Evaluating the efficacy of Decon 7 against four filamentous fungal species from representative classes of Ascomycetes

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Fungal species used in the study:

The purpose of this experiment was to obtain preliminary data related to the efficacy of Decon 7 (D7) against different fungal species from the phylum (division) of Ascomycota (also known as "sac fungi"), subphylum of Pezizomycotina (Fig. 1). The Ascomycota is the most import division of fungi that accounts for nearly 75% of the currently described fungi and includes the majority of plant, animal, and human fungal pathogens. Basidiomycota (also known as "club fungi") is the second most important phylum of fungi, which contains the known to all mushrooms. The phylum of Ascomycota is further subdivided to the subphylum of Pezizomycotina, which includes nearly all filamentous (hyphae-forming) species and the subphylum of Saccharomycotina, which contains the yeasts, including the baker's yeast *Saccharomyces cerevisiae* used in the bakery and brewing industry, as well as well-known human pathogens such as *Candida albicans*. Since the physiology of yeasts and filamentous fungi differs substantially, it is advisable that the activity of D7 is tested at one point against both fungal forms.

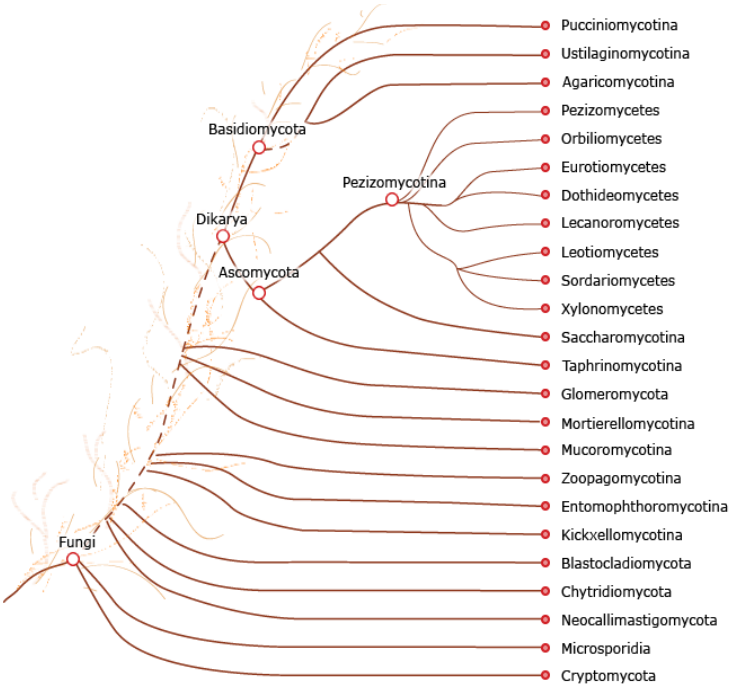

Figure 1. Phylogenetic classification of fungi

For our experiments we have selected four fungal species that represent three classes within the Pezizomycotina, namely the Eurotiomycetes, Dothideomycetes and Sordariomycetes (Fig. 1). The Eurotiomycetes include many pathogenic and mycotoxigenic fungi of mainly medical importance from the genus of *Aspergillus spp*, whereas Dothideomycetes and Sordariomycetes include most of the known plant pathogenic fungi. Missing from our tests were fungi from the class of Leotiomycetes, which contains many necrotrophic plant pathogens with broad host-ranges, including *Botrytis cinerea* and *Sclerotinia sclerotiorum*. The efficacy of D7 against fungi from this class can be examined in subsequent tests. Next to their phylogenetic placement, we have selected the four species listed below because of their importance from an agriculture and public health perspective. Specifically the four species that were selected for these preliminary studies were:

- ***Aspergillus flavus* and *Aspergillus parasiticus*** (*Phylogenetic classification: Kingdom: Fungi; Subkingdom: Dikarya; Phylum: Ascomycota; Subphylum: Pezizomycotina; Class Eurotiomycetes; Genus: Aspergillus).* These two species are well known **producers of aflatoxins,** a group of highly toxic and carcinogenic polyketide mycotoxins that are produced as secondary metabolites during fungal

development, mainly by species of *Aspergillus spp*. They are ubiquitous saprophytes in nature but under certain conditions they may also infect plants, particularly maize (Fig. 2) and peanut. Aflatoxins pose serious health hazards to humans and domestic animals, as they frequently contaminate agricultural commodities, including crops, dairy products, meat, and eggs. Grain crops, in particular, such as corn, rice, groundnut, cassava, and many nut crops are major sources of contamination of the food supply. It is estimated that over 25% of the global food supply is contaminated by aflatoxins resulting in a 64% reduction in food quality particularly in Africa (Source: WHO). Consequently, approximately 4.5 billion people are chronically exposed through their diet to aflatoxins (Source: WHO), which frequently leads to liver cancer as well as a variety of other ailments relating to immunosuppression. Acute toxicity caused by ingestion of large amount of aflatoxins from heavily contaminated food may also occur and leads to liver failure, blood clotting, vomiting and even death of the affected person. Consequently, there is a need to control aflatoxin contamination of food and feed grains and global efforts are currently underway to control these fungi.

Figure 2. Corn cobs infected with *Aspergillus flavus*.

- ***Fusarium oxysporum* f. sp. *lycopercisi* Race 3.** (*Phylogenetic classification: Kingdom: Fungi; Subkingdom: Dikarya; Phylum: Ascomycota; Subphylum: Pezizomycotina; Class: Sordariomycetes; Genus: Fusarium).* **Wilt and crown rot diseases** caused by soil-borne pathogens of the *Fusarium oxysporum* species complex create devastating crop losses worldwide to various crops. Efforts to eradicate pathogenic strains are confounded by tolerance to most chemical controls and a poor understanding of their persistence in soil. Over 100 plant pathogenic forms, or *formae speciales* (f. sp.), are recognized in the *F. oxysporum* species complex, with each *formae speciales* defined by its strict host specificity. Moreover, different physiological races are recognized within each form that are differentiated based on the range of the host cultivars that they can infect. Notorious, pathogens within this complex include, for example, *F. oxysporum* f. sp*. cubence Race 4 (Foc TR4),* which causes Panama disease (Fusarium wilt) of bananas and is able to infect bananas of the Cavendish group that are grown on a global scale for commercial production, as well as *F. oxysporum* f. sp. *fragariae*, causal agent of Fusarium wilt of strawberry*. Fusarium oxysporum* f. sp. *lycopersici* (Fol), a pathogen of worldwide importance, causes vascular wilt disease specifically in tomato (Fig. 3). Currently, three races of Fol have been reported, with Race 1 and Race 3, considered as the most dangerous ones. Race 3, in particular, is a major threat to the tomato production in California and other parts of the US and the world as there are no commercial resistance lines available against this Race yet. Fol is a soilborne pathogen and can remain in infected soils for up to 10 years. It is disseminated mainly via seed, tomato stakes, soil, infected transplants or infested soil adhering to transplants. Surface waters infested with spores of the pathogen as well as farm machinery, tools, cloths and footwear dirtied with contaminated earth are often another major source of pathogen inoculum. Therefore, we considered that D7 could potentially be used for decontamination of such sources of inoculum or other pathogen exclusion strategies.

Figure 3. Fusarium wilt in processing tomatoes

- ***Pseudocercospora fijiensis* (synonym** *Mycosphaerella fijiensis*). *Phylogenetic classification: Kingdom: Fungi; Subkingdom: Dikarya; Phylum: Ascomycota; Subphylum: Pezizomycotina; Class Dothideomycetes; Genus: Pseudocercospora).*This foliar pathogen causes the disease known as **black Sigatoka of banana** (Fig. 4), currently the economically most important disease of this crop. Infections from this pathogen can reduce banana yields by up to 50% and fungicide applications are at present the

only means of battling the disease, thus turning bananas in the most heavily sprayed crop in the world with more than 50 applications per year. The extensive use of fungicides in this crop can account for up to 40% of the overall production costs and next to the financial costs of approximately $ 450 million, the widespread aerial application of fungicides imposes a heavy toll on the environment and on the health of farm workers. The negative socio-economic impact of this disease is much higher in developing countries and communities that almost exclusively depend on the banana crop for their living. Therefore managing this pathogen is of urgent importance and is currently under critical public review for humanitarian, biosafety, and environmental reasons.

Figure 4. Black Sigatoka disease of banana

Fungal growth conditions and set-up of the experiment:

Aspergillus flavus strain NRRL 3357, *Aspergillus parasiticus* strain SU-1, and *Fusarium oxysporum* f. sp. *lycpoersici* Race 3 strain were grown on potato dextrose agar (PDA) medium, at 25°C in the absence of light. After 7 days, spores were collected from the plates and the spore concentration was adjusted to $1x10^6$ spores/ml in sterile ultrapure water. *Pseudocercospora fjiensis* strain C86 was grown on PDA medium for a period of 21 days at 27°C in dark. The fungus grows considerably slower as compared to all other fungi used in our study and fails to produce spores under in *in vitro* growth conditions. Therefore, mycelia of the fungus were harvested from the plates and the mycelial suspension was subsequently filtered through two layers of sterile cheesecloth. The concentration of mycelial fragments with an average size of 50-150 μm was then measured using a hemocytometer and their concertation was adjusted to $1x10^6$ fragments/ml in sterile ultrapure water.

The three components of Decon seven (D7) were mixed according to the supplier's instructions and the final product was tested in 5 concentrations diluted in sterile ultrapure water (V/V): 1, 0.5, 0.25, 0.1 and 0.05 % and 0% (control). For the toxicity assays, 100 μL of spore suspension from each of the fungal species was added separately to 900 μL of every D7 dilution treatment and incubated for 30 seconds and 5 minutes under gentle agitation. For *P. fijiensis* only one incubation time with D7 was used (90 sec), as there were not enough mycelia available. Following co-incubation of spores with D7, 25 μL of each mixture were transferred to 975 μL of ¼ Ringer's solution (40x diluted), and 100 mL of the final mixtures were plated on PDA and incubated at 25°C for 2 days, when fungal colonies were counted to analyze the proportion of surviving spores in each treatment. The experiment was repeated twice for *A. flavus*, *A. parasiticus*, and *F. oxysporum* f. sp. *lycopersici* and once for *P. fijiensis* with two technical replicates in each experiment. Toxicity against mycelial fragments of *P. fijiensis* was evaluated after 7 days, instead of two.

Results:

Results obtained by our tests are shown in Fig. 5 and Table 1. Results are shown only for the second test, as there were no considerable difference between the two biological replicates. Differences between the two technical replicates in each biological replicate are shown as % standard error.

D7 proved to be very toxic against all fungi tested, although small differences were observed among the species. In this respect, *Pseudocercospora fijiensis* and *F. oxysporum* f. sp. *lycopersici* were the most sensitive species, as spore germination and subsequent fungal growth was inhibited even at the lowest concentrations tested (0.05% v/v). In contrast, *A. flavus* and *A. parasiticus* were 10x more tolerant, as growth of these two fungal species was fully inhibited at 0.5% v/v after 5 min of contact time between the compound and the fungal spores.

Notably, a positive correlation with respect to the contact time between D7 and the fungal spores was observed, with longer contact times resulting in higher toxicity. It should be noted that with the exception of *P. fijiensis*, the assay is measuring the effect of D7 on spore germination, as this is the life form that is in contact with D7 during the initial incubation step. Spores are generally considered more resilient

structures than mycelia but differences may exist in sensitivity of the two life forms against toxic chemicals, as has been recorded for example with some fungicides (e.g. QoIs). Thus, in subsequent tests it is advisable that the sensitivity of spores and mycelia towards D7 is evaluated separately, in order to assess the full potential of the compound.

It is interesting to note that the two species of Aspergillus used in this study are almost equally sensitive towards D7. A comparison of the sensitivity of *F. oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *cubence* against D7, as determined by our and Dr Kema's assay, shows that these two fungal species are also almost equally sensitivity towards D7. Collectively, these results could suggest that species belonging to the same genus tend to be equally sensitive to D7, an important observation whose generality is worth further exploring.

Figure 5. Bar graphs and associated linear trend lines, showing the growth (defined as colony forming units (CFU)) of the different fungal species following incubation with D7 at different concentrations (0.05, 0.1, 0.25, 0.5, and 1.0 % v/v) and contact times (30 sec and 5 min), relative to growth in the untreated control (0 %, set to 100 %).

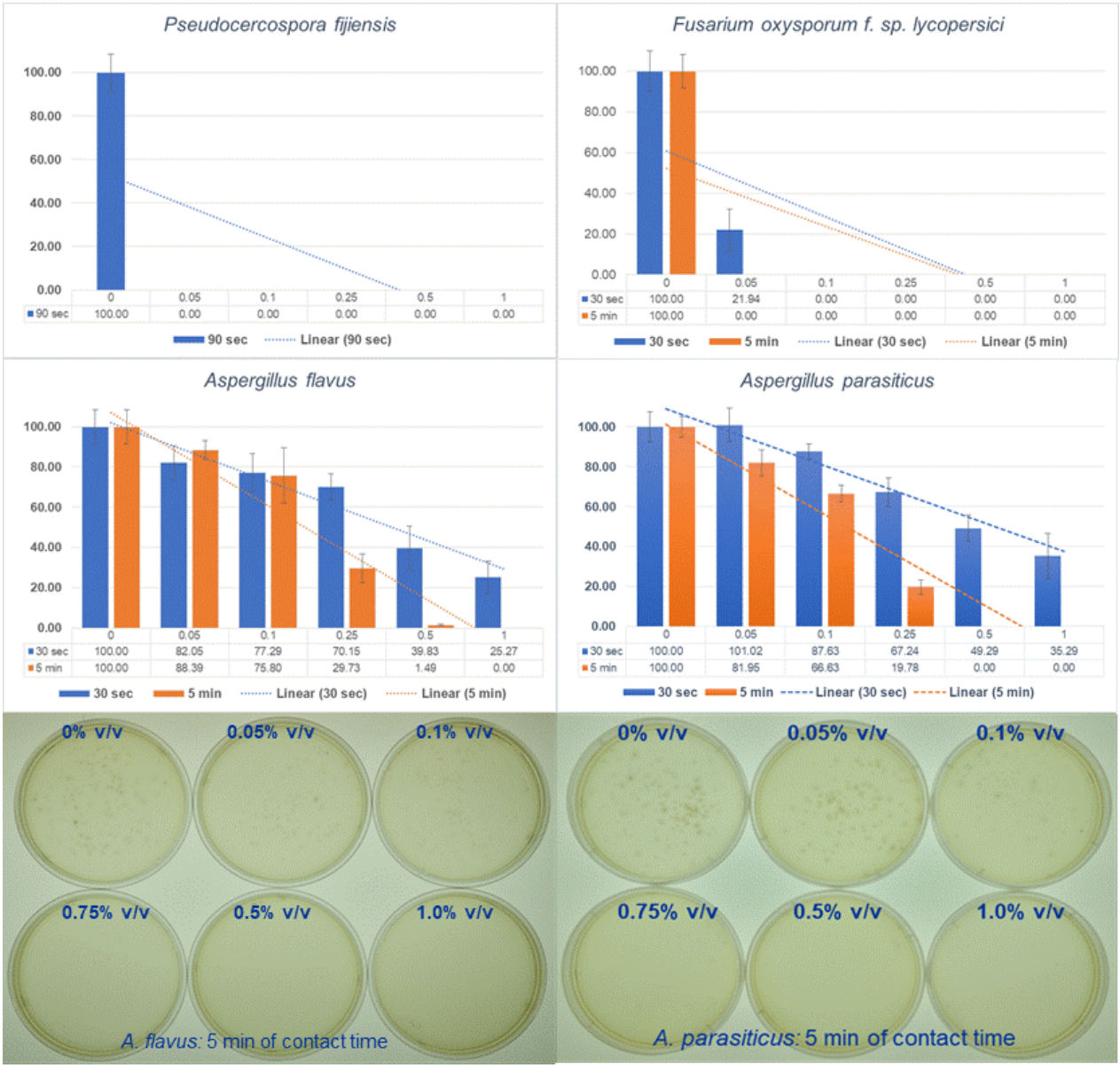

Table 1. Number of colony forming units (CFU) per concentration of D7 and contact time with fungal spores or mycelia (for *Pseudocercospora fuligena*) of the different fungal species tested in this study.

% v/v D7	0	0.05	0.1	0.25	0.5	1.0
***Aspergillus parasiticus*: 30 sec of contact time**						
CFU	164.33	166.00	144.00	110.50	81.00	58.00
% CFU	100.00	101.02	87.63	67.24	49.29	35.29
error %	7.68	8.40	3.74	7.32	6.67	11.40
***Aspergillus parasiticus*: 5 min of contact time**						
CFU	164.33	134.66	109.50	32.50	0.00	0.00
% CFU	100.00	81.95	66.63	19.78	0.00	0.00
error %	5.12	6.37	4.09	3.59	0.00	0.00
***Aspergillus flavus*: 30 sec of contact time**						
CFU	168.20	138.00	130.00	118.00	67.00	42.50
% CFU	100.00	82.05	77.29	70.15	39.83	25.27
error %	8.41	8.51	9.22	6.45	10.93	7.88
***Aspergillus flavus*: 5 min of contact time**						
CFU	168.20	148.66	127.50	50.00	2.50	0.00
% CFU	100.00	88.39	75.80	29.73	1.49	0.00
error %	8.40	4.81	13.66	7.01	0.53	0.00
***Fusarium oxysporum f*. sp. *lycopersici*: 30 sec of contact time**						
CFU	59.25	13.00	0.00	0.00	0.00	0.00
% CFU	100.00	21.94	0.00	0.00	0.00	0.00
error %	9.97	10.34	0.00	0.00	0.00	0.00
***Fusarium oxysporum* f. sp. *lycopersici*: 5 min of contact time**						
CFU	58.50	0.00	0.00	0.00	0.00	0.00
% CFU	100.00	0.00	0.00	0.00	0.00	0.00
error %	8.27	0.00	0.00	0.00	0.00	0.00
***Pseudocercospora fijiensis*: 90 sec of contact time**						
CFU	14.67	0.00	0.00	0.00	0.00	0.00
% CFU	100.00	0.00	0.00	0.00	0.00	0.00
error %	8.58	0.00	0.00	0.00	0.00	0.00