Effect of nutritional factors on the *Aspergillus nidulans* germination program

Efeito de fatores nutricionais sobre o programa de germinação de conídios de *Aspergillus nidulans*

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ABSTRACT

The development of conidia germination of the filamentous fungus *Aspergillus nidulans* can be used to evaluate the effects of different agents. The objective of the present work was to test eight compositions of the culture medium for the germination of conidia of the *bimeth* strain. Dormant conidia were inoculated in minimal medium with the following compositions: TN1 (MM + glyc 1% + VIT), TN2 (MM + glyc 0.1% + VIT), TN3 (MM + glyc 1% + VIT + casein), TN4 (MM + 0.1% gli + VIT + casein), TN5 (KH2PO4 + 1% gli + VIT), TN6 (KH2PO4 + 1% gli + casein), TN7 (NaNO3 + KCl, 1% gli + VIT) and TN8 (MC). Five hundred conidia were analyzed in each observation, determining the percentage of conidia in each germination phase, for a period of up to 24 hours. After that time, the percentages of death and malformation of the conidia were determined. The results showed changes in germinative development, in the generation of dead and malformed in all TNs that lacked nutrients, when compared to MM and MC. When testing two glucose concentrations, it can be seen that glucose is not consumed during germination, although it is necessary for breaking dormancy. The absence of casein showed that the *bimeth* strain, which has a requirement for methionine, germinated in the absence of methionine, therefore, it was able to synthesize at least a small amount of this amino acid, although in casein media the germination was better. The absence of phosphate totally inhibited the germination of the conidia, due to the lack of raw material

for DNA duplication. Some media that are poorer in nutrients than MM have shown to allow germination. The analysis of dead and malformed showed that TNs 3, 4 and 6 had a disrupting effect on polarized growth and TNs 1, 2 and 5 had a more drastic inhibitory effect, increasing cell death. The results of this work show that the conidia had changes in the development of germination in response to nutritional conditions, showing that the choice of constituents for the preparation of the medium to be used influences the responses of germination, death and malformations in conidia of *A. nidulans*.

Keywords: Nutritional constituents. Filamentous fungus. Culture media. Germination of conidia.

RESUMO

O desenvolvimento da germinação de conídios do fungo filamentoso *Aspergillus nidulans* pode ser usado para avaliar os efeitos de diferentes agentes. O objetivo do presente trabalho foi testar oito composições do meio de cultura para a germinação de conídios da linhagem *bimeth*. Conídios dormentes foram inoculados em meio mínimo com as seguintes composições: TN1 (MM+gli 1%+VIT), TN2 (MM+gli 0,1%+VIT), TN3 (MM+gli 1%+VIT+caseína), TN4 (MM+gli 0,1%+VIT+caseína), TN5 (KH_2PO_4+gli 1%+VIT), TN6 (KH_2PO_4+gli 1%+VIT+caseína), TN7 ($NaNO_3$+KCl, gli 1%+VIT) e TN8 (MC). Foram analisados 500 conídios em cada observação, determinando-se a porcentagem de conídios em cada fase da germinação, por um período de até 24 horas. Após esse tempo, foram determinadas as porcentagens de morte e malformação dos conídios. Os resultados mostraram alterações no desenvolvimento germinativo, na geração de mortos e malformados em todos os TNs que tinham carência de nutrientes, quando comparados ao MM e MC. Ao testar duas concentrações de glicose pode-se perceber que a glicose não é consumida durante a germinação, embora seja necessária para a quebra de dormencia. A ausência da caseína mostrou que a linhagem *bimeth*, que possui requerimento para metionina, germinou na ausência de metionina, portanto, foi capaz de sintetizar pelo menos uma pequena quantidade deste aminoácido, embora nos meios com caseína a germinação tenha sido melhor. A ausência de fosfato inibiu totalmente a germinação dos conídios, devido à falta de matéria prima para a duplicação do DNA. Alguns meios mais pobres em nutrientes do que o MM mostraram que permitem a germinação. A análise de mortos e malformados mostrou que os TNs 3, 4 e 6 tiveram efeito desregulador do crescimento polarizado e os TNs 1, 2 e 5 tiveram efeito inibidor mais drástico aumentando a morte celular. Os resultados deste trabalho mostram que os conídios tiveram alterações no desenvolvimento da germinação em resposta a condições nutricionais, mostrando que a escolha dos constituintes para o preparo do meio a ser utilizado influencia nas respostas de germinação, morte e malformações em conídios de *A. nidulans.*

Palavras- chave: Constituintes nutricionais. Fungo filamentoso. Meio de cultura. Germinação de conídios.

1 INTRODUCTION

The germination of *Aspergillus* (= *Emericella*) *nidulans* (EIDAM VUILL.) conidia presents 3 morphologically distinct phases: metabolic activation with isotropic growth, nuclear division and polarized growth of the germ tube (D´Enfert, 1997). These

phases have a morphogenesis with well-established temporal genetic regulation, although this control can be influenced by numerous environmental factors (Timberlake and Clutterbuck, 1994; Timberlake and Marshall, 1988).

In soaking (isotropic growth) (D´Enfert, 1997) there is an increase in the cell size of the wall, due to water absorption (Griffin, 1994), metabolism activation, increased protein synthesis, RNA, ATP and respiratory rate of spore, resulting in a cell whose diameter is two, or more times, larger than the resting spore. Then, the cell cycle phase occurs with the budding of the germinative bud that marks the first mitosis of the original nucleus of the conidia and the axis that is established at this moment is the manager axis for the polarized growth of the next phase, which is the germ tube. During this phase, the morphogenetic machinery is redirected to the polarization site (D´Enfert, 1997). Subsequently, the growth of the germ tube occurs, changing the functional organization of the hyphae tip area, which acquires the potential to perform mechanisms of endocytosis and exocytosis (Taheri-Talesh et al., 2008).

The fungus *A. nidulans* has been used as a model in tests to evaluate the effect of physical and chemical agents on the development of the different phases and has been considered a promising test organism for nutrigenomics.

Since the 1970s, studies have shown the importance and effect of nutritional factors on the development of fungi. Pass and Griffin (1972) showed the requirement of carbon and exogenous nitrogen for the germination of *Aspergullis flavus* conidia to happen. Abdel-Rahim and Arbab (1985) demonstrated differences in the germination of *Aspergillus niger* conidia in response to changes in the culture medium, varying the sources of carbon (glucose, sucrose and fructose) and nitrogen (glutamic acid and valine). With regard to *A. nidulans*, it has already been demonstrated that there is an influence on the preference of sexual or asexual development of the colony, due to nutritional changes (glucose and lactose) (Kap-Hoon et al., 2003). Another study compared the ability of some nutrients to trigger the germination of *A. niger* and *A. nidulans* and it showed that glucose serves as a trigger for the germination of the two fungi, galactose was shown to be efficient only for *A. nidulans*, whereas the source of nitrogen (amino acids) was an activator for *A. niger* (Hayer et al., 2014).

However, studies on basic compounds in culture media, such as inorganic salts, are rare. Evaluating the effect of these compounds on the germination of conidia can demonstrate the influence of nutrients on events related to cell cycle, nuclear division, apoptosis, cell repair mechanisms and malformations. Because of this, the objective of

the present work was to characterize qualitatively the influence of different compositions of *A. nidulans* culture medium in the process of germination of conidia.

2 MATERIAL AND METHODS

STRAINS AND CULTURE MEDIA

The *biA1methG1* strain of *A. nidulans* from the University of Glasgow (Scotland) was used in the present study. The culture media were liquid complete medium (LCM), liquid minimal (LMM) medium and solid complete medium (SCM), prepared in accordance with Pontecorvo et al. (1953) and six media for treatments.

PREPARATION OF NUTRITIONAL TREATMENTS

Eight nutritional compositions of the culture media were tested, identified as nutritional treatment (TN) 1, 2, 3, 4, 5, 6, 7 and 8 (Table 1).

Table 1 - Nutritional composition for 100 mL of medium from the nutritional treatments (TN) tested in the conidia germination of the *bimeth* strain of *A. nidulans.*

	TN1	TN2	TN3	TN4	TN5	TN6	TN7	TN8**
$NaNO_3$	0,6g	0,6g	0,6g	0,6g			0,6g	0,6g
KH_2PO_4	0,15g	0,15g	0,15g	0,15g	0,15g	0,15g		0,15g
KCl	0,05g	0,05g	0,05g	0,05g			0,05g	0,05g
$MgSO_4.7H_2O$	0,05g	0,05g	0,05g	0,05g				0,05g
$FeSO_4.7H_2O$	trace	trace	trace	trace				trace
$ZnSO_4.7H_2O$	trace	trace	trace	trace				trace
$CuSO_4.7H_2O$	trace	trace	trace	trace				trace
Glucose	1,0g	0,1g	1,0g	0,1g	1,0g	1,0g	1,0g	1,0g
Vitamins *	0,1mL	0,1mL	0,1mL	0,1mL	0,1mL	0,1mL	0,1mL	0,1mL
Hydrolyzed casein			0,15g	0,15g		0,15g		0,15g
Peptone								0,2g
Yeast extract								0,2g

*Biotin, pyridoxine, p-aminobenzoic acid, nicotinic acid, riboflavin and thiamine.
** Liquid complete medium.

CONIDIA GERMINATION ASSAY

Conidia from the strain were collected from 5-day-old growing colonies in solid complete medium at 37°C, and placed in a water solution with 0.01% (v/v) Tween 80. The suspensions was filtered in sterilized glass wool after heavy shaking. The conidia suspension was divided into eight experimental groups (TN1, TN2, TN3, TN4, TN5, TN6, TN7 and TN8). The concentration of conidia inoculated in each tube was $6,67x10^5$/mL.

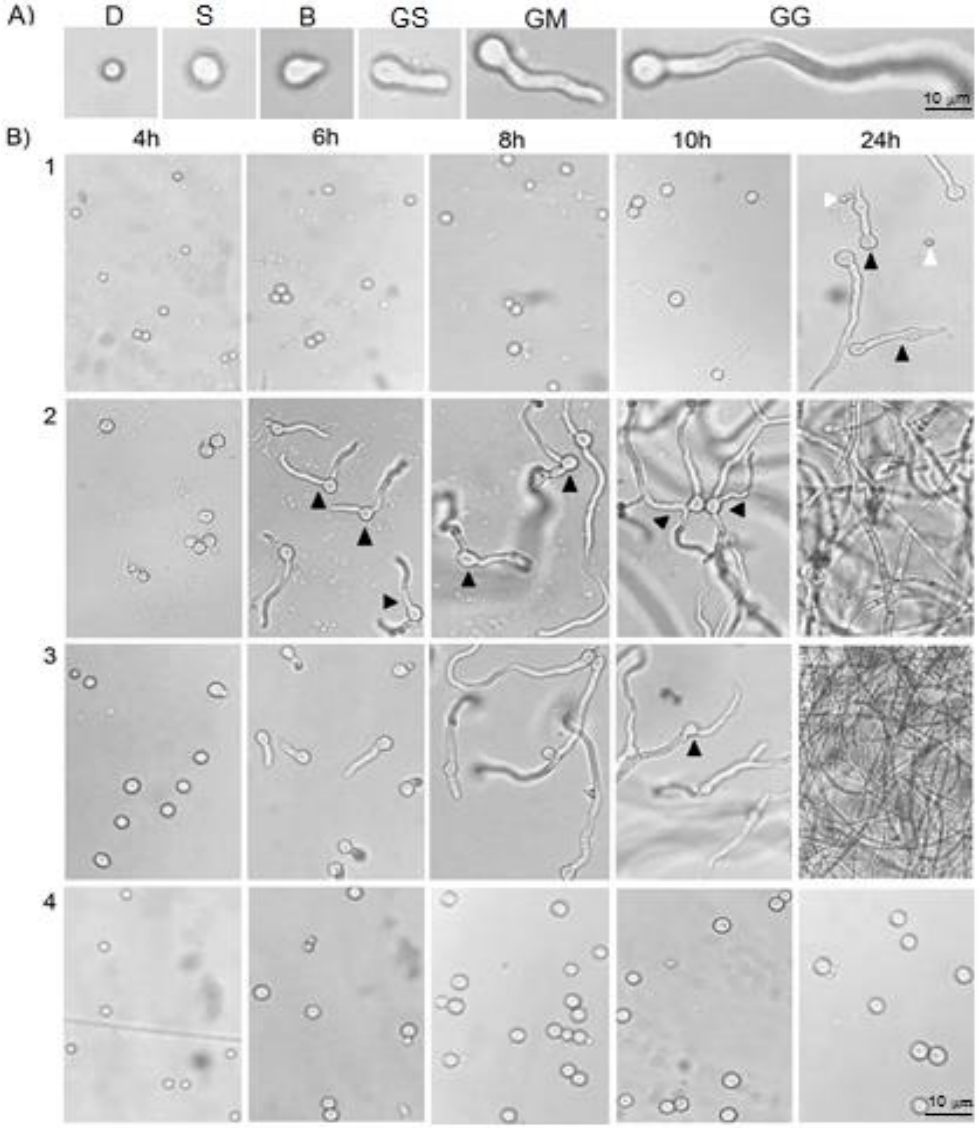
After preparing the test suspensions, 100 µL of the sample was transferred to sterile microscope slides and placed in petri dishes with moist paper. The plates were incubated at 37°C for 4 to 24 hours. After 4, 6, 8, 10 and 24 hours, two slides from each condition were analyzed. The cells were counted under optical microscopy, with a final magnification of 400x, by image capture (Canon EOS Rebel 3TI), with NDPL-2 (2x) SLR / DSLR adapter for binocular microscope.

At each time, were analyzed 500 conidia and determined the percentages of conidia in each phase of germination: dormant (D, 2,65±0,18µm), soaked (S, 4,11±0,58µm), button (B, 7,84±0,80µm), small germinated (GS, 22,71±6,38µm), medium germinated (GM, 50,22±4,24µm) and great germinated (GG, 83,64±11,18µm) (Fig. 1). At 10 and 24 hours the survival percentages were determined, considering alive only the conidia with germinative tube. At this time, was estimated the conidia GG with malformations of tubes. Conidial malformations were considered where the first germ tube bifurcated, or the second tube began to form early or at an angle other than 180^{o} or when more than 2 tubes were formed at the same time (Fig. 1). Small, medium and great germinated sizes, as well as the diameter of dormant and soaked conidia are shown in Table 1. This test was performed in triplicate in 3 independent assays for all experimental groups.

3 RESULTS

Analysis of the development of *A. nidulans* conidial germination showed differences between nutritional treatments in the development stages (D, E, B, GS, GM and GG), in speed of germination and in generation of dead and malformed (Figure 1).

Figure 1 – Conidia germination and growth of the *bimeth* strain in different nutritional treatments after 4, 6, 8, 10 e 24 hours, observed under an optical microscope (400X magnification). **A** Stages of conidia germination (D=dormant; S=soaked; B=button; GS=small germinated; GM=medium germinated; GG= great germinated); **B1** Representative micrograph of MM nutritional treatments that did not receive casein (TN 1, 2 e 5); **B2** Representative micrograph of MM nutritional treatments that received casein (TN 3, 4 e 6); **B3** Representative micrograph of nutritional treatment with MC (TN8); **B4** Representative micrograph of nutritional treatment that did not receive phosphate (TN7). The black arrows indicate malformed conidia and the white arrows indicate dead conidia.



Nutritional treatments 1 and 2 had all the nutritional compounds of the minimal medium (phosphate, chloride and sulfates), plus vitamins and glucose and had no casein. They differed only in the concentration of glucose (TN1 = 1g and TN2 = 0.1g). The analysis of the use of glucose was also made with TNs 3 and 4 which had all compounds of TN1 and TN2 plus casein and different concentrations of glucose (TN3 = 1g and TN4 = 0.1g).

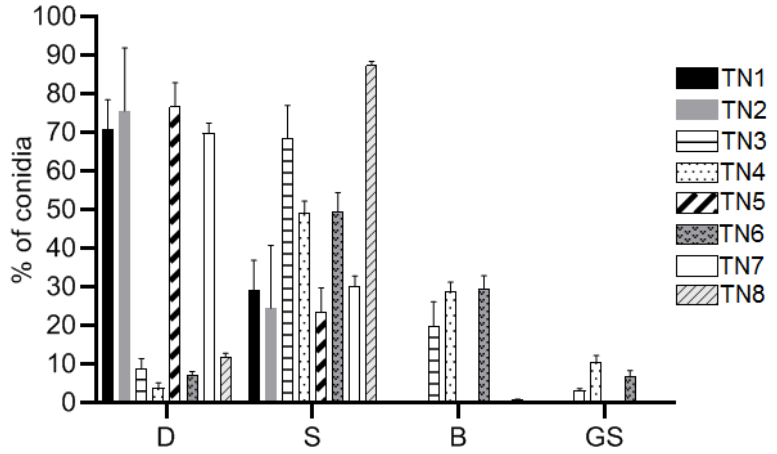
Nutritional treatments 5 and 6 had only the inorganic potassium phosphate salt, plus vitamins, glucose and casein, only differing in the concentration of glucose (TN5 = 1g and TN6 = 0.1g).

Nutritional treatment 7 had all inorganic salts, less potassium phosphate, more glucose and vitamins and had no casein.

Nutritional treatment 8 was the control of rich medium, with all salts, plus glucose, vitamins, casein, peptone and yeast extract.

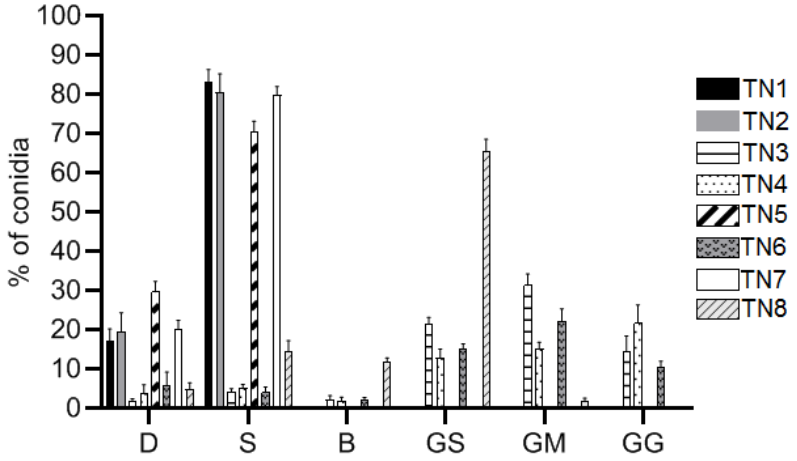
With four hours of analysis (Figure 2) it can be seen that TNs 3, 4 and 6 (all with casein) had a higher percentage of soaked than the group of TNs without casein, but already had conidia in the button phase and even small germinates, which is not normal at this time in the richest medium (TN 8). At this time, TNs 1, 2, 5 and 7 (all without casein) had fewer soaked conidia and approximately 70% of dormant conidia. TN8 (richer in nutrients) had more than 80% of its conidia soaked.

Figure 2 - Conidia of the *bimeth* strain in each phase of germination after 4 hours grown in nutritional treatments 1, 2, 3, 4, 5, 6, 7 and 8. D=dormant; S=soaked; B=button; GS=small germinated; GM=medium germinated; GG= great germinated. Error bars indicate standard error of the mean of three repetitions.



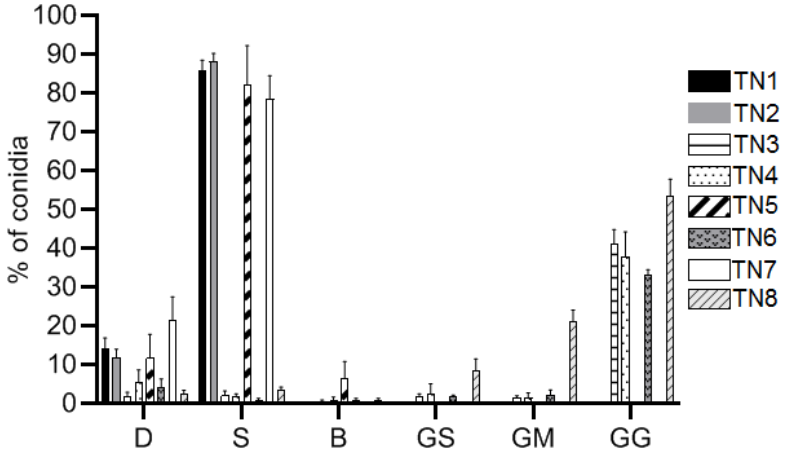
In six hours it is possible to observe the appearance of germinates in several nutritional treatments. However, the delay profile of TNs 1, 2, 5 and 7 still remained, since most of its conidia, approximately 80% in the four treatments, were still in soaking. Nutritional treatments 3, 4, 6 had small, medium and large germinates, with TN3 having more small and medium sprouts, TN4 had more large sprouts and TN6 had more small and medium sprouts. TN8 already had more than 60% of the small germinated conidia (Figure 3).

Figure 3 - Conidia of the *bimeth* strain in each phase of germination after 6 hours grown in nutritional treatments 1, 2, 3, 4, 5, 6, 7 and 8. D=dormant; S=soaked; B=button; GS=small germinated; GM=medium germinated; GG= great germinated. Error bars indicate standard error of the mean of three repetitions.



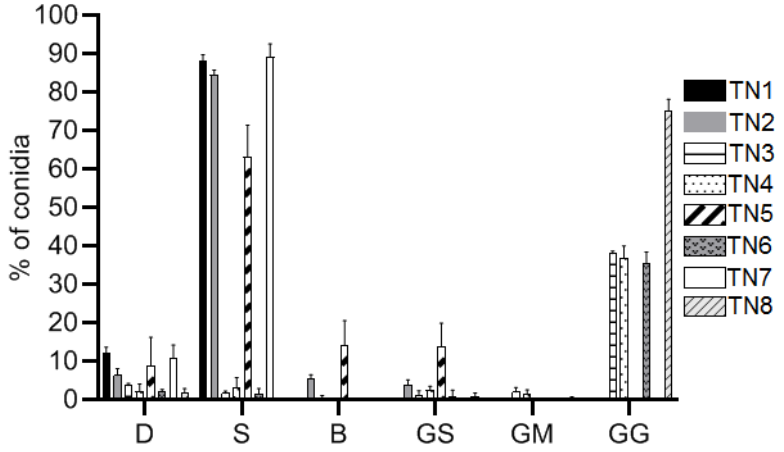
The delay profile in TNs 1, 2, 5 and 7 remained at eight hours, as all of these groups had more than 75% of their conidia soaked, while all other treatments were in later stages of development. Nutritional treatments 3, 4 and 6 had approximate amounts of large germinates, just over 30%. Nutritional treatment 8 already had more than 50% of its germinated conidia large (Figure 4).

Figure 4 - Conidia of the *bimeth* strain in each phase of germination after 8 hours grown in nutritional treatments 1, 2, 3, 4, 5, 6, 7 and 8. D=dormant; S=soaked; B=button; GS=small germinated; GM=medium germinated; GG= great germinated. Error bars indicate standard error of the mean of three repetitions.



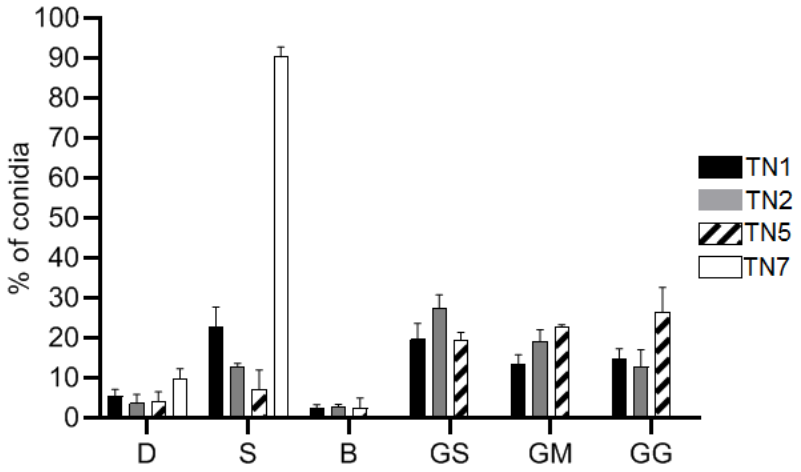
For ten hours, the response profile was very close to that of eight hours. Nutritional treatments 1, 2, 5 and 7 were still late, as most of their conidia were still only soaked, however, TNs 2 and 5 started to show small buds and small germinated conidia, with greater effect on TN 5. Nutritional treatments 3, 4 and 6 had very similar amounts of large germinates and TN 8 was the group that had more large germinates, reaching almost 80% (Figure 5).

Figure 5 - Conidia of the *bimeth* strain in each phase of germination after 10 hours grown in nutritional treatments 1, 2, 3, 4, 5, 6, 7 and 8. D=dormant; S=soaked; B=button; GS=small germinated; GM=medium germinated; GG= great germinated. Error bars indicate standard error of the mean of three repetitions.



The 24-hour analysis was performed on nutritional treatments that delayed the germination process, that is, those that did not receive casein (TNs 1, 2, 5 and 7). This analysis allowed to verify that the TNs 1, 2 and 5 had very close amounts of small, medium and large germinates after 24 hours. Nutritional treatment 1 still had a significant portion of soaked conidia, just over 20%. Nutritional treatment 2 had approximately 25% of small germinates. Nutritional treatment 5 had more large germinates. Nutritional treatment 7 had 100% of its conidia dormant and/or soaked (Figure 6).

Figure 6 - Conidia of the *bimeth* strain in each phase of germination after 24 hours grown in nutritional treatments 1, 2, 5 and 7. D=dormant; S=soaked; B=button; GS=small germinated; GM=medium germinated; GG= great germinated. Error bars indicate standard error of the mean of three repetitions.



The mortality analysis (Table 2) showed close values for NTs 3, 4 and 6. Nutritional treatment 1 had the highest mortality, followed by TN 2 and then TN 5. Nutritional treatment 7 had 100% mortality and TN 8 had the least mortality, only 1.84%.

Regarding the phase in which more conidia died, TNs 1, 2, 4, 5 and 7 showed higher mortality in soaked conidia and TNs 3, 6 and 8 in dormant conidia.

Table 2 - Percentage of dead conidia in each phase (D=dormant, S=soaked and B=button) in nutritional treatments 3, 4, 6 and 8 after 10 hours and in nutritional treatments 1, 2, 5 and 7 after 24 hours, in the *bimeth* strain at 37ºC.

Tratamentos		TN1	TN2	TN3	TN4	TN5	TN6	TN7	TN8
Germination	D	5,43	3,63	3,65	2,15	4,19	1,99	9,72	1,84
stages	S	22,80	12,89	1,59	2,97	7,09	1,51	90,28	0
	B	2,26	2,85	0,37	0	2,49	0,89	0	0
Total		30,49	19,37	5,61	5,12	13,77	4,39	100	1,84

The analysis of malformations, shown in table 3, shows that TN 6 was the one with the most malformations, followed by treatments 3 and 4, with very close values. Nutritional treatments 1, 2 and 8 showed close values of malformations, followed by TN 5. TN 7 did not present malformations because they all died. Nutritional treatments 1, 2 and 5, without casein, showed atypical malformations when compared to all other TNs (Figure 1).

Table 3 - Percentage of malformations in nutritional treatments 3, 4, 6 and 8 after 10 hours and in nutritional treatments 1, 2, 5 and 7 after 24 hours, in the *bimeth* strain at 37ºC.

Treatments	TN1	TN2	TN3	TN4	TN5	TN6	TN7	TN8
Malformations	21,61	21,36	53,30	54,24	17,37	60,04	0	21,75

4 DISCUSSION

Germination of *A. nidulans* conidia occurs in three stages: isotropic activation and growth, nuclear division and polarized growth. Each of these phases is controlled by specific phase genes that regulate the events related to germination and the time in which these changes must happen (D´Enfert, 1997). In this way, this control can be changed due to the influence of several external factors, among which, the available nutrients (Timberlake and Clutterbuck, 1994; Timberlake and Marshall, 1988).

In this work, eight nutritional compositions (for composition see Table 1) were tested to evaluate the effect of glucose concentration, different inorganic components and the source of amino acids, casein during the germination of *A. nidulans*.

Fungal spores contain nutrient reserves that can support growth for a limited period; however, the composition of the medium affects the germination process (Hassouni et al., 2007), as shown below, in all TNs tested in this research.

The ideal cultivation medium for *A. nidulans* is already well established in the literature, since 1953 by Pontecorvo et al. (1953), which brings the composition of

complete medium (CM) and minimum medium (MM) for growth of the fungus. When testing various nutritional compositions, it can be seen that the conidia germinated in less nutritious media than the MM proposed by the author, because the conidia of TNs 5 and 6 (with total absence of NaNO3, KCl, MgSO4 and trace elements) germinated (Figure 5 and 6).

The absence of MM constituents in TN 5 delayed the germination process, no doubt, as the conidia started to germinate only after ten hours (Figure 5), and some could be seen only later, with 24 hours (Figure 6). However, the total absence of these constituents did not inhibit the germination process, showing that minimal nutritionally poorer means can be used to make the germination of the conidia happen.

Nutritional treatments 6 was added with casein, showing that methionine was being missed for the full development of germination when compared to TN5 that does not have casein (Figures 2, 3, 4 and 5). This strain is a mutant for normal methionine synthesis. However, even though several components of the usual MM are missing, the conidia germinated. The favorable effect that casein caused on germination can be seen not only in TN 6, but also in TNs 3 and 4, which also have casein in their composition, and they all germinate faster when compared to all other nutritional treatments that did not receive casein (Figures 2, 3, 4 and 5).

Thus, TNs 1, 2, 5, presented delay in germination due to the lack of casein and other constituents of the medium (Figures 2, 3, 4, 5 and 6), because with ten hours (Figure 5) they had almost no germinated conidia and even after 24 hours, they still had a large portion of small and medium germinated conidia (Figure 6). As hydrolyzed casein acts as a supplier of amino acids, these results can easily be explained. The *bimeth* strain has requirements for biotin and methionine, and the vitamin suspension added to the medium provides biotin, but not methionine. Therefore, biotin is provided in treatments from the vitamin solution, but methionine comes from other sources, such as casein.

An interesting result obtained in this work was that, when testing nutritional treatments without methionine, it would be expected that the conidia would not germinate, as it is a strain with a requirement for methionine. However, as the conidia germinated, even with delay, in the absence of this amino acid, the conclusion is that the deficiency in the synthesis of methionine in this strain is partial.

The time required for the production of germ tubes in any strain varies widely, even when mature spores are tested and other factors are kept at their ideal level. Internal conditions, such as spore maturity, in addition to the innate propensity of strains, are

naturally also important in regulating the speed of germination (Gottlieb, 1950). Thus, when the factors of the culture medium were changed, there was a change in the speed of growth of the germ tube of *A. nidulans.*

Corroborating these results Ryan (194) demonstrated that mutant conidia of *Neurospora crassa* that lost the ability to synthesize some amino acids and that also required the presence of these amino acids for normal germination, in their absence, the germ tubes were produced only after long periods of time. Osherov and May (2000) demonstrated that the germination of *A. nidulans* conidia can be induced by a variety of carbon-containing compounds even in the absence of amino acids. Research on several species of fungi, including *Fusarium solani* (Nelson, 1991), *Pythium ultimum* (Nelson and Hsu, 1994) *A. niger* (Abdel-Rahim and Arbab, 1985) and *N. crassa* (Schmit and Brody, 1976) involved sugars and amino acids as general essential germination activators.

Nutritional treatment 8 is the complete medium (Table 1), proposed by Pontecorvo et al. (1953) as ideal for the growth of *A. nidulans*. This group presented a development different from all the other experimental groups (figures 2, 3, 4 and 5), which would be expected, as it is a medium rich in nutrients. This group has, in addition to the constituents of MM and vitamin, hydrolyzed casein, peptone and yeast extract. This treatment showed the largest number of large normal germinated conidia after eight hours (figure 4). Thus, it is clear that this culture medium, among those tested, is ideal for the development of *A. nidulans* germination.

Nutritional treatments 1, 2, 3 and 4 differ in their composition in glucose concentration (for differences see Table 1). When analyzing the germination response profile (TN1 versus TN2 and TN3 versus TN4), these groups showed similar speeds of soaking, bud sprouting and growth of small, medium and large germinates (Figures 2, 3, 4, 5 and 6). These results show that the glucose concentration does not seem to influence the speed at which germination occurs. However, when tested for germination with glucose 0% (data not shown) the conidia did not germinate. This demonstrates that in the total absence of glucose, germination does not happen.

Taken together, these results show that glucose should act as a signal. Nutritional treatments 1 and 3 had a standard concentration of 1% glucose in the formulation of the media according to Pontecorvo (1953). Nutritional treatments 2 and 4 had 1/10 of that glucose concentration (0.1%). Thus, if it is absent, the conidia does not germinate, and if it is present, germination happens, regardless of the concentration. However, even after 10 hours for TN 3 and 4 (Figure 5) and 24 hours for TN 1 and 2 (Figure 6), there were

almost no differences in the percentages of small, medium and large germinates among these groups, showing that even after 24 hours of germination, the conidia have not yet used glucose. Therefore, this saccharide is necessary for germination to happen, but it is only used in later stages of the development of hyphae.

According to Alves (2012) glucose is the main source of carbons, but there are other sugars that can serve as a carbon source for the growth of fungi. Of materials of known constitution, carbohydrates, such as glucose and sucrose, stimulate germination more frequently (Uppal, 1924; Lin, 1940; Lin, 1945).

Results of Shimoda (1980) corroborate those found in this work. The author showed that glucose is essential for the germination induction of *Schizosaccharomyces pombe*. Osherov and May (2000) showed that the omission of carbon was able to inhibit the germination of conidia, however, a combination of glucose and water are sufficient to initiate the germination, perform nuclear division and growth of hyphae in *A. nidulans*.

The results of this work demonstrate that although an external carbon source is necessary, in this case glucose, its consumption does not happen during germination, therefore, there is another carbohydrate used during germination. In fact, the germination of *A. nidulans* conidia requires an external source of carbon that will not be consumed, but that is used for the continuous degradation of the trehalose carbohydrate to occur, since non-metabolizable glucose analogues are sufficient to trigger this phenomenon, suggesting that trehalose decomposition is controlled by detecting the extracellular carbon source (D´Enfert, 1997). Thus, the results of this work prove that germination is dependent on the presence of a carbon source, in this case glucose, but that it is not degraded during germination, and that the metabolized sugar during germination events is trehalose, as proposed by D´Enfert already in 1997.

Trehalose is a non-reducing disaccharide that is accumulated by a wide variety of organisms during periods of reduced growth (Elbein, 1974). In fungal spores, trehalose can represent up to 15% of the dry mass and has been proposed as a protector against stress (Hottiger et al., 1994). A large amount of trehalose is essential during the resumption of spore growth (D´Enfert, 1997). After germination activation, the disaccharide is degraded (Thevelein, 1996) to form glycerol, which is indicative of active glycolysis (D´Enfert, 1997).

The results of the present study show that TN7 prevented the conidia from germinating, because even after 24 hours, the group did not show bud or germinated conidia (Figure 6). Nutritional treatments 7 differs from other groups, mainly due to the

absence of KH_2PO_4 (Table 1). Therefore, the lack of potassium phosphate (phosphorus source) clearly prevented the germination of *A. nidulans* conidia, regardless of the absence of other nutritional constituents, as TNs 5 and 6, which contain phosphate, but absence of all other salts inorganic (Table 1), germinated (Figures 4 and 5).

Phosphate is an indispensable raw material for DNA duplication and considered a basic element for the composition of ATP, ADP and nucleotides that play important roles in energy metabolism (Mcdowell, 1992) and phosphorus is an essential and indispensable component for cell multiplication (Yamada and Abdalla, 2004). The germination of the conidia is marked by the formation of the bud, which coincides with the first mitosis (D´Enfert, 1997), for this process to occur, nucleic acid synthesis is necessary. Thus, the absence of phosphate inhibited DNA synthesis, making the conidia lack raw material for duplication during the cell cycle. For this reason, all conidia reached the maximum soaked phase, prevented from entering nuclear division in TN7 (Figure 6).

Still regarding the phosphate, it is important to compare TNs 1, 2 and 5. Nutritional treatments 1 and 2 has all the inorganic salts, while TN 5 has only phosphate. Conidia germinated faster (Figure 5), showed lower mortality (Table 2) and less malformation (Table 3) in TN5. In other words, the medium that has the highest nutrient deficit but has phosphate was the one that most favored the development of germination. These results indicate that phosphate may be the main component of the minimal medium, which the fungus uses as a nutrient to germinate.

Starting from the idea that not all the traditional constituents of MM, proposed by Pontecorvo et al. (1953), were strictly necessary for the germination of conidia, one possibility is that as they are not necessary, they end up having undesirable effects, such as toxicity, when present, which would explain the change in germination and the increase in dead and malformed. Therefore, TN 5, with less nutritional constituents than TNs 1 and 2, seemed to present a better condition for the germination of *A. nidulans* conidia (Figure 5).

Similar results to this were found by Pulz and Massola Jr (2009), where the potato dextrose agar medium, traditionally used in the growth and sporulation studies of the *Alternaria* fungus species, had lower growth speeds for two species tested at work, when compared two other types of medium.

The analysis of mortality (table 2) and malformations (table 3) showed that TNs 3, 4 and 6, who received casein, showed less dead conidia and more malformed ones. In contrast, TNs 1, 2 and 5, which did not receive casein, had higher mortality than all casein

treatments, and close amounts of malformations. These results indicate that the lack of methionine compromises the development, favoring the death of the conidia in the initial stages of germination.

The lower number of malformations in the treatments that did not receive casein, can also be explained by the great delay in development, since with 24 hours, there were still significant amounts of small and medium germinates (Figure 6), which would certainly lead to an increase in the quantities of malformed when they reached the stage of large germination.

This aspect is relevant when observing TN 8, which presented the lowest amount of dead conidia (Table 2) and malformation values close to the values of TNs 1, 2 and 5 (Table 3). As TN 8 is the complete medium, certainly, the delay in germination of TNs 1, 2 and 5 explains the close amounts of malformations, since TN 8 already at 10 hours had almost 80% of its germinated conidia large, while TNs 1, 2 and 5, had practically no germination within 10 hours (Figure 5).

The relative mortality in each of the phases (Table 2) showed that TNs 1, 2 and 5, which do not receive casein, have more dead soaked conidia, these data may be related to the lack of this nutrient. Nutritional treatments 3, 4 and 6, who receive casein, did not seem to show any relation to the phase in which the conidia were dead. Glucose concentration also seemed to have no effect on the stage when the conidia are dead, because when comparing TN1 versus TN2 and TN3 versus TN4 there is no relationship. Therefore, only the absence of casein seemed to influence the mortality stage in which the conidia are.

These effects on the incidence of deaths and malformations are due to the absence of the different constituents of each composition of the medium. In the case of TNs 5 and 6, several natural constituents of MM are missing, and in TNs 1, 2, 3 and 4 the glucose concentrations and the presence or absence of casein are altered. But in general, all TN have somehow altered mortality and malformations. These results show that the complete absence of some constituents, reduced glucose or absence of casein, were not able to inhibit germination, but directly influenced processes related to polarized growth, which led to the production of malformed ones.

For polarized growth, there is the perception of signals, internal and external, which will define the necessary position for the new growth axis of the cell (Sudbery, 2008). Therefore, during polarized growth the conidia respond to external factors, as in

the case of the constituents of the culture medium, which in this work were able to cause several developmental changes and in the generation of dead and malformed people.

Nutritional treatments 1, 2 and 5, which did not receive casein, presented atypical malformations, different from the existing malformations in all other experimental groups, including TN 8, which is CM. These data again bring up the discussion about the need for methionine by the *bimeth* strain. Thus, the lack of methionine did not inhibit the germination of this strain, but caused malformations in the conidia of *A. nidulans* (Figure 1), demonstrating a harmful effect on the development of the conidia of the strain due to the lack of the amino acid.

In general, the concentration of amino acids made available by the culture medium can limit the maximum cell concentration of culture influence cell growth and viability, and can affect protein synthesis (Amable and Butler, 2008). Methionine is the main amino acid donor of methyl groups for the DNA methylation process, one of the mechanisms responsible for modulating gene expression, in addition to exercising functions in the constitution of proteins (Szyf, 2011). Therefore, its absence, possibly, altered genes and/or proteins that are related to the growth of hypha during germination, leading to the appearance of unusual malformations.

The results of this work show that changes in the constitution of the culture medium for the germination of *A. nidulans* conidia can have effects on the speed at which germination takes place and on the incidence of deaths and malformations.

Thus, changes in the constituents of the culture medium, used for the germination of *A. nidulans* conidia, led to changes in morphogenetic events synchronized with the development phases (soaking, bud and germinated), directly interfering in these phases.

These results constitute a contribution to research using *A. nidulans* and probably other filamentous fungi, reinforcing the need for careful standardization of environmental factors such as the composition of the culture medium, since classic formulations, used routinely in research laboratories since the last century may be introducing undesirable variables, especially when analyzing the development of these strains.

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