Efficacy of Disinfectants Against Tropical Race 4 Causing Fusarium Wilt in Cavendish Bananas

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Abstract

Fusarium wilt of banana (FWB), caused by a suite of *Fusarium* fungi, is among the most devastating plant diseases. The iconic FWB epidemic in the previous century lasted decades and was caused by so-called Race 1 strains that wiped out the dominant 'Gros Michel' banana plantations across Central America. Eventually, it was stopped because the Race 1-resistant 'Cavendish' banana variety replaced 'Gros Michel', which dominates global production (>50%) and trade (>95%). However, presently, the so-called Tropical Race 4 (TR4) threatens plantations of 'Cavendish' and many other banana varieties around the globe. Prevention is the first line of defense against the spread of TR4. Therefore, many disinfection units are installed to prevent the entry of TR4 in banana plantations. These foot and tire baths are filled with disinfectants, but limited knowledge is available on their efficacy. In this project, we evaluated 13 disinfectants commonly used in the Philippines. Our results show that the efficacy of these products depends on the type of fungal spores, the exposure time, and the replenishment frequency of the disinfection units. The resting spores of TR4 were resistant to all but one – unfortunately corrosive – disinfectant. Furthermore, we show that the actual contact time with disinfectants was far below the thresholds determined in laboratory experiments. Finally, muddy disinfection units reduced the efficacy of disinfectants. Taken together, we conclude that practices are inadequate to prevent the dissemination of TR4.

Keywords: corrosiveness, disinfectant, efficacy, propagule type, quarantine baths, TR4

The United Nations sustainable development goals indicate that crop production has to increase to meet future demands for food and feed (https://www.un.org/sustainabledevelopment/). With a continuously increasing global population, agricultural production has to increase while yield and postharvest losses should be reduced. Plant diseases in staple crops are of great concern with the reduced efficacies of disease control agents contributing to increasing yield gaps (Bebber et al. 2014; Fisher et al. 2012). Bananas (genus *Musa*) rank among the top-10 most important food crops with estimated annual consumption of 100 billion fingers as staple food or fresh fruit (Aurore et al. 2009; Churchill 2011). They include dessert and cooking types and are extensively grown in the tropics and subtropics, either in backyards, mixed-cropping systems, small-scale farms, or industrial monocrop plantations. Overall, 85% of the total production is for local consumption as staple crop and 15% enters international markets (FruitTrop 2017). In 2019, just over 20 million tons were exported (FAO 2020). The export market is dominated by Cavendish varieties (~90%), underscoring their importance as cash crop for agriculture-based economies.

The genus *Fusarium* contains a wide range of soilborne fungal pathogens, of which several *formae speciales* cause wilting diseases in manifold crops (Chakrabarti 2013). Fusarium wilt of banana (FWB) is one of the most devastating plant diseases, exemplified by the epidemic in 'Gros Michel' banana plantations in Panama in the mid-1900s by so-called Race 1 strains. The outbreak paved the way for the conversion of the 'Gros Michel'-based export trade to the contemporary 'Cavendish'-based industry (Pérez-Vicente 2004; Stover 1962), which quenched the epidemic due to the resistance of 'Cavendish' to FWB Race 1 strains. The re-emergence of FWB in 'Cavendish' bananas in the 1960s in Taiwan (Ploetz 2015) has now developed into a new pandemic that causes havoc in the industry (Aguayo et al. 2020; Chittarath et al. 2018; García-Bastidas et al. 2013, 2019a; Hung et al. 2018; Mostert et al. 2017; Ordóñez et al. 2015a; Özarslandan and Akgül 2019; Viljoen et al. 2020; Zheng et al. 2018). Taxonomical insights showed that the Race 1 strains that caused FWB in 'Gros Michel' comprise at least seven different *Fusarium* species, whereas FWB in 'Cavendish' and many local varieties (García-Bastidas 2019) is caused by Tropical Race 4 (TR4), a single clone that belongs to *Fusarium odoratissimum* (Maryani et al. 2019). So-called Subtropical Race 4 strains cause FWB in 'Cavendish' under abiotic stress conditions and are genetically different from TR4 (Ordóñez 2018). Unfortunately, there are no effective and long-term control measures to manage FWB. Moreover, contrary to the previous epidemic in 'Gros Michel', there is presently no resistant, widely accepted replacement for 'Cavendish' bananas. Hence, investigating methods to prevent TR4 incursions and strategies to manage FWB, once it occurs, are crucial for the global industry and millions of smallholders.

TR4 produces three types of spores: chlamydospores, macroconidia, and microconidia. Conidia are produced in large quantities in the xylem vessels and facilitate rapid distribution through the entire host plant (Pegg et al. 2019). Chlamydospores on the other hand, are thick-walled and are formed on senescing hyphae or germ tubes

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and in degrading host tissues (Pegg et al. 2019). They can persist in the soil in the absence of the susceptible host (Ploetz 2015). The primary risk factors for introducing TR4 into new areas are the transport of infected planting material and movement of infested soil. Plant material can be rhizomes or suckers (with attached soil) contaminated with mycelium, conidiospores, and/or chlamydospores. Hence, regional, international, and intercontinental traffic is a major risk factor for TR4 dissemination into new areas (García-Bastidas et al. 2013, 2019a; Ordóñez et al. 2015a; Özarslandan and Akgül 2019; Zheng et al. 2018). Robust biosecurity policies and implementing disease management strategies are top priorities aimed to control and limit the spread of inoculum in and across production areas. In the Philippines, the disease continues to spread after its incursion in the early 2000s (Molina et al. 2009), despite the implementation of biosecurity measures, employing disinfectants to decontaminate tools, footwear, vehicles, and equipment and planting less-susceptible Cavendish somaclones (Montiflor et al. 2019).

Disinfectants are organic or chemical agents used to decontaminate surfaces, footwear, or farm equipment (Cheah et al. 2009). The efficacy of disinfectants depends on their concentration (dilution effect), contact time, active ingredients, formulation, the presence of organic matter (acting as physical barrier for surface contact), degradation by chemical reactions, and the tissue of the target organism (Best et al. 1990; Gehr et al. 2003; Russell 2002; Rutala et al. 2008). Several in vitro studies have been conducted to verify the most effective disinfectant on different phytopathogenic propagules of the causal *Fusarium* spp. Nel et al. (2007) showed that quaternary ammonium-based disinfectants such as Sporekill and Prazin can easily (30-s exposure time) inhibit conidial growth of Subtropical Race 4 *Fusarium* strains (reclassified as *Fusarium phialophorum*; Maryani et al. 2019). Meldrum et al. (2013) confirmed the efficacy of Sporekill against TR4 conidia after at least 30-s exposure time. However, the efficacy of disinfectants is influenced by their access to propagules of *Fusarium* spp. in their natural habitat, such as soil. Therefore, it is of great importance to test efficacies on TR4 propagules in soil to simulate in-field scenarios in addition to in vitro conditions.

In this study, we tested the efficacy of disinfectants that are commercially available in the Philippines against TR4 under in vitro (mycelium, conidia, and chlamydospores) and in situ (chlamydospores in soil) conditions at various concentrations and exposure times. As the deployment of disinfectants in the field also depends on their corrosiveness (Bennett et al. 2011; James et al. 2012; Meldrum et al. 2013), we evaluated their capacity to oxidize aluminum (Al) and iron (Fe). Finally, we monitored entrance gates at a commercial farm to determine the actual exposure time to disinfectants while passing through obligatory foot and tire baths, and calculated the cost of maintaining quarantine baths in the field. These data provide an analysis of necessary versus actual quarantine measures to minimize on-farm, regional, and international dissemination of TR4.

Materials and Methods

TR4 isolate and inoculum preparation. Isolate Phil 2.6c from Davao del Norte, Mindanao, the Philippines, was previously characterized as TR4 by whole genome sequencing (Ordóñez et al. 2015b) and was therefore used throughout this study. A monoconidial culture of this isolate was grown on Komada medium (Komada 1975) at 25°C in the dark for 5 days, and mycelial plugs (0.5 cm diameter) from the margin of the culture as well as conidial suspensions (2×10^5 spores/ml), produced as described by García-Bastidas et al. (2019b), were used for in vitro efficacy tests. Chlamydospores were prepared following the protocol described by Borines et al. (2007) with modifications. 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 presence of chlamydospores was determined by microscopic observation and their concentration in CFU/g soil (CFU per gram of soil) was determined by a plate dilution technique on potato dextrose agar plates (PDA; Himedia, India; Gilchrist et al. 1973).

Disinfectants. Thirteen disinfectants, distributed over five chemical groups, were used in this study (Table 1) and comprise the major commercially available disinfectants in the Philippine market. All

Table 1. Disinfectants evaluated in vitro against *Fusarium odoratissimum* Tropical Race 4

Chemical group	Local trade name	Active ingredient	Strength	Recommended concentration[a]	Manufacturer
Quaternary ammonium	Biocit	Benzalkonium chloride	40%	3%	FKA Agri-Chemical Corporation (Philippines)
	Germ-X	Benzalkonium chloride	50%	3%	Shanghai Bosman Industries Co. Ltd. (China)
	GUAA	N-alkyl(C12-C16)(benzyl) dimethylamonium chloride	0.50 g/100 g	1%	GUAPEX (Czech Republic)
	Ivagard[b]	Di-C8-10-alkyldimethylchlroides + alkyl dimethyl benzyl ammonium chloride	48 + 32%	1%	Lonza Ltd. (Switzerland)
	Agresource	Benzalkonium chloride	40%	3%	Agresource Inc. (Philippines)
	Microvenum	Didecyl dimethyl ammonium chloride	80%	2%	Connect Chemicals GmbH (Germany)
	Gentrol	Benzalkonium chloride	40%	3%	TLV Agro-Sales Corporation (Philippines)
	Multisept	Quaternary ammonia (2) + aldehyde + alcohol	–	1%	Polara Chemical Corporation (Philippines)
Halogens	Hi-Chlon 70	Calcium hypochlorite	70%	6 ppm	Nippon Soda Co. Ltd.(Japan)
	Vanodine	Phosphoric acid + sulphuric acid + iodine + alcohol	20/10/5/20%	1%	Evans Vanodine International (England)
Alcohols	Ethanol	Ethyl alcohol	99%	None	Scharlab S.L. (Spain)
Diamidines	Formo	2,2-Dibromo-3-nitrilopropionamide	20%	1%	Texicon Agriventures Corporation (Philippines)
Aldehyde	Formalin	Formaldehyde	37%	None	–

[a] Recommended concentration of solution per liter.
[b] International trade name is Bardac 208M.

were provided as liquid formulations, except Hi-Chlon 70, which was provided as a powder. Before each experiment, fresh dilutions were prepared with sterile distilled water.

Experimental design. Inoculum source (mycelia, conidiospores, or chlamydospores in suspension and soil), disinfectant concentration, and exposure time were used as variables in a completely randomized block statistical design for this study. Disinfectant concentrations depended on the used inoculum sources. Incubations in sterile distilled water or on unamended PDA served as controls. Each experiment was conducted twice with five biological replicates for each of the above-mentioned variables.

Testing efficacy on mycelial growth. For efficacy tests on mycelial growth of TR4, 11 disinfectants were used as we excluded ethyl alcohol and Hi-Chlon 70. The former hampers media solidification at high concentrations and the latter was only available as a powder formulation, which is incompatible with PDA. The remaining disinfectants were added to autoclaved PDA after cooling to ~50°C, at 0.5, 1, 2, and 4 times the recommended rate. The disinfectant-amended media were aseptically dispensed into 90-mm-diameter plastic Petri dishes and allowed to solidify. Plugs of freshly grown mycelia of TR4 were placed at the center of the plates and were incubated for 5 days at room temperature (±27°C). Efficacies were determined by measuring the radial colony growth in millimeters (two cross-sectional measurements per colony per plate).

Testing efficacy on conidial spores. The efficacy on TR4 conidiospores was tested by adding 500-µl spore suspension to disinfectant solutions (at concentrations of 0.25, 0.5, 1, and 2 times the recommended rate) in a 1:1 ratio and incubation exposure times of 15, 30, 60, 120, and 300 s. Shorter exposure times were not applied, as the minimal handling time of samples/experiments was 15 s. After incubation, spore suspensions were immediately diluted 20× with buffer solution (1/4 strength Ringer, Honeywell Fluka, Seelze, Germany) and thoroughly mixed to stop and hence define a precise window of exposure to the disinfectant. Immediately thereafter, a 100-µl sample was plated on a PDA plate and subsequently incubated at 25°C in an incubator (Elbanton, Hettich Benelux B.V., Geldermalsen, The Netherlands). After 3 days the efficacy was determined as the number of developed TR4 colonies per PDA plate. Re-examination of the plates after prolonged incubation did not result in additional developing colonies. Controls were performed as described above to verify that the conidiospores (2×10^5 per ml) were viable.

Testing efficacy on chlamydospores. For testing the efficacy to chlamydospores, 0.5 g of soil (~10^7 chlamydospores/g) was suspended in 20 ml of 1/4 strength Ringer's solution. Chlamydospores were released into solution by vortexing for 10 min. Soil material was allowed to precipitate for 5 min and 500 µl of the resulting suspension was mixed in a 1:1 ratio with disinfectant solutions (at concentrations of 0.25, 0.5, 1, and 2 times the recommended rate) for 15, 30, 60, 120, and 300 s, respectively. To stop the treatment, samples of 50 µl were diluted 20× in 1/4 Ringer's solution and 100 µl was plated directly on Komada medium amended with 100 mg/liter streptomycin, which was then incubated at room temperature (±27°C) for 3 days. Efficacy data were collected by the number of growing TR4 colonies on each plate. The concentration and viability of the chlamydospores in the suspension was validated using the same procedure, and incubation in a 1:1 1/4 strength Ringer's solution.

The efficacy of disinfectants on chlamydospores embedded in soil was tested by mixing 0.5 g of infested soil (10^6 chlamydospores/g) and 5 ml of disinfectant solution at three concentrations (1, 2, and 4 times the recommended rate). We decided on one 15-s exposure time, which was considered as a maximum for practical on-farm situations. Directly afterward, 45 ml of 1/4 Ringer's solution was added to stop the treatment and the mixture was directly filtered (Durapore membrane filter 0.45-µm pore size, Millipore Ireland, Cork, Ireland) by vacuum infiltration (Sartorius, Göttingen, Germany). The filter with treated chlamydospores was then transferred into 20 ml of 1/4 strength Ringer's solution in a 50-ml plastic tube that was then vortexed (Mo Bio Genie 2 Vortex, USA Scientific, Ocala, FL, U.S.A.) for 30 min. The resultant chlamydospore suspension (100 µl) was subsequently plated on Komada medium amended with 100 mg/liter streptomycin and incubated at ±27°C for 3 days. At least three batches of the soil were tested with 1/4 Ringer's solution, following the same procedure, to validate the concentration and viability of the chlamydospores.

Assessment of disinfectants corrosiveness. All disinfectants except Hi-Chlon 70 and ethyl alcohol were assessed for their corrosiveness to aluminum (Al) and iron (Fe) at the Tadeco Inc. chemistry laboratory (Davao del Norte, Philippines), by a routine process using inductively coupled plasma optical emission spectrophotometry. Al and Fe samples were separately exposed to 375-ml disinfectant samples (1% per liter), two replicates per treatment, and monitored for 3, 6, 9 and 24 h. At each time point, 50 ml was pipetted for Al and Fe content analysis using inductively coupled plasma optical emission spectrophotometry (PerkinElmer, Shelton, CT, U.S.A.).

Measuring real-time exposure with disinfectants in the field. To monitor the potential efficacy of disinfectants under practical conditions, camera footage from three entries/exits of a large-scale commercial 'Cavendish' plantation in Davao del Norte, Philippines (6,613 ha planted) was used and analyzed to determine the duration of exposure in foot baths and vehicles tire bath stations (first contact to last contact in seconds; $n = 200$).

Determining cost of quarantine baths. In the same commercial banana plantation as mentioned above, the costs of installing and maintaining quarantine baths were calculated. There were 18 foot baths and tire bath stations installed in the major entry/exit points for the entire plantation and the estimated cost was calculated (in USD).

Data and statistical analysis. The efficacies of the disinfectants to TR4 spores were evaluated as radial growth for mycelia, as CFU/ml for conidia or as CFU/g soil. For all data, we assumed that CFU's resulted from individual conidia or chlamydospores. A nonparametric test using Kruskal–Wallis was employed (data did not satisfy the normality test using the Shapiro–Wilk test) to test the difference among groups. When the Kruskal–Wallis test is rejected, we used Dunn's test for pairwise multiple comparison with adjusted probability (P) values using the Bonferroni correction method. Statistical analysis was carried out using the R statistical tool (R v.3.6.3; https://cran.r-project.org/bin/windows/base/old/3.6.3/).

Results

Disinfectants inhibit mycelial growth. All quaternary ammonium-based disinfectants completely inhibited mycelial growth at 1× (recommended) and 0.5× rates, except for the GUAA and Multisept (Fig. 1). Both GUAA and Multisept showed significant reduction of mycelial growth relative to the control in all tested rates (P value < 0.01), but lacked total inhibition at the lower rates tested. All other compounds inhibited mycelial growth at all tested rates.

Conidiospores are sensitive to disinfectants even at low concentrations. All disinfectants showed excellent efficacy toward conidiospores at all rates and five exposure times, except for formalin and ethyl alcohol. For formalin, significant reduction of viable conidial spores was recorded at all rates and exposure times (P value < 0.01). At 5 and 7.5% formalin, only full efficacy was reached with a minimal exposure time of 300 and 60 s, respectively (Fig. 2). Similarly, a significant reduction of surviving conidial spores was achieved when exposed to ethyl alcohol at all time points tested (P value < 0.01). The one exception was the 15-s exposure of conidial spores to 35% ethyl alcohol (P value = 0.1857). One-hundred percent efficacy was achieved at 40% ethyl alcohol albeit at a 300-s exposure time (Fig. 2). All other disinfectants were additionally tested at 0.0625× and 0.125× rates and still showed full efficacy at all exposure times (data not shown).

Chlamydospores are sensitive to disinfectants in aqueous suspensions. Approximately 1×10^7 CFU/g soil for all water controls was observed across all experiments. The disinfectants Formo and Vanodine were effective at all concentrations tested (Fig. 3). All quaternary ammonium disinfectants were effective in preventing germination of chlamydospores in the suspension at all rates and after all exposure times with the exception of Multisept. Only at 0.5× or higher, Multisept prevented germination of chlamydospores after

30-s exposure time (P value < 0.01). In contrast, no significant reduction of germinating chlamydospores was observed for Hi-Chlon 70 at 0.25× rate at all exposure times (P value = 0.334). However, significant reduction of surviving chlamydospores was recorded at 1× and 2× rate for all exposure times (P value < 0.01). Also, formalin, which is frequently applied in the field by farmers, was fully effective at all concentrations tested, even at the shortest exposure times (P value < 0.01). Ethyl alcohol, routinely employed as disinfectant in the laboratory, and therefore included in this study, was fully effective at the 45 and 47.5% rate (vol/vol%), while lower concentrations (≤40%) required prolonged exposure times for full efficacy.

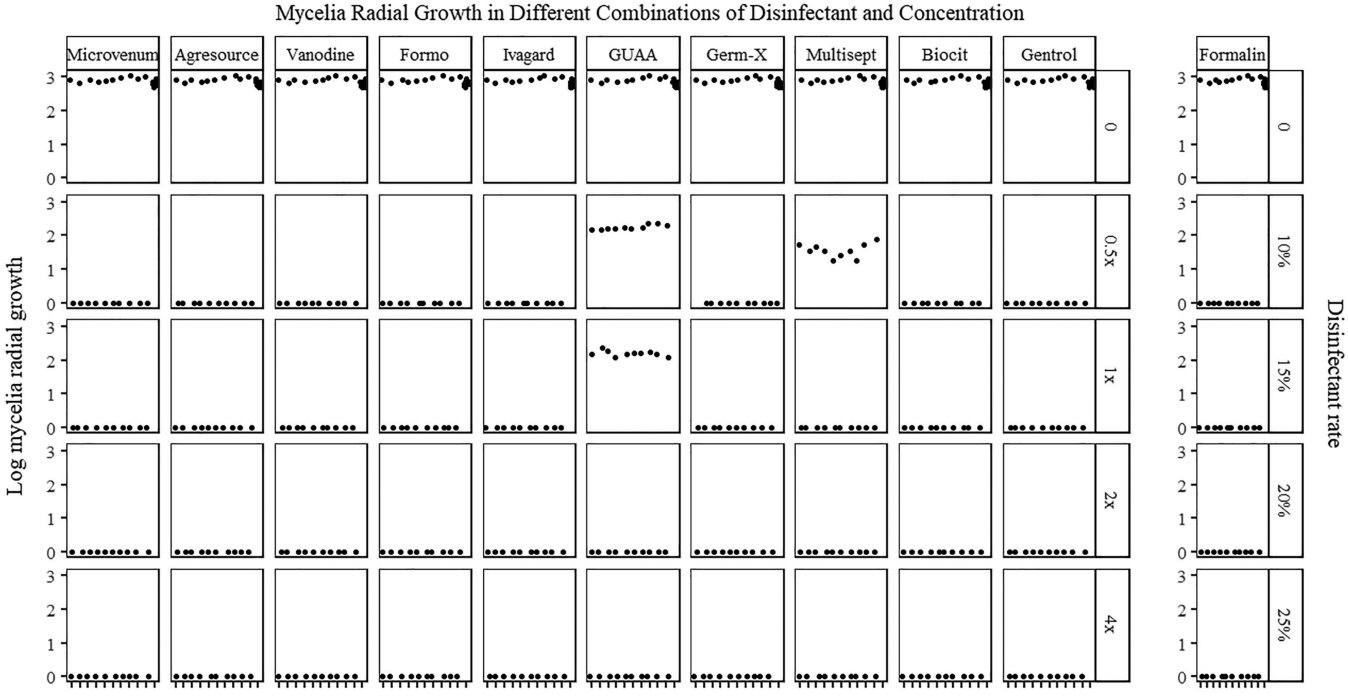

Fig. 1. Efficacy of 11 disinfectants on *Fusarium odoratissimum* Tropical Race 4, expressed as log-transformed mycelia radial growth in millimeters on amended potato dextrose agar medium in Petri dishes at 0, 0.5, 1, 2, and 4× the recommended rates. For formalin, we used concentrations of 10, 15, 20, and 25% (vol/vol). All experiments were conducted twice. Dots represent the 10 individual measurements for both experiments.

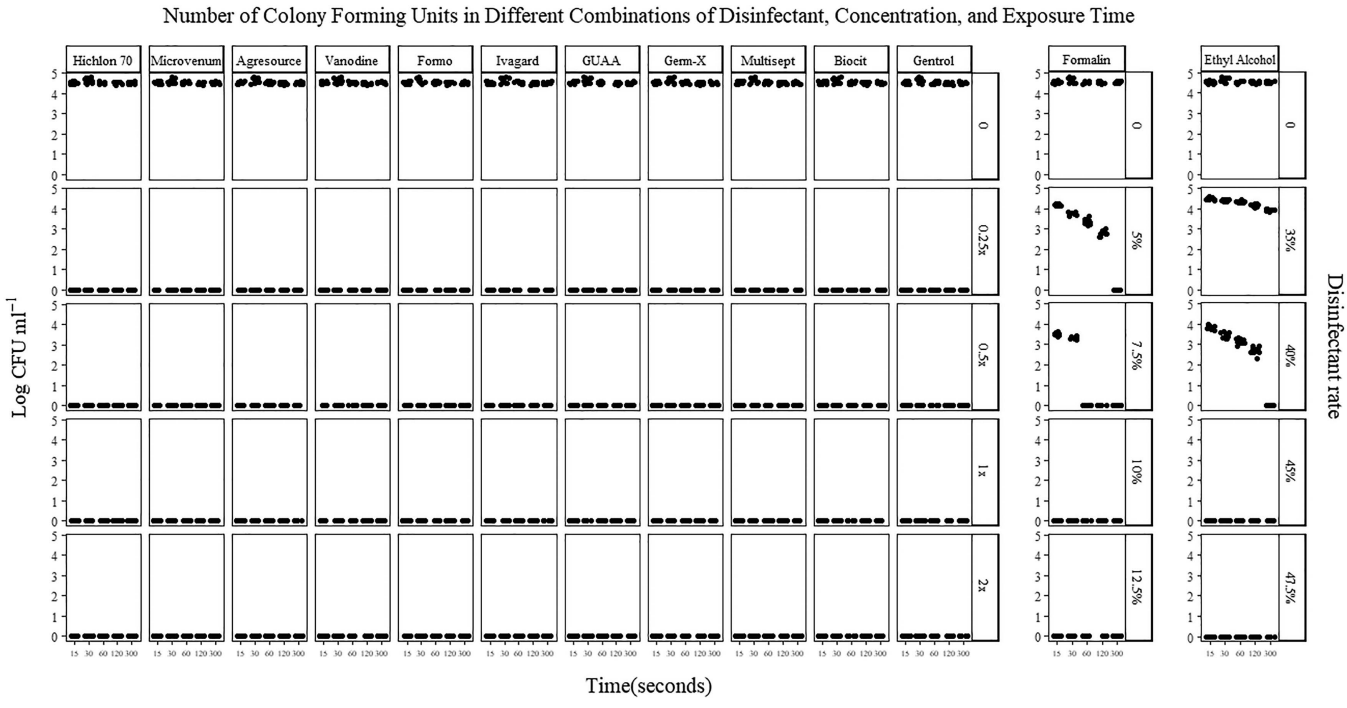

Fig. 2. Efficacy of 13 disinfectants on *Fusarium odoratissimum* Tropical Race 4 conidia (2×10^5 ml^{-1}) at 0, 0.25, 0.5, 1, and 2× the recommended rate and five exposure times (15, 30, 60, 120, and 300 s). For formalin and ethyl alcohol, we used concentrations of 5, 7.5, 10, and 12.5% (vol/vol) and 35, 40, 45 and 47.5% (vol/vol), respectively. Data are expressed as average log-transformed CFUs per applied milliliters of TR4 spore suspension on Petri dishes. All experiments were conducted twice and the means for both experiments are shown as dots.

Reduced efficacy of disinfectants to chlamydospores in soil. The efficacy of the tested disinfectants significantly declined against chlamydospores in soil after 15-s exposure time except for Formo (Fig. 4). Significant reduction of chlamydospores was recorded at the manufacturer's rate for all tested disinfectants (P value < 0.0125) with the exception of Hi-Chlon 70 (P value = 0.0191), Multisept (P value = 0.5202), and Germ-X (P value = 0.0694). Doubling the manufacturer's rate significantly improved the efficacy of Multisept and Germ-X along with the rest of the disinfectants, except for Hi-Chlon 70 (P value = 0.0311). Full reduction of surviving chlamydospores at this rate was, next to Formo, also observed for Gentrol, Microvenum, and Ivagard. At the highest rate (4×) tested, all disinfectants rendered significant reduction of surviving chlamydospores (P value < 0.0125). However, even at such rates, surviving chlamydospores were observed for Hi-Chlon 70, GUAA, Multisept, Vanodine, Formalin, and ethyl alcohol.

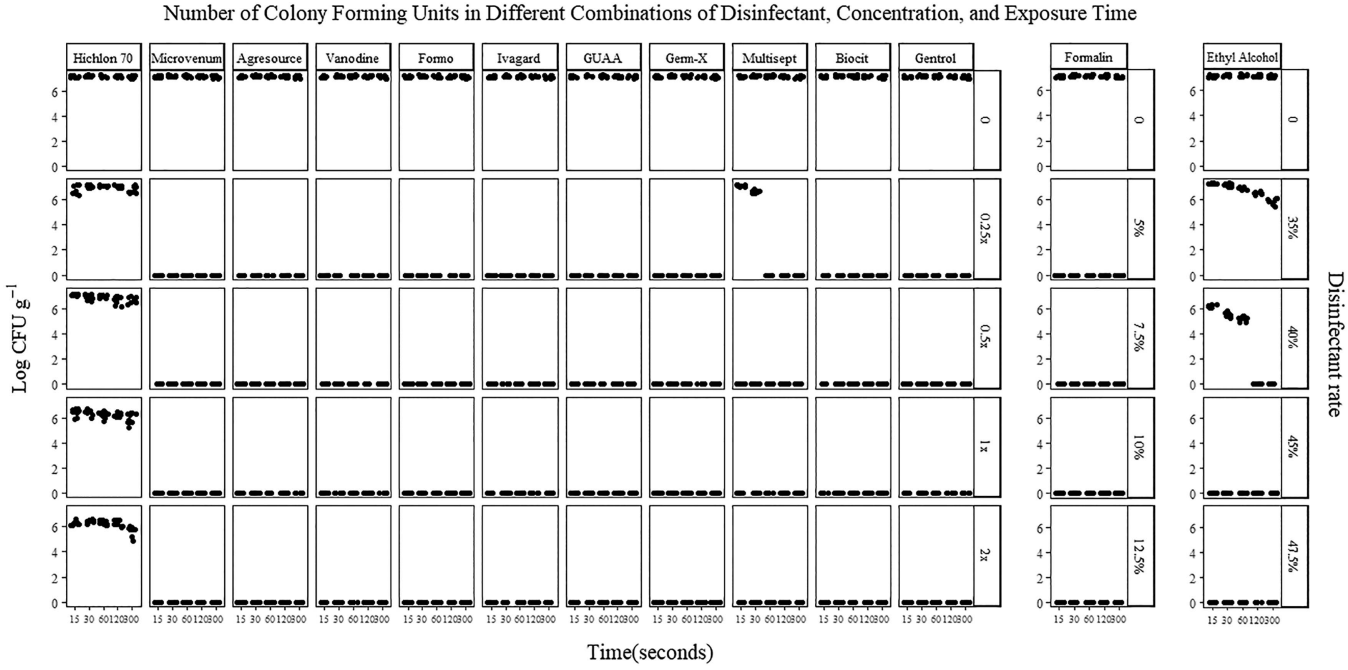

Fig. 3. Efficacy of 13 disinfectants on *Fusarium odoratissimum* Tropical Race 4 chlamydospores (10^6 chlamydospores.gram of soil^{-1}) in suspensions at 0, 0.25, 0.5, 1, and 2× the recommended rate and five exposure times (15, 30, 60, 120, and 300 s). For formalin and ethyl alcohol, we used concentrations of 5, 7.5, 10, and 12.5% (vol/vol) and 35, 40, 45, and 47.5% (vol/vol), respectively. Data were collected as log-transformed CFUs per gram of soil on Petri plates. All experiments were conducted twice and the means for both experiments are shown as dots.

Fig. 4. Efficacy of 13 disinfectants on *Fusarium odoratissimum* Tropical Race 4 chlamydospores (10^6 chlamydospores.gram of soil^{-1}) in soil at 1, 2, and 4× the recommended rate, an exposure time of 15 s and expressed as log-transformed CFUs per gram of soil on Petri plates. For formalin and ethyl alcohol, we used concentrations of 15, 20, and 25% (vol/vol) and 70, 80, and 95% (vol/vol) rates per liter, respectively. All experiments were conducted twice. Dots represent the individual count for both experiments.

Disinfectants are corrosive to aluminum and iron. Preference for specific disinfectants on farms also depends on the corrosiveness toward the surfaces of equipment and vehicles. Therefore, we tested whether the 11 disinfectants were reactive to aluminum (Al) and iron (Fe). This revealed that Vanodine is the most reactive disinfectant toward both Al and Fe. After 24-h exposure, the Al concentration reached nearly 20 parts per million (ppm) while for Fe the concentration increased just over 75 ppm (Fig. 5). Formo showed corrosiveness toward Fe, generating nearly 80 ppm, but hardly to Al (5 ppm), whereas all other disinfectants did not reveal any corrosiveness toward both metals (<5 ppm after 24 h). This was confirmed by conducting corrosiveness tests on farm tools exposed to these disinfectants at the manufacturer's recommended rate (data not shown).

In summary (Table 2), we conclude that nearly all disinfectants have appropriate efficacy for most TR4 propagules and have limited corrosiveness for Al and Fe. However, once chlamydospores are embedded in soil, efficacies significantly drop and only Formo showed the required capacity to significantly reduce TR4 viability even at short exposure times, but is among the two disinfectants, together with Vanodine, that have relatively strong corrosive capacity toward Fe.

Recording exposure to disinfectants under real-time conditions. Exposure time is crucial for the efficacy of any disinfectant. In all above-described in vitro and in situ experiments, a minimal exposure time of 15 s was used for experimental reasons, but it also approximates the required time to pass through footbaths or vehicle tire baths. Comparing this minimum required time lapse with actual live conditions revealed that the average exposure time for footbaths was 3.42 ± 0.82 s, measured from first contact of a person to the last contact with the disinfectant, and for vehicle tire baths, 5.07 ± 3.08 s (Table 3).

Cost of quarantine baths. In the commercial plantation, 18 concrete foot baths and tire bath stations are installed. Foot baths (2.5 × 0.6 × 0.05 m) are replenished with 10 liters of disinfectant every 3 days. Tire bath stations (9.3 × 3.8 × 0.2 m) are daily supplemented with 600 liters of disinfectant and were drained, cleaned, and refilled

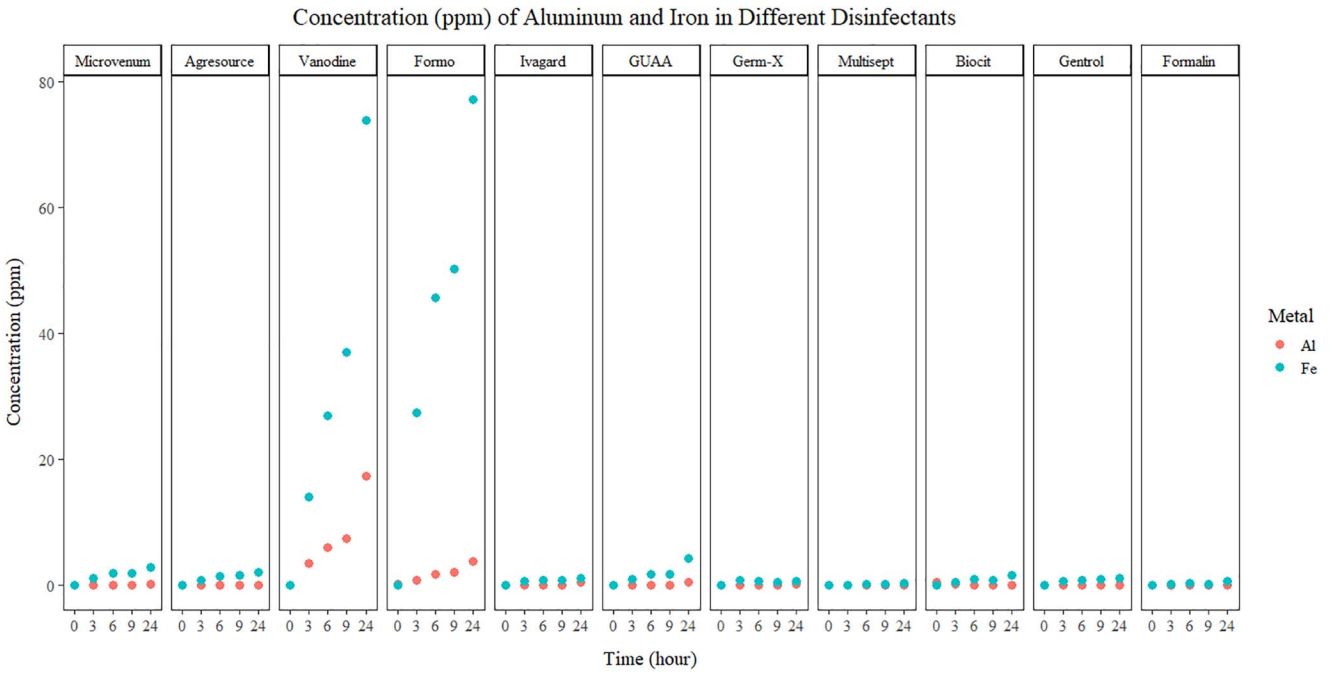

Fig. 5. Corrosiveness of disinfectants toward aluminum (Al) and iron (Fe) after 3-, 6-, 9-, and 24-h exposure to 11 disinfectants (1× dilutions) and expressed as concentrations of Al and Fe ions in parts per million (ppm). All experiments were conducted twice and the means for both experiments are shown as dots.

Table 2. Summary of the efficacy of disinfectants tested in vitro at manufacturers' recommended concentrations against different propagules of *Fusarium odoratissimum* Tropical Race 4 and their corrosiveness to metals

Disinfectant	Mycelium	Conidiospores	Chlamydospores (in suspension)	Chlamydospores (in soil)	Corrosiveness[a]
Hi-Chlon 70	NT	+[b]	–	–	NT
Microvenum	+	+	+	–	Slight
Agresource	+	+	+	–	Slight
Vanodine	+	+	+	–	High
Formo	+	+	+	+	High[c]
Ivagard	+	+	+	–	Slight
GUAA	–	+	+	–	Slight
Germ-X	+	+	+	–	Slight
Multisept	+	+	+	–	Slight
Biocit	+	+	+	–	Slight
Gentrol	+	+	+	–	Slight
Formalin[d]	+	+	+	–	Slight
Ethanol[e]	NT	+	+	–	NT

[a] Corrosive capacity to both Fe and Al metals.
[b] "+" denotes total killing and "–" denotes slight or minimal killing of TR4 propagules. NT = not tested.
[c] Highly corrosive on Fe metal, but only slightly corrosive on Al.
[d] Formalin tested at 15% (vol/vol) rate per liter.
[e] Ethanol tested at 45 and 70% (vol/vol) rate per liter for conidiospores, chlamydospores in suspension, and chlamydospores in soil.

every fortnight. As indicated in Table 4, installing quarantine baths across the main entrances of the investigated banana plantation required a budget of 207,000 USD, with annual maintenance costs of 63,000 and 279,000 USD for the replenishment of the disinfectants. Together, for 18 quarantine baths, this totals to 549,000 USD.

Discussion

Pests and diseases are major bottlenecks for food production and food security worldwide (Gurr et al. 2011; Oerke 2005; Oerke and Dehne 1997). Crop losses are prevented or reduced by either physical or biological control, cultural practices, and/or the use of pesticides. For soilborne pathogens, disease control is more complex, as traditional methods have variable success (Katan 2000; Weller et al. 2002) and chemical measures are increasingly restricted for economical and environmental reasons (Panth et al. 2020). Banana is an important food security and cash crop, but is seriously threatened by the soilborne pathogen TR4 causing FWB in 'Cavendish' varieties as well as in manifold locally preferred cultivars (García-Bastidas 2019; Ploetz 2015). The local and regional dissemination of TR4 is associated with regular farm operations, such as moving planting material (with attached soil), equipment, and irrigation water (Dita et al. 2018). International spread is elusive, but almost surely anthropogenic (Maymon et al. 2020; Zheng et al. 2018). Therefore, the prevention of further spread is crucial, as there is no known effective and straightforward solution to eliminate the pathogen, particularly in soil. This necessitates developing and adopting biosecurity measures within and between farms at regional, national, and international scales.

Verification of the efficacy of disinfectants used to decontaminate tools, footwear, machinery, and equipment against TR4 propagules is necessary to prevent the unconscious dissemination of TR4. Here, the efficacy of 13 commercially available disinfectants was tested against various propagules of TR4. We demonstrated that TR4 mycelia and conidia are sensitive to nearly all disinfectants at the manufacturer's recommended rates and in most cases even well below. Purified chlamydospores, despite their persistence in the soil, were also remarkably sensitive to the disinfectants when tested in suspension. However, a considerable reduction of efficacy was observed when disinfectants were tested against chlamydospores in soil. Overall, the diamidine-based disinfectant Formo was most efficacious at 15-s exposure time. However, this compound has high corrosiveness toward iron, making it less suitable for long-term usage, particularly in vehicle disinfection units. None of the other disinfectants – with limited corrosiveness – showed a sufficient efficacy toward chlamydospores in soil, except at extreme high concentrations (2 to 6 percent concentration of disinfectant solution per liter).

Contrary to Nguyen et al. (2019), who tested disinfectants in prepared mixtures of one part of soil and 20 parts of disinfectant and added chlamydospores in suspension, we tested chlamydospores suspensions as well as chlamydospores in their natural habitat (generated and maintained in the soil). In this way we simulate the actual practices of routine disinfection used in a farm operation. Our findings are in accord with the results by Bennett et al. (2011), who showed that disinfectants were able to significantly reduce viability of *F. oxysporum* f. sp. *vasinfectum* chlamydospores in suspensions, but most were not very efficacious in soil. Our findings, and the observations of Nguyen et al. (2019), showed that the presence of soil while testing disinfectants against chlamydospores in suspension reduced their efficacy, which underscores the importance of on-farm hygiene. As mentioned before, soil reduces the access of disinfectants to chlamydospores (Amass et al. 2001; Ewart 2001; Moore et al. 2001), likely by reducing effective concentrations, such as degradation by chemical reactions, or by physical exclusion (Bennett et al. 2011; Dvorak 2008; Ewart 2001; Morley 2002; Nguyen et al. 2019). Soil or organic matter also deactivates quaternary ammonium, iodine, and ethyl alcohol containing disinfectants (Chauret 2014; Rutala et al. 2008). This emphasizes the importance of precleaning all surfaces from soil before disinfectant exposure (Amass et al. 2000, 2001; Ford 1995). Hence, the adage "come clean, go clean" is a concise reminder to remove soil from footwear, machinery, and vehicles before disinfection.

In the study of Bennett et al. (2011), chlorine-releasing disinfectant Clorox effectively eliminated *F. oxysporum* f. sp. *vasinfectum* chlamydospores in soil, but in our study, Hi-Chlon 70 did not kill chlamydospores either in suspension or in soil. These opposing results might be from the different target organisms, the specific formulation, and/or concentration of each product or experimental conditions. Chlorine-releasing disinfectants are oxidizing agents that disrupt the cellular activity of proteins (Ascenzi 1996; Maris 1995), interrupt oxidative phosphorylation (Barrette et al. 1989), and other cellular membrane processes (Camper and McFeters 1979). Therefore, these disinfectants need to be in direct contact with the spore structure. However, in the presence of soil or any other organic matter, their efficacy is strongly reduced (Ewart 2001). This was also demonstrated by Cayanan et al. (2009), who showed that the free chlorine concentration rapidly declined in the presence of organic matter and other oxidizing agents when tested against *F. oxysporum* conidia in irrigation water. A higher concentration of chlorine (≥5 ppm) and longer exposure time (≥4 min) was required to disinfect high organic load dam water (Scarlett et al. 2015). Hence, chlorine merely reduces pathogen concentrations, but does not eradicate fungal propagules from complex substrates such as soil (Cayanan et al. 2009; Scarlett et al. 2015).

Because of the TR4 pressure in Mindanao, farmers are desperate and use virtually anything that is suggested to be functional toward FWB control. This is the reason formalin (37% solution of formaldehyde gas in water) is frequently used despite its highly reactive and mutagenic properties (Power 1997). Therefore, we included it in our study and observed that, indeed, it is effective against TR4 mycelia, conidiospores, and chlamydospores in suspension but not against chlamydospores in soil. This can be from over-dilution rendering it ineffective at 15% (vol/vol) rate per liter. Thus, despite the fact that this disinfectant is sporicidal, as based on its ability to penetrate fungal spores (Sykes 1970) and cross-linking to both proteins (Ewart 2001; Fraenkel-Conrat et al. 1945) as well as nucleic acids (Fraenkel-Conrat 1961; Maris 1995), its application under field conditions is essentially useless – as this study shows. We observed similar dose-dependent efficacies for soil treatments with the quaternary ammonium disinfectants Microvenum, Agresource, Ivagard, Germ-X, Biocit, and Gentrol.

We also showed that an exposure time of 15 s, the shortest exposure time manageable in our experiments, is seldom met under field

Table 3. Real-time exposure times (in seconds) at disinfections units for Fusarium wilt of banana caused by *Fusarium odoratissimum* Tropical Race 4 for persons and vehicles at an entry point of a large commercial plantation in Mindanao, the Philippines

Parameter	Footwear bath	Tire bath
Sample size	200	200
Average exposure time	3.42 ± 0.82	5.07 ± 3.08
Maximum exposure time	7.54	26.95
Minimum exposure time	1.85	2.21

Table 4. Estimated cost of quarantine baths per year in a large-scale commercial banana plantation in Mindanao, Philippines

	Estimated cost (USD[a])	
Expense	Per quarantine bath	Entire plantation[b]
Disinfectant	$15,500.00	$279,000.00
Labor maintenance	$3,500.00	$63,000.00
Installation[c]	$11,500.00	$207,000.00
Total	$30,500.00	$549,000.00

[a] Exchange value of 1 Philippine peso to 0.019218 USD.
[b] Total for 18 major footwear and tire baths.
[c] One-time cost.

conditions. Based on our test results, it is very unlikely that the reported efficacies are achieved at shorter exposure times. In any case, 15-s exposure time is far too short for any efficacy toward chlamydospores in a soil substrate, as shown in this study. To support the claim of inadequacy of disinfectants for in-field sanitation, we monitored the minimum soaking times in foot and tire baths at farm gates and observed average exposure times of 3.42 and 5.07 s, respectively. The discrepancy between the required and observed exposure times in such sanitation baths, particularly under wet and muddy conditions, needs to be explained and enforced on farms, otherwise disinfection practices will merely become token acts or "window dressing." This would clearly hamper any quarantine measure which is implemented at high cost, but still does not stop the further on-farm, regional, and international dispersal of TR4 (Zheng et al. 2018). We consider it striking that manufacturer's recommendations are limited to an optional concentration, but do not include minimal exposure times. Indeed, the required exposure times of various disinfectants vary, resulting in variable efficacies (Ewart 2001; McDonnell and Russell 1999). Some have residual activity (quaternary ammonium) while others are volatile, leaving behind no residue (ethyl alcohol). Nguyen et al. (2019) also showed that increasing exposure time from 30 s up to 5 min enhances the efficacy of quaternary ammonium, bio-flavonoids, oxidizing agent, and detergent-based disinfectants against chlamydospores in suspension. This accords with the observed decreasing efficacy of Farmcleanse to TR4 microconidia in a time series with decreasing exposure times from 15 min to 30 s (Meldrum et al. 2013). With 5 ppm of chlorine in deionized water, a minimum of 10-min exposure time is required to kill >99% of *F. oxysporum* mycelium, but is 20 min for chlamydospores (Scarlett et al. 2015). The bactericidal action of diamidine containing disinfectant was exposure-time–dependent against *Legionella pneumophila* suspension tested at 6 and 12 µg/ml (Skaliy et al. 1980). The survival of *Trichophyton mentagrophytes* arthroconidia in suspension media was also exposure-time–dependent, using a disinfectant containing 0.5% benzalkonium chloride (Gupta et al. 2001). James et al. (2012) demonstrated that time is a critical factor for ethyl alcohol exposure of *Phytophthora ramorum* sporangia germination. The quaternary ammonium-based disinfectant Vesphene (Steris, Mentor, OH, U.S.A.) was only sufficiently effective against *Coccidioides* spp. arthroconidia in aqueous suspensions at an exposure time of at least 20 min (Vogler et al. 2015). Clearly, such long exposure times are inadequate for on-farm use.

Taken together, our data show that disinfectant efficacies depend on the propagule of the target pathogen, substrate, exposure time, and concentration. Based on this study, we propose that minimal exposure times (which will clearly need to be longer than those measured for this study, and would be based on local test data) with disinfectants should be enforced during entry of uninfested areas to prevent TR4 incursions. Furthermore, manufacturer's recommendations should include minimal exposure times depending on the specific pathogen, weather, and local conditions as well as data on corrosiveness. For instance, the diamidines-based disinfectant Formo was efficacious but its corrosiveness toward iron is too high for practical use. Corrosiveness is a function of time, although environmental conditions such as temperature also influence these processes (Nguyen et al. 2019; Southcott et al. 1982). Hence, farmers need to be better informed to enable critical decisions on disinfectant preferences. Furthermore, the effectiveness of the disinfection bath requires explicit monitoring for remaining levels of active ingredients, which will decrease in time as a result of temperature, pH changes, and debris. Foremost, however, any material, vehicle, or footwear should be thoroughly cleaned of soil particles before entering disease-free areas by pressurized spraying and (car) wash installations. This removes organic load accumulation and affects the efficiency of disinfection baths. Alternatively, entry of non-site tools, vehicles, and footwear should be prohibited, which requires major additional logistic arrangements for on-farm transportation.

In conclusion, the rational choice for appropriate disinfectants should be facilitated by accurate data on efficacies under various environmental/temporal conditions, costs, and corrosiveness. Without such information, the implementation of effective quarantine measures is useless. The practical observations in the Philippines and elsewhere (Molina et al. 2009; Mostert et al. 2017; Zheng et al. 2018) are not promising as TR4 continues its dispersal to new locations (Aguayo et al. 2020; Chittarath et al. 2018; García-Bastidas et al. 2013, 2019a; Hung et al. 2018; Maymon et al. 2020; O'Neill et al. 2016; Ordóñez et al. 2015a; Özarslandan and Akgül 2019; Ploetz et al. 2015; Thangavelu et al. 2019), which may be driven by the flaws identified in this report as well as by the individual farm economies that enable or disable the procurement of costly disinfectants, and the installation and appropriate maintenance of sanitation units.

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