehret et al., 2001). However, none of these methods prevents dispersal of pathogens between plants in the same cultivation unit. This means that there is a need to monitor the health status of hydroponic systems in order to ensure that potential pathogen threats are detected early.

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http://dx.doi.org/10.1016/j.scienta.2014.04.036
0304-4238/

Since the number of pathogens that can infect tomato plants via the roots is large, a system looking at shifts in the entire microbial community, rather than searching for single pathogens, would be more efficient when looking at the health status of the crop. A well-established method to characterize microbial communities in different habitats is Community Level Physiological Profiling (CLPP), which is based on the metabolism of the microbial community (Rutgers et al., 2006). This method has promise for applications in commercial laboratories focusing on plant health. However, with culture-dependent methods such as CLPP, there are biases in the analysis towards microorganisms that are able to grow in a laboratory environment. Therefore, CLPP should be complemented with a culture-independent method, such as denaturing gradient gel electrophoresis (DGGE). Correa et al. (2006) and Suda et al. (2009) have both combined these two methods and shown that it gives a good picture of both the structure and function of microbial communities of both rhizospheres and phyllospheres, and changes in these caused by inoculation of a microorganism.

In the present study, the microbial communities on tomato roots inoculated with the plant pathogen *Pythium ultimum*, a commonly occurring pathogen in soilless tomato culture, were analyzed by CLPP and DGGE and compared with those on non-inoculated tomato roots. The culturable microbial population was also studied by the viable count method. The aim was to obtain insights into whether the CLPP method can be used to monitor changes in the rhizosphere microbial communities of tomato plants in hydroponic systems in relation to pathogen attack. The microbial community was analyzed at three different plant physiological stages, since one important aspect that needs to be taken into consideration is the changes that occur as the plant ages. As the plant grows, the root exudation rate increases until it sets flowers, while as it matures the rate decreases again (Aulakh et al., 2001). In tomato specifically, Kravchenko et al. (2003) found that swollen seeds contain more sugars than organic acids, while in the exudates of 4- and 14-day old seedlings the relationship switched to more organic acids than sugars. They also showed that the organic acids were more easily utilized by rhizobacteria than sugars were. Glucose, fructose, maltose and xylose, among others, are present in tomato root exudates. The ratios of these sugars were found to be highly dependent on plant developmental stage (Lugtenberg et al., 1999). These changes in exudate patterns will have an effect on the composition of the rhizosphere microbial communities.

The hypotheses of this study were that: (i) the microbial community of the roots changes in the presence of a pathogen in the system; (ii) certain organic nutrient sources are used faster in the presence of a pathogen and thus act as signature compound candidates; and (iii) the microbial community structure is affected by plant age, and this community change can be discriminated from the change caused by infection.

2. Materials & methods

The three different physiological plant stages of tomato chosen for comparison of their root microbiota were: seedlings (10 days), flowering plants (8 weeks), and plants with fruit (17 weeks).

2.1. Microorganisms and inoculum preparation

A kanamycin resistant *Pythium ultimum* isolate (Hultberg, 1999) used for plant inoculation in the present study was kept on potato dextrose agar (PDA, Difco Laboratories Inc., Detroit, Michigan) supplemented with 200 $\mu g\,mL^{-1}$ kanamycin during laboratory storage, and repeatedly reisolated from tomato seedlings to avoid loss of pathogenicity. The plants were inoculated with entire mycelium mats of *P. ultimum* which were produced in the same manner as described by Hultberg et al. (2011) for production of zoospores. Briefly, mycelium of *P. ultimum* grown on PDA was transferred to water agar. After 48 h 1 cm^2 pieces cut from the periphery of the *P. ultimum* colony were placed in petri dishes containing 15 mL of V8 broth and incubated in darkness at 25 °C for 72 h. The mycelium mat produced in the V8 broth was cleaned twice in an autoclaved mineral solution, and once in autoclaved distilled water. At this stage the mycelial mats were used to inoculate the plants. For inoculation of the seedlings, washed mycelium mats of *P. ultimum* were mixed with sterile distilled water, using a high-frequency blender (Polytron, Kinematica GmbH, Switzerland), to produce a solution with a mycelial fragment concentration of $2.4 \times 10^7\,L^{-1}$. The concentration was determined by counting the fragments in a haemocytometer. For inoculation of the flowering and fruit-bearing plants, the entire mycelium mat was added to the cultivation system.

Tomato plants sown in January (flowering stage) were inoculated two and six weeks after the sowing date with one mycelium mat per plant. Tomato plants sown in June (fruit-bearing stage) were inoculated 14 and 16 weeks after the sowing date. Later inoculation was used for experiments on fruit-bearing plants to avoid early plant death; these were given three mycelial mats each per inoculation. All of the plants were grown in the same greenhouse chamber and each experiment was conducted with five replicates per treatment, where one replicate equals one plant.

2.2. Cultivation of plants

2.2.1. Seedling experiment

Seeds of *Lycopersicon esculentum* cv. Tiesto were germinated at 24 °C on moist filter paper in petri dishes (18.5 cm) in darkness, with five replicates per treatment. Each petri dish had a density of 100 seeds which were incubated for 10 days. Five days after sowing, the seedlings were inoculated with *P. ultimum*. Each petri dish was given 5 mL of the mycelium fragment suspension described above, while the control dishes were given 5 mL tap water. After infection, the seedlings were left to grow for another 5 days before analysis.

2.2.2. Greenhouse experiment

For experiments on flowering plants, tomato seeds (*Lycopersicon esculentum* cv. Tiesto) were sown in January and germinated as described above. After seven days the seedlings were individually transferred to black, polyethylene foam mats (PEN 334, Åkesson, Mönsterås, Sweden) and placed in 1 L plastic hydroponic containers filled with nutrient solution. The nutrient solution was prepared in accordance with Sonneveld and Straver (1989) and contained KNO_3 8.01 mM, NH_4NO_3 0.6 mM, $MgSO_4$ 1.88 mM, $Ca(NO_3)_2$ 4.2 mM, Fe–EDTA 18.73 μM, $MnSO_4{\cdot}7H_2O$ 6.25 μM, $ZnSO_4{\cdot}7H_2O$ 12.5 μM, H_3BO_3 31.25 μM, $CuCl_2{\cdot}2H_2O$ 0.94 μM, and $Na_2MoO_4{\cdot}2H_2O$ 0.63 μM. The pH was approximately 5.7 and electrical conductivity 2.5 $mS\,cm^{-1}$. The nutrient solution was continuously aerated and the containers holding the plants were filled up once a week.

During the first six weeks the plants were grown in a climate chamber with a temperature set-point of 19 °C day and 18 °C night, and a 16 h photoperiod of 400 $\mu mol\,m^{-2}\,s^{-1}$ with fluorescent lamps. The plants were then moved to a 90 m^2 greenhouse chamber with a temperature set-point of 19 °C day and 18 °C night, and placed individually in 12 L buckets to give the roots more space. High pressure sodium lamps (200 $W\,m^{-2}$) were used for 12 h per day for extra lighting to compensate for the low natural light during early spring.

For experiments on fruit-bearing plants tomato seeds were sown in June, and placed directly in the greenhouse after germination. These plants were given additional light (high pressure