SaBios
Revista de Saúde e Biologia
ISSN: 1980-0002

SEÇÃO ARTIGOS COMPLETOS/ COMPLETE PAPERS

VEGETATIVE DEVELOPMENT PREDICTIVE PARAMETERS OF MUTAGENESIS AND ANTIMUTAGENESIS IN *Aspergillus nidulans*

PARÂMETROS DE DESENVOLVIMENTO VEGETATIVO PREDITIVOS DE MUTAGÊNESE E ANTIMUTAGÊNESE EM *Aspergillus nidulans*

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ABSTRACT

Aspergillus nidulans (Ascomycota) has been used as a model organism in research on eukaryote genetics for more than 50 years. Its sophisticated mechanism for regulating the expression of genes that control vegetative development make the analysis of conidial germination a relevant tool in assessing the possible effect of different factors on gene regulation mechanisms. Bromelain is a complex mixture of proteolytic and nonproteolytic substances, obtained from the stem and immature fruit of the pineapple plant (*Ananas comosus*). Its biological activities include anti-inflammatory, antithrombotic, fibrinolytic, anticancer and pro-apoptotic effects. The present study aimed to apply survival and germination tests as a preliminary analysis to assess the cytotoxic, mutagenic and antimutagenic potential of candidate agents. To that end, different concentrations of commercial bromelain were used as the test substance and ultraviolet (UV) light as a mutagenic agent. Bromelain showed no toxicity to the spore viability assay or mutagenicity in the germination test. However, a pro-apoptotic effect was observed in the treatment without UV light when the number of cell deaths increased in concentrations of 2.5 and 25 µg.mL-1, two times higher than the negative control. In addition, bromelain exhibited an antimutagenic effect in the germination test in the presence of UV light, halving the percentage of malformed conidia in relation to the positive control. The results indicated that survival and germination testing of *A. nidulans* conidia can be used as complementary tools for screening substances with possible cytotoxic, mutagenic or antimutagenic potential, since the combined analysis of survival, mortality and malformations allows inferences to be made regarding damage or protection by repair and/or apoptosis.

Key Words: cytotoxicity; genotoxicity; apoptosis; repair; survival; bromelain.

RESUMO

Aspergillus nidulans (Ascomycota) é um organismo modelo empregado há mais de 50 anos em estudos de genética dos eucariotos. Por possuir um sofisticado mecanismo de regulação da expressão de genes que coordenam o desenvolvimento vegetativo, a análise da germinação de conídios é uma ferramenta a ser considerada para avaliar o possível efeito de diversos fatores sobre os mecanismos de regulação gênica. A bromelina é uma complexa mistura de substâncias proteolíticas e não proteolíticas, obtida do caule ou de frutos imaturos de abacaxi (*Ananas comosus*). Suas atividades biológicas descritas incluem efeito anti-inflamatório, antitrombótico, fibrinolítico, anticâncer e pró-apoptótico. O presente trabalho propôs a utilização do ensaio de sobrevivência e de germinação de conídios de *A. nidulans*, como avaliação preliminar, para verificar o possível potencial citotóxico, mutagênico e antimutagênico de agentes candidatos. Para isso, foi utilizado diferentes concentrações de bromelina comercial, como substância teste, e a luz ultravioleta (UV) como agente mutagênico. A bromelina não mostrou toxicidade no ensaio de sobrevivência, nem mutagenicidade no ensaio de germinação. No entanto, apresentou efeito pró-apoptótico no tratamento sem luz UV, ao aumentar o número de mortos nas concentrações 2,5 e 25 µg mL-1, valor duas vezes maior que o do controle negativo. Além disso, a bromelina mostrou efeito antimutagênico no ensaio de germinação na presença de luz UV, reduzindo pela metade a porcentagem de conídios malformados, em relação ao controle positivo. Os resultados do presente trabalho indicaram que a análise de sobrevivência e o ensaio de germinação de conídios de *A. nidulans* constituem ferramentas complementares na triagem de substâncias com possível potencial citotóxico, mutagênico e/ou antimutagênico, uma vez que a análise associada de sobrevivência, mortalidade e malformações, permite inferir sobre danos ou proteção por reparo e/ou apoptose.

Palavras-Chave: citotoxicidade; genotoxicidade; apoptose; reparo; sobrevivência; bromelina.

INTRODUCTION

Genetic toxicology evaluates potential genotoxic effects since they are considered important prerequisites for the development of diseases such as cancer (1). Regulatory genetic toxicology tests involve a series of well-defined mutagenicity assays chosen to detect chemical and physical agents capable of inducing mutations frequently associated with the development of cancers and birth defects (2).

Several species of *Aspergillus* are commonly used in biotechnological processes, and in addition to their medical significance, have a well characterized genetic system widely used in studies on genetic mutations and regulation, being applied in genotoxicity tests due to their rapid growth in certain media, with phenotypically identifiable results (3).

Also known as *Emericella nidulans* when referring to its sexual form or teleomorph, it is one of the filamentous fungi of the phylum Ascomycota. It has been an important organism in the study of eukaryotic genetics (4) for more than 50 years (5) and is used in research on genetic recombination, DNA repair, mutation, cell cycle control, tubulin, chromatin, nucleokinesis, pathogenesis, metabolism (6) and developmental genetics (5,7). It is widely used in genetic studies due to its short life cycle, haploidy and well-defined morphogenesis (8,9).

The life cycle consists of three distinct stages: the vegetative phase, including spore germination and hyphal growth, producing the mycelium; the asexual phase or conidiogenesis, involving the production of asexual spores called conidia from conidiophores; and the sexual phase stage or ascosporogenesis, resulting in the formation of sexed spores (ascospores) in the cleistothecium, surrounded by Hülle cells (7).

Given its sophisticated regulatory mechanism for the expression of genes that coordinate germination, analysis of conidial germination is an important tool to study the effect of different factors on the regulatory mechanisms of this chain of events (10,11).

The vegetative phase begins with the disruption of spore dormancy, which occurs in the presence of water and a carbon source, such as glucose (7). Imbibition causes the spore to swell, forming the germination tube via mitosis (12), which exhibits apical growth and produces the septa. After a period of growth, the spore produces a second germ tube in an opposite direction to the first as well as a third lying perpendicular to the others, but all on the horizontally plane. These hyphae form a radial colony in a solid growth medium (10,13).

According to D'Enfert (11), each germination phase is marked by a set of morphogenetic events that reflect the expression of groups of genes and the influence of the environment during this stage. Imbibition coincides with the activation of spore metabolism, characterized by increased respiratory rate and high adenosine triphosphate (ATP) levels. The budding stage of germination marks the first mitosis of the conidia's original nucleus and the axis established in this stage guides the polar growth of the next phase.

In general terms, spore germination is an essential phase in the life cycle of all filamentous fungi. There are three important steps during spore germination: dormant spore activation in response to suitable environmental conditions; isotropic growth involving water uptake and the resumption of several metabolic activities; induction of the cell cycle and polar growth, resulting in the formation of a germination tube that produces the new mycelium (11).

Microbiological systems are frequently used to evaluate carcinogenicity and toxicity. These systems are faster, less expensive and produce valuable data. In addition, assessment of DNA damage is important in genetic toxicology (14).

Ames et al. (15), described an important method that uses *Salmonella typhimurium* to evaluate mutagenesis. According to Sousa et al. (16), *A. nidulans* is an effective alternative in the assessment of mutagenicity in that it is a eukaryotic cell with more complex genetic and cell mechanisms than *Salmonella thyphimurium*, used in the Ames' test. Despite the limitations involved in using these fungi as an alternative to mammals, including differences in the molecular environment and genetic interactions, *A. nidulans* allows rapid screening of the agents' biological activity, similarly to the Ames test.

Bromelain is a crude aqueous extract obtained from the stem and immature fruit of

the pineapple plant, *A. comosus* Merr. (Bromeliaceae), especially the Cayenne variety. It is a complex mixture of cysteine proteases (95%), and other non-protease components such as glycosidases, phosphatases, peroxidases, cellulase, carbohydrates and glycoproteins (17-19).

The main bioactivities described for bromelain include: anti-inflammatory, antithrombotic, fibrinolytic, and anticancer properties (19). The characteristic that contributes to these effects is its absorption in the human intestine without degradation, which would compromise its biological activity (20). Clinical observations and tests based on rat cell models suggest that bromelain acts systemically, affecting several cellular and molecular targets. Recent studies have demonstrated its ability to modulate the major pathways that trigger malignancy and directly impact the cancer cells in their microenvironments, as well as modulate the immune, inflammatory, and hemostatic systems (19).

Bromelain also exhibits pro-apoptotic activity, increasing p53 expression in mouse papilloma cells (21). Apoptosis was also reported in GI-101A breast cancer cells, with a dose-dependent effect. Bromelain increases levels of CK18 marker, which indicates programmed cell death and induces the apoptosis signal by activating a caspase pathway, resulting in the nuclear condensation and disintegration characteristic of cell death by apoptosis (22). Furthermore, Bhui et al. (23), demonstrated that bromelain causes intracellular glutathione depletion and the generation of reactive oxygen species, followed by depolarization of the mitochondrial membrane, leading to bromelain-induced cell cycle arrest of the G2/M phase.

The present study aimed to apply survival and germination tests to *A. nidulans* conidia in order to evaluate the cytotoxic, mutagenic and antimutagenic effect of compounds, substances or complex extracts from natural substances. Different concentrations of commercial bromelain were used as the test substance to analyze bioactivity, due to its anticarcinogenic and pro-apoptotic properties reported in the literature. Testing for protection against induced mutation used ultraviolet (UV) light as mutagenic agent.

MATERIAL AND METHODS

Strain and culture medium

The strain used was *biA1methG1* (*biA1* (I), a requisite for biotin and *methG1* (IV), a requisite for methionine) of green conidia, with normal growth and sporulation, donated by the University of Glasgow, Scotland. The strain was grown in a solid complete medium (CM), prepared according to Pontecorvo et al. (10), and Clutterbuck (24), and incubated at 37 ºC for five days.

Commercial bromelain concentrations

Commercial bromelain was acquired from BIO-CAT INC (Troy, Virginia), with no additives or preservatives, and diluted in distilled water to obtain concentrations of 2.5, 25 and 250 $\mu g.mL^{-1}$. The concentrations standardization was determined in survival tests on the *bia1methG1* strain of *A. nidulans,* based on the concentrations used in antiproliferative activity tests in neoplastic and normal cells, adopted by the Multidisciplinary Center for Chemical, Biological and Agricultural Studies of Universidade de Campinas, São Paulo - CPQBA-UNICAMP.

Spore suspension and controls

Conidia were collected from five-day-old colonies cultured in solid complete medium (CM) at 37 ºC in Tween 80 (0.01%) and filtered in glass wool. Half of the filtered volume was submitted to irradiation with UV light for ten seconds (0.24 mJ/cm^2). Distilled water was used for the negative and positive control, the latter irradiated with UV light.

Survival assay

Irradiated and non-irradiated conidial suspensions (≈1000 x 10^4 $conidia.mL^{-1}$) were treated with different bromelain concentrations for 2 h (hours) and diluted to ≈1000 $conidia.mL^{-1}$. After treatment, 100 µL of suspension from each condition were inoculated in ten glass plates (90x15 mm) containing solid CM and incubated at 37 ºC, for 72 h in a BOD (biochemical oxygen demand) germination chamber, using a randomized block design with three repetitions. Next, macroscopic counting of the colonies was carried out to estimate the survival of irradiated and non-irradiated conidia

Germination assay: analysis of dead and deformed conidia

The irradiated and non-irradiated conidial suspensions (≈500 x 10^4 spores.mL^{-1}) were inoculated in liquid CM in the presence and absence of each bromelain concentration (2.5; 25 and 250 µg.mL^{-1}), without the need for dilutions. After, 100 µL of suspension from each condition were transferred to microscope slides in a moist chamber and incubated at 37 ºC for 7 h.

After this incubation time, three slides from each condition were analyzed under optical microscope, with final magnification of 200x, using image capture (Canon EOS Rebel 3TI), with an SLR/DSLR NDPL-2 (2x) adaptor for a binocular microscope. At each reading, 200 conidia were randomly analyzed and the percentage of germinated, dead and deformed individuals calculated.

Statistical analysis

The results of survival and germination tests were submitted to comparison of means using the standard error of the mean (SEM) as reference, in accordance with Graveter and Wallnau (25), and analyzed by an one-way analysis of variance (one-way ANOVA).

Results' interpretation

In the germination assay, the estimate of survival considered living, that is, budding or germinated conidia; and dead, only dormant and embedded individuals; the estimate of deformed conidia considered those with abnormal morphology and growth in the second germination tube (Figure 1).

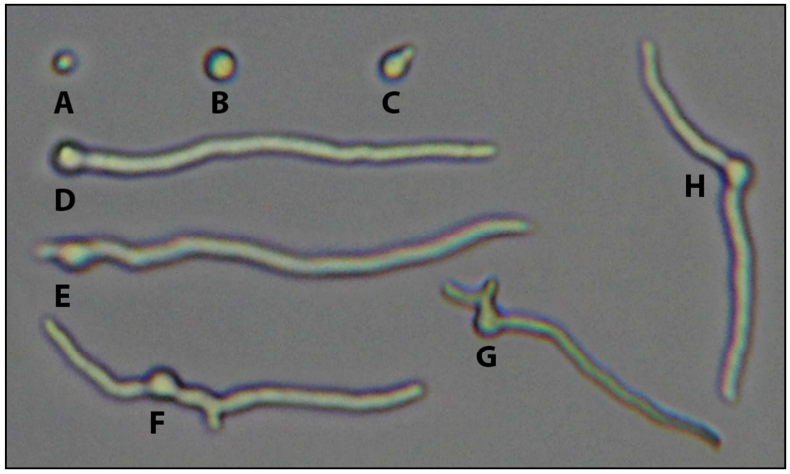

Figure 1. Germination phases of conidia from *A. nidulans* and morphology of deformed individuals. 100x magnification with SLR/DSLR NDPL-2 (2x) adaptor. **A**. Dormant; **B**. Embedded; **C**. Budding; **D** and **E**. Germinated; **F**, **G** and **H**. Deformed.

Interpretation of the germination analysis results of conidia from *A. nidulans* considers two situations: (a) analysis in the absence of UV irradiation for spontaneous mutation and (b) analysis in the presence of UV irradiation for induced mutation. The results can be interpreted according to the possibilities shown in Table 1.

Table 1. Interpretation of the germination analysis results of conidia from *A. nidulans.*

Result Observed	***Indication***
↑ nº. of dead and no change in the nº. of deformed	*Cytotoxic substance*
↑ nº. of dead and ↑ nº. of deformed	*Cytogenotoxic substance*
↑ nº. of dead and ↓ nº. of deformed	*Pro-apoptotic substance*
↓ nº. of dead and ↑ nº. of deformed	*Anti-apoptotic substance*
↓ nº. of dead and ↓ nº. of deformed	*Pro-repair substance*

nº. = number; ↑ = increase; ↓ = decrease.

Analysis of non-irradiated conidia indicates cytotoxicity when mean survival values are lower than those of the negative control, and mutagenicity when mean values

of conidia deformed by treatments are higher than those obtained for the negative control.

Protection of the test substance is confirmed when the mean survival values of conidia irradiated during treatment exhibit higher results than those of the positive control (irradiated), if there is repair, and lower than the positive control if there is apoptosis. For this to occur, the mean number of deformed individuals must be lower than that of the irradiated control.

Therefore, the test substance is considered mutagenic when the mean number of deformed conidia is greater than that of the positive control, and antimutagenic when the mean number of deformed conidia is lower. When irradiated conidia show a decrease in the number of deformed individuals and a simultaneous increase in the mean number of dead conidia, compared to the positive control, it suggests that the test substance is pro-apoptotic. This should be confirmed by additional testing, such as the pro-apoptotic activity test for *A. nidulans* proposed by Leles et al. (26).

RESULTS AND DISCUSSION

Survival assay

The results of the survival assay for different concentrations of bromelain, in the presence and absence of UV irradiation, are displayed in Table 2 and Figure 2. Examples of colonies obtained are shown in Figure 3. After 74 h of exposure to bromelain without UV radiation a decline was observed in colony survival for all bromelain concentrations, particularly at 2.5 and 25 µg mL^{-1}, although none of these results were statistically significant ($p < 0.05$).

Table 2. Average number of conidia per plate of *A. nidulans*, for UV irradiated and non-irradiated conidia (mean ± SEM), followed by treatment with bromelain.

Bromelain concentration (µg.mL^{-1})	Viability ± SEM	
	non-irradiated	irradiated
0	29.0 ± 3.3	16.3 ± 0.9
2.5	24.6 ± 1.5	14.8 ± 0.6
25	23.6 ± 0.4	10.9* ± 0.8
250	25.7 ± 2.5	19.3 ± 1.7

SEM: standard error of the mean; *Significantly different from control ($p < 0.05$).

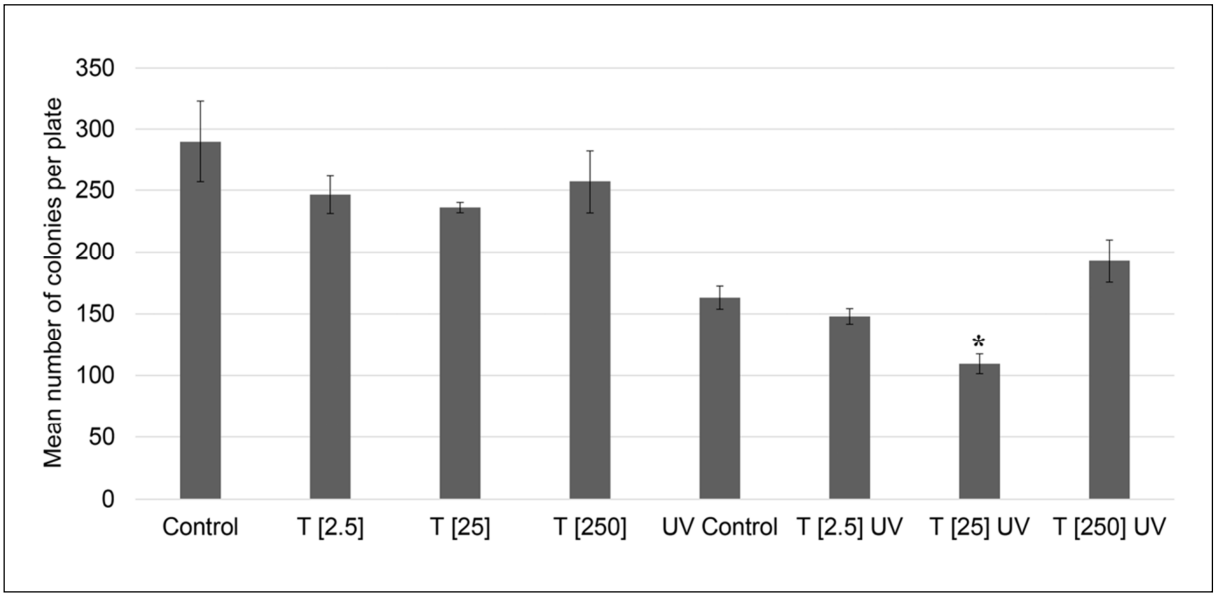

Figure 2. Average number of conidia per plate of *A. nidulans*, for UV irradiated and non-irradiated conidia, followed by treatment with different concentrations of bromelain. *Significantly different from control ($p < 0.05$).

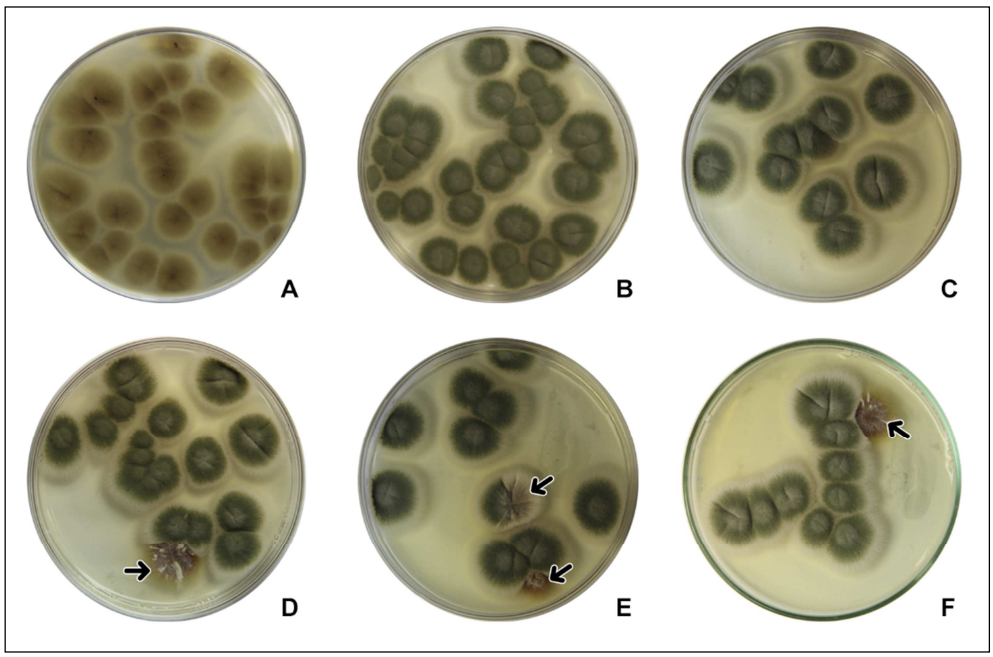

Figure 3. Normal and mutant *Aspergillus nidulans* colonies. **A.** Back of a negative control plate. **B.** Negative control colonies. **C.** Positive control colonies. **D.** Morphological mutant found in the positive control. **E** and **F. Arrow:** morphological mutants found in the 2.5 and 25 µg.mL^{-1} with UV irradiation, respectively. Mutant colonies identified according to criteria described by Lilly (27).

In survival analysis of the irradiated group, concentrations of 2.5 and 25 µg.mL^{-1} showed reduced viability, with the average number of colonies per plate in treatment with 25 µg.mL^{-1} statistically significant in relation to the positive control. These results indicate two different possible interpretations: (a) survival declined due to bromelain cytotoxicity or mutagenicity; (b) damaged conidia were eliminated by the pro-apoptotic effect of bromelain.

Though not a statistically significant result, survival increased at 250 µg.mL^{-1} in the irradiated group in relation to the positive control. This indicates two important aspects: (I) the deaths observed up to that point are likely not caused by the mutagenesis or cytotoxicity of bromelain, since this concentration recorded the lowest number of deaths, even in the non-irradiated group; (II) bromelain can activate repair mechanisms, protecting damaged conidia and allowing them to survive. The first aspect corroborates the possible pro-apoptotic effect, while the second confirms another protective property for bromelain, that of pro-repair.

In rat cardiomyocytes, bromelain increased cell survival and decreased apoptotic cell death, leading to protection against ischemia-reperfusion injury. Bromelain acts by activating *Akt*, a mediator of cell survival, deactivating the *Akt*-dependent pro-apoptotic regulator FOXO3 (28).

Estimate of dead and deformed conidia

The germination test contradicts survival and adds another important parameter for analysis in conjunction with death indication of damage. After 7 h of exposure to different bromelain concentrations, an increase in conidial death was observed after exposure to 2.5 µg.mL^{-1} (3.1%), double that found in the negative control, followed by 25 µg.mL^{-1} (2.7%).This increase is repeated in the irradiated group, where 2.5 µg.mL^{-1} led to an average rise of 13.5% in relation to the positive control, followed by 25 µg.mL^{-1} (13.5%), according to data in Table 3 and Figure 4.

The increase in mortality rate was statistically significant for all the concentrations cited. These results corroborate those obtained in macroscopic survival analysis, demonstrating the reliability of both tests.

Table 3. Percentage of dead conidia per plate of *A. nidulans*, for UV irradiated and non-irradiated conidia (mean ± SEM), followed by treatment with different concentrations of bromelain.

Bromelain concentration (µg.mL^{-1})	Mortality ± SEM	
	non-irradiated	irradiated
0	1.5 ± 0.1	11.9 ± 0.6
2.5	3.1* ± 0.2	13.7* ± 0.2
25	2.7* ± 0.4	13.5* ± 0.1
250	1.7 ± 0.2	12.0 ± 0.5

SEM: standard error of the mean; *Significantly different from control ($p < 0.05$).

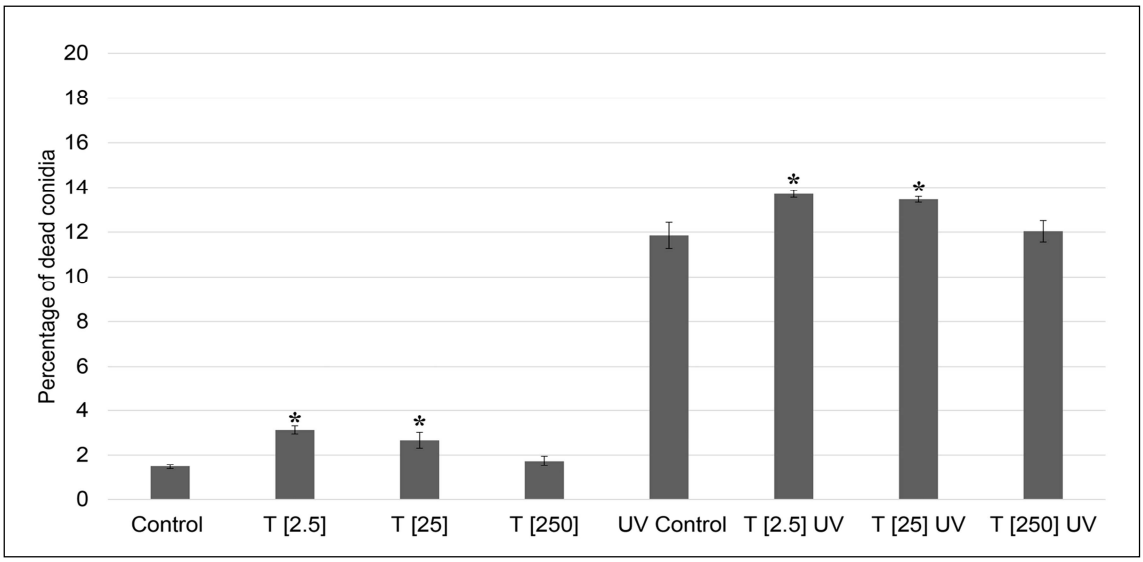

Figure 4. Percentage of dead *A. nidulans* conidia, irradiated and non-irradiated with UV, followed by treatment with different concentrations of bromelain. *Significantly different from control ($p < 0.05$).

Estimated deformed conidia after 7 h exposure to different bromelain concentrations in the non-irradiated group showed no reduction in relation to the negative control at 2.5 and 25 µg.mL^{-1}.

In the irradiated group, however, a considerable reduction was observed in the average number of deformed conidia at 2.5 µg.mL^{-1} (14.1%), the most bioactive concentration, reducing the mean percentage of deformed conidia by half in relation to the positive control, as per data from Table 4 and Figure 5. At 25 µg mL^{-1}, although not significant, the result also indicated a reduction in deformed conidia.

The decline in deformed conidia in this study may be correlated with the increase in dead conidia via programmed cell death induced a priori by the pro-apoptotic action of the commercial bromelain, corroborating the results of the macroscopic survival test.

Though not statistically significant, the results for deformed conidia in the irradiated and non-irradiated groups treated with 250 µg.mL^{-1} of bromelain showed a decline compatible with protection. Once again, these findings demonstrate the reliability of this test as a complement to the survival assay, indicating no reduction in viability for both treatments in either test, suggesting possible induction of the repair mechanism. The results for deformed conidia support this hypothesis.

Table 4. Percentage of deformed *A. nidulans* conidia irradiated and non-irradiated conidia with UV (mean ± SEM), followed by treatment with different concentrations of bromelain.

Bromelain concentration (µg.mL^{-1})	Deformation ± SEM	
	non-irradiated	irradiated
0	16.4 ± 1.5	28.2 ± 2.6
2.5	16.9 ± 0.9	14.1* ± 1.9
25	16.6 ± 0.4	23.8 ± 2.0
250	15.1 ± 1.5	24.0 ± 2.5

SEM: standard error of the mean; *Significantly different from control ($p < 0.05$).

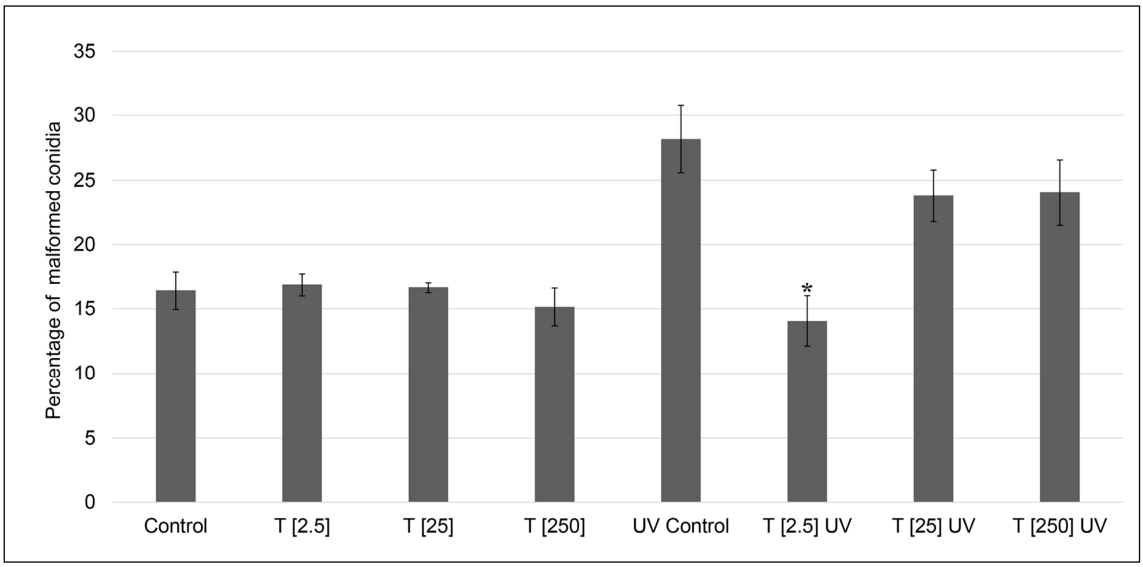

Figure 5. Percentage of deformed *A. nidulans* conidia, irradiated and non-irradiated with UV, followed by treatment with different concentrations of bromelain. *Significantly different from control ($p < 0.05$).

Bromelain has been shown to increase the expression of p53 and is a good activator of apoptosis in mouse papilloma cells (21). The bromelain cytotoxic effects on GI-101A breast cancer cells, inducing apoptosis, are dose-dependent. Bromelain increases levels of CK18, a marker that indicates programmed cell death and induces the apoptosis signal by activating a caspase pathway, resulting in the nuclear condensation and disintegration characteristic of cell death by apoptosis (22).

Bhui et al. (23) demonstrated that bromelain causes intracellular glutathione depletion and generation of reactive oxygen species, followed by depolarization of the mitochondrial membrane. This leads to bromelain-induced cell cycle arrest on the G2/M phase, which was mediated by modulation of *cyclin B1*, *fosfo-cdc25C*, *Plk1*, *fosfo-cdc2*, and *myt1*.This event is followed by apoptosis, evidenced by the formation of membrane blebs, the modulation of *Bax-Bcl-2*, *Apaf-1*, *caspase-9*and*caspase-3*; chromatin condensation, increased caspase activity and DNA fragmentation. This apoptotic feedback indicates that bromelain plays a complex role at different molecular levels of cellular metabolism, leading to protection and cellular homeostasis.

Finally, commercial bromelain may operate in the cellular and tumor microenvironments (19); in the case of *A. nidulans* colonies, in the microenvironment of the culture plates. The systemic action of bromelain, affecting different cellular and molecular targets, has been reported by Chobotova et al. (19). As such, proteolytic activity and other bromelain non-proteolytics may act synergistically, favoring apoptosis or cell survival via metabolic pathways or gene expression modulation, or by facilitating the absorption of nutrients by the cell wall and/or plasma membrane, either through the activation of repair mechanisms or bioprocesses that promote cellular homeostasis.

Bromelain showed no toxicity in the survival assay or mutagenicity in the germination test. On the other hand, it

exhibited a pro-apoptotic effect by increasing the number of deaths, a fact widely supported by the literature, and an antimutagenic effect by reducing the percentage of deformed conidia in the germination test.

The survival assay is efficient in indicating the toxicity of substances such as bromelain. Based on the estimated dead and deformed conidia, the germination test is an effective indicator that identifies toxicity, mutagenicity, cell repair and apoptosis.

The estimated deaths in the germination test corroborated the cytotoxicity data obtained in the survival assay, demonstrating its complementary nature in terms of determining death rates, and the mutagenic and antimutagenic potential of substances such as bromelain.

CONCLUSION

Based on the results presented in this study, survival analysis and germination testing are important tools for screening substances with cytotoxic, mutagenic or antimutagenic potential, given that it is possible to identify a relationship between survival data, mortality and deformed germinated conidia.

The two analyses are complementary and provide alternatives to the use of mammals since they are eukaryotic cells. They are also fast, practical, efficient, and low-cost tools to analyze the bioactivity of chemical and physical agents in biomonitoring, providing reliable, reproducible data.

ACKNOWLEDGEMENTS

The authors are grateful to the Coordination for the Improvement of Higher Education Personnel (CAPES) for its financial support.

REFERÊNCIAS

(1) RIBEIRO, L.R.; SALVADORI, D.M.F.; MARQUES, E.K. **Mutagênese Ambiental**. Canoas: ULBRA, 2003.

(2) RIBEIRO, L.R. Teste do micronúcleo em medula óssea de roedores *in vivo.* In: RIBEIRO, L.R.; SALVADORI, D.M.F.; MARQUES, E.K. (Eds.). **Mutagênese Ambiental**. Canoas: ULBRA, 2003. p. 173-198.

(3) ROCHA, C.L.M.S.C.; AZEVEDO, J.L. Use of the methG1 reversion system of *Aspergillus nidulans* for the detection of mutagenicity of the pyrrolizidine alkaloid integerrimine. **Revista Brasileira de Genética**, v. 9, p. 393-405, 1986.

(4) OSMANI, S.A.; MIRABITO, P.M. The early impact of genetics on our understanding of cell cycle regulation in *Aspergillus nidulans*. **Fungal Genetics and Biology**, v. 41, p. 401-410, 2004.

(5) MARTINELLI, S.D. *Aspergillus nidulans* as an experimental organism. In: MARTINELLI, S.D.; KINGHORN, J.R. (Eds.). ***Aspergillus*: 50 years on**. New York: Elsevier, 1994. p. 33-58.

(6) NIERMAN, W.C.; MAY, G.; KIM, H.S.; ANDERSON, M.J.; CHEN, D.; DENNING, D.W. Whatthe *Aspergillus* genomes have told us? **Medical Mycology**, v. 43, p. 3-5, 2005.

(7) TIMBERLAKE, W.E.; CLUTTERBUCK, A.J. Genetic regulation of conidiation. In: MARTINELLI, S.D.; KINGHORN, J.R. (Eds.). ***Aspergillus*: 50 years on**. Elsevier, New York, 1994, p. 383.

(8) TIMBERLAKE, W.E.; MARSHALL, M.A. Genetic regulation of development in *Aspergillus nidulans.* **Trends in Genetics**, v. 4, p. 162-169, 1986.

(9) MILLER, B.L. The developmental genetics of asexual reproduction in *Aspergillus nidulans*. **Seminars in Cell and Developmental Biology**, v. 1, p. 207-2019, 1990.

(10) PONTECORVO, G.; ROPER, J.A.; CHEMMONS, L.M.; MACDONALD, K.D.; BUFTON, A.W.J. The genetics of *Aspergillus nidulans.* **Advances in Genetics**, v. 5, p. 141-238, 1953.

(11) D'ENFERT, C. Fungi spore germination: insights from the molecular genetics of *A. nidulans* and *N. crassa*. **Fungal Genetics and Biology**, v. 21, p. 163-172, 1997.

(12) YUILL, E. The numbers of nuclei in conidia of *Aspergilli*. **Transactions of the**

British Mycological Society, v. 33, p. 324-31, 1950.

(13) AXELROD, D.E. Kinetics of differentiation of conidiophores and conidia by colonies of *Aspergillus nidulans*. **Journal of General Microbiology**, v. 73, p. 181-184, 1972.

(14) FENECH, M. The in vitro micronucleus technique. **Mutation Research**, v. 455, p. 81-95, 2000.

(15) AMES, B.N.; DURSTON, W.E.; YAMASAKI, E.; LEE, F.D. Carcinogens are mutagens: a simple test system combining liver homogenates for activation and bacteria for detection. **Proceedings of the National Academy of Sciences**, v. 70, p. 2281-2285, 1973.

(16) SOUSA, G.D.; ZUCCHI, T.D.; ZUCCHI, F.D.; MILLER, R.G.; ANJOAS, R.M.A.; POLI, P.; ZUCCHI, T.M.A.D. *Aspergillus nidulans* as a biological system to detect the genotoxic effects of Mercury fumes on eukaryotes. **Genetics and Molecular Research**, v. 8, p. 404-413, 2009.

(17) COOREMAN, W. Bromelain. In: RUYSSEN, R.; LAUWERS, A. (Eds.). **Pharmaceutical enzymes-properties and assay methods**. Belgium: Story-Scientia Scientific Publishing, 1978, p. 107-121.

(18) ROWAN, A.D.; BUTTLE, D.J. Pineapple cysteine endopeptidases. **Methods in Enzymology**, v. 244, p. 555-568, 1994.

(19) CHOBOTOVA, K.; VERNALLIS, A.B.; MAJID, F.A. Bromelain's activity and potential as an anti-cancer agent: current evidence and perspectives. **Cancer Letters**, v. 28, p. 148-156, 2010.

(20) LENARCIC, B.; RITONJA, A.; TURK, B.; DOLENC, I.; TURK, V. Characterization and structure of pineapple stem inhibitor of cysteine proteinases. **Biological Chemistry Hoppe-Seyler**, v. 373, p. 459-464, 1992.

(21) KALRA, N.; BHUI, K.; ROY, P.; SRIVASTAVA, S.; GEORGE, J.; PRASAD, S.; SHUKLA, Y. Regulation of p53 nuclear factor kappa B and cyclooxygenase-2 expression by bromelain through targeting mitogen-activated protein kinase pathway in mouse skin. **Toxicology and Applied Pharmacology**, v. 226, p. 30-37, 2008.

(22) DHANDAYUTHAPANI, S.; PEREZ, H.D.; PAROULEK, A.; CHINNAKKANNU, P.; KANDALAM, U.; JAFFE, M.; RATHINAVELU, A. Bromelain-indiced apoptosis in GI-101A breast cancer cells. **Journal of Medicinal Food**, v. 15, p. 344-349, 2012.

(23) BHUI, K.; TYAGI, S.; SRIVASTAVA, A.K.; SINGH, M.; ROY, P.; SINGH, R.; SHUKLA, Y. Bromelain inhibits nuclear factor kappa-B translocation, driving human epidermoid carcinoma A431 and melanoma A375 cells through G(2)/M arrest to apoptosis. **Molecular Carcinogenesis**, v. 51, p. 231-243, 2012.

(24) CLUTTERBUCK, A.J. ***Aspergillus nidulans***. In: KING, R.C. (Ed.). Handbook of Genetics. New York: Plenum Publishing, 1974, p. 447-510.

(25) GRAVETTER; F.J.; WALLNAU, L. **Statistics for the Behavioral Sciences**. Wallingford: West Publishing Co, 1995, p. 12-28.

(26) LELES, S.M.; FACTORI, R.; C.L.M.S.C, ROCHA. *In situ* analysis of apoptosis in *Aspergillus nidulans* with ethidium bromide and acridine orange. **Genetics and Molecular Research**, v. 12, p. 2895-2901, 2013.

(27) LILLY, L. An investigation of the suitability of the suppressors of methG1 in *Aspergillus nidulans* for the study of induced and spontaneous mutations. **Mutation Research**, v.2, p. 192-195, 1965.

(28) JUHASZ, B.; THIRUNAVUKKARASU, M.; PANT, R.; ZHAN, L.; PENUMATHSA, S.V.; SECOR JR, E.R.; SRIVASTAVA, S.; RAYCHAUDHURI, U.; MENON, V.P.; OTANI, H.; THRALL, R.S.; MAULIK, N. Bromelain induces cardioprotection against ischemia-reperfusion injury through Akt/FOXO pathway in rat myocardium. **American Journal of Physiology-Heart and Circulatory Physiology**, v. 294, p. 1365-1370, 2008.

Enviado: 02/04/2019
Revisado: 20/04/2020
Aceito: 06/05/2020