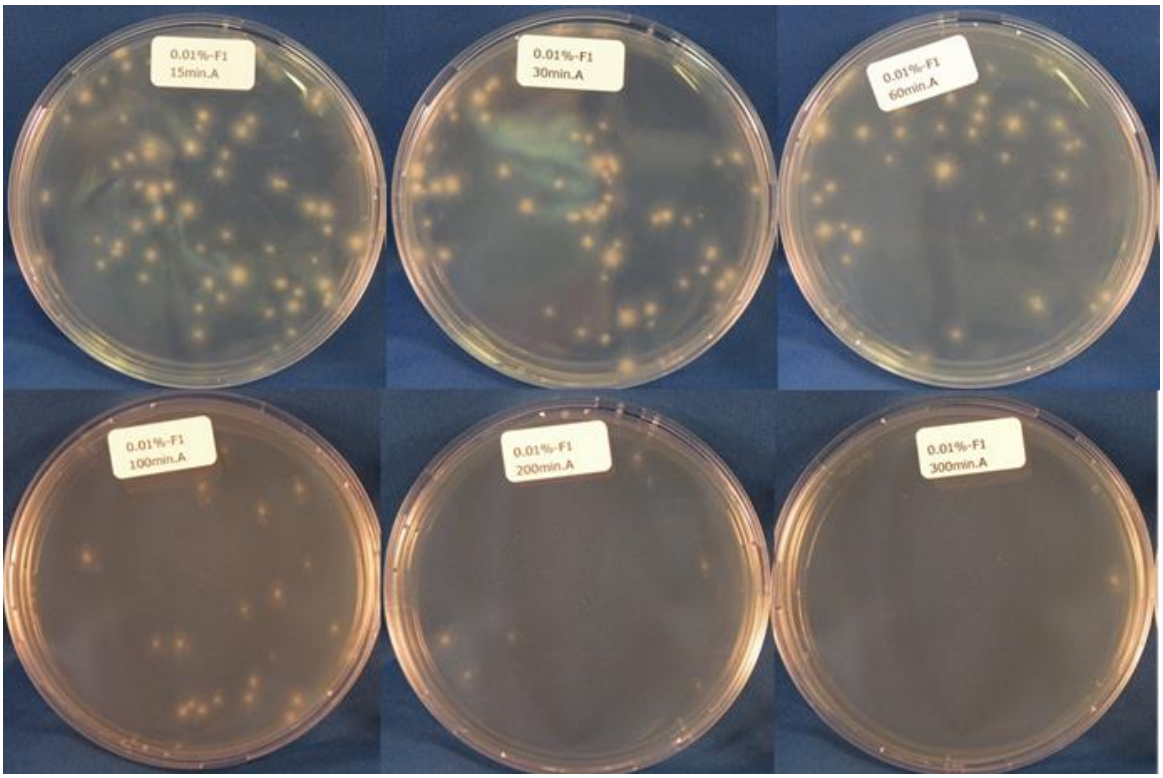
Efficacy of EcoClearProx against Tropical Race 4



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EcoClearProx efficacy against TR4

Introduction

Fusarium spp. are soil-borne plant pathogens invading plants directly or via wounds at the roots. *Fusarium oxysporum* spp. causes wilting diseases in a range of plants, crops and ornamentals. For each plant there is a race or *forma specialis* (f.sp.), specialized on its host plant. On banana (Musa) plants the soil-borne fungus *Fusarium oxysporum* f.sp. *cubense* (Foc) can be pathogenic, depending on the interaction between the individual strain and Musa variety. In the previous century, a Foc strain caused a devastating disease on the, at that time, globally cultivated banana species "Gros Michel", commonly referred to as Panama disease or Fusarium wilt. The Gros Michel variety dominated the banana industry but the rapid spread of that Foc strain forced the industry to shift to the "Cavendish" variety since it was found to be resistant. The Foc strain was later designated as "Race 1" (Foc R1) when it became more clear that other Foc strains exist with pathogenicity towards other Musa species, as determined in limited panel trials on various Musa types. Recent molecular research recently revealed that Foc R1 actually comprises a number of strains with similar properties shifting and challenging further the paradigm of race based classification [1, 2].

After the shift to the Cavendish variety, locations that were contaminated with Foc R1 remained uninfected for an extremely long time, which is exceptional in agriculture. So far no Cavendish plant has succumbed due to the presence and infection of this Foc R1 despite the millions of plants that have been placed on Foc R1 infested soil.

In the 1960's Fusarium wilt symptoms were reported for the first time on Cavendish plants in Southeast Asia [3] but it took till 1994 to identify the causal strain as a *Fusarium oxysporum* f.sp. *cubense*, Vegetative Compatibility Group "VCG01213", often referred to as Tropical Race 4 (TR4). More recently the strain has been described as a member of a new species, *F. odoratissimum*. Albeit the nomenclature added some controversy, it is clear that TR4 is a clonal *Fusarium* lineage that is genetically dissimilar from original Foc strains that are infecting banana and thus should be considered as an independent species.

After the first reports in Taiwan and Southeast Asia, TR4 started to spread in the region, in the early 1990's it was reported from Indonesia and Malaysia, followed by Australia (Northern Territory) and China. In 2005 the Philippines, a main producer in the region, was reached and in 2012 an unconfirmed report was derived from Oman. By 2013 the first confirmed outbreak outside Southeast Asia in Jordan was published (Garcia,2013) closely followed by Lebanon, Pakistan in 2015 [4]. Although contained in the Northern Territory for long, first report of emergence of TR4 in Queensland Australia was filed in 2015. Since then, outbreaks in the Tulley Valley are reported on six commercial banana farms till July 2022 (shorturl.at/eiJX4). Global dissemination further continued with "remaining" countries in Asia (Vietnam, Laos, Myanmar, India and Thailand), as well as Israel and Turkey [5-8]. In 2013, some rumours indicated that TR4 was identified in Mozambique but the first scientific evidence lasted till 2020 [9] stating that the fungus

was present on two commercial export plantations, but that the fungus was contained by strict measures to enforced this. Notably, this did not function well [10]. In the meantime, TR4 was reported to have emerged in Mayotte, an island next to Mozambique [11]. The major blow for the banana industry are the recent incursions in Latin-America. First it was reported in Colombia [12]) and more recent in Peru [13]. Although at the same continent, based on genetic data the incursions originate from two different sources but which origin they have remains to be solved [14]. Taken together, TR4 is now identified in all banana producing regions in the world and will spread likely within these regions to all banana producing area's.

As other *Fusarium* spp. TR4 produces, next to mycelial mass, asexual spores. These are conidiospores (macro- and microconidia) and chlamydospores. The conidiospores are facilitating the fungal spread within the plant by being released into the major vessels and using the sap stream to be transported. Conidiospores can be formed on alternative hosts and survive for limited time in the soil or when being transported in water, e.g. during floods. Chlamydospores are the tough survival propagules, produced both internally and externally in infected plants throughout the fungal lifecycle. They are resistant to desiccation and can survive for decades in the soil in the absence of susceptible hosts [15].

With the arrival of TR4 at all banana producing regions globally, management strategies are primarily focussed on halting the introduction of fungal spores into uninfested farms. This starts with usage of clean planting material from tissue cultures, clean tools and machinery that is not exchanged between farms. Strict regulations should be in place to avoid introduction of spores via (irrigation) water or soil attached to shoes or any other transport vector [15]. Once introduced, the focus shifts to quarantining infected areas. Thus far, eradication of spores by application of biological, chemical or other means have, at highest ,only temporal positive effects (Salacinas 2019).

As indicated before, one of the potential dissemination routes, once the fungus is established in a region, is by contaminated water. Contamination of water occurs when infested banana plants are subject to flooding, during rainy periods, hurricanes or typhoons [15]. Release of spores from decaying material results in water facilitated transport and consequently, downstream located (uninfested) banana farms are at high risk. They become easily infected during irrigation with contaminated water. A study on river water showed indeed that occasionally TR4 spores can be retrieved [16] and from aerial views the correlation between flooding area's and TR4 spread is obvious (Meijer, personal observation). This urges for strict quality control of irrigation sources and for the pro-active eradication of spores that are potentially present. It should be noted that disinfection treatments need tailoring depending on the sensitivity and type of pathogen propagule [17].

The EcoClearProx® (ECP) product is a patented biodegradable organic stabilized hydrogen peroxide (H2O2) product which has been tested and found effective against a

range of bacteria, fungi, yeasts and viruses. In contrast to many other hydrogen peroxide products ECP is not based on stabilization by silver. As a result it leaves no residues or contaminants and decays fully by degradation to water and oxygen.

In preceding tests, ECP was briefly tested for its efficacy against TR4 conidiospores. Short exposure was needed with ECP concentrations that were relative high whereas at lower concentrations the product was effective when longer exposure times were taken. Whereas in a first test a minimal concentration of 1% was taken, effective after 15 min on conidia, a follow up study learned that the ECP concentrations could be lowered to 0.025% when the compound was permitted to exert for five hours. In addition, a brief study on the influence of the presence of organic matter revealed that a clear correlation was found between ECP efficacy and the amount of conidia.

This study describes further insight in the efficacy of ECP. The conidia were tested for the efficacy of ECP in liquid an additional time to confirm previous results and they were tested when they were included in soil columns to establish if ECP is functional in soil. Furthermore, the experiments were broadened to chlamydospores, studying the ECP efficacy in liquid and on static soil. The data show that ECP application to liquid environments is extremely functional whereas limited efficacy is shown in soil, depending on soil density and, at least partially, the presence of organic material.

Materials and Methods

Fusarium odoratissimum strain Tropical Race 4 was derived from the Wageningen University and Research collection and maintained on Potato Dextrose Agar (PDA) plates.

Individual batches of TR4 conidia were generated as described [18], each inoculated from an individual full grown PDA plate to warrant fully independent experiments. Liquid cultures containing the conidia were filtered over a double layer of sterile cheesecloth. The concentration of the spores was determined using a Kova Glasstic slide and diluted to testing concentrations. Soil columns were prepared by mixing a conidia solution in a ration of $1E^6$ conidia g^{-1} soil. Columns were generated from plastic sheets that were formed into tubes that could contain ~250 ml of soil with a column hight of at least 25 cm. The columns were closed at their bottom with a gauze to prevent emptying. After the mixing (>1 hr) of the conidia and the soil, the columns were filled and divided over control and test tubes.

Chlamydospores were prepared according to the protocol described by [19]. In short, mycelial plugs were aseptically added to a twice autoclaved (121°C for 60 min) substrate composed of mesh-filtered and washed sandy soil, corn meal, and distilled water in a 500-ml Erlenmeyer flask. The flask was incubated at 25°C with a 12-h photoperiod for 15 days and the content was daily homogenized by shaking. Subsequently, 200 g of autoclaved sandy soil were added and thoroughly mixed, and flasks were then incubated for another 6 weeks under the same conditions. After incubation, soil with chlamydospores was dried for 3 days at 30°C and then stored until usage at 4°C. The concentration of chlamydospores was determined as CFU/g soil (CFU per gram of soil) by a plate dilution technique on PDA plates.

For testing the efficacy of ECP to chlamydospores in liquid medium, 0.5 to 1 g of chlamydospore infested soil was suspended in 25 ml of Ringer solution in a 50 ml tube. Chlamydospores were released into the solution by stringent hand shaking for 1 min followed by 30 min on a lab bench roller. After an additional 1 min stringent manual shaking the soil particles were allowed to pellet for at least 2 min. The supernatant was taken as the liquid chlamydospore solution. Repeated plating showed equal distribution of chlamydospores in the solution.

ECP testing

For the experiment with chlamydospores in liquid, the same procedure was followed as described in the previous report [20]. ECP dilutions were generated in Ringer solution and chlamydospores were added to the solution in a 1:9 ratio, resulting in the final concentration of the ECP as indicated. Then the solution was incubated for the times indicated, at room temperature. At the times indicated, samples were taken and diluted immediately in Ringer to reduce the effect of the initial concentration of ECP. The diluted chlamydospore solution was subsequently plated, with replicates, on PDA plates. Plates were incubated for 2 days at 25°C and developing colonies were counted as CFU's. Plates were photographed and then incubated again to be rechecked after an additional 24-48 h (mainly to observe if plates that had no or very few colonies showed additional colonies). Due to the unequal distribution of chlamydospores in the soil taken, the CFU values were converted to percentage, based on the maximum amount of chlamydospores detected within one batch (usually in control sample).

Chlamydospores embedded in soil were tested by taking ~0.5 g of soil and load into a 12-wells titer plate. After application of 3 ml of various concentrations of ECP, small samples were taken (~50-100 mg) by spatula and taken into 1 ml of Ringer fluid in an Eppendorf tube. The tube was shaken for 1 minute manually and then rotated (40 rpm) for 30 min. Thereafter 100 µl of the supernatant, after settling of the soil particles, was plated in duplicate on PDA plates and processed as described above.

For the experiments with columns, two individual batches of TR4 conidia were generated, each inoculated from an individual full grown PDA plate to warrant fully independent experiments. Columns were flooded with 1% of ECP fluid until run-through. The columns were then left overnight and thereafter soil samples were taken with a spatula ~1 cm below the top of the column, at the middle and ~ 1 cm above the bottom of the column. Sampling was performed in a flow cabinet after sterilizing the column at the outside to avoid cross-contamination. The samples (~0.5 gr) was put in MQ (40 ml) and shaken for at least 15 min to release surviving spores. Thereafter 100 µl of the supernatant was placed in duplicate on PDA plates and processed as described above.

Result

Conidia in liquid culture

The efficacy of ECP towards conidia in liquid was tested previously (Meijer, 2022) in duplicate. For validation of the sensitivity of conidia the experiment was conducted an additional time. ECP was tested in a range of 0.01% up to 1% with a minimum exposure time of 15 min and a maximum of 300 min as described before (Meijer, 2022). The data confirms the observed effects that ECP is fully effective at 1% within 15 min and that the lowest effective concentration was 0.025% after 300 min. The data for the triplicate experiments is shown before per time point tested (Figures 1 - 6). Notably, all data matches with earlier measurements confirming the stability of the ECP solution at room temperature.

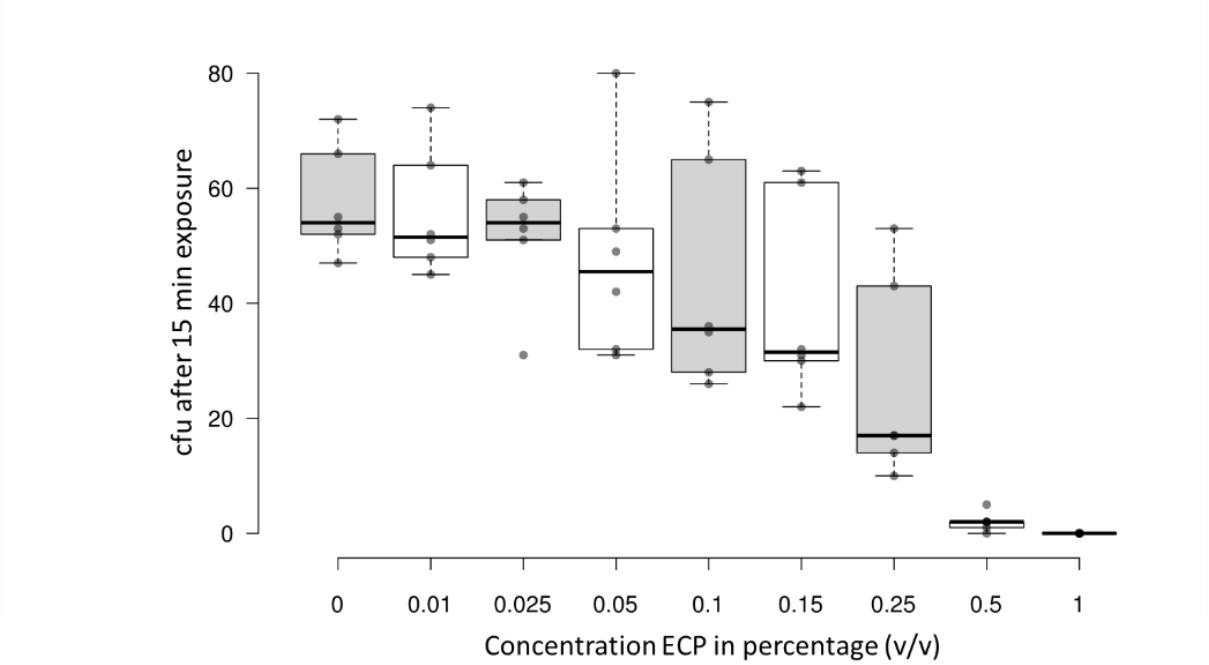

Figure 1: Colony growth (CFU after 48 h) from TR4 conidia exposed for 15 min to concentrations of ECP. Center lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles data points are plotted as open circles. n = six sample points (three batches with replicates).

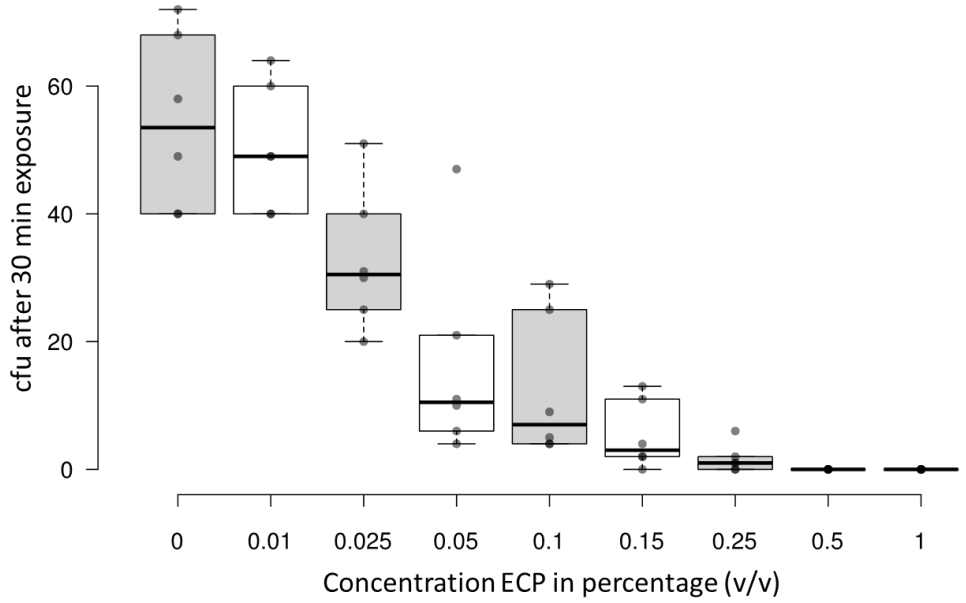

Figure 2: Colony growth (CFU after 48 h) from TR4 conidia exposed 30 min to concentrations of ECP. Center lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles data points are plotted as open circles. n = six sample points (three batches with replicates).

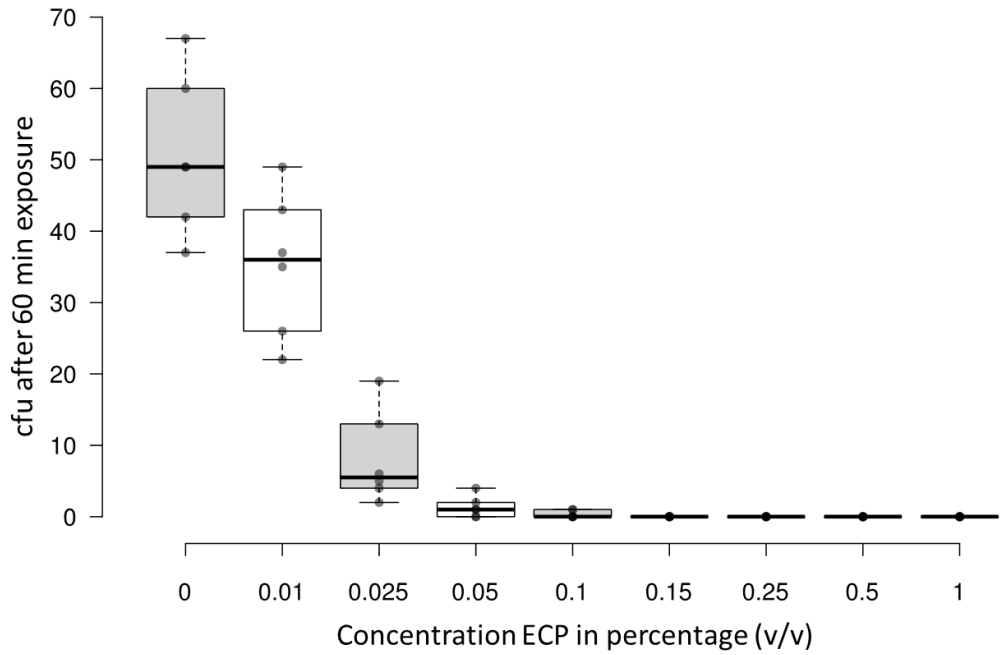

Figure 3: Colony growth (CFU after 48 h) from TR4 conidia exposed 60 min to concentrations of ECP. Center lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles data points are plotted as open circles. n = six sample points (three batches with replicates).

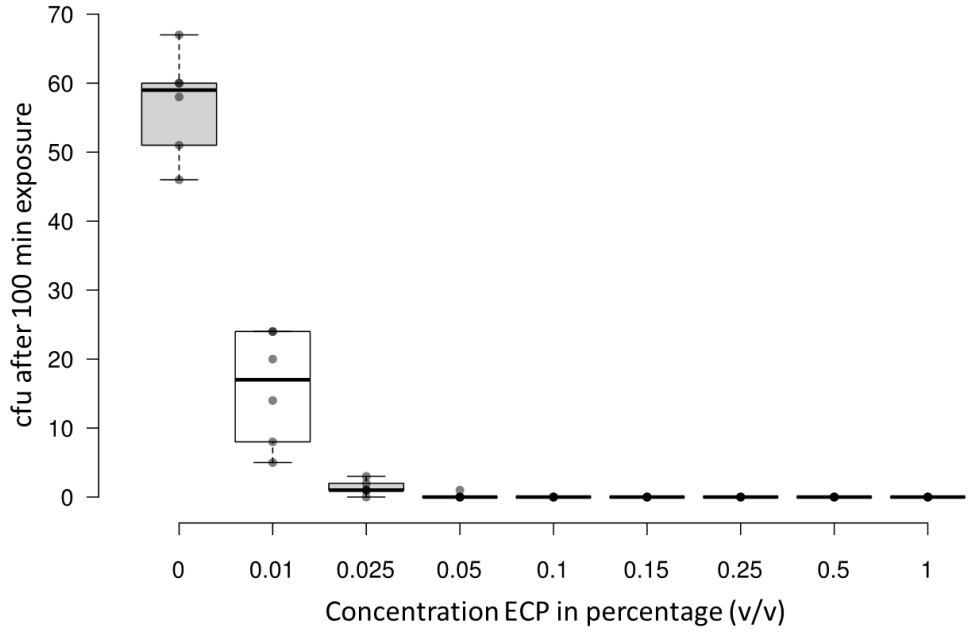

Figure 4: Colony growth (CFU after 48 h) from TR4 conidia exposed 100 min to concentrations of ECP. Center lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles data points are plotted as open circles. n = six sample points (three batches with replicates).

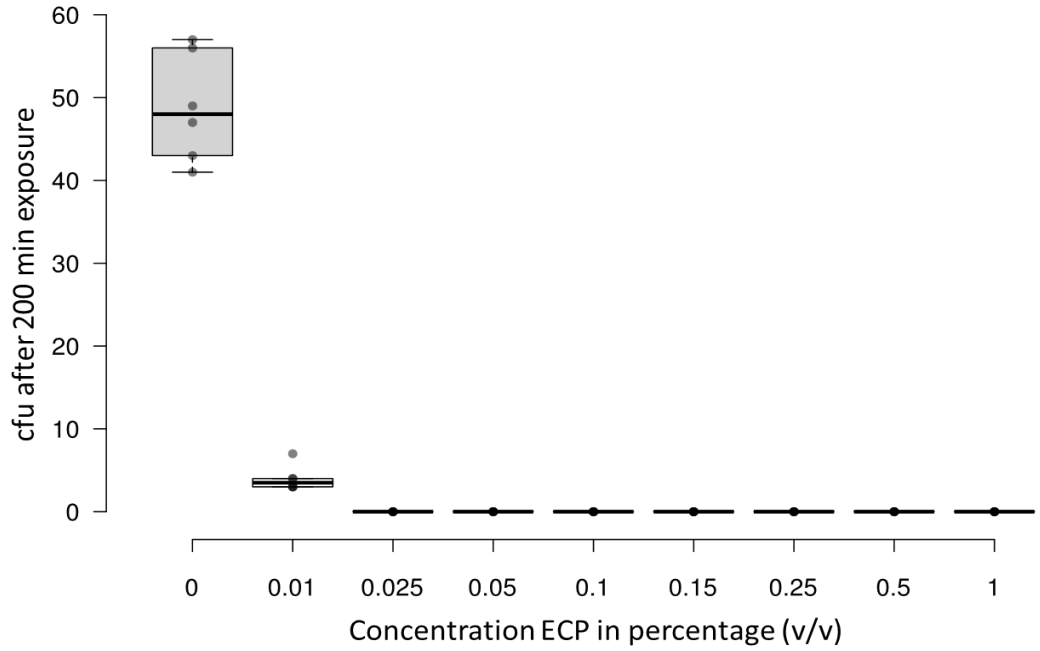

Figure 5: Colony growth (CFU after 48 h) from TR4 conidia exposed 200 min to concentrations of ECP. Center lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles data points are plotted as open circles. n = six sample points (three batches with replicates).

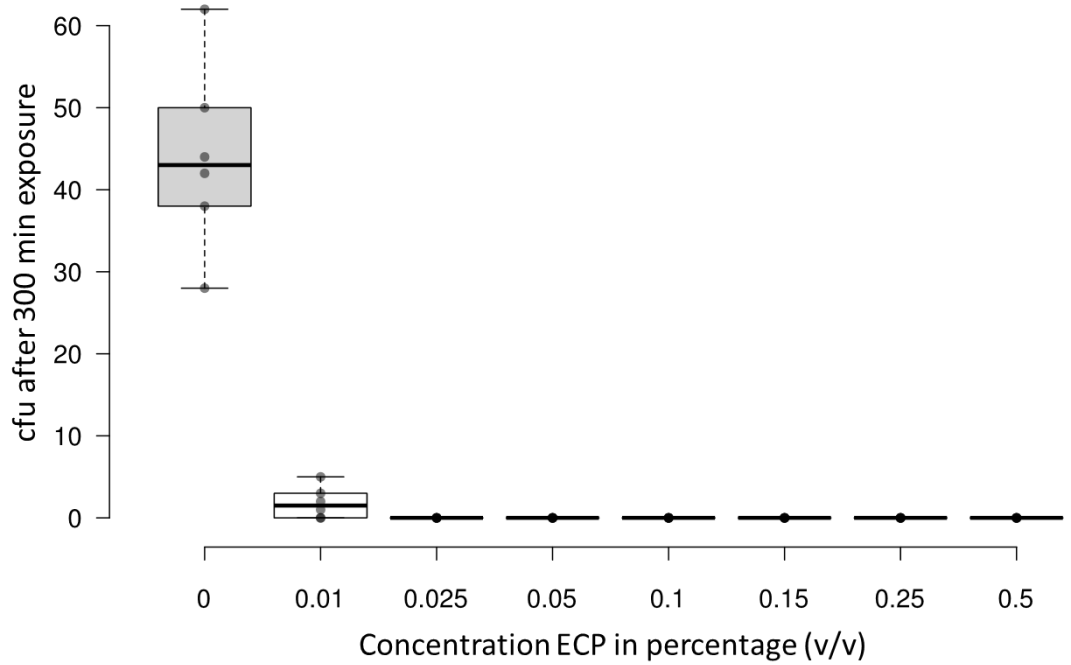

Figure 6: Colony growth (CFU after 48 h) from TR4 conidia exposed 300 min to concentrations of ECP. Center lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles data points are plotted as open circles. n = six sample points (three batches with replicates).

The one concentration illustrating the positive effect of prolonged exposure to ECP is the treatment with 0.01% (v/v) (Figures 7 & 8). At 15 min the number of colonies are comparable with those of the control. However, with increasing exposure time a strong reduction of surviving colonies was observed, continuing up to 300 min.

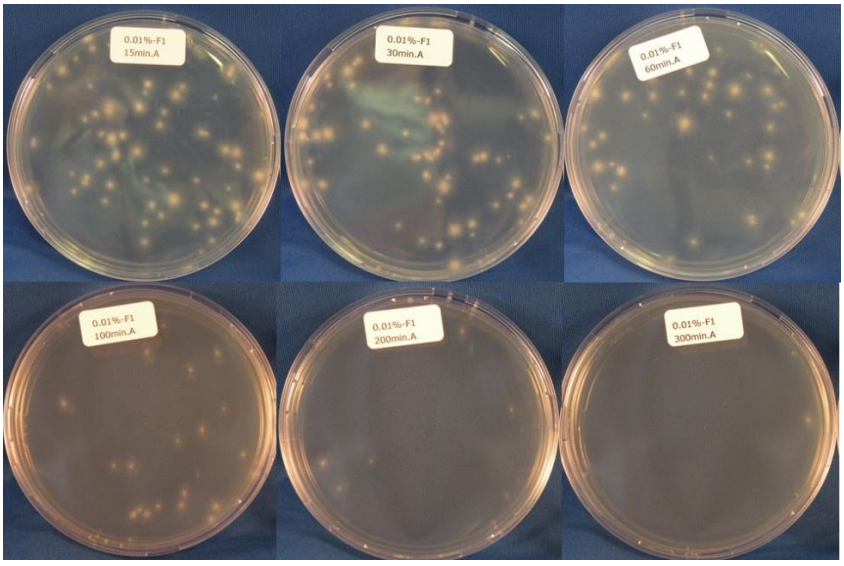

Figure 7: Colony growth after exposure of conidia to 0.01% ECP for the times indicated, after 48 h incubation. One representative series is shown. (Figure is retaken from [20]).

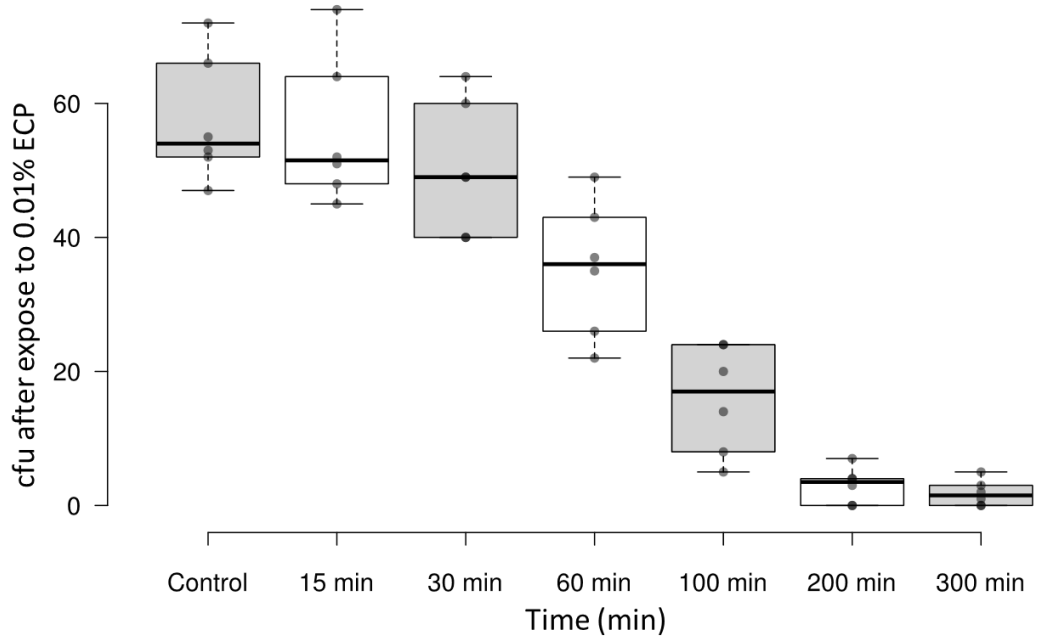

Figure 8: Colony growth (CFU after 48 h) from TR4 conidia exposed in time to a concentrations of 0.01% ECP. Control represents the number of colonies in the absence of ECP at 15 min. Center lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles data points are plotted as open circles. n = six sample points (three batches with replicates).

Chlamydospores in liquid culture

The efficacy of ECP, in the range 0.01% up to 1%, against TR4 chlamydospores in liquid cultures was tested between a minimum of 15 min and a maximum 300 min exposure time. As expected, due to unequal distribution of chlamydospores, the number of CFU per batch varied. Therefore the data is presented in percentages (%) to make merge all three independent experiments.

After 15 min incubation of chlamydospores, ECP was nearly effective at the highest concentration (1% ECP) tested, but lower concentrations had less effect Figure 9. The data is shown in boxplot format in Figure 9.

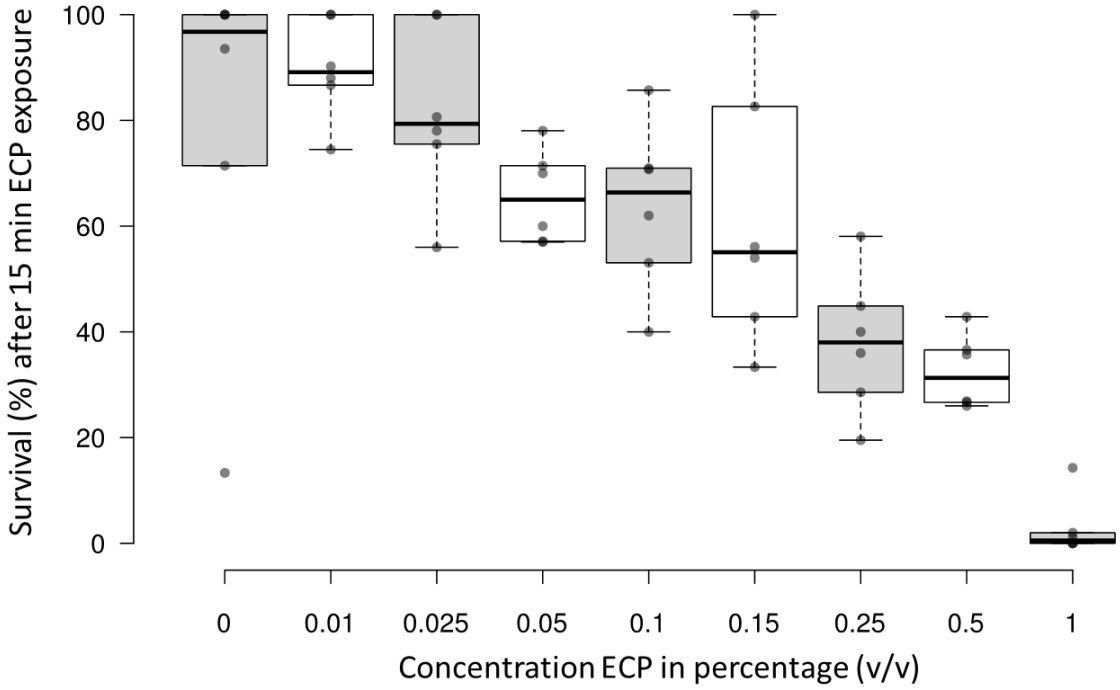

Figure 9: Percentage of survival of TR4 chlamydospore exposed for 15 min to concentrations of ECP. Center lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles data points are plotted as open circles. n = six sample points (three batches with replicates).

Prolonged exposure of the chlamydospore solution in the presence of ECP reduced the number of surviving chlamydospores witnessed as growing colonies after incubation. After 30 min, no colonies were obtained for 1% ECP and the average reduction for 0.5% ECP was over 80% on average (Figure 10). With a 60 min incubation, 0.5% ECP was 100% effective (Figure 11), but the same level efficacy for the next concentration (0.25%) was achieved only after 200 min (Figure 12 & 13) together with 0.15% ECP. At the longest time tested (300 min), the minimal required concentration for ECP was found to be 0.1% (Figure 14).

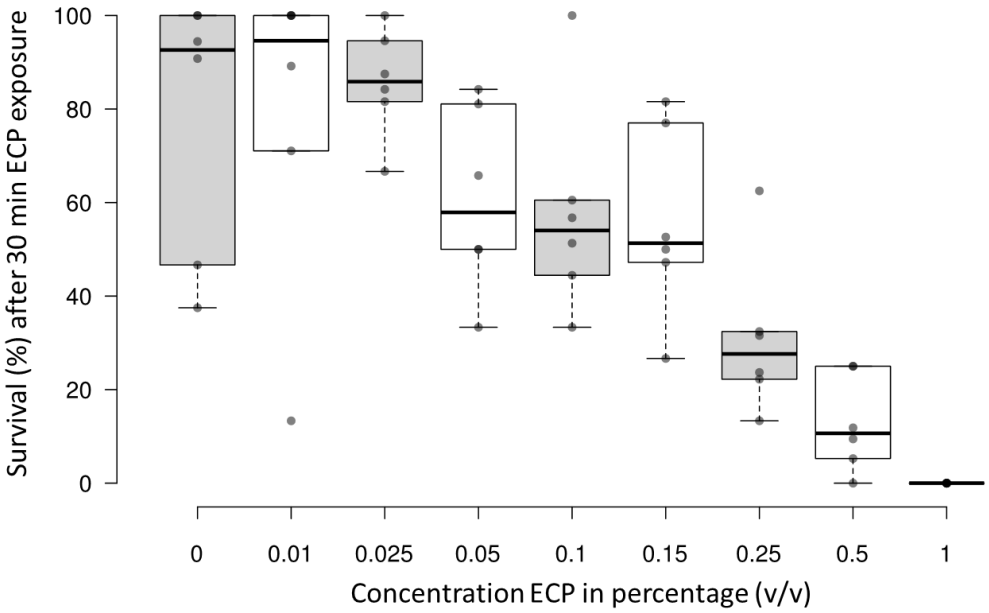

Figure 10: Percentage of survival of TR4 chlamydospore exposed for 30 min to concentrations of ECP. Center lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles data points are plotted as open circles. n = six sample points (three batches with replicates).

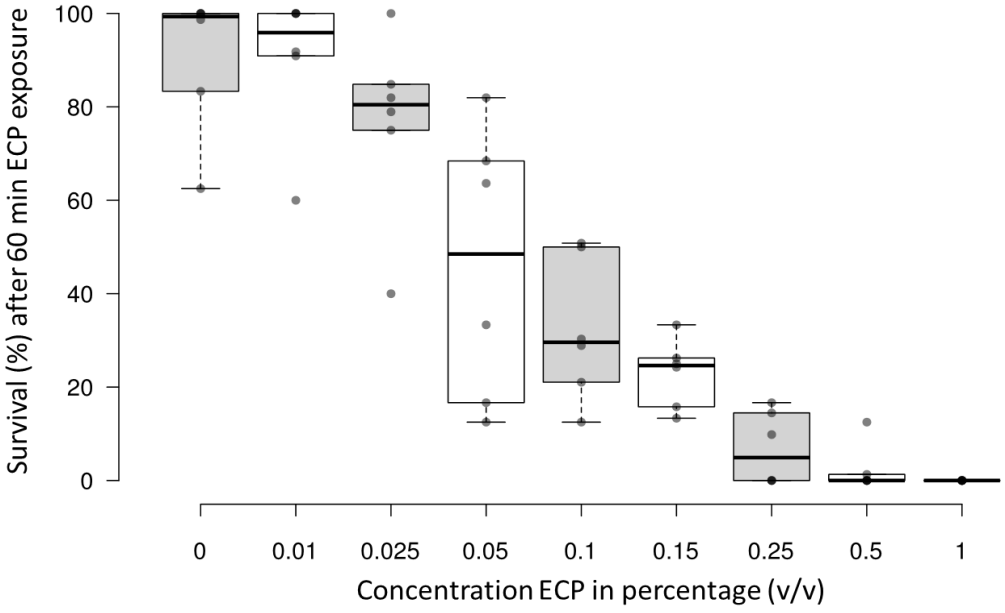

Figure 11: Percentage of survival of TR4 chlamydospore exposed for 60 min to concentrations of ECP. Center lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles data points are plotted as open circles. n = six sample points (three batches with replicates).

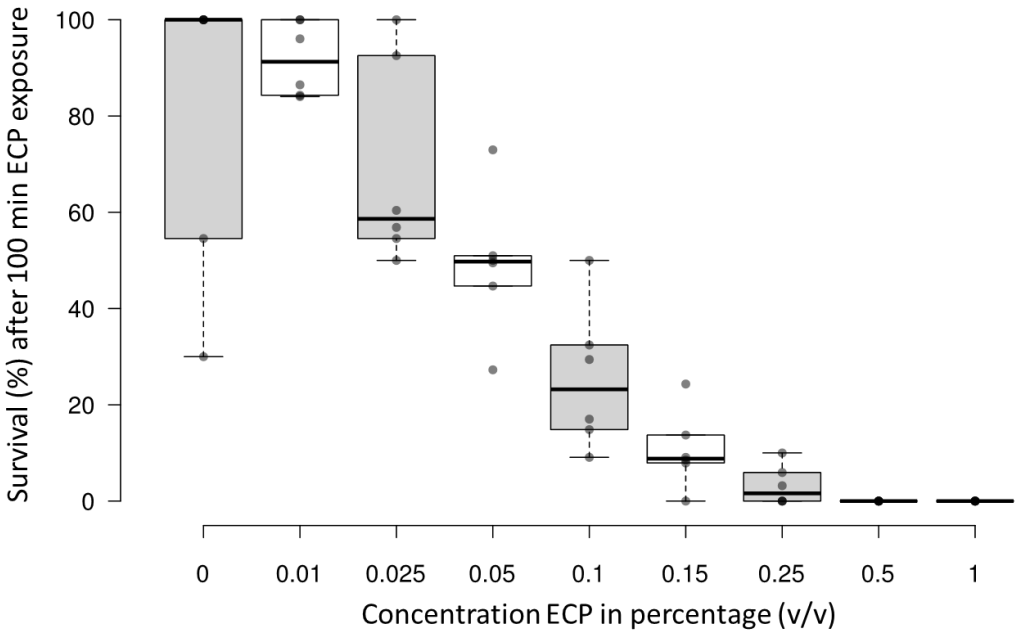

Figure 12: Percentage of survival of TR4 chlamydospore exposed for 100 min to concentrations of ECP. Center lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles data points are plotted as open circles. n = six sample points (three batches with replicates).

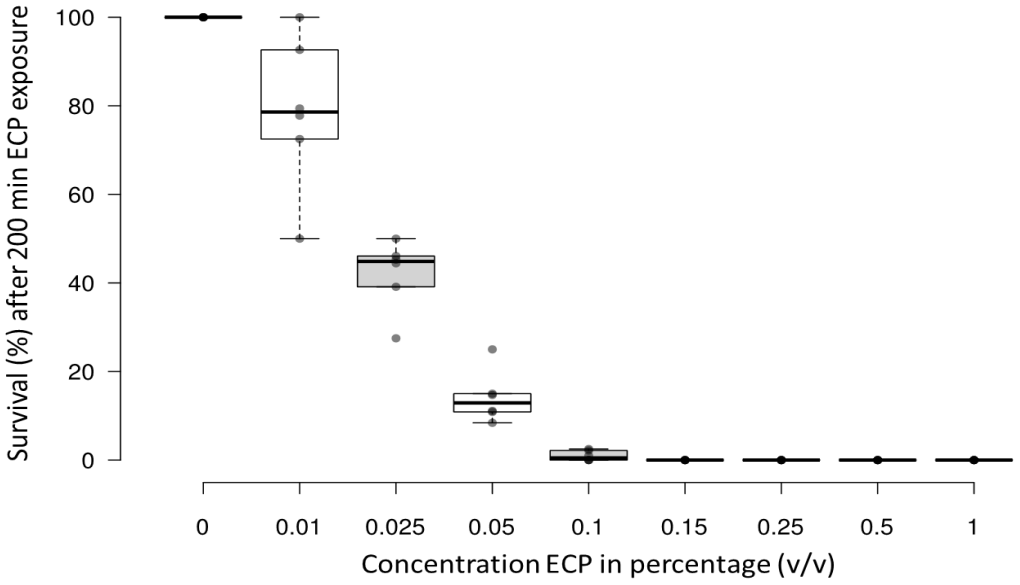

Figure 13: Percentage of survival of TR4 chlamydospore exposed for 200 min to concentrations of ECP. Center lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles data points are plotted as open circles. n = six sample points (three batches with replicates).

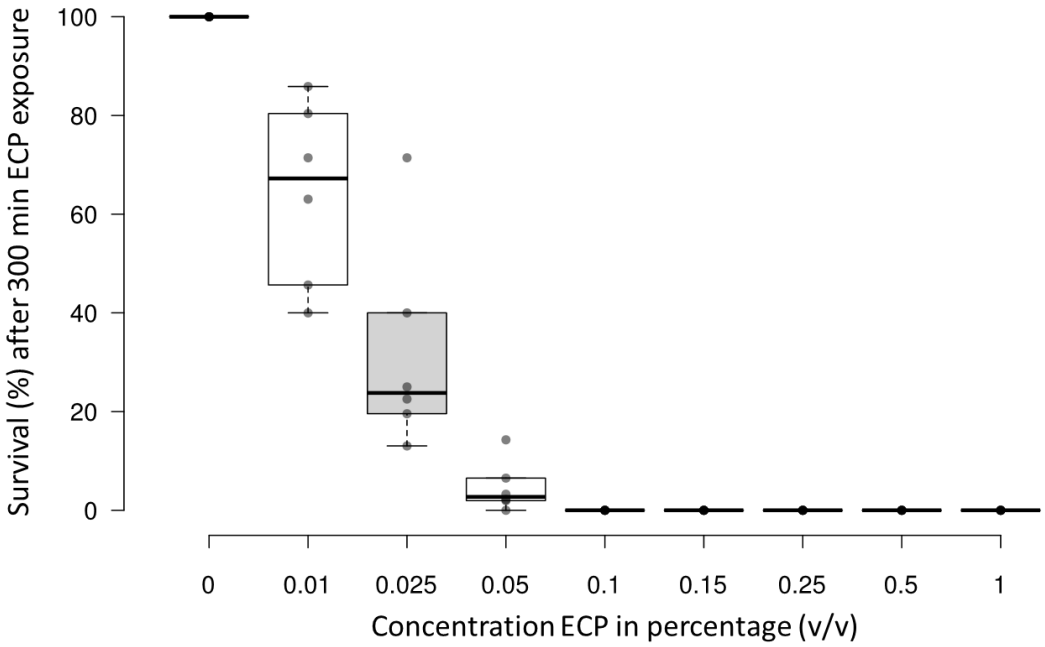

Figure 14: Percentage of survival of TR4 chlamydospore exposed for 300 min to concentrations of ECP. Center lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles data points are plotted as open circles. n = six sample points (three batches with replicates).

For all concentrations, decrease of surviving chlamydospores was thus consistently observed in time, as visualized for 0.1% (Figure 15).

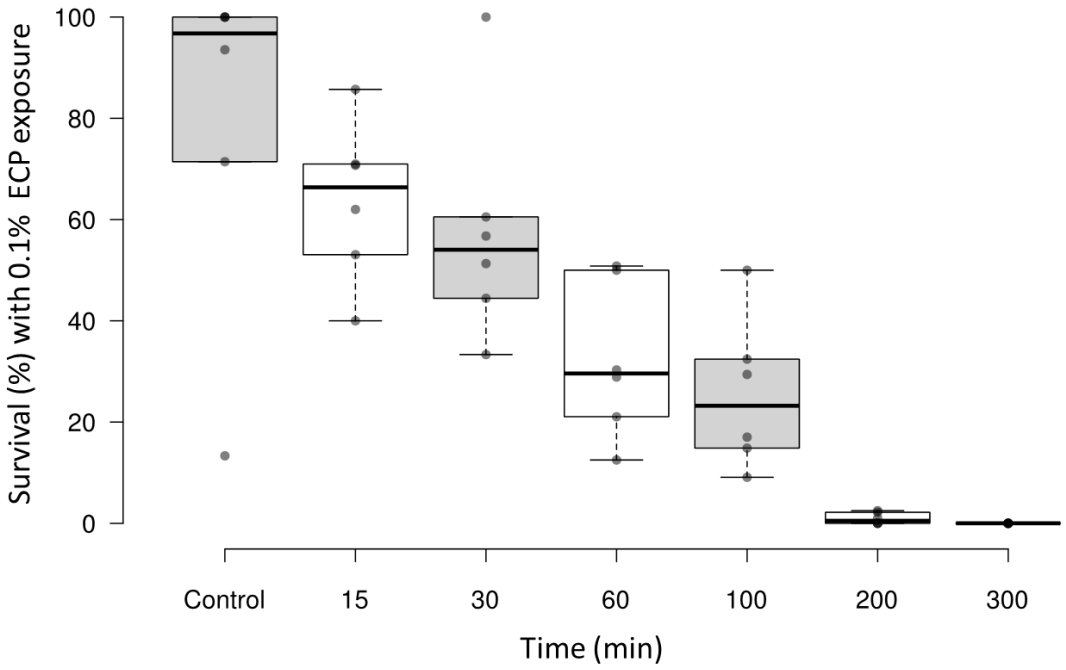

Figure 15: Percentage of survival of TR4 chlamydospore exposed to a concentrations of 0.1% ECP in time. Control represents the number of colonies in the absence of ECP at 15 min. Center lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles data points are plotted as open circles. n = six sample points (three batches with replicates).

Chlamydospores in soil

Next to chlamydospores being present in water, they also are formed and embedded in soil(-particles). To test whether ECP can penetrate soil, soil samples were taken, in which chlamydospores were grown, and placed under a volume of ECP solution. An overview of the incubation is shown in Figure 16.

Figure 16: Overview of steps during incubation of soil. Top panel: titer plate filled with amounts of TR4 containing soil particles.
Middle panel: titer plate filled with the soil and the ECP solutions. Three replicates per solution.
Lower panel, close-up of the highest concentration tested, clear interaction (bubble formation) observed at all higher concentrations tested.

EcoClearProx efficacy against TR4

Samples were taken at various times and processed as described in Materials and Methods. As indicated before, distribution of chlamydospores was very unequal (as expected) due to uneven colonization of soil particles and lumps, so the experiment was repeated in total 5 times, with concentrations of ECP up to 1%. Plates with the resulting colonies were scored on presence or absence in a range of 0 to 1% of ECP, and at various times up to 3 days. It should be noted that the composition of the soils in which the chlamydospores are varied, as observed in Figure 16, with some have a more sandy structure whereas others have are more lumps. Overall, there seems a tendence of reduction of resulting colonies in time at the higher concentrations of ECP. More importantly, resulting colonies are observed, even after 3 days of incubation with ECP. In some cases the highest number of growing colonies were indeed found with high concentrations of ECP (e.g. 0.5% ECP, 240 min), depending on the conditions described above. A number or representative pictures is shown in Figure 17 with for the A batch, a decrease of CFU while in the C batch an "increase" is observed. For the B batch several plates turned out to be empty despite the material analysed but, as indicated in the inset some of the plates showed colony development. All together the results suggest that higher concentrations of ECP are capable to reduce the number of surviving colonies, potentially due to the eradication of chlamydospores positioned at the outside of soil particles, but this is not fully effective in full eradication up to at least 1%, even after a significant amount of time.

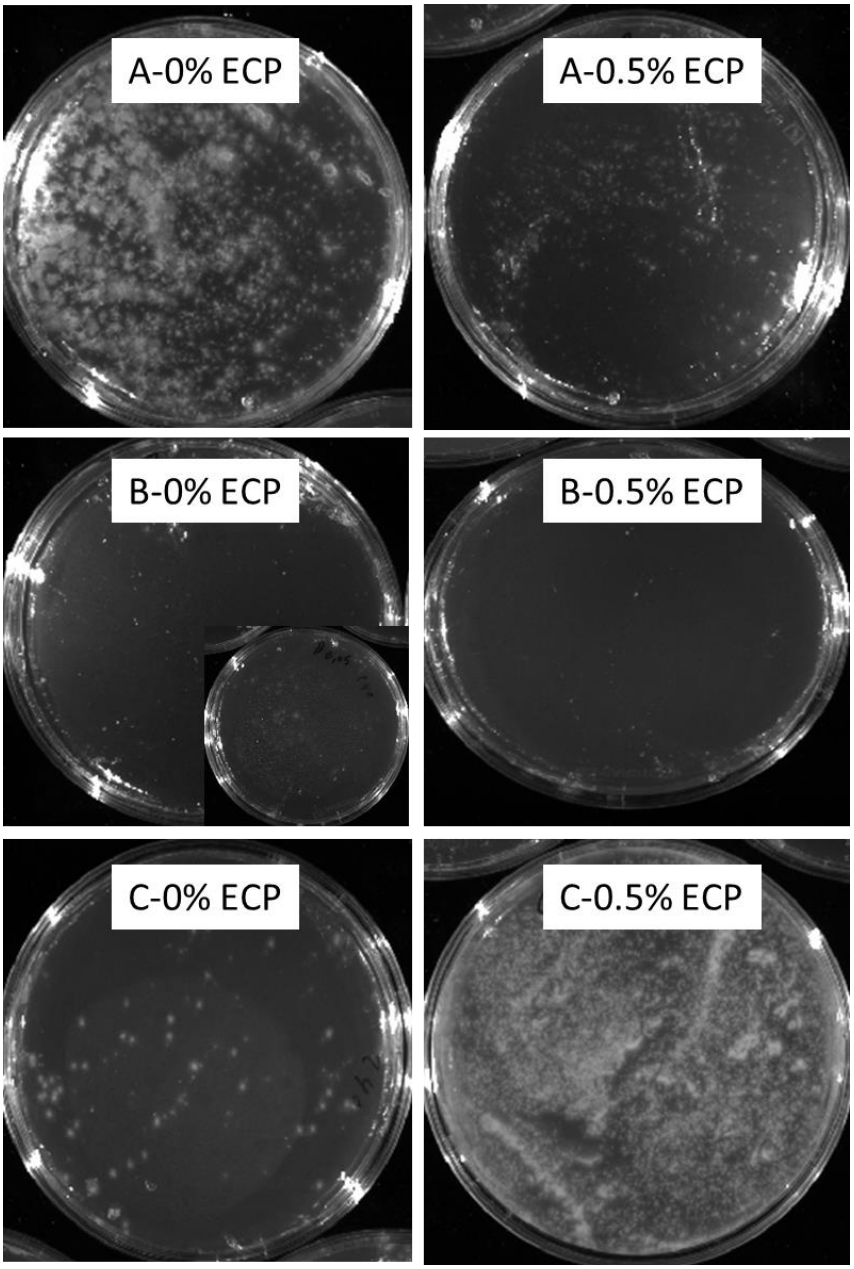

Figure 17: Shown are representative plates after 240 min of incubation with no (0) or 0.5% ECP. Batches of chlamydospores are indicated as A-C. Due to unequal distribution among samples taken, variation occurs due to presence of chlamydospores or to the effect of ECP. The inset of B-0%ECP shows ~20 CFU after incubation for 240 min with 0.05% ECP , confirming the presence of chlamydospores in the starting material (also at other concentrations and their duplicates colonies were grown). Detailed quantification was not effective.

Conidia in soil

Since conidia are sensitive to relative low concentrations of ECP (Figures 1- 6), columns with soil well mixed with TR4 conidia were challenged overnight with water (control) or a 1% ECP solution. Notably, reactions during the addition of ECP were observed (formation of bubbles), clearly showing the effect of the ECP addition. Soil samples were taken and surviving conidia were extracted from the soil and plated on PDA plates. A representative result is shown with representative pictures in Figure 18. So, qualitatively for all samples, colonies were grown. However, in comparison with the water treated columns the ECP treated columns showed visually a slight reduction of the resulting number of colonies. The experiment was repeated with a slightly different mixture of soil (less dense), with similar outcome, establishing the validity of the results. These results suggest that the concentration of ECP used (1%) is insufficient to deeply penetrate soil that was inoculated with TR4 conidia.

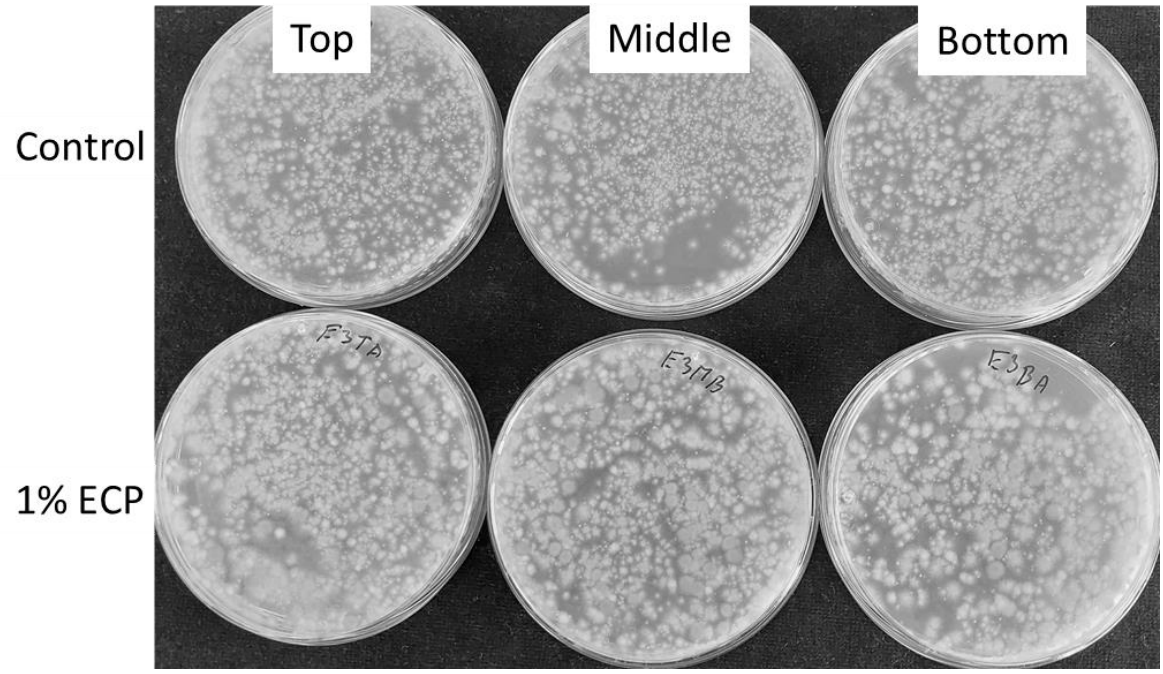

Figure 18: Effect of ECP on conidia in a soil column. Columns were generated with autoclaved standard soil and mixed with TR4 conidia. After overnight exposure to water or ECP samples were taken and conidia released into solution and subsequently plated on PDA plates. The resulting colonies from one control and one ECP treated column is shown representative for 5 columns with independent conidia batches. Top part are the controls, lower part ECP treated. Soil samples were taken from top, middle and bottom of the column.

Discussion

In this report the effect of EcoClearProx® on TR4 conidia and chlamydospores in solution and soil have been further tested *in*-vitro. *Fusarium odoratissimum* TR4 conidia were shown before to be sensitive to ECP in a previous quicktest (Meijer 2021) and more extensively based on time and concentration during a full test (Meijer 2022). This resulted in the observation that ECP is a promising product when the concentration and time are provided to eradicate conidia. Since it is a stable product, long exposure times are indeed permitted and thus, relative low levels of ECP are function, e.g. 0.01% ECP is nearly fully effective after 300 min towards a conidial culture although this is dependent on the amount of conidia (representing organic load). In this study, the focus was to establish functionality towards TR4 chlamydospores. They represent the more resilient spore of *Fusaria* species, that can stay dormant for years in e.g. the soil.

In order to validate the ECP results from the previous study (Meijer 2022), a single replicate experiment was performed as previously described. The results were merged with the previous duplicate experiment (Meijer, 2022). As expected, the results are in line with the previous report and confirm and validate the outcome of these experiments. ECP was very efficient towards the conidia during short incubation times at higher concentrations, and during longer incubation times at lower concentrations. Again, the 0.025% ECP is fully functional after an exposure time of 200 min which is a 40 fold reduction of the required 1% ECP that was needed to be effective within 15 min. Notably the efficacy per concentration versus time is not fully linear in such a way that lower concentrations are more effective than the fold dilution multiplied by the time of incubation. Thus, time is more effective than the ECP concentration. Another conclusion from this repeat is that the ECP is extremely stable under laboratory conditions (~20°C), which is not generally observed for other H_2O_2 based products. Stability of ECP should be monitored during field usage where temperatures are higher.

Since the conidia are very sensitive to ECP concentrations in solution, the efficacy of ECP against chlamydospores in solution was further tested. Previous studies already showed that TR4 chlamydospore are generally less sensitive to many compounds including various fungicides when challenged for a (relatively) short period of time (e.g. [19]). Indeed, from results in this study a clear shift is observed to higher concentrations necessary for ECP and more prolonged incubation in order to be effective against TR4 chlamydospores in solution. Whereas after 300 min 0.025% of ECP is necessary for conidia, the percentage of ECP needs to be increased to 0.1% to eradicate chlamydospores at similar time points. It should be noted that the chlamydospore concentrations might be not equally correlated with the biological load as observed for conidia and likely less biomass is present in the chlamydospore trials. Since the ECP efficacy is affected by such load this might influence the results. Nevertheless, these results clearly show that ECP is effective against chlamydospores in solution and that reduced levels of ECP can be used if time permits and stresses again the time dependency for its efficacy to these fungal propagules.

The next step was to observe if ECP is capable to penetrate soil particles and lumps. This was tested by placing soil containing conidia under ECP solution, without stirring. In previous tests, this was shown to be extremely challenging since many products either are "consumed" during the penetration process or lack compounds that facilitate the penetration process. With several repeats on various batches of chlamydospores, we conclude that ECP, at a concentration up to 1%, is unable to consistently eradicate chlamydospores during all repeats. Potentially, the various types of soil might influence some the minor reduction observed but it stresses that under field conditions, ECP is insufficient to eradicate the TR4 chlamydospores present. It was observed, although not quantified, that in general slightly reduced levels of surviving colonies were obtained after a period of incubation but it was also noted in one case that the plate with most colonies was retrieved after long incubation with a high concentration of ECP. These results match results of fungicide treatments using the same approach which showed that those functional against chlamydospores in suspension were at least strongly reduced in efficacy towards chlamydospores in soil. In fact, none of the non-corrosive disinfectants was capable to eradicate the spores except at extremely high concentrations (up to 6% v/v) making them inappropriate to be used for such treatment in the field. Similar conclusions were drawn for other *Fusarium* species once spores were soil embedded ([19] and references therein).

Since conidia were more sensitive to ECP than chlamydospores, the column experiment was thought to provide more insight. The conidia are relatively short mixed with soil, ensuring that they are not completely embedded into particles and their distribution in the soil is more equal. As stressed before, many products loose activity extremely quickly during their progress into the soil. Here we tested whether conidia, mixed in soil (so not "inserted" into soil particles) were sensitive to the ECP concentration (1%, which is 40* the minimal required concentration in liquid) that was applied from the top. With standard potting soil (double autoclaved), it was aimed that isolation of remaining TR4 from just under the top, middle and bottom of the column would clearly show an effect of the ECP application. However, in all cases (control and 1% ECP), TR4 colonies grew from all the soil samples taken. This suggests that even under flow through conditions the ECP is unable to reach all conidia. Indeed, as observed with the chlamydospores, some reduction of the remaining conidia (witnessed as growing colonies) was observed, resulting as slightly bigger colonies, which is not sufficient under field conditions to reduce the pathogen's load significantly.

One of the main sayings in agricultural systems is "come clean, go clean" and the results for both chlamydospores and conidia in soil is an excellent reminder also to remove soil (particles) from footwear, machinery, and vehicles before any disinfection.

Our results clearly indicate that the application of ECP in the field should revolve around its usage in liquid systems such as the irrigation system. Water taken in, which might be contaminated with TR4 (or probably other (pathogenic) micro-organisms), would benefit

from an addition of ECP to reduce any potential threat. Notably, pretreatment of the water should involve a filtering (or centrifuging) step, to remove all (non-)organic load as much as possible. This aids in the reduction of biomass, the risk of TR4 being embedded in ECP impenetrable soil particles and lowers the necessary amount of ECP needed. Furthermore, ECP should be allowed sufficient time to be effective at the lowest concentration possible, achievable by the usage of water basins, water silos or reservoirs. That also enables the ECP to wear out in time before application in the field. Notably, in the studies so far, long term application of ECP to (banana) plants or the regular microbiome was not analyzed. Such a study could be conducted to ensure the impact of ECP and highlight potential risks for direct application and/or the benefits on (potential) surface disinfection.

Of course, Fusarium wilt is not the only major disease in banana. Others are also caused by fungi, such as Black sigatoka, a leaf disease caused by the plant pathogen *Pseudocercospora fijiensis* whereas in various parts of the banana producing region bacterial diseases might play a major role. For example Moko disease, a devastating bacterial disease on banana caused by *Ralstonia solancearum* race 2. Notably the latter enters the banana farms also by irrigation water and a future study might aim at the effect of ECP on this banana pathogen to validate that this bacterium is also eradicated from contaminated irrigation water.

In conclusion, our reports, describing the efficacy of ECP towards TR4 propagules, clearly show and confirm that ECP is very effective against TR4 conidia and chlamydospores when these are in suspension. Notably, for chlamydospores ECP is effective albeit higher concentrations that are needed for conidia, taken into account also the combination of proper exposure time and the amount of biomass present in a given solution. When it comes to conditions in which soil is playing a role, the efficacy of ECP is strongly reduced. The presence of soil (clay, sand, organic material, other) is sufficient to shield TR4 propagules and as a result, only a minor reduction of surviving colonies is observed and eradication is not established. Therefore, ECP in its current formulation, shows only sufficient efficacy in watery systems and its application benefits from adequate filtering in order to reduce biomass and (among other) soil particles. As a result the dissemination by irrigation of TR4 and potentially several other threats to banana plants can be strongly reduced or fully halted. In order to achieve this all other suggested quarantine measures necessary need to be in place and executed correctly to avoid parallel entry into the farm.

Future work or research on improvement of ECP could be targeting the penetration capabilities into soil particles. Furthermore, testing under field conditions as an additional step in the water cleaning process is envisioned in which also other plant pathogens can monitored to determine if the usage of an ECP step is beneficial to avoid dissemination via irrigation water.

References

1. Maryani N, Lombard L, Poerba YS, Subandiyah S, Crous PW, Kema GHJ. Phylogeny and genetic diversity of the banana Fusarium wilt pathogen *Fusarium oxysporum* f. sp. *cubense* in the Indonesian centre of origin. Stud Mycol. 2019;92:155-94. Epub 2018/08/21. doi: 10.1016/j.simyco.2018.06.003. PubMed PMID: 30122796; PubMed Central PMCID: PMCPMC6086327.
2. Ordóñez N, Seidl MF, Waalwijk C, Drenth A, Kilian A, Thomma BP, et al. Worse comes to worst: bananas and Panama disease-When plant and pathogen clones meet. PLoS Path. 2015;11(11):e1005197. Epub 2015/11/20. doi: 10.1371/journal.ppat.1005197. PubMed PMID: 26584184; PubMed Central PMCID: PMC4652896.
3. Ploetz RC. Panama disease, an old nemisis rears its ugly head: Part 1, the beginnings of the banana export trades. Plant Health Progress. 2005;doi:10.1094/PHP-2005-1221-01-RV.
4. Ordoñez N, Garcia FA, Laghari H, Akkary M, Harfouche EN, Al Awar BN, et al. First report of *Fusarium oxysporum* f. sp. *cubense* tropical race 4 causing Panama disease in Cavendish bananas in Pakistan and Lebanon. Plant Dis. 2015:http://dx.doi.org/10.1094/PDIS-12-14-356-PDN. doi: 10.1094/pdis-12-14-1356-pdn.
5. Maymon M, Sela N, Shpatz U, Galpaz N, Freeman S. The origin and current situation of *Fusarium oxysporum* f. sp. *cubense* tropical race 4 in Israel and the Middle East. Sci Rep. 2020;10(1):1590. Epub 2020/02/02. doi: 10.1038/s41598-020-58378-9. PubMed PMID: 32005853.
6. Özarslandan M, Akgül DS. First Report of *Fusarium oxysporum* f. sp *cubense* Race 4 causing Fusarium wilt disease of banana in Turkey. Plant Dis. 2019;104(3):974. doi: https://doi.org/10.1094/PDIS-09-19-1881-PDN.
7. Thangavelu R, Mostert D, Gopi M, Devi PG, Padmanaban B, Molina AB, et al. First detection of *Fusarium oxysporum* f. sp. *cubense* tropical race 4 (TR4) on Cavendish banana in India. Eur J Plant Pathol. 2019;154(3):777-86. doi: 10.1007/s10658-019-01701-6.
8. Zheng S-J, García-Bastidas FA, Li X, Zeng L, Bai T, Xu S, et al. New geographical insights of the latest expansion of *Fusarium oxysporum* f.sp. *cubense* Tropical Race 4 into the Greater Mekong Subregion. Front Plant Sci. 2018;9:457. doi: 10.3389/fpls.2018.00457.
9. Viljoen A, Mostert D, Chiconela T, Beukes I, Fraser C, Dwyer J, et al. Occurrence and spread of the banana fungus Fusarium oxysporum f. sp. cubense TR4 in Mozambique. S Afr J Sci. 2020;116(11/12). doi: 10.17159/sajs.2020/8608.
10. van Westerhoven AC, Meijer HJG, Houdijk JdlP, E., Luntadila Matabuana E, Seidl MF, Kema GHJ. Dissemination of Fusarium wilt of banana in Mozambique caused by *Fusarium odoratissimum* Tropical Race 4. Plant Dis. 2022;doi.org/10.1094/PDIS-07-22-1576-SC.
11. Aguayo J, Cerf-Wendling I, Folscher AB, Fourrier-Jeandel C, Ioos R, Mathews MC, et al. First report of *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 (TR4) causing banana wilt in the Island of Mayotte. Plant Dis. 2021:219. doi: 10.1094/PDIS-06-20-1196-PDN.
12. Garcia-Bastidas F, Quintero-Vargas J, Ayala-Vasques M, Schermer T, Seidl MF, Santos-Paiva M, et al. First report of Fusarium wilt Tropical Race 4 in Cavendish bananas caused by *Fusarium odoratissimum* in Colombia. Plant Dis. 2019;104(3):994. doi: https://doi.org/10.1094/PDIS-09-19-1922-PDN.
13. Acuna R, Rouard M, Leiva AM, Marques C, Olortegui JA, Ureta C, et al. First Report of *Fusarium oxysporum* f. sp. *cubense* Tropical Race 4 Causing Fusarium Wilt in Cavendish Bananas in Peru. Plant Dis. 2022:PDIS09211951PDN. Epub 2021/12/18. doi: 10.1094/PDIS-09-21-1951-PDN. PubMed PMID: 34918946.
14. Reyes-Herrera PH, Torres-Bedoya E, Lopez-Alvarez D, Burbano-David D, Carmona SL, Bebber DP, et al. Genome sequence data reveal at least two distinct incursions of the tropical race 4 (TR4) variant of Fusarium wilt into South America. bioRxiv. 2022;doi.org/10.1101/2022.01.17.476647. doi: 10.1101/2022.01.17.476647.
15. Dita M, Barquero M, Heck D, Mizubuti ESG, Staver CP. Fusarium Wilt of Banana: Current Knowledge on Epidemiology and Research Needs Toward Sustainable Disease Management. Front Plant Sci. 2018;9:1468. Epub 2018/11/09. doi: 10.3389/fpls.2018.01468. PubMed PMID: 30405651; PubMed Central PMCID: PMCPMC6202804.
16. Salacinas M. Spot on: managing Panama disease of banana in the Philippines. Wageningen: Wageningen University and Research; 2019.
17. Scarlett K, Collins D, Tesoriero L, Jewell L, van Ogtrop F, Daniel R. Efficacy of chlorine, chlorine dioxide and ultraviolet radiation as disinfectants against plant pathogens in irrigation water. Eur J Plant Pathol. 2016;145(1):27-38. doi: 10.1007/s10658-015-0811-8.
18. García-Bastidas FA, Van der Veen AJT, Nakasato-Tagami G, Meijer HJG, Arango-Isaza RE, Kema GHJ. An Improved Phenotyping Protocol for Panama Disease in Banana. Front Plant Sci. 2019;10:1006. Epub 2019/08/27. doi: 10.3389/fpls.2019.01006. PubMed PMID: 31447871; PubMed Central PMCID: PMCPMC6691145.
19. Salacinas M, Meijer HJG, Mamora SH, Corcolon B, Mirzadi-Gohari A, Ghimire B, et al. Efficacy of disinfectants against Tropical Race 4 causing Fusarium wilt in Cavendish bananas. Plant Dis. 2022;106:966-74. doi: 10.1094/PDIS-08-20-1814-RE.
20. Meijer HJG. Efficacy of EcoClearProx against conidia of Tropical Race 4. 2022.