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Programmed Necroptosis in *Aspergillus salvadorensis* under Oxidative Stress Conditions Caused by Hydrogen Peroxide in Conidias

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Abstract:

This study demonstrates the possible pathways used by the *Aspergillus* fungus in its growth and cell death using the KEGG result of DNA sample analysis sent by MACROGEN INC South Korea in 2024. Three concentrations of H_2O_2 (3%, 5% and 10%) were evaluated by direct microscopy applied to cultures in Saboraud samples of *Aspergillus salvadorensis* aliquots. This paper presents a procedure to induce and record the morphological changes and analyzes their relevance in research on pathogenicity and mechanisms of tolerance to oxidative stress. The results show a progressive deterioration of hyphal and conidial integrity, with marked structural loss at higher concentrations. In observations of dry fresh preparations, the hyphae show localized thinning and decreased turgor, while the conidiophores experience gradual deformations and the vesicles show irregularities in their shape and loss of uniformity, as well as in the conidia. Necroptosis in fungi of this species is manifested by the rupture of the wall, the release of cytoplasmic contents and the generation of granular detritus. The transition from a morphologically integral state to cell disintegration at 10% hydrogen peroxide occurs. Necroptosis in this fungus is strongly influenced by the intensity of oxidative stress, damage is not immediate except for inflammation of the conidia at its onset. Complete results are seen in five days at non-lethal concentrations, on average 15 days that the lethal invasive attack of destruction of *Aspergillus* is observed in the dry smears.

Keywords: *Aspergillus salvadorensis*, hydrogen peroxide, Necroptosis, conidia, ROS.

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INTRODUCTION

Necroptosis is a specific scientific term that describes a type of programmed cell death that occurs under conditions of induced stress, characterized by following a controlled genetic pathway, despite presenting morphological features typical of necrosis, such as cell lysis and the release of cytoplasmic contents. In the literature, this process is also often referred to as programmed necrosis, differing from apoptosis, which is a form of programmed cell death executed in an orderly manner by the cell itself without causing inflammation. Due to the release of intracellular components, necroptosis is considered a form of pro-inflammatory cell death, as is pyroptosis, which is mainly associated with responses to infections, especially in cells of the immune system. These classifications have been established to distinguish the different subroutines of programmed cell death according to their molecular mechanisms and pathophysiological consequences (Cookson and Brennan, 2001; Galluzzi *et al.*, 2012; Vandenabeele *et al.*, 2010).

Oxidative stress in filamentous fungi shows complex responses to the presence of reactive oxygen species (ROS), including hydrogen peroxide. These molecules can induce damage to proteins, lipids, and cell membranes, affecting the viability and functionality of the microorganism.

In fungi, including species of the genus *Aspergillus*, the existence of programmed cell death processes similar to the Apoptosis and Necroptosis observed in higher eukaryotic organisms has been reported. Exposure to hydrogen peroxide is an effective method to induce these cell death pathways by generating a redox imbalance that directly affects the integrity of the hyphal and other structures (Chen *et al.*, 2021).

In fungi, manifestations such as necroptosis are very complex, it is a process of violent and inflammatory programmed cell death that combines characteristics of necrosis and apoptosis. Three proteins are mainly involved: RIPK1, RIPK3 and MLKL. The MLKL protein travels to the cell membrane and pierces it, causing the cell to swell and explode, releasing all its contents to the outside. Pyroptosis is specifically triggered by signs of infection in humans. Necrosis is due to the absence of oxygen in the tissues seen in humans. Apoptosis corresponds to a programmed cell death, controlled by the cell itself, which occurs in an orderly manner and does not induce inflammation, since it develops silently through the activation of caspases. On the contrary, necrosis is an unprogrammed process, the result of external aggression or cell damage, in which the membrane is passively broken, causing the release of cell contents and a marked inflammatory response. Necroptosis, although it shares with necrosis its inflammatory character, differs because it is a form of regulated cell death, mediated by specific signals in which proteins participate. In this way, necroptosis is situated as an intermediate mechanism: it is controlled like apoptosis, but with inflammatory consequences similar to those of necrosis (Aguirre *et al.*, 2006; Gonçalves *et al.*, 2020; Paoletti and Saupe, 2009; Shlezinger *et al.*, 2017; Vandenabeele *et al.*, 2010). Under laboratory conditions, it is observed that at 100x the object cover rises in mm due to the inflammation of the conidia, it is difficult to focus on observation under normal conditions, so it is necessary to wait several days for drying.

This study aims to describe the morphological effects generated by different concentrations of H_2O_2 on *Aspergillus salvadorensis*, allowing to visualize in a comparative way the progression towards necroptosis induced by oxidative stress.

MATERIALS AND METHODS

Preparation of the Fungal Culture

Initial Culture: Seed spores of *A. salvadorensis* in a Saboraud culture medium and allow growth to the exponential phase or beginning of the stationary phase (usually 24-48 hours at 25−37 °C with agitation).

Density Adjustment: Dilute the culture to obtain a standardized concentration of mycelium, vesicle, and conidia (e.g., 1×10^6 conidia/mL) prior to treatment to induction of stress and necroptosis. The most likely inducing agents that promote non-apoptotic cell death in fungi include: Oxidative Stress with Hydrogen Peroxide (H_2O_2): It is a common inducer of oxidative stress. Concentrations vary widely between 3.5 and 10%. Hydrogen peroxide is a strong candidate for dysregulating fungal metabolism and causing cell death.

Induction Procedure: Divide the crop into vials. Add the H_2O_2 inducer at a pre-determined concentration of 3.5 and 10 %), leaving a control vial without an inductor as a control. Incubate at the optimal growth temperature for one week and make observations with cotton blue lactophenol with multiple fresh preparations and observe the same dry smears performed previously after 5 to 15 days.

RESULTS

Table (1) demonstrates the KEGG result of the possible pathways used by the fungus in its growth and death provided by MACROGEN INC found in the *Aspergillus* sample sent in 2024. The KEGG analysis reveals a marked predominance of orthologs linked to the category of Cellular Processes, indicating that the evaluated gene repertoire is mainly involved in functions essential for the maintenance and regulation of the cell. Within this group, the subcategory of cell growth and death is the most representative, covering routes related to cell cycle control, senescence, and different types of programmed cell death. The identification of pathways such as Apoptosis and Necroptosis highlights the body's ability to modulate cell survival and respond in a regulated manner to adverse stimuli or stress conditions. In addition, the presence of cell cycle pathways described in different model organisms, such as yeasts and other eukaryotes, suggests a high degree of evolutionary conservation of these mechanisms.

These results provide a solid functional basis for interpreting complex cellular responses and guide future studies aimed at the detailed characterization of the molecular pathways involved. In a dry assembly after 15 days of the same initial sheet, where the peroxide has already acted and the fungus is fixed. The hyphae are flattened, dull, as if they have completely lost their hydration. Conidia, on the other hand, look swollen. The conidiophore looks like a stiff, brittle tube, with areas where the wall is fractured into small segments. The gallbladder is just a malformed, flattened, opaque sphere, without the regular contours of a healthy gallbladder. The phylalides are almost indistinguishable; they look like crushed or torn points, like the remains of a structure that no longer retains shape. Conidia are the best-preserved structures, but even they show deformations: some are wrinkled, others fragmented, or partially collapsed. The whole conveys a feeling of total degradation, with the fungal structures reduced to a rigid and unvitality pattern, marked by previous peroxide damage.

In the first condition (Aspergillus in the basal state) fully conserved morphological structures are observed, characteristic of a filamentous fungus in active growth. Septate hyphae have continuous, homogeneous cell walls without signs of degradation. The conidiophore maintains its upright axis with complete structural integrity, reflecting cytoplasmic stability and cytoskeletal functionality. The vesicle is spherical and shows a uniform distribution of phylalides and conidia, which have regular contours and absence of morphological artifacts. This condition represents a stable physiological state, without the intervention of oxidative stress or mechanisms of cell death.

In the second condition (*Aspergillus* subjected to hydrogen peroxide) alterations compatible with sublethal oxidative stress are identified. Hyphae show partial loss of cell wall definition, with focal thinning and areas of optic pallor indicating damage to structural polysaccharides such as chitin and glucans. Regions of intracellular vacuolization are evidenced, suggesting accumulation of reactive oxygen species (ROS)

and organelle dysfunction. The conidiophore shows slight deformation and decreased turgor, while the gallbladder reveals superficial irregularities attributable to oxidation of membrane lipids. Several conidia appear retracted, deformed, or detached, reflecting a stress response prior to the activation of unregulated programmed cell death pathways.

In the third condition (hydrogen peroxide-induced necroptosis) a morphological pattern marked by advanced structural destruction is observed. Hyphae exhibit frank rupture of the cell wall, with extravasation of cytoplasmic contents and presence of granular debris, characteristic findings of fungal necroptosis. The conidiophore is collapsed and presents axial fragmentation, associated with the complete loss of mechanical integrity. The gallbladder shows severe deformation, with collapse of the curvature and evidence of membrane disruption. Conidia are absent, destroyed, or irregularly dispersed, suggesting de-anchoring by generalized lysis. This scenario represents a terminal state, compatible with activation of ROS-mediated regulated necrosis pathways and degradation of essential cellular components.

Table 1. Result of the sequencing of *Aspergillus salvadorensis* 2024. MACROGEN INC.

KEGG summary: Orthology Frequency within Main and Sub-Categories

* KEGG: Database for understanding biological functions and utilities from molecular-level data
* Level1, Level2, Level3: Hierarchical classification of KEGG pathways, from broad categories to specific processes.
* {Sample}: Gene counts specific to each sample, indicating the presence of genes related to each pathway.

Level1	Level2	Level3
Cellular Processes	Cell growth and death	Apoptosis
Cellular Processes	Cell growth and death	Apoptosis - fly
Cellular Processes	Cell growth and death	Cell cycle
Cellular Processes	Cell growth and death	Cell cycle - Caulobacter
Cellular Processes	Cell growth and death	Cell cycle - yeast
Cellular Processes	Cell growth and death	Cellular senescence
Cellular Processes	Cell growth and death	Meiosis - yeast
Cellular Processes	Cell growth and death	Necroptosis
Cellular Processes	Cell growth and death	Oocyte meiosis
Cellular Processes	Cell motility	Cytoskeleton in muscle cells
Cellular Processes	Cell motility	Flagellar assembly
Cellular Processes	Cell motility	Motor proteins

Result of peroxide induction

The cells maintain a conserved morphology, with intact membranes and a homogeneous distribution in the control condition (Figure 1). No signs of significant structural damage are observed, indicating the absence of cell death and, therefore, the absence of necroptosis. When cells are exposed to 3% H_2O_2, minor morphological changes associated with oxidative stress begin to be evident. Some cells show mild alterations, such as contour irregularities and discrete aggregation, but most retain their integrity. This suggests an early stage of cell damage, in which cell death pathways may be activated, although necroptosis is not yet predominant. In the sample treated with 5% H_2O_2, more marked changes were observed: loss of normal cell shape, increase in granular material and presence of cellular debris. These characteristics are compatible with necroptosis, a type of programmed cell death that occurs with inflammation and progressive rupture of the plasma membrane, but still retains certain structural features before complete lysis. Finally, in the 10% H_2O_2 condition, cell damage is extreme. Most cells have completely lost their integrity, with few recognizable cellular

elements. This pattern corresponds more to unregulated necrosis, the result of excessive oxidative stress, in which cell destruction occurs rapidly and uncontrollably, overcoming the regulated mechanisms of necroptosis. Taken together, the results indicate that necroptosis manifests more clearly at intermediate concentrations of H_2O_2 (5%), while low concentrations induce limited damage and high concentrations cause massive necrosis. It is clarified that necroptosis is not immediate, the results are seen in five days in non-lethal concentrations, it is on average 15 to 30 days that the invasive attack of destruction is observed in *Aspergillus*.

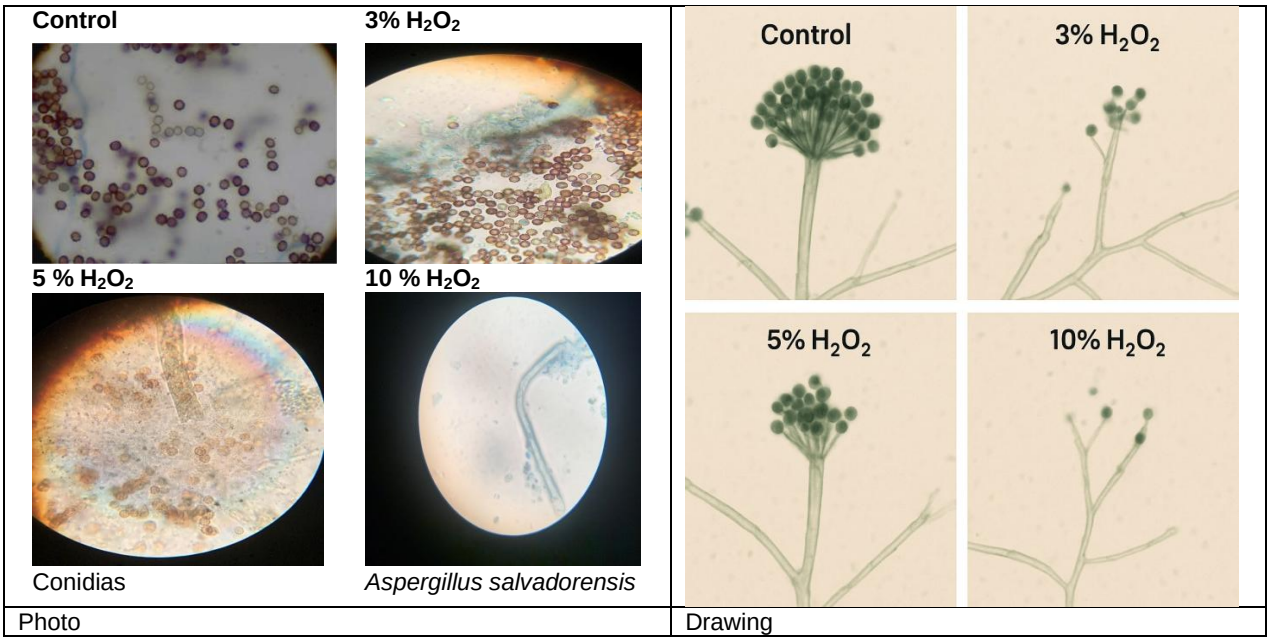

Fig. 1. Photo and drawing of *Aspergillus salvadorensis* at different concentrations of hydrogen peroxide. 100x.

Morphological analysis of deterioration due to necroptosis

Microscopic evaluation of *Aspergillus salvadorensis* under different levels of necroptosis revealed progressive alterations in the integrity of the conidial head and in the density of the associated conidia. Microscopic analysis revealed a progressive degradation of reproductive structures as a function of the percentage of induced necroptosis. In specimens with minimal damage (3%), the preservation of fungal architecture was observed, highlighting swollen globose conidial heads, with circular morphology and high sporal density; In this phase, the conidia remain compact with a uniform grainy texture on a structurally sound conidiophore. The reproductive structures presented a practically preserved morphology. The conidial head was spherical, voluminous and uniformly delimited, with a surface densely covered by conidia. These conidia maintained regular spherical morphology, with homogeneous distribution and no evidence of premature separation or disorganization. The conidiophore was intact and robust, adequately supporting the apical vesicle. The conidia are complete, with a normal development in their structure (spherical and with an elongated pedicel). This suggests that necroptosis has a slight impact on the morphology of conidia at this concentration. When the level of necroptosis increased to a moderate range (5%), a volumetric reduction of the conidial head and a loss of cohesion in the

spore mass were evidenced. This state is characterized by the appearance of surface irregularities and erosion zones where the density of the conidia decreases, leaving areas of the conidiophore partially exposed. Moderate morphological alterations were detected. The conidial head exhibited a visible reduction in its diameter, with partial loss of its structural homogeneity. Areas with decreased density of conidia were identified, generating irregular and discontinuous areas. The surface took on a less compact appearance, with interruptions in the usual spherical pattern. Although the conidiophore remained structurally stable, the gallbladder showed signs of peripheral deterioration. The conidia are complete, with a normal development in their structure (spherical and with an elongated pedicel). This suggests that necroptosis has a slight impact on the morphology of conidia at this concentration. As the concentration of necroptosis increases, it can be observed that the conidia begin to change slightly, showing a decrease in size or a deformation, but still maintains a relatively intact shape. At 10% necroptosis, the damage was markedly severe. The conidial head was almost completely absent, leaving the apical region of the conidiophore exposed. The conidia showed a drastic loss, with only a few sporadic remnants remaining without spatial organization. The absence of the typical globular structure indicates advanced collapse of the conidial vesicle and complete disruption of the spore-producing apparatus. At this stage, the morphology corresponds to a naked conidiophore, lacking reproductive functionality. At higher concentrations of necroptosis, conidia show more obvious damage, possibly presenting abnormal elongation or even fragmentation. This indicates that at high concentrations of necroptosis, the conidia suffer greater structural damage.

Overall, the findings show that even modest increases in necroptosis generate progressive and quantifiable changes in the architecture of conidia, affecting first the density of conidia and then the total integrity of the vesicle.

In summary, necroptosis is being observed in *Aspergillus salvadorensis* at different concentrations, we could interpret the following:

There is evidence of necroptosis:

If the necroptosis process is ongoing, a disintegration or alteration of cellular structures should be observed, such as an abnormal expansion of the cell, rupture of the cell membrane, or the release of cellular content into the extracellular space. The fungus seems to undergo changes in its structure as the concentration of the chemical agent increases, causing oxidative stress. If we are talking about necroptosis, what could be happening is that, at higher concentrations, the stress caused by the agent triggers the necroptosis process, which prevents the cell from continuing to function normally and leads to its death. At lower concentrations, the cell may not be as severely affected, and only slight alterations may be seen, which could indicate a beginning of the necroptosis process, but not be completely irreversible.

Interpretation of morphological changes:

At 3% concentration, the cell appears intact, which could indicate that the necroptosis process has not begun or that the damage is very slight. At 5%, alterations in morphology could suggest a process of necroptosis in its initial stages, where some cells begin to decompose, but not yet completely. At 10%, the disappearance of the spherical structure and the elongation of the stem indicate advanced necrosis or necroptosis, suggesting that the cells have reached a point where their structure is severely affected, with rupture of their components (See Table 1).

Table 1. Comparative summary table of morphological indicators.

Structure	Status basal (control)	H_2O_2 oxidative stress (sublethal)	Necroptosis H_2O_2-induced (lethal)
Hyphae	Septate, homogeneous walls, dense cytoplasm	Focal thinning, pallor, moderate vacuolization	Cell wall rupture, lysis, debris release
Conidioforo	Erectic, cylindrical, whole	Mild deformation, partial loss of turgor	Collapsed, fragmented, unrecognizable structure
Vacuolization	Spherical, well-defined	Uneven, with areas of oxidation and loss of gloss	Deformed and collapsed, obvious membrane rupture
Conidia	Abundant, spherical, uniform	Deformation, retracted, partial detachment and swelling.	Absent, destroyed or dispersed by lysis
Cytoplasm	Dense, continuous	Vacuolized, slight granularity	Granular, extravasated, cellular debris
Cell wall	Intact, without discontinuities	Weakened, rusty, thinning	Broad discontinuities, rupture, and collapse
General appearance	Stable and functional morphology	Initial progressive alteration	Total disintegration, non-apoptotic cell death

Figure (2) shows a sample of *Aspergillus salvadorensis* with an *Aspergillus* conidiophore with a morphology altered by a process of induced necroptosis. The stem of the conidiophore rises straight, but its texture appears denser and more granular than usual, a sign of structural damage. At the upper end, a darkened vesicle and few conidia can be distinguished, with poorly defined contours, which indicates condensation of the cellular contents and loss of integrity. Around the vesicle emerge radially distributed phylalides, although several show shortening, partial collapse and an irregular arrangement, reflecting the deterioration produced by oxidative stress. Spherical conidia are observed on the phylalides, some well-formed, but others with diffuse borders, superficial rupture or unequal separation, which reinforces the presence of cell death due to oxidative damage. The whole set has a grainy appearance, with areas of greater density and light sectors around the vesicle, indicative of the release of intracellular content. The image in its entirety represents the typical structure of a conidiophore, but profoundly affected by a process of necroptosis.

An Aspergillus conidiophore subjected to an advanced process of cell damage compatible with necroptosis induced by oxidative stress is clearly visible. The main structure, the axis or stem of the conidiophore, is relatively straight, although it has a more intense brown color and a rougher and grainier internal texture than normal, indicating alterations in the cell wall and abnormal accumulation of intracellular material. This loss of homogeneity in the stem suggests that free radicals have deteriorated the consistency of the hyphae that originates it.

At the distal end of the conidiophore is the vesicle, a sphere that under normal conditions exhibits a clearer and more uniform appearance. In the image, on the contrary, the vesicle appears markedly darkened, with a dense center and partially irregular borders, features that reflect an internal structural collapse and a possible condensation of the cytoplasm as a response to stress, in others the absence of a vesicle is observed. That darkening, along with the lack of uniformity on the surface, is characteristic of significant cell damage.

Surrounding the gallbladder are the phylalides, which are normally arranged in an orderly fashion forming a symmetrical crown. In this projection it is observed that the phylalides are present, but their length and shape are irregularly compromised: some are seen as thin and elongated, while others are visibly

shortened, deformed or with a collapsed tip, which suggests loss of turgor and damage to the cell membrane. This asymmetry in the phylalidic architecture is typical of a degenerative process.

At the ends of the phylalides are conidia, spores that in a healthy conidiophore should be spherical, homogeneous and symmetrically distributed. In this image, while many conidia maintain their rounded shape, others have blurred contours, areas of translucency, rough surface, or signs of incomplete detachment, indicating alterations in sporal wall synthesis or direct damage by reactive oxygen species. The uneven distribution of conidia also suggests a disruption of the sporulation process.

Around the entire structure there is a clear halo, possibly caused by the release of intracellular content of the fungus after the rupture or weakening of the membrane. This halo reinforces the interpretation of necroptosis or cell death associated with oxidative damage. In addition, small granular areas or cytoplasmic bubbles are observed in the gallbladder and in some phylalides, a typical feature of deterioration caused by exposure to hydrogen peroxide or other ROS-generating agents.

In a direct fresco smear of *Aspergillus* treated with hydrogen peroxide and observed at 100x, it is possible to see only general changes in the structure of the fungus. At this magnification and without staining, the hyphae may appear darker, grainy or partially collapsed, and it is possible to distinguish an altered conidiophore with a vesicle that appears less defined and denser than normal. Scattered conidia and some obvious deformations can also be seen, such as irregularity in the shape of the sprinkler head or areas of rupture in the hyphae. However, the level of structural detail such as the precise shape of the phylalides, the individual separation of the conidia or the fine changes in the surface of the gallbladder is not clearly observed at 100x with a cool setup, as this type of preparation only allows the identification of the overall morphology and the most obvious signs of damage. Therefore, what is visualized is a general image of morphological deterioration due to the effect of oxidative stress, but not a clear definition of each component of the conidiophore.

With hydrogen peroxide applied directly to a fresh Aspergillus smear, the microscopic image would show clear signs of immediate oxidative damage, but without the fine sharpness of a stained preparation. When the hydrogen peroxide is placed, the structure of the fungus begins to show an irregular darkening in the hyphae, as if parts of the cytoplasm were condensing into small opaque clumps. Hyphae, which are usually transparent and sharply edged, take on a more broken and grainy appearance, with areas that appear to thin and others that inflate slightly before collapsing. Some are seen with a more matte or yellowish tone, a sign that the cell wall is beginning to be damaged.

The conidiophore can still be seen as a vertical prolongation, but with a surface that loses uniformity. The gallbladder at the tip is no longer visible as a sharp sphere; It appears denser, darker and with blurred contours, as if it is losing its internal tension. Around it, the phylalides are only partially distinguished, often shortened or poorly defined, as a result of the immediate damage caused by the oxidant.

The conidia, normally spherical and shiny, are scattered and some lose their circular shape, taking on a deformed or collapsed appearance, almost like small bubbles that have deflated. Sometimes, a clear halo can form around the fungus, as a result of the interaction between H_2O_2 and the cellular components released into the environment.

Overall, there is an impression of disorganization and rapid deterioration, where the fungus retains its general shape, but presents multiple signs of severe stress: darkening, partial collapse, loss of structural definition and irregular dispersion of conidia (See Table 1).

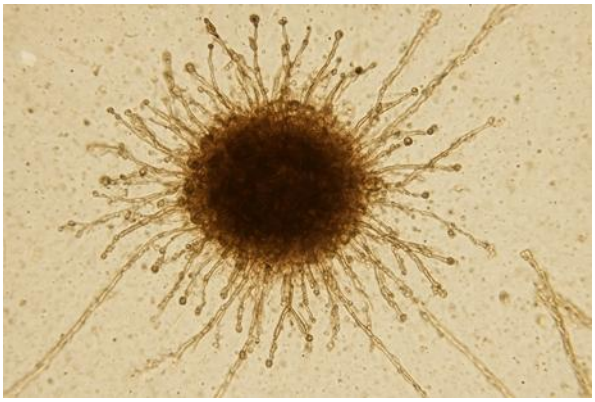

Fig. 2. *Aspergillus* sample vesicle morphology, conidifiora and conidia with necroptosis.

Stained (Lactophenol Blue or Blue Cotton)

The genus *Aspergillus* is a eukaryotic fungus; therefore, its cells have a true nucleus delimited by a nuclear membrane, in addition to other organelles such as mitochondria, endoplasmic reticulum and Golgi apparatus. The structure of this species is not colored in its entirety because its components do not allow the entry of the dye, this because the genus *Aspergillus* corresponds to filamentous fungi that present a complex structural organization, especially in their cell wall and in their reproductive structures. The cell wall plays a key role in the protection and stability of the fungus and is mainly made up of chitin, which confers rigidity, and polysaccharides such as β-glucans, which form the basic structural support. In addition to these components, galactomannans and various glycoproteins are involved in adhesion processes and in the interaction with the host's immune system. In certain species, the presence of melanin in the cell wall contributes to increased resistance to adverse environmental factors and to the body's defensive mechanisms.

With staining, the fungus's reaction to peroxide becomes more apparent. Hyphae are dyed a deep blue since the possible peroxide softens the wall, conidia are more resistant to color due to their thickness, but they present clear interruptions where the wall is damaged, visible as pale or thinned areas. The conidiophore appears sharper, but its outline is not smooth; it appears rough, like the surface of an eroded fiber. The gallbladder is intensely stained, but its perfect roundness is altered: it is slightly collapsed on one side, with a sunken appearance. The phylalides are seen as short, irregular projections; some are well tinted and others appear only as shadows. Conidia tend to stain homogeneously, but many shows clear areas or small tears, indicators of superficial rupture. Overall, the fungus looks defined, but damaged, with a clear contrast between the stained and broken parts.

Under a concentration of 3% H_2O_2, *Aspergillus salvadorensis* maintains a practically intact morphology, with maximum values of cell wall and conidia integrity, while vacuolization is minimal, indicating a state close to baseline with mild oxidative stress (Table 2). When the concentration increased to 5%, a transition to sublethal stress is evidenced, characterized by a moderate decrease in the integrity of the cell wall and conidia, accompanied by a clear increase in vacuolization, which suggests the activation of adaptive mechanisms and compartmentalization of oxidative damage. Finally, at 10% H_2O_2, morphological progression culminates in a severe state, where the integrity of the cell wall and conidia is drastically reduced, while vacuolization reaches high values, reflecting advanced structural disorganization and functional collapse compatible with a process of cell death induced by oxidative stress.

Table 2. Signs of conidia at different concentrations (%) of hydrogen peroxide.

H_2O_2 (%)	Cell wall integrity (%)	Vacuolization (%)	Conidia Integrity (%)
3	100	5	100
5	60	40	50
10	10	90	5

Figure (3) shows a progressive sequence of morphological alterations in *Aspergillus* as the oxidative stress caused by H_2O_2 intensifies. In the control condition, cells show a stable physiological state, characterized by total cell wall and conidia integrity, along with very low vacuolization, indicating a conserved cell structure. Under sublethal stress, noticeable changes begin to manifest: the integrity of the cell wall and conidia decreases moderately, while vacuolization increases, suggesting the activation of cellular mechanisms of response and adaptation to oxidative damage. In the necroptosis stage, structural deterioration is marked, with an almost complete loss of cell wall and conidia integrity, accompanied by a maximum increase in vacuolization. This pattern reflects a transition from an initial adaptive response to a state of irreversible cellular damage and death, showing an inverse relationship between structural conservation and vacuolization as oxidative stress progresses.

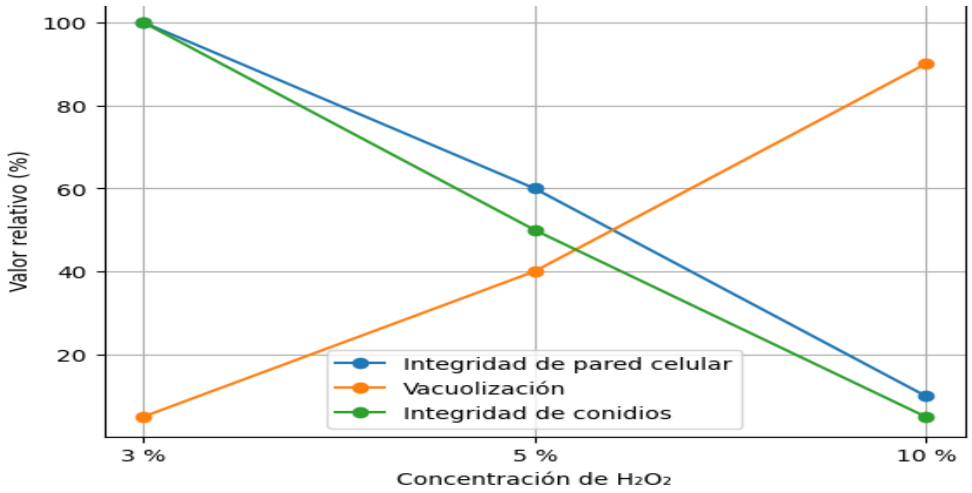

Fig. 3. Morphological progression to oxidative stress with hydrogen peroxide.

Figure (4) summarizes the structural response of *Aspergillus salvadorensis* to an oxidative stress gradient induced by hydrogen peroxide, evaluating six fundamental morphological variables. The overall pattern shows progressive deterioration of cellular integrity as oxidant exposure progresses from physiological conditions to a scenario of complete necroptosis. Under control conditions, Aspergillus presents an integral morphology, characterized by a functional cell wall and conidia, high hyphal turgor, high cytoplasmic density and minimal vacuolization. When the mycelium is exposed to 3% H_2O_2, a state close to baseline is observed, with general preservation of structural integrity and only mild indications of oxidative stress, reflected in incipient vacuolization without significantly compromising cell viability.

When the concentration of hydrogen peroxide increases to 5%**,** the fungus enters a phase of sublethal stress, evidenced by a moderate reduction in the integrity of the cell wall, conidia and vesicles, along with a decrease in hyphal turgor and cytoplasmic density. In this condition, vacuolization increases markedly, suggesting an adaptive response aimed at compartmentalizing damage and temporarily maintaining cellular homeostasis.

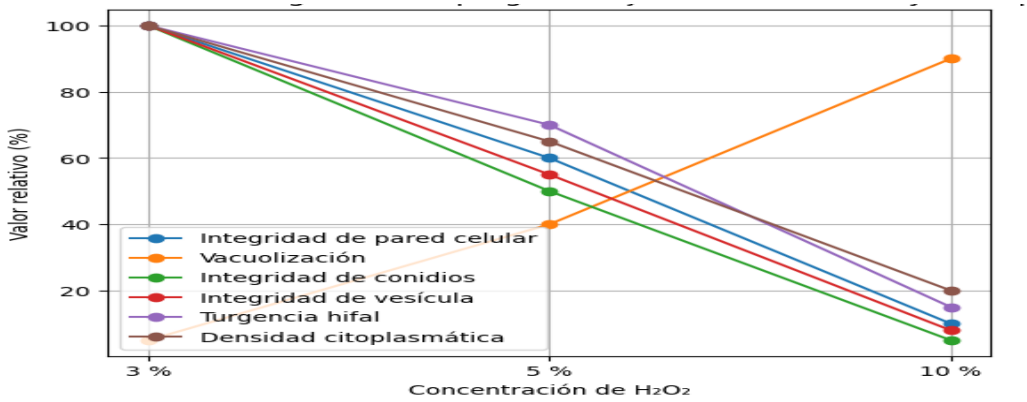

Fig. 4. Morphology to oxidative stress with necroptosis.

At 10% H_2O_2, morphological progression culminates in a state compatible with necroptosis, where there is a severe loss of cell wall, conidia and vesicles integrity, accompanied by a marked collapse of hyphal turgor and a pronounced decrease in cytoplasmic density. In contrast, vacuolization reaches maximum values, indicating advanced cellular disorganization and irreversible failure of oxidative stress adaptation mechanisms.

This pattern describes a clearly dose-dependent response of H_2O_2, in which Aspergillus transitions from a baseline state, through a sublethal adaptive phase, to a necrooptic outcome characterized by structural and functional collapse of the mycelium.

At baseline, all variables start from a relative value of 100%, reflecting the typical morphology of the intact fungus: firm cell walls, turgid hyphae, dense cytoplasm and functional conidiophores with fully developed vesicles and conidia. The minimum level of vacuolization (5%) is consistent with metabolically active cells with no signs of stress.

Under sublethal oxidative stress, a simultaneous shift is observed in all metrics. Vacuolization increases by up to 40%, reflecting a state of response to damage, while the integrity of the wall, vesicle, conidia, and cytoplasm falls by 50–70%. Hyphal turgor decreases to 70%, suggesting impairment of osmotic balance and structural support. This set is typical of a reversible state, in which the fungus activates antioxidant mechanisms and partially repairs ROS-induced lesions. The structure is still functional, but visibly compromised.

The state of necroptosis shows a drastic break in the cell architecture: wall integrity (15%), gallbladder integrity (8%), turgor (15%) and conidia integrity (5%) fall to minimum levels. Cytoplasmic density is reduced to 20%, indicating massive extravasation of the internal contents and rupture of compartmentalization. In contrast, vacuolization reaches 90%, which represents one of the clearest indicators of severe dysfunction and collapse of cellular maintenance systems. The combination of these values confirms a pattern of non-apoptotic cell death, characterized by lysis, osmotic rupture and irreparable degradation of reproductive structures.

The behavior of the indicators shows that necroptosis in filamentous fungi can be quantified by morphological metrics, and the graph reflects this with a synchronized decrease in most structural variables in the face of an explosive increase in vacuoles. This relationship allows us to differentiate three biological states: normality, compensated stress and terminal collapse. In addition, it highlights the cell wall and gallbladder as the elements most sensitive to oxidation, while hyphal turgor and cytoplasm

are robust markers of the level of functional integrity.

Comparative analysis shows that *A. salvadorensis* presents a progressive transition from a physiologically stable state to one of moderate oxidative stress and, finally, to a terminal form of regulated cell death. The intensity and distribution of structural damage in each condition reflect the sensitivity of the fungus to strong oxidants and the possibility of inducing necroptosis by targeted disturbance of the intracellular redox balance. These findings provide a consistent visual and experimental framework for understanding the cellular dynamics of *A. salvadorensis* in the face of chemical stimuli, and constitute a solid basis for further studies focused on the molecular characterization of oxidative resistance pathways and programmed death mechanisms in filamentous fungi.

DISCUSSION

The response of *A. salvadorensis* to oxidative stress is dependent on H_2O_2 concentration, which coincides with patterns observed in other fungi. In other fungi, reactive oxygen species (ROS) play a determining role in the activation of programmed cell death pathways, acting as intracellular signals that trigger responses dependent on fungal metacaspases and MAPK signaling pathways (Carmona-Gutierrez *et al.*, 2018; Ikner and Shiozaki, 2005; Madeo *et al.*, 2009). At low concentrations of the oxidizing agent, cell structures maintain their integrity; however, at a concentration of 5%, morphological alterations typical of emergent programmed cell death are observed, which is consistent with processes mediated by pathways similar to fungal caspases and MAPK mechanisms previously described in fungi (Dagenais and Keller, 2009).

The almost complete disintegration at 10% suggests that oxidative stress overwhelms the fungal capacity for adaptation, activating necroptosis pathways that lead to hyphal collapse and generalized structural loss. Similar phenomena have been documented in other *Aspergillus* and pathogenic fungi under elevated ROS conditions (Carmona-Gutierrez *et al.*, 2018; Dagenais and Keller, 2009; Latgé, 1999; Shlezinger *et al.*, 2017).

The results provide a line of morphological evidence that supports the existence of processes comparable to necroptosis in filamentous fungi, opening the door to molecular studies involving redox regulation, membrane integrity and stress signaling.

On the other hand, the detection of processes associated with cell motility, including components of the cytoskeleton, flagellar assembly, and motor proteins, highlights the relevance of structural organization and intracellular movement in cell dynamics (Alberts *et al.*, 2022; Pollard and Cooper, 2009). Taken together, this functional profile indicates a strong gene investment in the regulation of growth, cell death, and internal architecture, supporting the idea of a highly controlled and adaptable cellular system (Elmore, 2007; Vale, 2003).

In the process of asexual reproduction, *Aspergillus* develops a conidiophore that culminates in a globose-shaped vesicle. This structure functions as a platform for the production of spores, since the phylalides are arranged on its surface, either directly or by means of metulas, depending on the species. The phylalides are the cells responsible for the formation of conidia, and the arrangement and morphology of the vesicle are important criteria for the taxonomic identification of the fungus (Samson *et al.*, 2010).

Conidia represent the asexual spores responsible for the dissemination of the microorganism. They have a multilaminar cell wall rich in chitin and glucans, frequently pigmented with melanin, which gives them remarkable resistance to unfavorable environmental conditions. In addition, they have a hydrophobic outer layer that facilitates their dispersion through the air. Inside they contain essential cellular elements, such as nucleus and

mitochondria, as well as energy reserves. These properties explain their ease of inhalation and their relevance in the development of infections caused by *Aspergillus* (Latgé, 1999).

In *Aspergillus* hyphae, nuclei are usually numerous and distributed along the filament, separated by septa (septa) that have pores, allowing the movement of cytoplasm and organelles between compartments. Likewise, conidia also contain one or more nuclei, which allows them to germinate and form new hyphae when conditions are favorable. In summary, *Aspergillus* is a multicellular eukaryotic organism with nucleated cells, a fundamental characteristic that differentiates it from bacteria and other prokaryotic organisms (Deacon, 2013; Kwon-Chung and Bennett, 1992; Latgé, 1999).

From a comparative approach, these proteins allow inferring the ability of the fungus to activate cell death processes under stress conditions, especially oxidative stress, which in fungi triggers accumulation of reactive oxygen species, mitochondrial damage and activation of proteolytic factors that lead to the loss of membrane integrity. In a complementary way, the vegetative incompatibility pathway, mediated by HET proteins, can generate a regulated cell death response that shares morphological traits with necroptosis, such as cell lysis and the release of cytoplasmic content (Glass *et al.*, 2000; Paoletti and Saupe, 2009; Saupe, 2020).

The conceptual bioinformatic analysis of regulated cell death in *Aspergillus salvadorensis* allows us to understand how this species, like other filamentous fungi, could activate mechanisms functionally equivalent to the necroptosis processes described in higher organisms, even though it lacks the classical genes associated with this pathway in animals (Carmona-Gutierrez *et al.*, 2018). In fungi, programmed cell death is articulated through particular pathways involving metacaspases, HET-like vegetative incompatibility proteins, NACHT/WD40 domains associated with intracellular sensors, and ATG systems linked to autophagy, forming a molecular network distinct from animal apoptosis and necroptosis (Glass *et al.*, 2000; Paoletti and Saupe, 2009).

From this perspective, a first theoretical step consists of the identification of candidate genes by searching for orthologs in phylogenetically related species such as *Aspergillus fumigatus* and *Aspergillus nidulans*, as well as in the detection of conserved functional domains such as the C14 domain in metacaspases, HET domains involved in vegetative incompatibility, and NACHT/WD40 domains related to the detection and transduction of signals from Stress (Carmona-Gutierrez *et al.*, 2018; Glass *et al.*, 2000; Paoletti and Saupe, 2009).

Currently, evidence suggests that fungal necroptosis may be an important mechanism in host-pathogen interaction. A key identified inducer for necroptosis in host cells (*in vitro*) is the exopolysaccharide secreted by *A. fumigatus* (such as galactomannan-galactan, GAG). However, to induce necroptosis in the fungus itself (in *A. salvadorensis*), the focus is on environmental/chemical stress that dysregulates survival mechanisms.

Necroptosis in filamentous fungi describes a form of non-apoptosis programmed cell death, whereby mycelial colonies can manage extreme damage by controlling compromised hyphae, thereby contributing to the survival of the mycelium as a whole (Glass *et al.*, 2000; Paoletti and Saupe, 2009). In *Aspergillus salvadorensis*, comparative evidence with related species suggests the existence of an integrated system involving redox sensors and NOD/NACHT receptors, MAPK stress response cascades such as SakA/Hog1 and MpkC, as well as kinases with functional similarity to RIP, capable of coordinating regulated cell death responses under adverse conditions (Daskalov *et al.*, 2015; Kawasaki *et al.*, 2002).

The activation of these pathways would lead to signaling towards membrane executors of the HeLo/HELL type proteins capable of oligomerizing and inserting into the plasma membrane forming pores, which causes loss of cellular integrity, lysis and release of cytoplasmic

content, a characteristic mechanism of regulated lytic death in fungi (Daskalov *et al.*, 2015; Saupe, 2020). Together, these elements support that *A. salvadorensis* does not develop necroptosis in the strict sense described in animals, but rather a set of functionally equivalent responses, integrated within a fungal molecular network specialized in the controlled elimination of damaged cells as an evolutionary strategy of adaptation to stress (Saupe, 2020).

CONCLUSION

Aspergillus salvadorensis presents a morphological response clearly dependent on H_2O_2 concentration, in which increased oxidative stress leads to progressive and well-defined cellular changes. Exposure to concentrations of 5 % and 10 % triggers morphological alterations typical of necroptosis, suggesting that cell death induced by oxidative stress is a relevant mechanism in the cell biology of this fungus. The effect of stress manifests itself in a stepwise manner, beginning with potentially reversible modifications, such as increased vacuolization and partial loss of structural integrity, before progressing to extensive and irreversible cell destruction. In this process, vacuolization emerges as the most sensitive early indicator, as it increases before global structural collapse. The cell wall and vesicle are identified as the components most susceptible to oxidative damage, functioning as a critical threshold between repairable damage and definitive cell death. Necroptosis, in advanced stages, is characterized by a massive loss of internal compartmentalization, minimal turgor and almost complete destruction of reproductive structures. Together, these metrics allow us to define a quantitative profile of morphological progression, with potential applications in toxicity studies, antioxidant defense mechanisms and fungal pathogenicity. The concentration of 10% is lethal and induces necroptosis with loss of integrity and function of the fungus.

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CONFLICT OF INTEREST

The author declares that he has no conflicts of interest.

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