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DOI: https://doi.org/10.5169/seals-738300

Assessing efficacy of ultra-filtration and slow filtration in soil-less production by molecular detection of *Pythium oligandrum* and *Bacillus subtilis* as model organisms

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Ms. received 14th june 2007, accepted 20th november 2007

Abstract

Greenhouse crops are mainly grown on artificial substrates in soil-less systems. Re-circulating nutrient systems offer an advantageous alternative for controlling nutrient solutions leaching from greenhouses into the environment, which represents an environmental issue in greenhouse production areas. However, the potential for the rapid spread of plant pathogens, is the main hindrance to the general adoption of re-circulating nutrient systems by the greenhouse industry. Here we assessed by real time PCR the efficacy of filtration for eliminating oomycete and bacterial pathogens, artificially added in the re-circulating nutrient solution, by using a tangential ultra-filtration system, a slow sand filter (SSF) and a slow Rockwool granule filter (SRF). The oomycete Pythium oligandrum *and the bacterium* Bacillus subtilis *were used as model organisms because of their easy culture, their lack of pathogenicity and their similarity with common oomycete and bacterial plant pathogens. Using real time PCR and species specific oligonucleotides showed that ultra-filtration and SRF filter were highly effective in removing* P. oligandrum *and* B. subtilis, *while the SSF, although completely removing* P. oligandrum, *was less effective for* B. subtilis *removal. Real time PCR showed to be a much quicker alternative to classical counting on agar plate culture.*

Keywords: *recycled nutrient solution, real time PCR,* Bacillus subtilis, Pythium oligandrum.

Introduction

For environmental and economic reasons, recycling irrigation runoff is becoming a common practice in greenhouse crop production. Most greenhouse crops are grown on artificial substrates in soil-less systems because of the improved control over water, aeration, nutrition and root distribution. These systems were developed as drain-to-waste or open systems, in which excess nutrient solutions were allowed to drain to the soil and groundwater. Concern about environmental pollution caused by the green house industry, and questioning about the sustainability of such an activity has been resulting in the promotion of closed re-circulating nutrient systems. Although re-circulation of nutrient solutions proved to be economically rewarding, possible contamination of irrigation tanks and re-circulating systems by important plant pathogens such as *Pythium* and *Phytophthora* species, is listed among the main limitations. Contamination of re-circulating nutrient solutions could also be a potential way for contaminating fruits

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and vegetables with food borne pathogens or fungal toxins (Powell et al. 2002; Steele and Odumeru 2004).

Development of closed systems required effective disinfection of plant nutrient solutions (Ehret et al. 2001) and several active and passive methods have been tested in the past years: UV treatment, heat treatment, ozonization, membrane filtration, such as ultra-filtration or slow filtration (Runia 1995).

Ultra-filtration (membrane filtration) may remove up to 6 logs of bacteria, viruses or parasites (Taylor and Wiesner 1999). On the other hand, slow sand filtration (SSF) techniques, involving filtration through a sand bench, present advantages of low installation and maintenance costs, but do not totally eliminate the microflora. It appears however that *Pythium* and *Phytophthora* could be totally removed by SSF (Runia 1995), while bacterial removal was less effective at 80 to 98.8% of total bacteria (Wohanka et al. 1999).

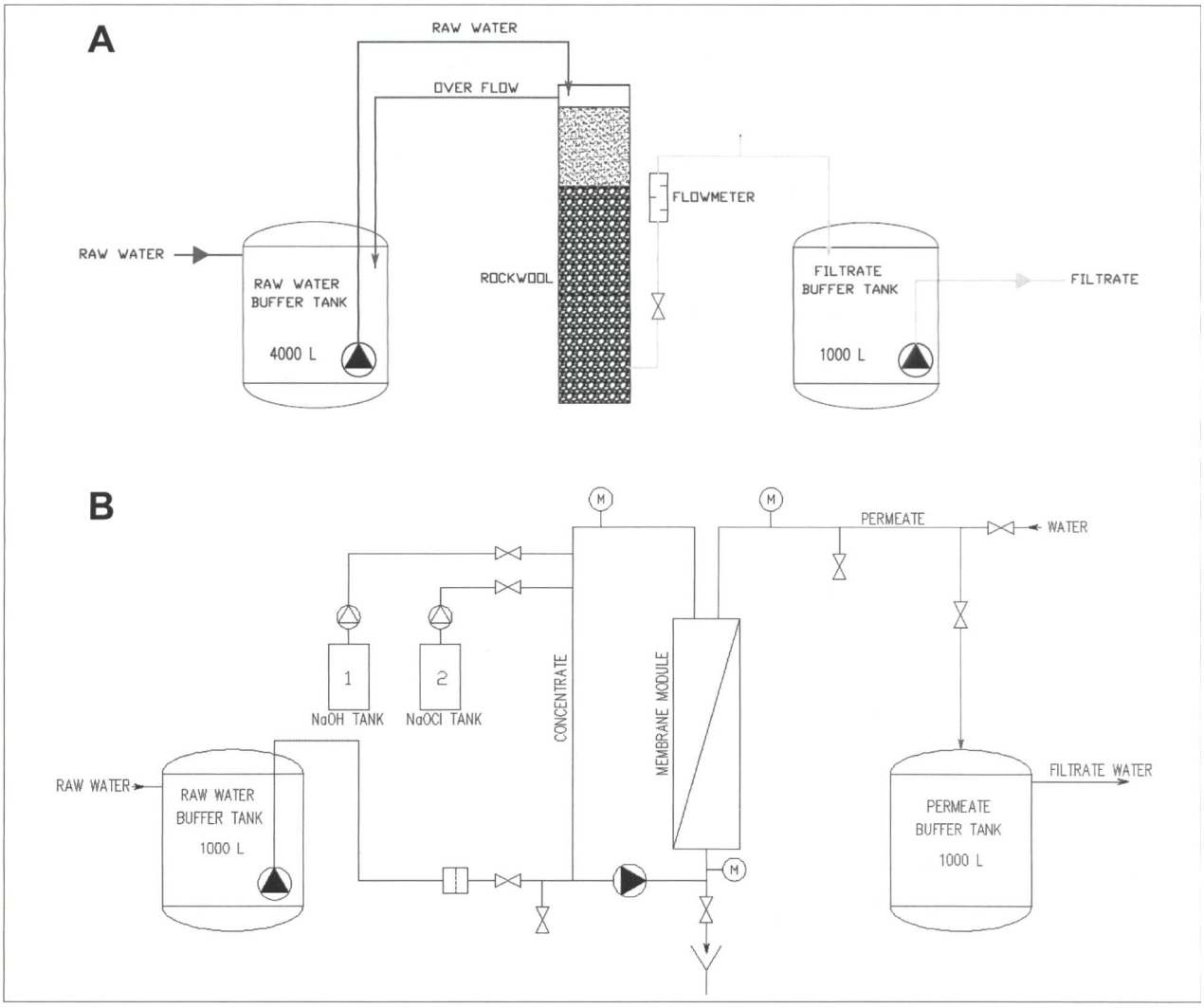
In the present study, using specifically designed real time PCR, we assessed the efficacy of ultra-filtration and SSF filtration on tomato and eggplant greenhouse soil-less cultures for removing microorganism (*Pythium oligandrum, Bacillus subtilis*) artificially added in the circulating nutrient solution.

Materials and methods

Greenhouse trials

Experimental trials on closed soil-less systems were carried out at the Vegetable Crop section of the Professional Training Centre of Lullier (Jussy, Switzerland). Tomato plants, hyb. Temptation (Enza Zaden BV, The Netherlands), 30 days old were grown on Rockwool cubes (Rockwool, Mid-Glamorgan, UK) and transplanted on Rockwool substrate (Rockwool) during the year 2005. Eggplant plants, hyb. Combo (Rijk Zwaan Zaadteelt en Zaadhandel BV, The Netherlands) and hyb.

Fig. 1: Schematic representation of the filtration units used in this study. A. Biofiltration unit. B. Ultrafiltration unit.



Cristal (Semillas Fito S.A., Spain) were grown in a similar system, in which Rockwool was replaced by coco peat (type BLG 943 DUO, 100 cm x 20 cm x 12 cm, weight 2,4 kg, Biogrow, Montescot, France). Plants were maintained on concrete benches with a drip irrigation system (one water emitter for each plant, flow rate of 2 L/h) in a soil-less culture. General information on water, fertilizers and the nutrient solution adopted during the trials was reported by Garibaldi et al. (2003).

Slow-filtration

The slow Rockwool filtration (SRF) system was constructed as described by Wohanka et al. (1999) with Grodan granules of hydrophile type 012-519 (Grodan, Hedehusene, Denmark). The slow Rockwool filter (SRF) is schematically described in Fig. 1A and shown on Fig. 2C. The drain water was pumped into the Rockwool filter to maintain a water layer of 35 cm. Air forced by pressure into the filter enhances efficiency of the system. The effluent flow rate from the filter was adjusted by a flow-meter located between the filter and the disinfected water storage tank, obtaining a final flow rate of 40 $L.m^{-2}.h^{-1}$.

Fig.2: C. Slow rockwool filter (SRF).

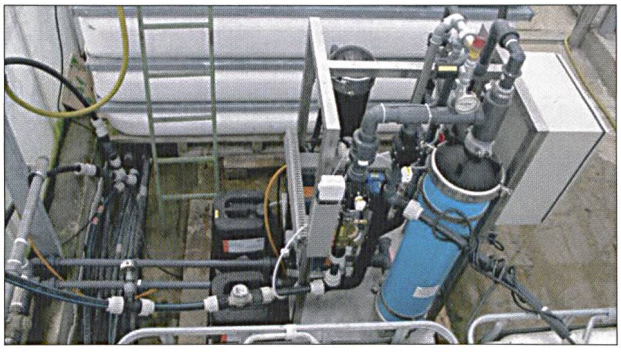
Fig.2: A: Slow sand filter (SSF).

The slow sand filter (SSF) was a BIO AQUA FILTER supplied by Rossat SA (Payerne, Switzerland) and is shown on Fig. 2A. The BIO AQUA FILTER is based on the use of quartz particles (Vasilgrit quality 25/16 (0,4-0,8mm) and Vasilgrit quality 16.14.1 (0,6/1,2 mm), Sifraco, France). The drain water was pumped into the coral filter. The effluent flow rate from the filter was adjusted by a water valve located between the filter and the disinfected water storage tank, obtaining a final flow rate of 100 $L.m^{-2}.h^{-1}$. Both slow filters were allowed to ripe during 4 weeks of functioning after starting before the experiment.

Ultra-filtration

The ultra-filtration system was supplied by Membratec SA (Sierre, Switzerland). The pilot ultra-filtration unit adopted is schematically described in Fig. 1B and shown on Fig. 2B. The capillary ultra-filtration membrane (HYDRAcap, Hydranautics, USA) has a capillary configuration with inside diameter of 0.8 mm and outside diameter of 1.2 mm. Molecular weight cut-off of the HYDRAcap capillary membrane is 100.000-150.000 Daltons. The fiber polymer is polyethersulfone, modified to maintain a hydrophilic property. The flow pattern is inside out (feed water enters bore of the capillary).

Fig.2: B. Ultrafiltration filtering station



Isolates, culture conditions and inoculation

Pythium oligandrum Drechsler, Strain No. 1133 (Provided by Yves Tirilly, Ecole Supérieure de Microbiologie et Sécurité Alimentaire, Université de Bretagne occidentale), was grown on V8 medium or on pea broth medium at 24°C in the dark and regularly sub-cultured. For the production of inoculum, *P. oligandrum* was cultured in a liquid pea broth medium (Le Floch et al. 2003). Plastic bottles containing 150 ml of culture medium autoclaved at 121 °C for 15 min were inoculated with 4 disks of *P. oligandrum* (10 mm in diameter), then incubated in the dark for 14 days at 25 °C. Mycelial mats were then removed and fragmented into distilled water using a Waring blender (Le Floch et al. 2003).

Bacillus subtilis subsp. *spizizenii* (strain DSMZ 6405) used in this study was obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH: Braunscheig, Germany) and maintained on Luria-Bertani (LB) medium. For inoculation of nutrient solution, fresh bacterial cultures have been centrifuged and re-suspended in sterile water shortly before use.

These organisms were used as model organisms instead of pathogenic organisms since the cultures in the greenhouse installation were used for horticulture teaching purposes and could not be wasted by artificially inoculated diseases. Estimation of *P. oligandrum* and *B. subtilis* cell concentrations was obtained by counting in a Thomas cell-chamber.

Inoculation was carried out by adding 10^4 propagules of *Pythium oligandrum* per litre of water or 10^4 cells of *Bacillus subtilis* per litre of water in the raw water buffer tank.

Sampling of the nutrient solution before filtration was performed by collecting 1 L samples from the raw water buffer tank (Fig1) for each installation (SSF, SRF and ultrafiltration). Filtered nutrient solution samples (1 L) were taken by a deviation on the filtered water from the filtrate water buffer tank (Fig 1) for each installation (SSF, SRF and ultrafiltration). Samples (5 L) were concentrated by passing through a 47 mm diameter Durapore® 0.22 µm mesh membrane. Bacteria present on the filter were then released by vortexing the filter in sterile water (2 ml) and serial dilutions plated on agar plates in 90 mm diam. Petri dishes. Bacteria colonies were checked visually for colony shape and morphology.

Plate counting

Counting was carried out on Petri plates after spreading diluted samples on V8 medium supplemented with ampicillin, pimaricin and rifampicine for *P. oligandrum* in order to suppress bacteria and fungal growth. Counting was carried out on LB medium supplemented with streptomycin for *B. subtilis* growth.

DNA extraction

DNA was purified from fresh harvested oomycete mycelium or bacterial cultures with the use of the DNA-Easy Plant Mini kit (Qiagen, Basel, Switzerland), according to manufacturer's specifications. DNA was extracted from the nutrient solution following a protocol derived from Kong et al. (2003). Quality was checked by visualization under UV light following electrophoresis with a molecular mass standard (HindIII/EcoRI DNA Marker, Biofinex,

Table 1. Comparison of real time quantitative PCR (qPCR) and plating on selective media for detection of P. oligandrum *and* B. subtilis *in 1 L ultrafiltered nutrient solution (TRNS = tomato recycled nutrient solution; ERNS = eggplant recycled nutrient solution). Before means before filtration: the sample is taken in the raw water buffer tank. After means after filtration: the sample is taken in the permeate buffer tank.*

		N° of *P. oligandrum* spores				N° of *B. subtilis* cells			
		qPCR		Plating		qPCR		Plating	
Time of UF run	Sample[a]	Before	After	Before	After	Before	After	Before	After
0 hour	TRNS	10453	ND	9876	ND	11545	ND	10987	ND
	ERNS	10143	ND	8765	ND	9865	ND	8769	ND
1 hour	TRNS	1521	0	1231	0	953	0	824	0
	ERNS	1654	0	1178	0	987	0	795	0
30 Hours	TRNS	1590	0	1354	0	874	0	764	0
	ERNS	1421	0	1289	0	1112	0	652	0
90 Hours	TRNS	1478	0	1374	0	956	0	764	0
	ERNS	1456	0	1167	0	798	0	873	0

ND, Not determined

Switzerland) in 1% agarose (Biofinex) gel in 1xTBE buffer, subjected to 100 V for 1 h and stained with ethidium bromide (0.5 mg.ml^{-1}). Concentrations were assayed in a S2100 Diode Array spectrophotometer (WPA Biowave, Cambridge, UK).

Primers, PCR amplification and Real Time PCR conditions

Primers for the detection of *Pythium oligandrum* (Po-F: 5'gctttgcgctggtgggcgacttcggttagg3'; Po-R: 5'ggtgttgtctcctttacctaccgaagcaggcg3') were designed in the ITS2 region of the rRNA, after alignment and comparison of all ITS rDNA sequences available for *P. oligandrum* and related *Pythium* species. The PCR product obtained had a size of 219 bp (Fig 3C). Primers were specifically designed to discriminate *P. oligandrum* from related *Pythium* species, particularly those found currently in irrigation water. The specificity of potential primers was further tested by BLAST (Altschul et al. 1997) searching the GenBank database (National Center for Biotechnological Information, NCBI, USA) for compatible sequences. confirmed the results (Table 1). No *P. oligandrum* could be detected in the outflow water. In order to enhance the sensitivity of the real time PCR approach, DNA samples from ultra-filtration outflow water were submitted to a preliminary amplification using the primers DC6 and ITS4 for specifically amplifying *Pythiales* and *Peronosporales* (Cooke et al. 2000). Following amplification, a 1 µl aliquot of the reaction product was substituted to the nutrient solution DNA as template in real time PCR reactions described above. No amplification products corresponding to *P. oligandrum* were detected confirming that ultra-filtration removes completely oomycete pathogens.

Primers specific of *Bacillus subtilis* (Bs-F: 5' cctggagagccgcctacagtagaaaatgcg3'; Bs-R: 5' agcaaggatttctcctgtttcaggatcaacaagc3'.) were designed in the RNA polymerase beta subunit (rpoB) gene, after alignment and comparison of all rpoB sequences available for *B. subtilis* and related *Bacillus* species. The PCR product obtained had a size of 189 bp (Fig. 3C). Primers were specifically designed to discriminate *B. subtilis* from related *Bacillus* species. The specificity of potential primers was further tested by BLAST searching the GenBank database for compatible sequences.

Following the identification of suitable target regions in the ITS2 sequence for *P. oligandrum* and in the rpoB gene for *B. subtilis*, primers were tested in real time PCR reactions against total DNA extracted form 1 L samples of recycled nutrient solution (data not shown).

Amplification mixtures (20 µL final volume), were made using the LightCycler Fast Start DNA Master SYBR Green I kit (Roche diagnostics, Switzerland) to which 2 ng of template DNA and primers at a final concentration of 5 µM were added. Amplifications were carried out in capped capillary tubes in a Lightcycler 2.0 Real time PCR systems (Roche Diagnostics) using an initial denaturation at 95°C for 10 min, followed by 45 cycles of 95°C for 10 s, 70°C for 5 s and 72°C for 15 s No post PCR manipulations, such as gel electrophoresis analysis, were required, hence removing many of the contamination problems associated with conventional PCR.

The presence of contaminants in DNA from environmental samples can cause PCR inhibition, autofluorescence, or quench fluorescence, which can be problematic for quantitative PCR. In order to overcome these potential problems, a DNA dilution series of the samples was prepared by using the recommendations of Stults et al. (2001) who used this method to accurately quantify DNA from *Geobacter* spp. in aquifer sediments. The results obtained with *Pythium* and *Bacillus* primers showed that 10-fold dilution of DNA resulted in a 10-fold reduction in the quantity of target DNA detected in a manner identical to that observed with the pure *P. oligandrum* and *B. subtilis* DNA standards. This indicated that there were no major potential inhibitors in the nutrient solution sample that would have been diluted out at the lower dilution. The results used were averages of four readings, which were used for calculating the amounts of DNA in the samples. The measurements were transformed into the number of cells by assuming that each *Pythium* cell contains 40-80 fg of DNA (Kamoun 2003).

Results

Optimization of Real Time PCR for detection of P. oligandrum and B. subtilis

In order to assess and compare the efficiency of ultra-filtration and slow filtration for disinfecting nutrient solutions from bacteria and oomycete populations, we used certified strains of *P. oligandrum* and *B. subtilis* as model organisms. Identity of these strains was confirmed by sequencing the ITS region for *P. oligandrum* (AY986954) and the 16S ribosomal RNA for *B. subtilis* (DQ195067). Real time PCR protocols were developed for these 2 organisms and species specific primers targeting the ITS region produced a 219 bp fragment for *P. oligandrum*, while specific primers targeting the RNA polymerase beta subunit (rpoB) gene produced a 189 bp fragment (Fig. 3C). No amplification occurred when amplifying total DNA extracted from recycled nutrient solutions prior to inoculation, proving that these 2 species were not

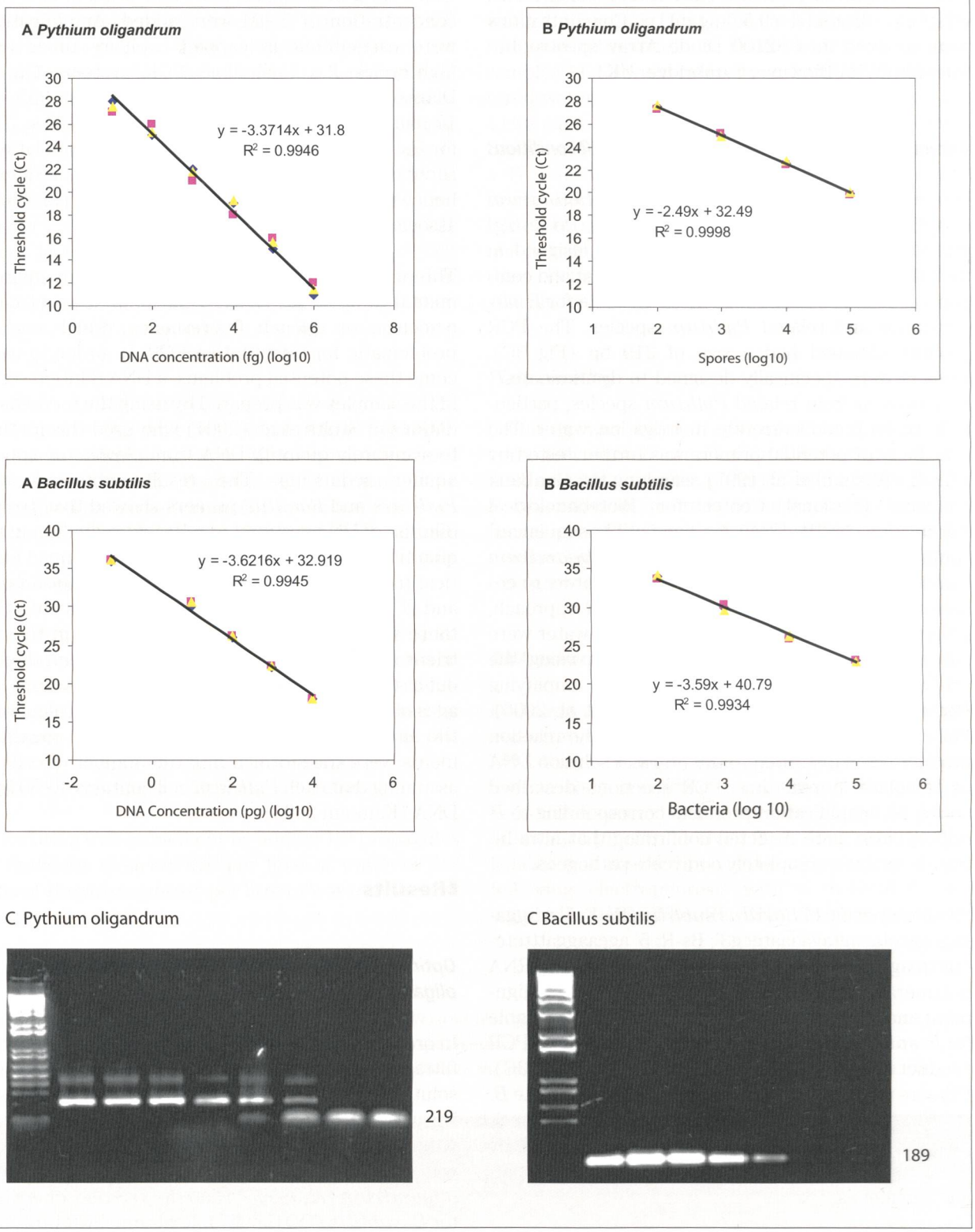


Fig. 3: Standard curves quantifying DNAs of Pythium oligandrum *and* Bacillus subtilis *obtained by real-time PCR with SYBR-Green as the fluorescent dyè. Ct values, corresponding to the increase in template DNA above the background level, were plotted against the log of genomic DNA standards of known concentrations. DNA amplification was done with the primer combination Po-F and Po-R for ribosomal RNA gene for* Pythium oligandrum *and the primer pairs Bs-F and Bs-R for the single-copy RNA polymerase beta subunit* rpoB *gene respectively. (A) Standard curves obtained with DNA extracted from mycelium or bacteria in the range of 10 fg to 10 ng per reaction. (B) Standard curves obtained with DNA extracted from a cell suspension in the range of 102 to 105 spores or bacteria L-1. (C) Gel electrophoresis of real time PCR products for* Pythium oligandrum *(219 bp) and* Bacillus subtilis *(189 bp) respectively.*

naturally present in the cultures (data not shown). Sensitivity of detection was tested with serial dilutions of genomic DNA ranging from 2 ng to 0.02 fg for *P. oligandrum* and *B. subtilis*. The minimum level of detection was 20 fg for *P. oligandrum* and 200 fg for *B. subtilis* (Fig. 3A and 3B). For *P. oligandrum*, this level of detection (20 fg) is equivalent to ca. 0.05 propagules assuming that the equivalent genome of *P. oligandrum* is between 40 to 80 fg, since rDNA is present in multiple copies allowing high levels of sensitivity of the protocol. However the level of sensitivity of 200 fg for *B. subtilis* is equivalent to 20 cells, since only one copy of the target RNA polymerase beta subunit gene is present in the genome of *B. subtilis* and assuming a genome equivalent for this species of 10 fg. Successive increases in *Ct* values with the expected melting temperature were observed as the concentration of template DNA in the samples decreased (Fig. 3A).

The detection limit of the quantitative real-time PCR assay was determined by testing triplicate sets of genomic DNA prepared from serial dilutions of *P. oligandrum* propagules ($2.5\ 10^2$ to $2.5\ 10^8$) and *B. subtilis* (10^2 to 10^8). Positive signals were found in all dilutions having more than 25 propagules per liter for the oomycete and in dilutions having more than 10^2 bacteria per liter.

A melting curve analysis of the final PCR products was also carried out at the end of the quantitative PCR; the amount of fluorescence was measured while the temperature of the sample was slowly increased to 95°C. Since SYBR green binds only to double-stranded DNA, a sharp decrease was observed when the product melted. When the rate of the decrease in fluorescence was plotted against temperature, a sharp peak was visible, which represented the melting temperature of the PCR product. The analysis of the melting curve for all samples and standards showed a single peak, indicating the absence of non specific products, such as primer dimers.

Ultra-filtration efficacy against oomycete and bacterial pathogens

Ultra-filtration efficacy was determined by testing for the removal of *P. oligandrum* propagules from the water passing through the membrane module (Fig. 1). In three different runs, efficacy of ultra-filtration reached 100% removal of zoospores. In the different runs about 425 zoospores per liter were counted in the re-circulating nutrient solution compared to no zoospores counted in the outflow water. In the different runs, filtration was continued for 1 month, during which time full efficacy was maintained..

Ultra-filtration was also very effective in removing bacteria as was shown by real time PCR experiments (Table 1.). Bacterial count confirmed that up to 4 logs of bacteria have been removed since as few as 10 bacteria have been counted in outflow solution. Moreover, real time PCR with DNA extracted from these bacteria gave negative signals.

Slow filtration efficacy against oomycete and bacterial pathogens

Slow filtration efficacy was determined by testing for the removal of *P. oligandrum* propagules from the water passing through the slow Rockwool filter (SRF) and the slow sand filter (SSF) where quartz particles replace Rockwool fibers.

Table 2. Comparison of real time quantitative PCR (qPCR) and plating on selective media for detection of P. oligandrum *and* B. subtilis *in 1 L bio-filtered nutrient solution (TRNS = tomato recycled nutrient solution; ERNS = eggplant recycled nutrient solution). Before means before filtration: the sample is taken in the raw water buffer tank. After means after filtration: the sample is taken in the filtrate buffer tank.*

		N° of *P. oligandrum* spores				N° of *B. subtilis* cells			
		qPCR		Plating		qPCR		Plating	
Time of SSF run	Sample[a]	Before	After	Before	After	Before	After	Before	After
0 hour	TRNS	12398	ND	10780	ND	13650	ND	12456	ND
	ERNS	11243	ND	9873	ND	11456	ND	10987	ND
1 hour	TRNS	1876	0	1456	0	987	122	888	111
	ERNS	1768	0	1322	0	956	113	896	109
30 Hours	TRNS	1980	0	1279	0	789	135	743	112
	ERNS	1871	0	1387	0	1234	221	983	187
90 Hours	TRNS	1378	0	1134	0	1253	136	1098	122
	ERNS	1673	0	1476	0	1544	167	1234	108

ND, Not determined

In the SRF, ultra-filtration efficacy reached 100% for zoospores removal in three different runs and 100% for bacteria removal (not shown). Real Time PCR analysis confirmed the results (Table 2). Filtration was then continued for 2 months, during which full efficacy was achieved, since no *P. oligandrum* and no *B. subtilis* could be detected in the outflow water.

The slow sand filter (SSF) provided similar results, suggesting that oomycetes could be removed efficiently by slow sand filtration. However SSF was less effective in removing bacteria as was shown by real time PCR experiments (Table 2). The result corresponds to a two logs of *B. subtilis* population reduction. *B. subtilis* counting confirmed real time PCR results.

Discussion and conclusion

Quantitative PCR with a LightCycler 2.0 (Roche Diagnostics) sequence detection system showed a detection limit of 20 fg and 200 fg respectively for *P. oligandrum* and *B. subtilis*, indicating that the PCR was efficient and reproducible.

The measure of the efficacy by real time PCR, specifically targeting *Bacillus subtilis* and *Pythium oligandrum*, confirmed that slow sand filtration (SSF), slow Rockwooll filtration and ultra-filtration are able to significantly reduce micro-organisms present in re-circulating nutrient solution. However SSF failed to remove totally bacteria, while ultra-filtration and SRF performed equally. Real time PCR applied for checking the presence of micro-organisms, *P. oligandrum*, and *B. subtilis,* proved to be quicker and more precise for detecting micro-organisms than classical counting on agar plates. Real time PCR methods could become a tool of choice for pinpointing specific organisms, human or plant pathogens for safety control.

SSF has been successfully used to remove a wide range of plant pathogens from contaminated irrigation water, including *Cylindrocladium* spp., *Fusarium* spp., *Phytophthora* spp., *Pythium* spp., *Thielaviopsis* spp., *Verticillium dahliae*, *Xanthomonas* spp., (Berkelmann et al. 1994; Postma et al. 1999). Biological processes are known to be central to the removal of plant pathogenic *Xanthomonas* spp. (Brand and Wohanka 2000), and the same could be true for the removal of oomycete zoospores retained by the filter.

The dilution plate count results should also be interpreted as underestimates of the true numbers of pathogenic organisms present. Counted micro-organisms and real time PCR estimates were similar, but counted micro-organisms represent all cultivable organisms in the given culture conditions, which means that plate counting underestimated the real number of micro-organisms present in a sample. This has already been observed; for instance 20-fold difference between the quantitative PCR estimate and the number of CFU was reported for *Legionella* species (Calvo-Bado et al. 2003a; Wellinghausen et al. 2001).

Practical use of the membrane technology revealed problems such as clogging and leaking already mentioned by van Os (1999).

Based on the technical problems and the comparable high investment, membrane filtration has not been used widely in the horticulture industry and would certainly be restricted for large surfaces of glass houses, where it is economically rational. *Pythium* zoospores are the key elements of disease spreading in water. The efficient removal of zoospores by SRF indicates that SRF use would be able to suppress *Pythium* diseases in closed soil-less systems.

Providing an effective and cheap disinfection method of the nutrient solution makes SRF an alternative of choice in closed soil less growing systems for small scale nurseries, or in countries where pressures on irrigation water resources are increasing.

Acknowledgements

We are grateful to Prof. Yves Tirilly (Ecole Supérieure de Microbiologie et Sécurité Alimentaire, Université de Bretagne occidentale) for providing *Pythium oligandrum* Dreschler, Strain No. 1133. This work was financially supported by the Strategical Research Fund of the University of Applied Sciences of Western Switzerland, Rossat SA (Payerne, Switzerland) and Membratec SA (Sierre, Switzerland).

References

- **Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25: 3389-3402.
- **Berkelmann B, Wohanka W, Wolf G.** 1994. Characterisation of the bacterial flora in recirculating nutrient solutions of a hydroponic system with rockwool. Acta Hortic. 361: 372–381.
- **Brand T, Wohanka W.** 2000. Importance and characterization of the biological component in slow filters. Acta Hortic. 554: 313–321.
- **Calvo-Bado L A, Morgan JA, Sergeant M, Pettitt TR, Whipps JM.** 2003. Molecular characterization of *Legionella* populations present within slow sand filters used for fungal plant pathogen suppression in horticultural crops. Appl. Environ. Microbiol. 69: 533-41.
- **Cooke DEL, Drenth A, Duncan JM, Wagels G, Brasier CM**. 2000. A molecular phylogeny of *Phytophthora* and related oomycetes. Fung. Genet. Biol. 30: 17-32.
- **Ehret D L, Alsanius B, Wohanka W, Menzies JG, Raj Utkhede R.** 2001. Disinfestation of recirculating nutrient solutions in greenhouse horticulture. Agronomie, 21: 323-339
- **Garibaldi A, Minuto A, Grasso V, Gullino ML.** 2003. Application of selected antagonistic strains against *Phytophthora cryptogea* on gerbera in closed soilless systems with disinfection by slow sand filtration. Crop Prot. 22:1053-1061.
- **Kamoun S.** 2003. Molecular genetics of pathogenic oomycetes. Eukaryot. Cell. 2: 191-9.
- **Kong P, Hong CX, Jeffers SN, Richardson PA.** 2003. A species-specific polymerase chain reaction assay for rapid detection of *Phytophthora nicotianae* in irrigation water Phytopathology, 93:822-831
- **Le Floch G, Rey P, Déniel F, Benhamou F, Picard K, Tirilly Y.** 2003. Enhancement of development and induction of resistance in tomato plants by the antagonist *Pythium oligandrum.* Agronomie, 23: 455–460.
- **Postma J, Os EA, Kritzma G.** 1999. Prevention of root diseases in closed soilless growing systems by microbial optimization. Med. Fac. Landbouww. Univ. Gent, 64: 431–440.
- **Powell DA, Bobadilla-Ruiz M, Whitfield A, Griffiths MW, Luedtke A.** 2002. Development, implementation, and analysis of an on-farm food safety program for the production of Greenhouse vegetables. J. Food Prot. 65: 918-923.
- **Runia WT.** 1995. A review of possibilities for disinfection of recirculating water from soilless culture. Acta Hortic. 382: 221–229.
- **Steele M, Odumeru J.** 2004. Irrigation water as source of foodborne pathogens on fruit and vegetables. J. Food Prot. 67: 2839-2849:
- **Stults JR, Snoeyenbos-West O, Methe B, Lovley DR, Chandler DP.** 2001. Application of the 5' fluorogenic exonuclease assay (TaqMan) for quantitative ribosomal DNA and rRNA analysis in sediments. Appl. Environ. Microbiol. 67: 2781-2789.
- **Taylor JS, Wiesner MW.** 1999. Membranes. *In*: Water Quality & Treatment, Letterman RD (ed.), McGraw-Hill, New York, p. 11.1-11.71.
- **van Os E A, Amsing JJ, van Kuik AJ, Willers H.** 1999. Slow sand filtration: a potential method for elimination of pathogens and nematodes in recirculating nutrient solution from glasshouse-grown crops. Acta Hortic. 481: 519-526.
- **Wellinghausen N, Frost C, Marre R.** 2001. Detection of *Legionellae* in hospital water samples by quantitative real-time LightCycler PCR. Appl Environ Microbiol. 67: 3985-3993.
- **Wohanka W, Luebke H, Ahlers H, Luebke M.** 1999. Disinfection of recirculating nutrient solutions by slow sand filtration. Acta Hortic. 481: 539–544.