The identity of a Wine Cellar Mould and its susceptibility to Path-Away.

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A mould isolated from a winery cellar was cultured and DNA sequence analysis of the conserved ITS region identified the predominant organism as a *Penicillium* species. The cultured *Penicillium* was susceptible to Path-Away™ in diffusion assays, and with a minimum lethal concentration of 0.2% (of concentrate by volume) in microdilution assays.

Identification of the winery mould.

A mould isolated from a winery cellar was supplied as scrapings. The mould was cultured from the scrapings on Difco YPD agar (Yeast Extract-Peptone- Dextrose, cat no. 242720, Fort Richard Laboratories, Auckland). Culture was over 3 plus days at room temperature.

To identify the organism present, DNA was isolated from both the original scrapings and mycelia and conidia from the cultured organism. Purified genomic DNA (50 ng per reaction) was subjected to PCR using KAPA HiFi HotStart ReadyMix (2X) (KAPA Biosystems) and primer sets (Macrogen) for the fungal ITS region:

`ITS(f) 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCTTGGTCATTTAGAGGAAGTAA`

`ITS(r) 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCTGCGTTCTTCATCGATGC`

The primers recognise highly conserved regions of the chromosome, allowing amplification of DNA with variable regions that can be used to identify the species. Amplified DNA was purified using an Agencourt AMPure XP kit (Beckman Coulter), confirmed by agarose gel electrophoresis and quantified (Qubit Fluorometric Quantitation (Thermo Fisher Scientific)). The DNA was subjected to Sanger sequencing (DNA Sequencing Centre, University of Auckland) and identified using BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch). Both DNA samples identified a best hit with *Penicillium brevicompactum*.

The susceptibility of the winery mould to Path-Away.

To test the effectiveness of Path-Away against the isolated *Penicillium* mould a selection of assays was employed.

Diffusion assay. Fungal spores were isolated by washing a YPD plate with YPD broth and diluted to approximately 10^7 spores / ml after counting in a haemocytometer. Approximately 10^6 spores were spread on a YPD agar plate and a plug approximately 0.5cm in diameter was cut from the centre of the plate and discarded to leave a well for samples. Path-Away concentrate or 5% Path-Away were mixed 1:1 with molten YPD agar at 55^{o}C and pipetted into the well. Triplicate plates were prepared for each Path-Away treatment and a negative control with only YPD agar. After the agar had set in the well the plates were incubated at room temperature for one week, after which photographs showing zones of inhibition were taken.

The results (Figure 1) show a clear zone of inhibition for the Path-Away concentrate and a smaller zone for the 5% Path-Away. Zones of inhibition represent the minimum inhibitory concentration of the active agent and are dependent on how well the active agent diffuses through the agar.

Spreading assay. As a variation on the diffusion assay, a spreading assay that is able to compare different agents on the same agar plate was performed. Six filter papers soaked in either the active agent or controls were dried and placed at equidistant positions around a central well in a YPD plate filled with a plug taken from an agar plate of actively growing mould. The isolated *Penicillium* species or the control mould *Aspergillus niger* ATCC 16404 were tested. Filter papers were autoclave sterilised and then soaked in Path-Away concentrate, 5% Path-Away or YPD broth. Triplicate agar plates with 2 of each filter paper were prepared and incubated at room temperature for one week before being photographed. Path-Away concentrate soaked filters inhibit spreading growth of *Aspergillus*, but as the *Penicillium* does not spread as well it is difficult to say how effective Path-Away is at inhibiting the spread of the *Penicillium* (Figure 2).

Microdilution assay. To quantify the antifungal effect of Path-Away for the *Peniciillium* isolate 3% and 5% solutions of Path-Away concentrate were tested. Doubling dilutions in YPD broth were made in a microwell plate leaving 50 μl of antifungal solution in each well. The final well was YPD alone. To each well 50 μl of a fungal spore suspension (approx. 5 $x10^5$ spores) in YPD broth was added. The microplates were incubate in a humid box for 2 days, after which time 10ml was removed from each well to determine whether viable fungus remained by spotting to YPD agar, which incubation at room temperature for one week. The experiment was also run with *Aspergillus niger* ATCC 16404 as a control.

A preliminary assay just looking at inhibition of growth is shown in Figure 3, where it can be seen that down to 0.04% path-Away inhibits the growth of the *Penicillium*. The assay here does not say whether those spores used as the inoculum have been killed. Table 1 shows the combined results of replicate assays for the minimum lethal concentration (MLC). The results show lethal activity of Path-Away in the pre-prepared 5% solution and a 3% solution made in the laboratory by diluting 30ml Path-Away concentrate in a final volume of 1l sterile milliQ water. It is clear from these experiments that Path-Away is lethal to the *Penicillium* isolate and that the isolate appears more sensitive than *Aspergillus niger* ATCC 16404, a commonly used test strain for anti-mould agents.

Conclusion.

Path-Away at 5% (pre-prepared) or 3% (self-prepared) from concentrate are active against the *Penicillium* species isolated from the wine cellar. The activity of Path-Away against the *Penicillium* in the laboratory is comparable to that against a test strain of *Aspergillus niger*. Path-Away have commissioned and/or published independent testing studies (http://path-away.com/downloads.html) that demonstrate efficacy against *A. niger* which suggests that Path-Away will be effective against the *Penicillium* from the winery. The MLC is at least 10 fold below the in use concentration, however this is in suspensions in the laboratory, whereas in reality fungal spores will contact Path-Away aerosol droplets and so it is good to have a safety margin. The kinetics of killing have not been determined for the different concentrations of Path-Away, and higher concentrations may effect a faster kill.

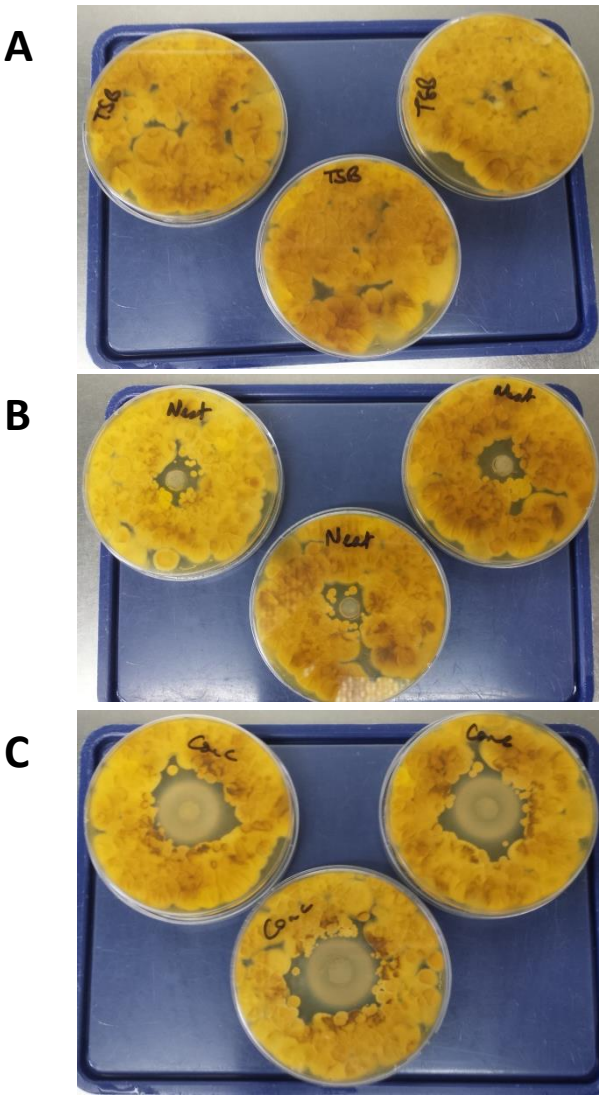

Figure 1. Diffusion Assay for Path-Away. A 1:1 mixture of YPD broth and YPD agar (A), 5% Path-Away and YPD agar (B) or Path-Away concentrate and YPD agar (C) were set into a YPD agar plate inoculate with spores of a *Penicillium* isolated from a winery cellar. Zones of inhibition are apparent after 1 week of incubation at Room temperature for plates with wells containing Path-Away. Larger zones are observed for higher concentrations of Path-Away.

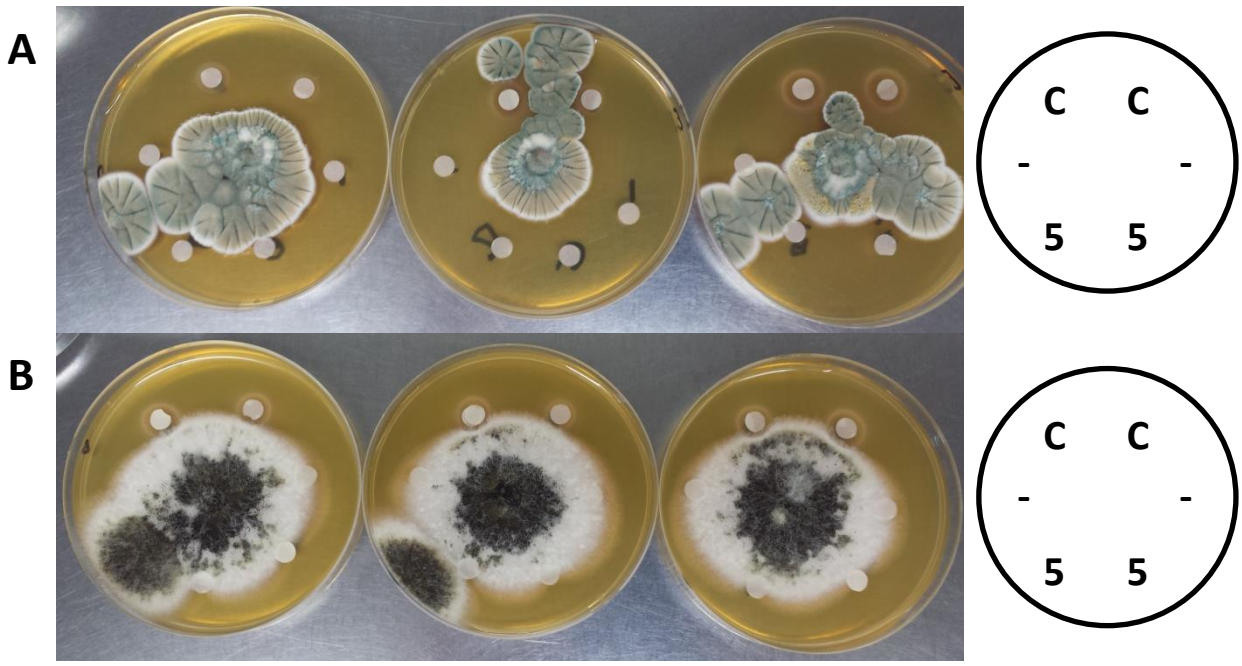

Figure 2. Spreading assay for Path-Away. Filter papers soaked in Path-Away concentrate (C), 5% Path-Away (5), or YPD broth (-) were placed on an agar plate inoculated with the winery *Penicillium* isolate (A) or *Aspergillus niger* ATCC 16404 (B). Plates were incubated for one week at room temperature. There is a retardation of spreading seen for the filter paper soaked in Path-Away concentrate for *Aspergillus*. The *Penicillium* does not spread well and it is difficult to conclude any effects.

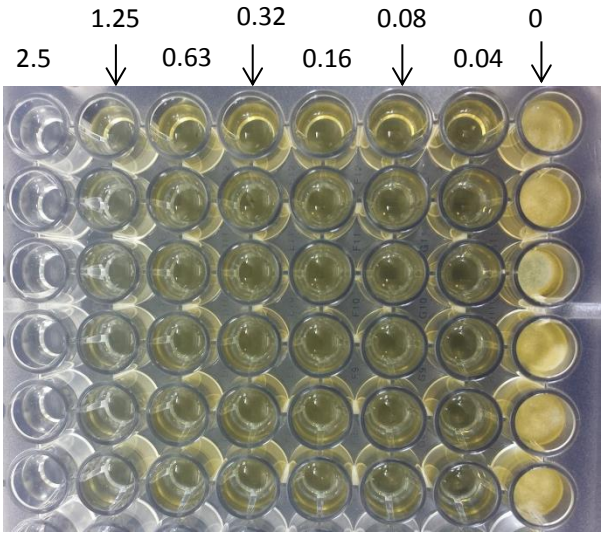

Figure 3. Microdilution assay. Doubling dilutions of Path-Away inhibit the growth of Penicillium spores down to 0.04% v/v of the concentrate. There is clear growth in the control ‘0’ well.

Table 1. MLC of Path-Away. Starting with 3% or 5% Path-Away by volume, the median concentration and the upper range required to kill spores of *Aspergillus* and *Penicillium* is recorded. Results are from at least triplicate samples tested on three occasions.

	3%		5%	
	Median MLC	Upper Range	Median MLC	Upper Range
Penicillium	0.05	0.19	0.16	0.16
Aspergillus	0.09	0.19	0.16	0.31