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ORIGINAL ARTICLE

Inactivation of plant infecting fungal and viral pathogens to achieve biological containment in drainage water using UV treatment

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Keywords

Barley stripe mosaic virus, chemical inactivation, elimination of a plant virus by dilution, filtration, lethal dose, microbial disinfection, plant pathogenic micro-organisms, ultraviolet disinfection, ultraviolet kill.

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Abstract

Aim: To explore whether ultraviolet (UV) light treatment within a closed circulating and filtered water drainage system can kill plant pathogenic species.

Methods and Results: Ultraviolet experiments at 254 nm were conducted to determine the inactivation coefficients for seven plant pathogenic species. At 200 mJ cm^{-2}, the individual species log reductions obtained for six Ascomycete fungi and a cereal virus were as follows: *Leptosphaeria maculans* (9·9-log), *Leptosphaeria biglobosa* (7·1-log), *Barley stripe mosaic virus* (BSMV) (4·1-log), *Mycosphaerella graminicola* (2·9-log), *Fusarium culmorum* (1·2-log), *Fusarium graminearum* (0·6-log) and *Magnaporthe oryzae* (0·3-log). Dilution experiments showed that BSMV was rendered noninfectious when diluted to >1/512. Follow-up large-scale experiments using up to 400 l of microbiologically contaminated waste water revealed that the filtration of drainage water followed by UV treatment could successfully be used to inactivate several plant pathogens.

Conclusions: By combining sedimentation, filtration and UV irradiation within a closed system, plant pathogens can be successfully removed from collected drainage water.

Significance and Impact of the Study: Ultraviolet irradiation is a relatively low cost, energy efficient and labour nonintensive method to decontaminate water arising from a suite of higher biological containment level laboratories and plant growth rooms where genetically modified and/or quarantine fungal and viral plant pathogenic organisms are being used for research purposes.

Introduction

In agricultural research laboratories where plants are routinely grown in pots within environmentally controlled growth rooms, crop and model plants are often inoculated with one or more plant pathogenic species when studying disease resistance mechanisms or pathogen spread. These experiments can generate low-level contaminated drainage water, which needs to be inactivated before drainage to the sewerage system to prevent potential spread into the environment. When experiments involve foreign plant pathogens, genetically modified pathogens or genetically modified plants, national authorities in most countries regulate and require effective waste water treatment procedures from agricultural research laboratories. Such treatments can include mechanical separation, steam sterilization in autoclaves, chemical treatment and/or ultraviolet (UV) irradiation.

Ultraviolet light treatment is an increasingly used alternative to chemicals for the microbiological disinfection of drinking water, waste water and industrial waters (Bolton and Linden 2003; Hijnen *et al.* 2006). Potentially, a UV disinfection system together with mechanical separation and filtration could be utilized as an environmentally

friendly and low-cost treatment system to inactivate fungal and viral plant pathogens when present as low-level contaminants in drainage water. The most effective wavelength for UV disinfection at 260 nm falls in the ultraviolet C range (between 200 and 280 nm) (Meulemans, 1987) and is close to the 254 nm output of a typical low-pressure UV lamp used in UV appliances (Sonntag and Schuchmann 1992).

Germicidal UV light is absorbed by nucleotides and causes phytochemical damage because of the formation of covalent bonds between adjacent nucleotides (Wang 1976). Accumulation of sufficient numbers of nucleotide dimers within an organism prevents DNA and RNA replication, which is a prerequisite for cell division and viral replication.

The UV dose requirements for microbial inactivation can be determined using a calibrated bench scale UV light apparatus. Typically, a small dish containing a water sample is irradiated using a UV source of known intensity, and UV dose–response relationships are established by varying the time of exposure. The kinetics of microbial UV light inactivation is a negative exponential function defined as: $N = N_0 \cdot e^{-klt}$ (Gray 2005), where N is the number of surviving microbes after UV exposure from a starting population N_0, e is the Euler's number, k is the microbial inactivation rate constant, and the product of UV intensity (I) and time (t), measured in mJ cm^{-2}, defines the UV dose (fluence). The UV dose which kills 90% of the initial microbial population is called the 1-log reduction dose. Higher population reduction values are referred to as x-log decrease, which means that the number of organisms would be expected to be reduced by a factor of 10^x (US Department of Agriculture Food Safety and Inspection Service 1999). The probability that a given organism will survive a x-log$_{10}$ lethality reduction can be calculated as $P = (1/10^x)$. Several studies on fungal spore and virus elimination by UV radiation using a spectrum of model and indicator organisms showed that 1-log reduction doses vary considerably between species and are typically in the range of 20–200 mJ cm^{-2} for RNA viruses and filamentous *Aspergilli* fungi, respectively (Tothova and Frankova 2001; Simonet and Gantzer 2006).

Very limited data exist on UV inactivation dose rates or development of low-energy waste water treatment systems for plant pathogens (Zhang and Tu 2000; Clauss 2006). Plant pathogenic microbes of agricultural importance increasingly investigated in research laboratories globally include the Ascomycete fungi, *Fusarium graminearum* (teleomorph *Gibberella zeae*) and *Fusarium culmorum* which cause ear blight disease of small grain cereals, *Mycosphaerella graminicola* (anamorph *Septoria tritici*) which causes Septoria leaf blotch disease of wheat, *Magnaporthe oryzae* the causal agent of rice blast disease, and the oilseed rape pathogens *Leptosphaeria biglobosa* and *Leptosphaeria maculans*. As a research tool, the RNA virus *Barley stripe mosaic virus* (BSMV) is studied as the vector for virus-induced gene silencing (VIGS; Scofield *et al.* 2005). The BSMV viral genome consists of three ssRNA components protected by a protein coat. All seven organisms are currently studied at Rothamsted Research in various research projects. Non-European and genetically modified strains need to be effectively inactivated when accidentally released in drainage water for environmental protection.

This study describes the establishment of a drainage water treatment system within a suite of research laboratories and plant growth rooms where six Ascomycete fungal pathogens of cereals and oilseed rape, and/or the viral pathogen BSMV are used as experimental organisms. The UV 1-log reduction doses were first determined for each species in bench scale experiments. UV inactivation of the seven plant pathogens was then tested in a 400-l-capacity custom-built UV inactivation batch system. The UV treatment system was linked to a chemical Dosatron inactivation system, and efficacy of one biocide was tested. Finally, we explored the effect of physical dilution on virus viability.

Materials and methods

Plant pathogenic fungal strains used and their propagation

The fungal species tested and relevant details pertaining to each strain are given in Table 1. Each fungal species was grown *in vitro* on suitable diagnostic media and conditions required to generate abundant quantities of viable asexual spores. Specifically, *Fusarium* species and *M. oryzae* were grown using blue/white light at 20°C for 10 days on synthetic nutrient-deficient agar (SNA) medium or oatmeal agar, respectively (Crawford *et al.* 1986; Urban *et al.* 2002). *Mycosphaerella graminicola* spores were harvested from 7-day-old culture on yeast extract peptone dextrose plates (Motteram *et al.* 2009). *Leptosphaeria maculans* and *L. biglobosa* were grown on V8® vegetable juice plates (West *et al.* 2002). Viability of fungal spores was assessed for *Leptosphaeria* species and *M. oryzae* on potato dextrose broth (PDB) plates and PDB plates containing hygromycin (100 μg ml^{-1}), respectively. For *Fusarium* species, SNA plates containing 0·05% tergitol (Sigma-Aldrich, Dorset, UK) were used.

Plant material and growth conditions

Triticum aestivum cultivar Riband was used in the virus experiments for viral propagation. *Chenopodium amaranticolor*

Table 1 Species and strain list

Species	Vegetative spore size (appearance)	Strain (accession*)	Origin	References	Growth medium and conditions	
					For inoculum production	For viability test
Barley stripe mosaic virus	Three modal lengths: 150–160, 126 and 109 nm; diameter 20–25 nm	ND18 (a gift from S. R. Scofield, Purdue University, West Lafayette, IN, USA)	North Dakota, USA	Petty *et al.* (1989)	10-day-old seedlings of wheat cultivar Riband	3- to 4-week old *Chenopodium amaranticolor* plants
Fusarium graminearum	41–60 × 4·5–5·5 μm (5-septate)	10FG131 (NRRL54110)	Germany	Dawson *et al.* (2004)	SNA, blue/white light at 20°C	SNA with 0·05% tergitol added, blue/white light at 20°C
Fusarium culmorum	32–50 × 4·8–7·5 μm (5-septate)	Fc98/11 (NRRL54112)	United Kingdom	Urban *et al.* (2002)	SNA, blue/white light at 20°C	SNA with 0·05% tergitol added, blue/white light at 20°C
Leptosphaeria biglobosa	4–5 × 1·5–2 μm (unicellular)	Lb UK29	United Kingdom	Unpubl.	V8, 20°C	PDB, 20°C
Leptosphaeria maculans	3·5–4·5 × 1·5–2 μm (unicellular)	Lm UK20	United Kingdom	Unpubl.	V8, 20°C	PDB, 20°C
Mycosphaerella graminicola	30–80 × 1·5–2 μm (unicellular)	IPO323 (CBS115943)	The Netherlands	Motteram *et al.* (2009)	YEPD, 16°C	YEPD, 20°C
Magnaporthe oryzae	17–28 × 6–9 μm (2-septate)	Guy-11 (H3:eGFP hyg^R)	French Guyana	Veneault-Fourrey *et al.* 2006)	Oatmeal agar, blue/white light at 20°C	PDB with 100 μg ml^{-1} Hygromcyin added

PDB, potato dextrose broth; SNA, synthetic nutrient-deficient agar; YEPD, yeast extract peptone dextrose.

*Accession numbers are as follows: NRRL, ARS Culture Collection, Peoria, IL, USA; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; Lm UK20 and Lb UK29 are field isolates obtained from J. West, Rothamsted Research, Harpenden, UK.

was used as an indicator/local lesion host to quantify the numbers of viable virus particles present in water samples and to identify the virus preparation dilution endpoint (Fig. S1) (Hollings 1956; Scofield *et al.* 2005). The *C. amaranticolor* seed was provided by the Scottish Crop Research Institute, Dundee, UK. Plants were grown in a controlled environment at 23°C (day) and 18°C (night), 50% relative humidity and a light intensity of 300 μmol m^{-2} s^{-1} for 16 h.

Barley stripe mosaic virus (BSMV) purification and plant inoculation

Thirty grams of young upper uninoculated leaves from the wild-type BSMV ND18 inoculated wheat cultivar Riband were collected at 10 dpi (days postinoculation) and used for virus purification as described (Lawrence and Jackson 1998). The resulting virus pellet was resuspended at a concentration of 3 mg ml^{-1} in 10 mmol l^{-1} potassium phosphate buffer (pH 6·8).

For wheat cultivar Riband inoculation, the first leaf of 10-day-old seedlings was used, because wheat plants at the young seedling stage are the most susceptible to virus infection. For *C. amaranticolor,* 4-week-old plants were used, and fully expanded leaves 3 and 4 were infected. Both leaves provide a large area for scoring virus-induced local lesions. Thirty microlitres of the virus inoculum supplemented with 1% coarse Celite® 545 AW (Sigma-Aldrich) was rubbed onto the adaxial side of a leaf, then misted with tap water and covered with clear plastic bags overnight. The development of disease symptoms (wheat) or the appearance of local lesions (*C. amaranticolor*) was observed and recorded (Figs S1 and S2).

Bench scale UV irradiation, viral dilution and chemical inactivation experiments

The UV sensitivities at 254 nm of each target organism were determined in small-scale experiments similar to that recommended by the EPA US Environmental Protection Agency *guidance manual* (2006). As the UV light source, a Stratalinker 1800 (Stratagene, La Jolla, CA, USA) was used to emit controlled amounts of short wavelength UV light with an energy level of 8 W. This apparatus is fitted with a UV light energy sensor and has the significant advantage of producing measured levels of irradiation, which automatically compensates for ageing light bulbs. The light bulbs were prewarmed for 2 min prior to use. For viral irradiation experiments, a crystallizing beaker (Fisher, FB35101) fitted with a magnetic stir bar was filled with 5 ml of BSMV particles suspended in water at a concentration of 187·5 μg ml^{-1} and placed on top of a magnetic stirrer plate (VELP, Milan, Italy) contained within the Stratalinker 1800. The beaker was kept on ice, and the suspension was stirred at 100 rev min^{-1} during UV irradiation. UV doses were applied cumulatively and are detailed for each species in Table S1. After each irradiation step, a 100-μl aliquot was removed. For fungal irradiation experiments, frozen spore stocks were adjusted to 10^6 viable spores per ml using a hemacytometer (Brightline, Hausser Scientific, Horsham, PA, USA). A 5-ml aliquot of spore suspension was then transferred into the beaker, irradiated at room temperature and sampled as described above. The viability of spore solutions was determined by plating serial dilutions onto diagnostic agar plates and counting the resulting fungal colonies over the subsequent 4–14 days. The fungal UV irradiation experiments were repeated at least 3 times for each of the six species (Table 2).

For the viral dilution experiments, a 3 mg ml^{-1} BSMV preparation was diluted from two times to 4096 times with distilled water. After the irradiation or dilution treatments, the virus was rub-inoculated onto wheat or *C. amaranticolor* leaves to assess its infectivity. Virus dilution experiments were repeated three times.

The chemical disinfectant MicroSol 3+ concentrate was obtained from Anachem, Luton, UK (MIC-203). It contains the quaternary ammonium compound

Table 2 Estimated ultraviolet (UV) inactivation coefficients from fitting an exponential dose–response curve, derived 1-log decrease values and lethal chemical inactivation concentrations for seven agricultural pathogens*

	UV irradiation			
Organism	$-k \times 10^{-3}$†	*n*‡	1-log decrease§	MicroSol 3+ dilution ratio¶
Magnaporthe oryzae	3·651 ± 0·371	3	630·6 ± 64·1	1/100
Fusarium graminearum	7·338 ± 0·868	3	313·8 ± 37·1	1/200
Fusarium culmorum	13·48 ± 1·61	4	170·9 ± 20·4	1/300
Mycosphaerella graminicola	33·0 ± 15·9	4	69·8 ± 33·8	1/300
Barley stripe mosaic virus	47·19 ± 3·92	4	48·8 ± 4·05	NT
Leptosphaeria maculans	82·0 ± 76·4	3	28·1 ± 26·4	1/500
Leptosphaeria biglobosa	113·0 ± 149·0	5	20·3 ± 27·2	1/500

*The table has been ranked by the calculated 1-log decrease values.
†Estimated UV inactivation coefficient k ± SE.
‡Number of replicated measurements.
§Fluence for 1-log decrease in surviving microbes is given in mJ cm^{-2} ± SE.
¶Ratio of MicroSol 3+ biocide concentrate to water sufficient to kill a population of $\geq 10^4$ viable spores within 24-h contact time.

didecyldimethylammonium chloride as the active ingredient. For the MicroSol 3+ chemical inactivation experiment, spore stocks containing 10^4–10^6 viable spores were incubated at room temperature for 12 h with MicroSol 3+ at various dilutions ranging from 1 : 500 to 1 : 25 in a 1- ml volume. Samples were filtered through 1·2-μm nitrocellulose filter discs (Millipore, Dundee, UK). Membranes were flushed with 10 ml of water to remove residual biocide decontaminant, placed upside down on diagnostic agar for 2–4 days to allow the transfer of growing fungal colonies and then monitored during the next 14 days. Spore viability was determined as described previously. Each experiment was repeated three times.

Four hundred litres capacity UV irradiation and Dosatron chemical treatment system

The system was set up within a purpose-build environmentally controlled containment facility in a room maintained at a negative air pressure of −15 Pa. The system has the capacity to treat continuously a 400-l batch of waste water, whilst at the same time collecting a further 400-l batch for treatment. Two units of the system are installed at Rothamsted Research, in parallel, to be able to treat a maximum of 800 l in 24 h. The system is fed by drainage water originating from humidity extraction units from growth rooms, hand washing and laboratory sinks within the containment facility. The water is collected via underground pipes which feed a sump connected to the UV irradiation system. Particulate material is removed with sedimentation units (soil traps) attached to the sinks and 1000-, and 500-μm inline filters fitted to the treatment system to remove any remaining contaminating peat/soil particles, plant tissue fragments and break-up microbial clumps. Once in a 24-h interval, the entire contents of one water collection tank is automatically pumped over to a water treatment tank which feeds into a UV light microbiological deactivation unit. The water circulates repeatedly through a GK-75 UV irradiation chamber (ATG UV Technology, UK) fitted with a 120-cm long low-pressure UV lamp (Licht & Design, Nagold DE; Philips TUV 75W GE G75 T8 UV-C) at a flow rate of 29·5 l min^{-1} for a period of 24 h. UV lamp functionality is ensured by electronic monitoring and replacing UV lamps before reaching the end-of-life UV output. Post UV treatment, the waste water is pumped to a chemical Dosatron inactivation unit using MicroSol 3+ (Anachem) as decontaminant and treated for further 24 h before being pumped to drain.

Large-scale UV irradiation system tests

Spores of a single wild-type fungal species were added into the collection tank of either A or B of the custom-built UNIGRO microbiological deactivation system (Fawkham, UK). This was carried out by adding 10^5 spores into 400 l water to produce a final spore concentration of 250 spores per litre. Spore viability in untreated water was determined in serial dilutions on agar plates. The spores were retained untreated in the collection tank for 18 h to permit some spore germination. This protocol was adopted to ensure there were both germinated and nongerminated spores in the test solution and because the UV lamp in the treatment system does not have an off switch. The inoculated water was then pumped to the treatment tank to start a batch UV inactivation process. Post UV irradiation, the treated water was pumped to the chemical inactivation tank and water samples of 1·33 l each were drawn from early-, mid- and late stage of the effluent. Samples were combined, and 500-ml aliquots were filtered through 1·2-μm Millipore filter discs. Each of the eight filter discs containing the filtered microbes was placed upside down on a separate diagnostic agar plate and incubated under the appropriate conditions to permit the growth of each tested fungal species. The filters were removed after 4 days to allow the visual inspection of outgrowing colonies for up to 14 days. Contaminating species were expected, and those most frequently observed and identified using spore morphology diagnostics were *Cladosporium sphaerospermum*, *Penicillium* species, *Aspergillus fumigatus* and *Fusarium redolens*. For each pathogen species, the two independent UV treatment systems were tested on at least one separate occasion.

For the virus tests, the UNIGRO microbiological deactivation system was set up to circulate 60 l of tap water containing 3 mg of purified BSMV. The solution was circulated for 4 h at a flow rate of 20 m^3 per hour. Then, a 10-l aliquot of the treated virus solution was reduced to 20 ml in 24 h using a slightly modified replica of a previously described ultrafiltration recovery system (Polaczyk *et al.* 2008) (Fig. S3). The virus was then precipitated by ultracentrifugation at 80 000 ***g*** for 3 h. The resulting virus pellet was resuspended in 200 μl of 10 mmol l^{-1} potassium phosphate buffer (pH 6·8). As a control, 3 mg of UV-untreated virus preparation was diluted to 60 l with tap water and prepared as described above. Both experiments were repeated twice.

Transmission electron microscopy (TEM) of virus particles

Formvar/carbon-coated copper grids were placed onto 20 μl drops of the virus suspension in a closed humid atmosphere at room temperature. After 1 h, the grids were removed and placed same face down onto 20 μl drops of 2% phosphotungstic acid (PTA) for 15 min. The grids were removed from the PTA solution, and excess

stain was drawn off using filter paper. They were then placed on a silicon mat in a covered petri dish to air dry. Examination was performed using a transmission electron microscope – FasTEM 2011 (JEOL Ltd, Tokyo, Japan) operating at 200 kV. For each grid, at least three fields of approximately 200 μm^2 were viewed. Images were captured and analysed using the US1000 CCD camera and Gatan Digital Micrograph software (Gatan Inc., Warrendale, PA, USA).

Calculation and statistical analysis

For the calculation of the 1-log UV reduction doses, a nonlinear dose–response regression model was fitted to data derived as the ratio (within individual replicates) of the counts of fungal colonies after a given dose, $N_{Dose,}$ to the initial count in the control situation before treatment (N_0). The model was $y = \exp(-k_i \text{ Dose})$, where $y = N_{Dose}/N_0$ and k_i is the exponential rate of decline in this ratio (from unity) with increasing dose, for each of $i = 1\ldots 7$ pathogens. The model was set up and fitted using the GenStat® 12th Edition (VSN International Ltd, Hemel Hempstead, UK) statistical software, employing a nonlinear optimization (Ross 1990). Estimates and standard errors (SE) of the k_i were provided, from which 1-log reduction doses were derived as: $1\text{-log}_{10} = -\log_e(0{\cdot}1)/k_i$ also with standard error, given the nonlinear optimization. The *F*-test was used to assess whether the k_i were statistically significantly different, having fitted a model with a common k for all pathogens for comparison.

To test the UV effect on the width of the BSMV viral axial canal, analysis of variance (anova) was applied to the natural log-transformed axial canal width of 53 randomly selected viral RNA strands, numbering 22 for each of control and Stratalinker-treated samples and nine for the UV treatment. The transformation ensured homogeneity of variance across the three treatment groups. Following anova, means were compared using the significant difference (LSD) at the 5% level of significance.

Results

Small-scale testing to explore the ultraviolet light and MicroSol 3+ chemical decontaminant dose ranges required to inactivate various plant pathogenic species

The species-specific UV inactivation rates were recorded for six plant pathogenic fungal species, namely *F. graminearum* (teleomorph *Gibberella zeae*), *F. culmorum*, *M. graminicola* (anamorph *Septoria tritici*), *M. oryzae*, *L. biglobosa* and *L. maculans*, and the RNA viral pathogen BSMV. Negatively exponential inactivation curves were fitted by combining the data from the (at least three) independent replicates for each species (Figs 1 and S1). Nonlinear regression analysis was used to calculate the inactivation coefficients k and SE (Table 2). Interestingly, the *F*-test statistical analysis showed that the k exponential rates of decline in the ratio were significantly different for the seven species ($F_{6,168} = 47{\cdot}46$; $P < 0{\cdot}001$). Decreasing 1-log reduction values were obtained in the following order: *M. oryzae*, *F. graminearum*, *F. culmorum*, *M. graminicola*, BSMV, *L. maculans* and *L. biglobosa*. Thus, *M. oryzae* was approximately 60 times more resistant to UV than *L. biglobosa*. A further observation was that when fungal growth occurred at nonlethal doses of UV treatment, no melanization of vegetative mycelium or altered growth characteristics were observed. Similarly, none of the remaining nongerminated spores had visibly melanized as a result of the UV treatment.

For the same plant pathogenic species, chemical inactivation was investigated using the broad-spectrum biodegradable disinfectant MicroSol 3+. MicroSol 3+ is a formulated blend of quaternary ammonium compounds and amphoteric biocides. The effective dilution of MicroSol 3+ for inactivation was determined, and a ≥4-log reduction to ≥6-log reduction was obtained after 24-h contact time (Table 2). Regression analysis revealed that UV inactivation and MicroSol 3+ chemical sensitivity in these small-scale tests were highly correlated ($r = 0{\cdot}92$, $P < 0{\cdot}001$).

BSMV inactivation using UV irradiation and dilution in water

Inoculation of the plant species *C. amaranticolor* with the untreated BSMV resulted in appearance of >200 local lesions, whereas no local lesions were observed when total UV doses of ≥100 mJ cm^{-2} were applied (Fig. 1 and Fig. S2). The BSMV UV 1-log reduction value was similar to the fungus *M. graminicola* (Table 2).

We determined the dilution endpoint of purified virus in water at 25°C using inoculation of an indicator plant *C. amaranticolor*. When the original BSMV preparation at a concentration of 3 mg ml^{-1} was inoculated, numerous local lesions developed within 7–10 days (Fig. 2). Serial dilutions of original BSMV preparation in water induced fewer local lesions, and no local lesions were observed on leaves inoculated with the virus diluted 512 times or more. Three independent experiments gave the same outcome (Fig. 2).

UV irradiation of microbial populations in 400 and 60 l of drainage water

The fungal spore tests

Ultraviolet irradiation and mechanical separation by deposition and filtration is used in the drinking water

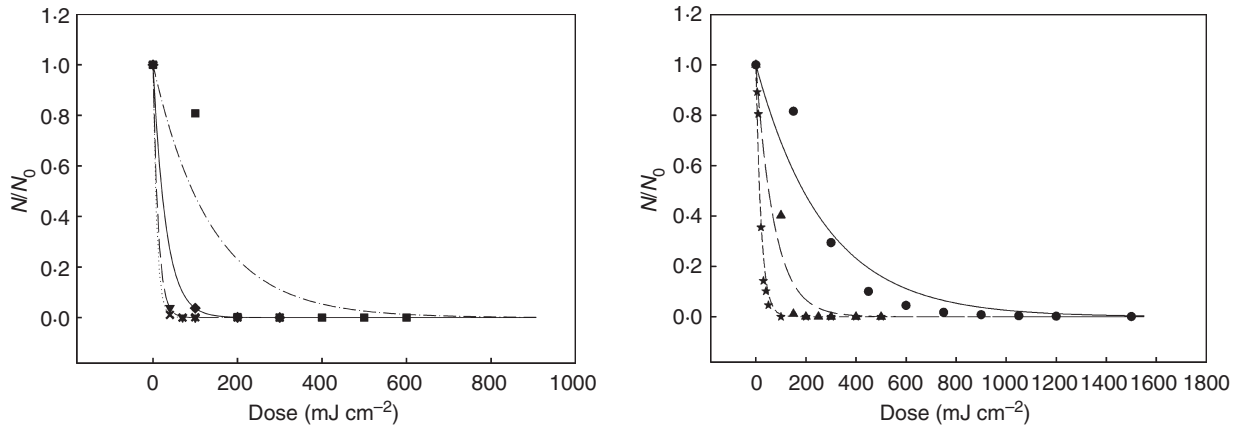

Figure 1 Mean inactivation dose–response curves for all species. Standard errors have not been plotted to improve clarity and are given in Table 2. Each data point represents at least three independent small-scale irradiation experiments. (◆——) *Mycosphaerella graminicola*; (▼– – –) *Leptosphaeria maculans*; (× ·····) *Leptosphaeria biglobosa*; (■ –·–·–) *Fusarium graminearum;* (● ——) *Magnaporthe oryzae*; (▲ – – –) *Fusarium culmorum* and (⋆ ------) *Barley stripe mosaic virus*.

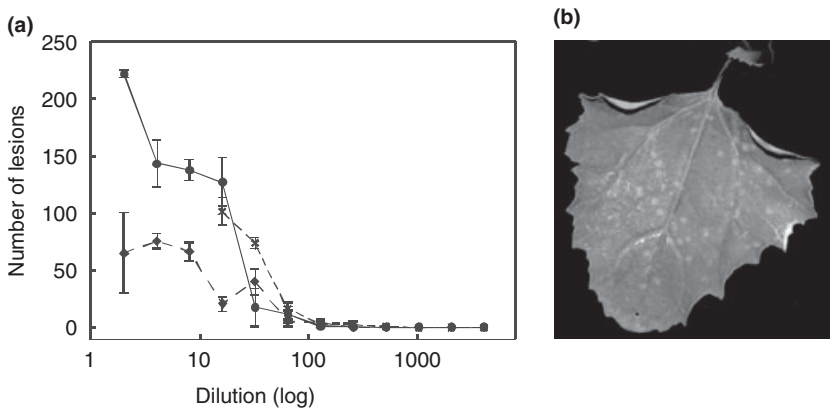

Figure 2 Endpoint dilution experiment of a 3 mg ml^{-1} BSMV preparation. (a) Assessment of infection potential for each serial BSMV dilution on *Chenopodium amaranticolor* leaves (–•–) Experiment 1; (--×--) Experiment 2; and (–⋆–) Experiment 3 and (b) typical appearance of BSMV-induced local lesions on a *C. amaranticolor* leaf.

and horticultural industries to treat drainage water (Meulemans 1987; Runia 1994) and can be used to decontaminate plant pathogens generated in agricultural research laboratories. To explore the possibility that UV irradiation can be used as a first inactivation step in a two-step large-scale decontamination procedure, we tested a duplicated set of custom-built UV treatment systems supplied by UNIGRO each capable of treating 400-l batches of waste water (Figs 3 and 4). Each treatment system first collects drainage water from a sump in tank A. Once in a 24-h period, the content is pumped to tank B for treatment and then circulates continuously through the UV irradiation chamber. Using the 1-log reduction rates determined earlier for the seven agricultural pathogens, we calculated that after 24-h UV treatment, all seven species had received a UV dose sufficient to cause ≥12-log reductions (Table 3). Thus, the estimated probability to

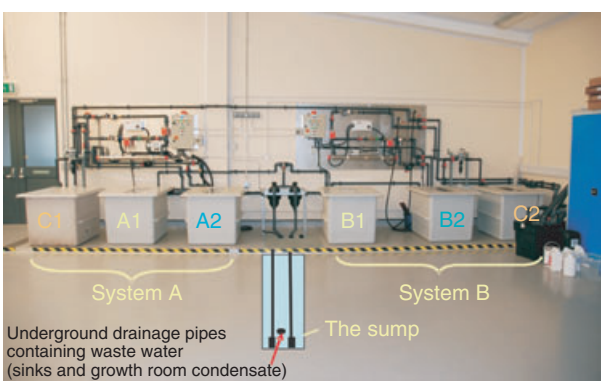

Figure 3 The two independent UV and chemical treatment systems for decontamination of waste water inside the environmentally controlled containment facility. A1, B1 – water collection tanks; A2, B2 – treatment tanks for batch UV irradiation; C1, C2 – Dosatron chemical inactivation tanks.

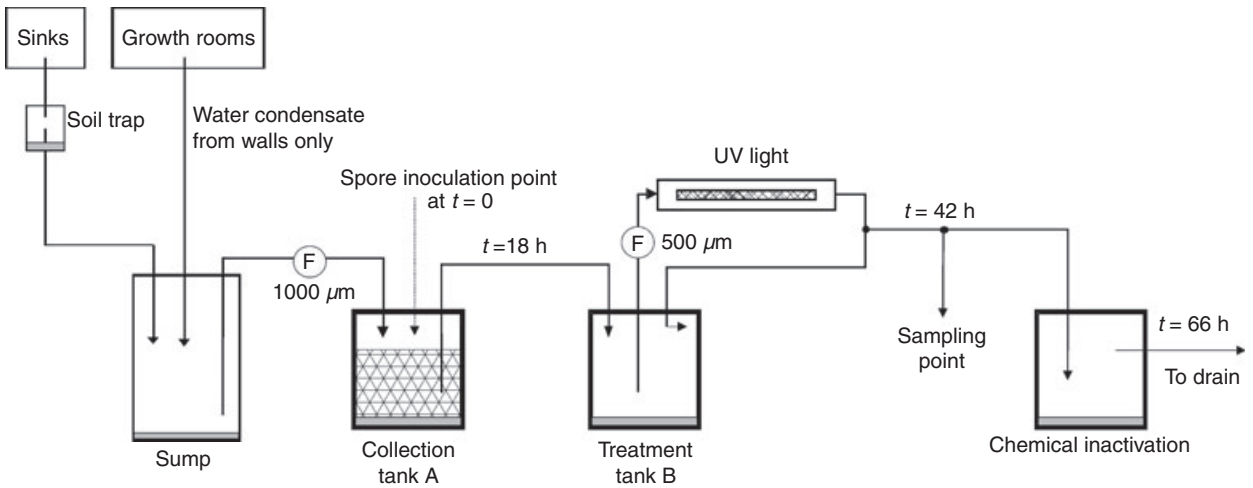

Figure 4 Two-step microbial inactivation process of 400-l water batches using UV irradiation and chemical decontamination. Only one of two replicated systems is shown. The operation is described in Materials and Methods. The experimental spore inoculation point and flow times through the system for testing are indicated. The system is not drawn to scale. The circles designated (F) indicate the position of the inline filters.

detect any viable micro-organism was negligible. We tested this hypothesis by direct inoculation of 10^5 viable spores into 400 l of water contained in tank A to produce a concentration of 250 spores per litre. The spore dose was chosen to mimic an accidental low-level contamination of waste water, as the intentional introduction of plant pathogens into the drainage system will be strictly avoided by the use of other restrictive measures. Water samples were analysed for the presence of viable microbes after 24-h UV irradiation. Although the exact number of spores circulating through the UV irradiation system for 24 h was not determined, dead spores were found microscopically on the final Millipore collection filters. All emerging fungal colonies were analysed using microscopy and plant host inoculations. In most cases, colonies were found to be ubiquitous nonpathogenic contaminants. Interestingly, for *F. graminearum*, *M. oryzae* and *M. graminicola*, following UV treatment, no viable propagules were detected in at least three replicate 400 l experiments, while for *L. maculans*, *L. biglobosa* and *F. culmorum*, water samples retained viable microbes in at least one test (Table 3).

Table 3 Calculated probabilities for surviving live microbes using the experimentally determined 1-log reduction values and the actual test results obtained in the 400-l fungal and 60-l viral inoculation experiments*

Organism	Log reductions	Probability of survivors	Number of replicates	Positive tests†
Magnaporthe oryzae	11·57	$2{\cdot}7 \times 10^{-07}$	2	0
Fusarium graminearum	23·26	$5{\cdot}5 \times 10^{-19}$	5	0
Fusarium culmorum	42·70	$2{\cdot}0 \times 10^{-38}$	5	2
Mycosphaerella graminicola	104·32	$4{\cdot}8 \times 10^{-100}$	6	0
Barley stripe mosaic virus	149·03	$9{\cdot}3 \times 10^{-145}$	2	0
Leptosphaeria maculans	260·80	$1{\cdot}6 \times 10^{-256}$	1	1
Leptosphaeria biglobosa	365·13	0	3	2

*The table has been ranked by the calculated log reductions.
†The diagnostic test used does not allow quantification of surviving microbes.

The BSMV *tests*

For these tests, only 60 l of tap water was used and a final BSMV concentration of 50 $\mu g\ l^{-1}$ was either UV irradiated for 4 h or just circulated without treatment. This low water volume was deliberately used to eliminate the possibility that excessive dilution would influence the subsequent infectivity of the virus, and treatment time was reduced proportionally. Also, a threshold virus concentration of 6 $\mu g\ ml^{-1}$ was required to retain virus particle infectivity (see Fig. 2). After the period of treatment/nontreatment, the virus particles were recovered using a specially devised ultrafiltration system (Fig. S3) followed by ultracentrifugation and tested for infectivity on the local lesion host plant *C. amaranticolor*. The control UV-untreated virus samples induced numerous lesions on the inoculated leaves. In contrast, no local lesions developed following the inoculation with suspensions of the UV-treated virus (Fig. 5 and Table 3). The experiment was repeated once with the same outcome. These results

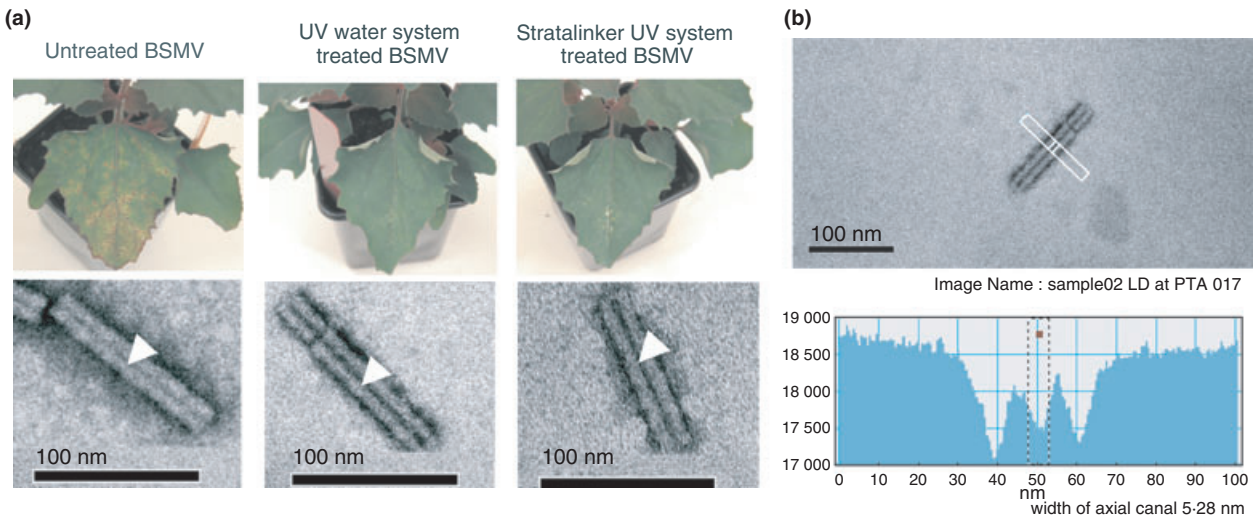

Figure 5 Effects of UV irradiation on BSMV plant infectivity and viral morphology. The BSMV particles were recovered after treatment either in the large-scale irradiation system or in the UV Stratalinker (1000 mJ cm^{-2}), or after circulating in the large-scale irradiation system without receiving UV treatment. (a) Upper panels, the three different virus preparations were inoculated onto *Chenopodium amaranticolor* and representative leaves photographed at 8 dpi. For each treatment, six leaves from three independent plants were used and the experiments were repeated twice with similar results. Lower panels, the three different virus preparations were stained with phosphotungstic acid and analysed by TEM. The central axial canal of the virus particle penetrated by stain is indicated with a white triangle. The scale bar in each panel is 100 nm. (b) Gatan Digital Micrograph software was used to measure the width of the central axial canals in virus particles. Upper panel indicates the position of a line scan where information was obtained across the viral particle in the image. Lower panel displays the line scan as a histogram based on the intensity profile and allows very accurate measurements to be made on features in the acquired image.

indicated that the UV water treatment system inactivates BSMV.

TEM microscopy of UV-irradiated virus particles

The physical appearance of the irradiated virus particles was examined using TEM. Considerable damage to the viral protein coat was evident in the treated viral preparations retrieved from both the small-scale and large-scale UV irradiation systems compared to the untreated particles (Fig. 5). The degree of microscopy stain penetration around and into the virus particle was increased in UV-treated particles. ANOVA revealed overall significant difference in the degree of staining between the three treatments ($F_{2,50}$ = 354·4; $P < 0·001$). The means (on log-scale) were as follows: control – 1·020 (n = 22), UV water system treated – 1·439 (n = 9) and Stratalinker treated – 1·481 (n = 22). The LSD (5%) values were as follows: 0·0482 for UV water system treated vs control or Stratalinker and 0·0367 for Stratalinker *vs* control. There was no significant difference ($P > 0·05$, LSD 5%) between UV water system and Stratalinker treatments.

Taken together, we conclude that the batch water UV system tested can reduce the plant pathogenic species *F. graminearum*, *M. graminicola* and *M. oryzae* to undetectable levels. In contrast, the species *L. maculans*, *L. biglobosa* and *F. culmorum* have the ability to pass through the system independent of their UV sensitivity.

Discussion

In this study, we investigated the suitability of UV irradiation, chemical treatment and dilution to inactivate a range of fungal plant pathogens and one viral plant pathogen in small- and large-scale experiments. First, we determined the species-specific UV irradiation coefficients in small-scale liquid suspension (Table 2). The data show that all the species are sensitive to UV light. The highest UV resistance observed in this study was for *M. oryzae* (630 mJ cm^{-2}). To our knowledge, this UV resistance value is the highest value reported to date for any organism and is about twofold higher than values reported for blue-green algae at 300 mJ cm^{-2} (Legan 1980; Groocock 1984). *Magnaporthe oryzae* produces dark, heavily melanized tripartite spores. In *Alternaria alternata*, a fungal pathogen of pears, mutants affected in melanin biosynthesis showed dramatically increased sensitivity to UV irradiation (Kawamura *et al.* 1999). Similarly, it was suggested that melanin in *M. oryzae* is a protectant against UV radiation (Talbot 1995; Henson *et al.* 1999). In addition, the high resistance level of *M. oryzae* to UV light can

be explained by the fact that a single infectious spore consists of three cells able to form viable progeny. Multiple UV damage needs to occur in all three cells simultaneously before a single spore is killed.

Fusarium graminearum and *F. culmorum* have intermediate resistance to UV and show significantly different UV log reduction coefficients. This result was not unexpected, because vegetative *Fusarium* spores are translucent, have relatively thin walls and lack protective melanins and other pigmentation (Leslie and Summerell 2006). However, vegetative *Fusarium* spores are curved and consist of four to five cells, which potentially can shield each other against UV irradiation from certain directions. For *Fusarium oxysporum* f. sp. *lycopersici*, a lower UV dose of 84 mJ cm^{-2} is sufficient to achieve a 99·9% reduction in propagules (Runia 1994). The observation that UV sensitivities within the same genus can be significantly different was also found for *Aspergillus* species. For *Aspergillus niger*, a threefold higher log reduction rate was reported compared to *Aspergillus flavus*, 180 and 54 mJ cm^{-2}, respectively (Tothova and Frankova 2001; Simonet and Gantzer 2006). The spores of *L. maculans*, *L. biglobosa* and *M. graminicola* are nonmelanized and single-celled. These species were found to be moderately susceptible to UV irradiation (20–70 mJ cm^{-2}). Interestingly, we observed UV resistance for the viral pathogen BSMV at 47 mJ cm^{-2}, which is slightly higher than resistance levels reported for MS2 and GA bacteriophages at 38 mJ cm^{-2} (Simonet and Gantzer 2006). Microbial inactivation for all of the seven plant pathogens followed a negative exponential curve, and we did not observe any deviation at high-energy doses, which might be attributed to the clumping of microbes (Parker and Darby 1995). Slight deviation at low-energy doses were only seen for *M. oryzae* and the two *Fusarium* species and can be explained using series-event kinetics (Severin *et al.* 1984). It seems plausible that an initial energy threshold has to be overcome in these species before sufficient DNA damage has occurred killing all of the multiple cells contained in a single spore.

Chemical disinfection is used worldwide to treat water contaminated with a range of microbes. However, the most frequently used agent sodium hypochlorite, contained for example in household bleach, releases free chlorine and is hazardous to human health and the environment. We explored the suitability of a less toxic, biodegradable disinfectant called MicroSol 3+ (Anachem) which contains quaternary ammonium compounds. Prior to this study, MicroSol 3+ efficacy data for the seven plant pathogenic species investigated did not exist. Table 2 shows that all tested pathogens are effectively inactivated at a dilution rate of ≤1 in 100 in a contact time of 24 h.

Current regulations in the United Kingdom require a higher level of biological containment and the treatment of drainage water, when doing research using foreign and/or transgenic agricultural pathogens. Generally, a robust two-step inactivation procedure is recommended to establish an error-proof decontamination system (HSC Advisory Committee on Genetic Modification 2000). Such a system could consist of chemical inactivation using bleach followed by steam sterilization. However, both methods have disadvantages regarding their environmental impact and energy demand. We set up and explored, as an alternative option, the use of UV irradiation followed by chemical inactivation using the biodegradable MicroSol 3+ decontaminant, within a closed and filtrated drainage system (Figs 3 and 4). This approach reduces both treatment and energy costs.

Large-scale water batches were inoculated and sampled after UV irradiation to detect the remaining viable microbes. For three fungal species, *F. graminearum*, *M. graminicola* and *M. oryzae*, the replicated experiments conducted for two independent UV treatment systems confirmed that a 24-h period of treatment eliminated the presence of viable propagules (Table 3). The two experiments with BSMV to test both UV systems also confirmed UV treatment eliminated the presence of viable virus particles. All these tests were designed to simulate low-level contamination of the drainage water with plant pathogens that would have arisen primarily from hand washing, rinsing, and wiping down laboratory equipment as well as from the condensate collected from the growth room walls. Although fungal spores were initially added to the treatment tank, by 18 h, considerable spore germination had occurred prior to the onset of the UV treatment. Therefore, although the experiment was started solely with spores, to quantify the inoculum applied, these tests also included the exposure of fungal mycelium to UV. For *L. maculans*, *L. biglobosa* and *F. culmorum*, the water samples collected following the large-scale UV treatment retained some viable microbes. This unexpected result conflicted with the data obtained in the bench scale UV sensitivity tests, where these three species showed intermediate to low resistance to UV. For these species, physical and biological properties appear to be sufficiently different compared to the other tested organisms. Currently, we have no mechanistic insight into the underlying causes of these species differences. The most likely explanation for the retention of viability is the formation of fungal biofilms under certain conditions (Marques *et al.* 2006). In bacterial disinfection facilities, the formation of biofilms was suggested as a chronic source of microbial contamination (Peng *et al.* 2002). This hypothesis could be tested in future experiments by tracking fungal reporter strains of each species, which have been constructed to constitutively express at a high level different fluorescent marker proteins (Eckert *et al.* 2005).

Ultraviolet irradiation is a relatively new decontamination technique. However, the possibility already exists to increase the efficiency of the eradication further by modifying the UV treatment. For example, the addition of hydrogen peroxide or ozone during UV treatment, or the use of other oxidation technologies is under investigation (Clauss 2006; Bohrerova *et al.* 2008; Sichel *et al.* 2009). It seems likely that the UV treatment system described in this study could be adapted in the future to disinfect even higher densities of microbial populations that have accumulated in waste water.

The BSMV dilution tests (Fig. 2) verified results reported earlier that this tripartite virus species can be inactivated by dilution in water alone (Kassanis and Slykhuis 1959). In fact, all plant viruses described in the Virus Identification Data Exchange database are known to be inactivated by dilution in water, and the dilution endpoint reported for BSMV in extracted plant sap is 1/2048, but varies with environmental conditions (Boswell *et al.* 1986). In a typical virus–plant inoculation test, the experimenter handles a solution of virus particles at a concentration of 1 mg ml^{-1} and a total volume of 5 ml. Therefore, even if the entire inoculum intended for an experiment accidentally entered the drainage system, the batch water collection system would dilute this inoculum into a volume of waste water ranging from 100 to 400 l. This would achieve dilutions in the range of 15 000–60 000 times. Our data show that dilution of BSMV (3 mg ml^{-1}) even to 512–1024 times was sufficient to ensure the complete loss of virus infectivity *in planta* (Fig. 2). The genetically modified strain of BSMV used as the vector, for example for VIGS experiments, is even less infectious than the wild-type strain. Also, genetically modified BSMV containing inserted 'foreign' sequences are highly unstable *in planta*. The 'foreign' inserts are frequently deleted during the infection process and the virus reverts to the wild type (Bruun-Rasmussen *et al.* 2007). Most VIGS inoculation experiments would use fresh viral RNA transcripts generated *in vitro* at concentration of 50 μg viral RNA per ml (equals 1·2 mg ml^{-1} virus particles) and a total volume of 3 ml. Overall, we consider that in the biological containment facility, the entry of fragments of infected plant tissue into the drainage system is potentially the most likely route by which the waste water could become contaminated with virus particles. We conclude that in research facilities where BSMV is the only plant pathogen being studied, viral dilution alone would be sufficient to inactivate BSMV. However, in facilities where BSMV is being used for VIGS experiments on plants in combination with other plant pathogens such as fungi, additional decontamination procedures are required for drainage water.

In summary, UV irradiation to inactivate *F. graminearum*, *M. graminicola*, *M. oryzae* and BSMV was successfully employed using a worst case scenario. Large numbers of viable propagules of a single plant pathogenic organism were added directly to the treatment water tanks immediately prior to UV irradiation. During the treatment process, large numbers of spores also germinated. Our data indicate that the UV irradiation/filtration system could remove four plant pathogenic species from treated drainage water to the point that no viable and/or infectious propagules were detected in a range of diagnostic follow-up tests. The use of a UV irradiation/filtration unit linked to a chemical treatment unit using MicroSol 3+ is, therefore, considered to be a viable option to decontaminate drainage water containing agricultural wild-type and genetically modified strains from these species.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Cumulative UV irradiation doses applied to each species in bench scale experiments.

Figure S1 The responses of wheat (*Triticum aestivum*) and *Chenopodium amaranticolor* following mechanical inoculation with BSMV.

Figure S2 Effects of UV irradiation on the viability of BSMV.

Figure S3 Virus enrichment from 10 litres of water.

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