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A simplified colorimetric method to assess drug sensitivity in oomycetes

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Introduction

Oomycete are a diverse group of fungus-like filamentous eukaryotic microbes, also referred to as water molds. These organisms were initially grouped with fungi due to similarities in their morphology and lifestyle. Later, they were found to be evolutionarily distinct from fungi through molecular and phylogenetic analyses leading to its classification as members of the stramenopiles. Unlike fungus, the hyphae of oomycetes are rarely septate. They can reproduce both asexually and sexually. The asexual spores called zoospores are the main dispersive form while the sexual spores (oospores) are primarily for surviving the adverse environmental conditions. While many species of oomycetes are plant pathogens, few species, for example members of the genus, *Saprolegnia* can cause severe disease in fish.

Oomycete species exhibits variable susceptibility to antifungal and anti-oomycete compounds; hence drug sensitivity testing will help to find the most effective agent against a specific species or isolate. In the case of oomycetes, the effect of drug is either determined through radial growth inhibition assay or inhibition of germination and colonization using zoospores. In the radial growth inhibition assay, the relative retardation in hyphal growth under the effect of an anti-oomycete agent as compared to untreated control at different time intervals is measured. In the other method that determines the inhibition of germination and colonization, a particular number of zoospores are incubated in broth containing the test drug and hemp/sesame seeds. After incubation at suitable temperature, germination of the zoospores and colonization on the seeds are observed under microscope. Here, we report a simple colorimetric method for determination of minimum inhibitory concentration (MIC) of anti-oomycete agent using oomycete hyphae.

Methodology

The oomycetes (*Saprolegnia* and *Achlya* species) were cultured on glucose-yeast extract agar (GYA) plates and incubated at 20 ± 1◦C. Subculture was done by transferring a piece of

agar excised from the advancing edges of the colony to a new GYA plate. In all the experiments, 4-day old culture was used to maintain uniformity. In a 48-well plate, 400 µl of glucose yeast extract broth (GYB) was added in all the wells of first row. In the 2nd well, 400 µl of a particular concentration of the test drug was added. The contents were mixed and 400 µl was transferred to the next (3rd) well. The same procedure continued till 7th well and 400 µl was discarded from it. Then agar plug of 4 mm diameter from the 4 days old cultured GYA plate was added in all the wells except the 1st well which served as sterile control. The last (8th) well containing only GYB and the agar plug served as growth control. The same procedure was performed in the 2nd and 3rd row of the plate to make triplicate of each test. The plate was incubated at 20 ± 1◦C for 48 h. Thereafter, 100 µl of freshly prepared resazurin (300 µM in phosphate buffer) was added to each well. The plate was further incubated and observed for change in colour from blue to pink. Once there is pink colour in the growth control well, the result can be recorded.

Result interpretation

Resazurin gets reduced to pink-coloured resorufin during aerobic respiration of metabolically active cells making it a reliable indicator of cell viability. Therefore, the viability of the oomycete hyphae under the test conditions can be monitored visually on the colour of the wells. In the drug sensitivity assay of oomycetes, the minimum concentration of the test compound at which the colour remained blue was recorded as MIC. The absorbance of the wells can be recorded at 570 nm and 600 nm to calculate the relative percentage of dye reduction.

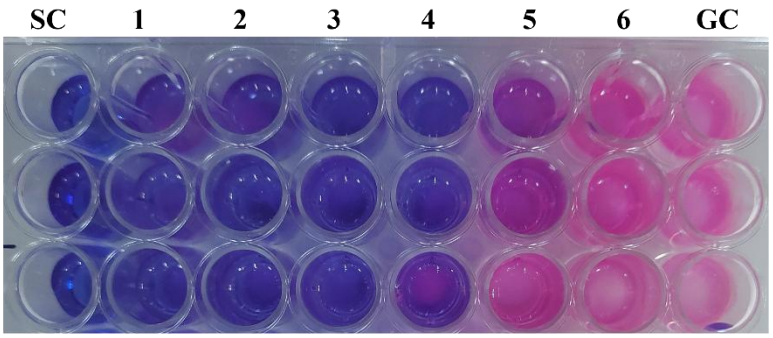
Critical points

1. The colour of resazurin is pH dependent, appearing blue to purple at pH above 6.5 and orange at pH below 3.8. So, resazurin based method for determination of MIC may not be suitable for drugs or solutions having lower pH.
2. If the sterile control turns pink, the media may be contaminated. The experiment should be repeated.
3. If the growth control well remained blue or develop pink colour slower than the treated wells, the result is not reliable. The experiment should be considered invalid and repeated with fresh reagents and inoculums.

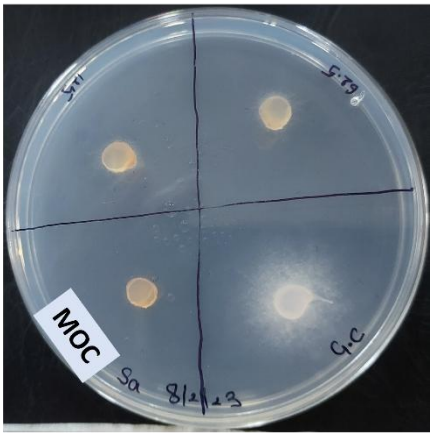
Uniqueness of this method

1. Unlike radial growth inhibition assay, there is no need of preparing large number of agar plates containing different concentration of the test compound.
2. The methodology uses hyphae instead of zoospores, so the additional steps of zoospores induction and its counting are not required, making it a simple method for determination of MIC.
3. After determination of MIC, the same agar plugs can be used for determination of minimum oomyceticidal concentration. This can be done by incubating the agar plugs

on fresh GYA and observing radiating hyphae (if any) from the plugs after 24 h of incubation.



Determination of MIC of test drug against *Saprolegnia* using resazurin (SC- Sterile control, GC- Growth control, 1-6: Different concentrations of the test drug)



Determination of MOC

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