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Short Communication

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Effects of Membrane Filtering of Tomato Root Exudates on Conidial Germination of *Fusarium oxysporum* f. sp. *lycopersici*

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Abstract

The effect of different membrane filters on the bioactivity of tomato root exudates was tested in an *in vitro* assay addressing the germination of microconidia of the soil-borne fungus *Fusarium oxysporum* f. sp. *lycopersici*. Membrane filtration of unsterile root exudates with filters of different membrane materials and filter brands resulted in an increased microconidia germination. This effect varied depending on the used membrane filter but was lacking when sterile root exudates were used. The alteration of the bioactivity of unsterile root exudates therefore seems to be due to the presence of microbial contaminants. The varying effects of different filter brands may be due to their differential potential of retaining inhibitory compounds. When working with root exudates, such effects of membrane filtration have to be taken into account.

Introduction

In the past years, the role of root exudates in the interaction between plants and fungi became more and more a matter of interest. Plant roots release a large variety of organic and inorganic compounds which are involved in complex biological processes in the rhizosphere; however, the role of most chemical signals in belowground interactions is still poorly understood (recently reviewed by Bertin et al., 2003; Bais et al., 2006). Root exudates are difficult to study because of the environment in which they occur, e.g. the nature and the activity of rhizosphere microbes, considerably influences the chemical composition of root exudates. Moreover, root exudates can be masked by metabolic by-products of micro-organisms. To avoid microbial activity, a series of methods are available to produce and collect root exudates under sterile conditions (e.g. Dhingra and Sinclair, 1995). In some studies on rhizospheric plant–microbe interactions, sterile plant cultivation can hardly be performed (e.g. studies with inoculated plants or over a whole growing period). Thus, the treatment of root exudates with membrane filters, immediately after exudate collection, is a widespread method to prevent undesirable microbial effects (Poulin et al., 1993; Dhingra and Sinclair, 1995; Aulak et al., 2001).

Förster et al. (1983) reported that filtering through filter paper can alter the effect of solutions on the germination of *Phytophthora* oospores; however, no data are available yet on possible alterations of the bioactivity of root exudates after the use of membranes used in sterile filtration. Thus, in the present work, we studied the effect of membrane filtration of root exudates with different membrane filters in an *in vitro* assay looking at the spore germination of the soil-borne fungus *Fusarium oxysporum*.

Materials and Methods

Collection of root exudates

Tomato seeds (*Solanum lycopersicum* L. cv. Micro Tom) were surface-sterilized (5 min) with household bleach (3.8% NaOCl), rinsed four times in sterile-distilled water and placed in autoclaved perlite. Fourteen days after, seeding plants were individually transplanted into plastic pots (volume 630 ml) containing moist, sterilized perlite. Throughout the experiments, plants were watered with a nutrient solution (Steinkellner et al., 2005). Experiments were performed at 24°C in a growth chamber (York International) with a photoperiod of 16 h light/8 h dark (light intensity 296 μmol m^{-2} s^{-1}). Plants were grown for 22, respectively, 50 days.

In addition to the plant cultivation in perlite, surface disinfested tomato seeds were arranged in Petri dishes (145-mm diameter, seeds seeds/Petri dish), containing 0.2% Knop medium and lined with a glass fibre filter, under sterile conditions. Thereafter, the Petri dishes were sealed with Parafilm and cultivated for 22 days in a plant growth chamber.

Thereafter, the plants were harvested non-destructively by gently washing roots free of perlite with tap water. Plants were inserted in a beaker (the beaker was covered by an aluminium foil to keep roots in the dark) containing the sterile-distilled water, such that roots were completely submerged. The beaker was placed in a plant growth chamber for 24 h at 24°C. Thereafter, plants were removed from the beaker and the exudate solution was adjusted with sterile-distilled water to 1 g root fresh weight per 20-ml exudate solution (w/v) (Stevenson et al., 1995; Pinior et al., 1999). Exudates were immediately frozen and stored at −20°C until the germination experiments were performed. Root exudates from plants cultivated in Petri dishes were collected in the same manner, but under sterile conditions.

Root exudate and water treatments

Root exudates, which were collected after 50 days, were separated into 12 equal parts and treated with the different membrane filters. Sterile-distilled water was also treated with different membrane filters. The following membrane filters were used: (1) Sartorius Minisart cellulose acetate membrane (0.2 μm, product number 17597); (2) Sartorius Minisart polyethersulfon (PES) (0.2 μm, product number16532) (Sartorius, Vienna, Austria); (3) Pall Supore membrane (0.2 μm, product number 4612); (4) Pall polyvinylidene difluorid membrane (0.2 μm, product number S4406); (5) Pall GHP membrane (0.2 μm, product number S4564) (VWR International, Vienna, Austria), (6) Roth PES membrane (0.22 μm, product number P668); (7) Roth MicronSep, Cellulosic, non-sterile filter discs (0.22, product number E02WP04700) (Lactan, Graz, Austria); (8) Millipore Steriflip, Durapore membrane (PVDF) (0.45 μm, product number SE1M003M00); (9) Millipore Millex HV, Durapore membrane (PVDF) (0.45 mm, product number SLHV033RS) (Millipore, Vienna, Austria); (10) Nalgene, Nylon membrane (0.2 μm, product number 195–2520); (11) Nalgene, polyethersulfone membrane (0.2 μm, product number 180–1320); (12) Nalgene syringe prefilter plus, CA membrane (0.2 μm, product number 192–2520) (Bartelt, Vienna, Austria).

Root exudates collected after 22 day (sterile and non sterile) were separated into two parts each. While one part was left untreated, the other part was membrane filtered (Roth MicronSep).

Germination experiments

Fusarium oxysporum f. sp. *lycopersici* (isolate Fol 007) was kindly provided by B. J. Cornelissen (Institute for Molecular Cell Biology, Amsterdam, The Netherlands). The fungus was stored as a microconidia suspension in 30% glycerol at −80°C. The microconidia suspension was transferred regularly to a Czapek Dox medium (Duchefa Biochemie, Haarlem, The Netherlands) and the fungus was grown in the dark at 24°C. After 2–3 weeks, fungal cultures were flooded under sterile conditions with water and the suspension obtained was filtered through three layers of filter paper (20–150 μm pore diameter) (Vliesscheiben für Kannenfilter, Laporte Ges.m.b.H., Wels, Austria). The resulting microconidia suspension was adjusted to 1.0×10^7 microconidia/ml water using a haemocytometer.

The germination assay was performed in sterile culture plates (24 wells, Greiner bio-one, Nr. 662160, Frickenhausen, Germany). Aliquots of 500 μl of root exudate or water, respectively, were mixed with 100 μl of the microconidia suspension and incubated at 24°C in the dark while shaking with 200 rpm. A sterile water control was included in the tests. After 20-h, lactophenol cotton blue was added to stain the fungus and to facilitate the quantification of the microconidia germination (200 spores were counted). A microconidium was considered germinated if the germ tube length was at least as long as the spore. The present data of each treatment are the means of two independent experiments with three replicates.

The statistical analyses were performed using STATGRAPHICS PLUS 5.0 (StatPoint, Inc., Herndon, VA, USA).

Results and Discussion

Facing the minimal amount of substances exuded by plant roots proper collection techniques and sensitive analytical methodologies are essential for studying signalling compounds exuded by plant roots. In this context, membrane filtration is known as an accurate technique to separate micro-organisms, cells and debris from liquids without absorbing substances from solution (Dhingra and Sinclair, 1995). However, testing the effect of root extracts on the germination of *Phytophthora* oospores, Förster et al. (1983) reported that filtering through filter paper can enhance germination, indicating that filtration can alter the bioactivity of solutions.

In our study, the untreated root exudate did not stimulate microconidia germination in comparison with the water control, while membrane filtered root exudates, independently of the used filter, always resulted in a clearly enhanced microconidia germination of *F. oxysporum* f.sp. *lycopersici*. The germination rate in membrane filtered root exudates was 3.2–5.3 times higher than in the untreated root exudate. (Fig. 1). This clearly shows that membrane filtration can alter the bioactivity of root exudates. This change of bioactivity of the root exudates could be due to (1) compounds released by the membrane filter material, (2) the presence of microbial contaminants in the exudates, or (3) the removal of inhibitory compounds.

Testing the microconidia germination in sterile-distilled water, treated with different membrane filters,

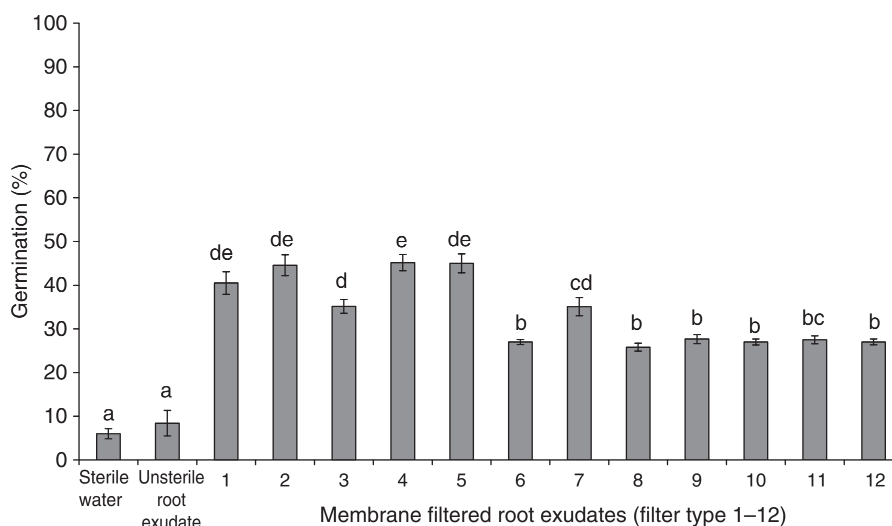

Fig. 1 Germination rate of *Fusarium oxysporum* f. sp. *lycopersici* in the presence of sterile water, unsterile and membrane filtered tomato root exudates. The following membrane filter types were tested: 1 = Sartorius Minisart (cellulose acetate membrane, 0.2 µm), 2 = Sartorius Minisart (polyethersulfon membrane, 0.2µm); 3 = Pall (Supore membrane, 0.2 µm); 4 = Pall (polyvinylidene difluorid membrane, 0.2 µm), 5 = Pall (GHP membrane (0.2 µm), 6 = Roth (polyethersulfon membrane, 0.22 µm); 7 = Roth (MicronSep membrane, 0.22 µm); 8 = Millipore Steriflip (Durapore membrane,0.45 µm); 9 = Millipore Millex HV (Durapore membrane, 0.45 mm); 10 = Nalgene (Nylon membrane, 0.2 µm); 11 = Nalgene (Polyethersulfone membrane, 0.2 µm); 12 = Nalgene syringe prefilter plus (cellulose acetate membrane, 0.2 µm). Bars indicate SE of the mean; columns with different letters are significantly different ($P < 0.001$) based on Kruskal–Wallis H test ($H = 72.609$)

showed no effect on microconidia germination (Fig. 2), thus excluding the release of bioactive compounds from the membrane filter material.

Microbial contaminants in the exudates could release bioactive compounds or could act directly on the microconidia of *F. oxysporum*. Testing the effect of root exudates from tomato plants cultivated under sterile and non sterile conditions (Fig. 3), we found a clear difference on microconidia germination. Root exudates from tomato plants cultivated under sterile conditions showed a clear stimulatory effect, whereas root exudates from tomato plants cultivated under non-sterile conditions showed a similar effect as the water control. This indicates that the presence of microbial contaminants in root exudates alters their bioactivity and thus results in a reduced microconidia germination.

Interestingly, the enhanced microconidia germination after membrane filtration of the exudates seemed to depend on the membrane filter type which was used in the exudates treatment (Fig. 1). When we tested 12 brands of membrane filters, we found different effects of the membrane filters on microconidia germination, possibly resulting from a different removal⁄adsorption of specific compounds from the root exudate depending on the membrane filter type. From our data, we cannot conclude that the tested membrane filter materials such as polyethersulfon, cellulose acetate, polyvinylidene difluorid or nylon are responsible for this effect.

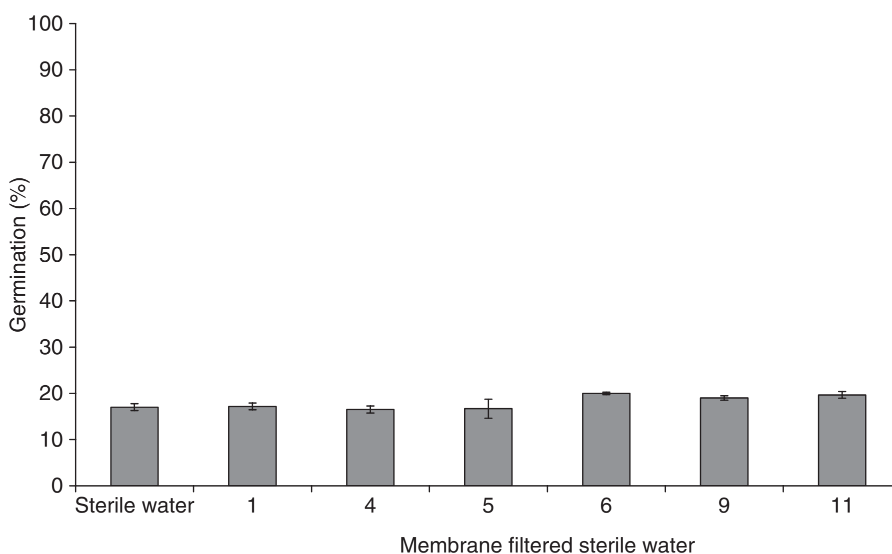

Fig. 2 Germination of *Fusarium oxysporum* f.sp. *lycopersici* microconidia in sterile water (H_2O) and membrane-filtered sterile water. The following membrane filter types were tested: 1 = Sartorius Minisart (cellulose acetate membrane, 0.2 µm), 4 = Pall (polyvinylidene difluorid membrane, 0.2 µm), 5 = Pall (GHP membrane (0.2 µm), 9 = Millipore Millex HV (Durapore membrane, 0.45 mm); 11 = Nalgene (Polyethersulfone membrane, 0.2 µm). Bars indicate SE of the mean. Values did not show significant differences (ANOVA, $F = 2.33$, $P = 0.089$)

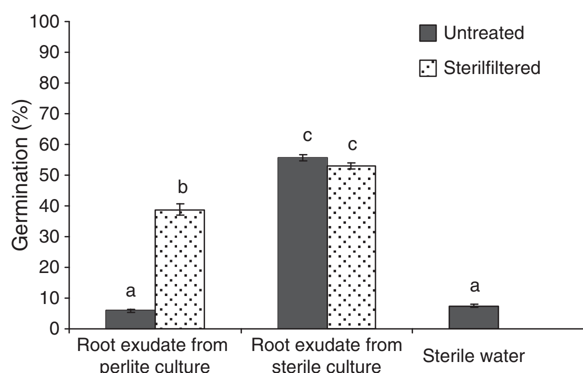

Fig. 3 Effect of tomato root exudates obtained under sterile and non-sterile conditions on microconidia germination of *Fusarium oxysporum* f.sp. *lycopersici*. Untreated and membrane filtered (Roth MicronSep membrane, 0.22 μm) root exudates were tested. Columns with different letters are significantly different (ANOVA, $F = 460.63$, $P < 0.001$)

Filtration with cellulose acetate membranes resulted either in a high microconidia germination (Filter 1; Fig. 1) or in an intermediate microconidia germination (Filter 12; Fig. 1). A similar pattern was observed with the polyethersulfon membranes. Depending on the brand, we found a high-microconidia germination (Filter 2; Fig. 1) or low-microconidia germinations (Filter 6 and 11; Fig. 1). Information on compounds adsorbed to membranes is scarce. Carlson and Thompson (2000) reported on the phenomenon of membrane filter adsorption of drug compounds in high-performance liquid chromatography. They found an effect on acidic compounds as well as on basic and neutral compounds. From our data, we cannot conclude which substances present in root exudates are removed/adsorbed by membrane filters. Although the stimulation of soil-borne fungi due to root exudates is well known, an identification of all root exudate compounds is still missing. Among sugars and organic acids as potential fungal stimuli unfortunately only limited data are available about the signalling compounds in tomato root exudates (Lugtenberg et al., 1999; Kravchenko et al., 2003).

In conclusion, based on microconidia germination of *F. oxysporum* f. sp. *lycopersici*, we found that conditioning of root exudates by membrane filtering significantly influence the response in root exudate-fungus bioassays. Thus, special attention has to be paid to a potential loss of active compounds present in root exudates in quality and quantity during sample preparation using membrane filters.

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