Visualizing Nuclear Migration during Conidiophore Development in *Aspergillus nidulans* and *Aspergillus oryzae*: Multinucleation of Conidia Occurs through Direct Migration of Plural Nuclei from Phialides and Confers Greater Viability and Early Germination in *Aspergillus oryzae*

Kazutomo I SHI, Jun-ichi MARUYAMA, Praveen Rao JUVVADI, Harushi NAKAJIMA, and Katsuhiko KITAMOTO

Department of Biotechnology, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

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Nuclear migration is indispensable for normal growth, differentiation, and development, and has been studied in several fungi including *Aspergillus nidulans* and *Neurospora crassa*. To better characterize nuclear movement and its consequences during conidiophore development, conidiation, and conidial germination, we performed confocal microscopy and time-lapse imaging on *A. nidulans* and *Aspergillus oryzae* strains expressing the histone H2B-EGFP fusion protein. Active trafficking of nuclei from a vesicle to a phialide and subsequently into a conidium provided the mechanistic basis for the formation of multinucleate conidia in *A. oryzae*. In particular, the first direct visual evidence on multinucleate conidium formation by the migration of nuclei from a phialide into the conidium, rather than by mitotic division in a newly formed conidium, was obtained. Interestingly, a statistical analysis on conidial germination revealed that conidia with more nuclei germinated earlier than those with fewer nuclei. Moreover, multinucleation of conidia conferred greater viability and resistance to UV-irradiation and freeze-thaw treatment.

**Key words:** *Aspergillus*; nuclear migration; conidiation; multinucleate conidium; conidial germination

*Aspergillus oryzae*, a well known koji mold, by virtue of its ability to secrete a wide variety of useful proteins, has long been commercially exploited in the Japanese food and fermentation industries during the production of sake, miso, and soy sauce. Efficient germination and greater viability of *tane-koji* (pure conidia of *A. oryzae*—the starter for the production of fermented foods) are crucial properties that influence fermentation processes. Therefore, studies on conidiation and germination are beneficial for productive utilization of *A. oryzae*.

Conidia, in general, are dormant asexual spores produced by several species of filamentous fungi, and can survive over long time periods in extreme environments. For instance, in response to suitable environmental conditions, *Aspergillus nidulans* starts conidiating by differentiating into structures in the following sequential order: foot cell, stalk, vesicle, metulae, phialide, and conidia. Among the lower eukaryotes, the distribution of nuclei during growth by nuclear migration is of fundamental importance for cellular differentiation and development. It is presumed that defective nuclear migration not only causes distorted conidiophore formation but also results in a reduction of spore numbers. Evidences supporting these studies on nuclear distribution in conidiophores and at various developmental stages in *Aspergillus niger* and *A. nidulans* were earlier obtained by transmission electron microscopy and DAPI staining followed by fluorescence microscopy. While these microscopic analyses were performed in fixed cells, nuclear migration during conidiophore development and conidiation using living hyphal cells has not been traced in real time. Hence, the mechanism underlying nuclear migration and nucleation of conidia during uninucleate or multinucleate conidia formation remains to be understood.

While *A. nidulans* propagates by uninucleate conidia, *A. oryzae* is known to produce multinucleate conidia and to maintain the multinucleate state throughout its life cycle. Recently we confirmed this by microscopic visualization and scoring of nuclei using FACS in the *A. oryzae* strain expressing histone H2B-EGFP fusion protein. Multinucleate conditions were also reported in conidia of other species of Aspergilli, an arbuscular mycorrhizal fungus, and in macroconidia of *Neuro-
spora crassa and Microsporum gypseum.11,12 While these observations indicate that filamentous fungi maintain a multinucleate state throughout their life cycle, knowledge on the mechanism of multinucleation in conidia is limited. It is, therefore, envisaged that visualizing nuclear migration during the development of conidiophore would provide vital information on the onset of multinucleate conidia formation. Moreover, since the primary step after conidiation is conidial germination, it is pertinent to examine a correlation between the two important processes of conidiation and conidial germination, specifically with regard to the number of nuclei in a conidium. Although it is well known that sensing of nutrients by the conidium provokes processes leading to conidial germination, it may be noted that a sharp contrast of characteristics exists between different fungi with regard to the initiation of germination and mitotic processes. While germination precedes mitosis in A. fumigatus both in rich and poor environments, in A. nidulans mitosis occurs ahead of germination in rich media and vice versa in poor media.14,15 It is therefore interesting to verify the relation between the two processes in multinucleate conidia as well.

In order to critically understand the process of multinucleate conidia formation and its significance, we visualized nuclear migration during conidiation in A. nidulans (known to form uninucleate conidia) and multinucleate conidiation in A. oryzae living cells by expressing the H2B-EGFP fusion protein and constructing time-lapse 3D images captured by confocal laser scanning microscopy. In addition to obtaining clear visual evidence on multinucleate conidia formation, we examined the advantages of multinucleate conidia of A. oryzae for germination and resistance against UV-irradiation and freeze-thaw treatments.

**Materials and Methods**

**Strains and culture conditions.** A. nidulans HGR1 and A. oryzae NHG10 strains expressing A. nidulans histone H2B-EGFP fusion protein were used for microscopic observation of nuclear migration during conidiophore development, conidiation, and germination. The A. nidulans HGR1 and A. oryzae NHG10 strains were cultured in liquid MM (0.6% NaNO₃, 0.05% KCl, 0.15% KH₂PO₄, 0.05% MgSO₄-7H₂O, 1.0% glucose, vitamin, and Hunter’s trace elements, pH 6.5) and Czapek–Dox (CD) medium (0.3% NaNO₃, 0.2% KCl, 0.1% KH₂PO₄, 0.05% MgSO₄-7H₂O, 0.002% FeSO₄-7H₂O, and 2% glucose, pH 5.5) respectively, at 30°C overnight, and transferred onto a thin layer (<1mm) of solid agar medium devoid of glucose between a glass bottom dish (Iwaki Glass, Japan) and a cover slip (Fig. 1A). The set-up was incubated at 30°C on the stage of an inverted microscope. For microscopic observation of conidial germination, the NHG10 strain was cultured on CD agar medium at 30°C for 7 d. Conidia were collected by addition of 10ml sterile distilled water to the CD agar medium plate, and the resulting conidial suspension was filtered through miracloth (Calbiochem, U.S.A.). Conidia were inoculated into DPY liquid medium (2% dextrin, 1% polypeptone, 0.5% yeast extract, 0.5% KH₂PO₄, and 0.05% MgSO₄-7H₂O; pH 5.5) and incubated at 30°C from 0 to 6h. The conidial suspension was diluted to about 1 x 10⁶ conidia/ml, and the number of nuclei was counted in more than 100 conidia with or without a germ tube. The experiment was repeated 4 times. Survival rates of conidia were measured in the A. oryzae RIB40, 128, 177, 609, and 647 strains (provided by the National Research Institute of Brewing, Japan) after UV-irradiation and freeze-thaw treatment.

**Staining of nuclei in conidia and microscopy.** Conidial nuclei of the A. oryzae RIB40, 128, 177, 609, and 647 strains were stained with 4’,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, U.S.A.) as described previously and visualized using a BX52 microscope equipped with an UPlanApo 100 x objective lens (1.35 numerical aperture) and BH-DMU ultraviolet excitation cubes. In order to visualize nuclei during conidiophore development, conidiation, and conidial germination, time-lapse EGFP images were captured using an IX70 inverted microscope (Olympus, Japan) equipped with an UPlanApo 40 x (1.00 numerical aperture) or an UPlanApo 100 x (1.35 numerical aperture) objective lens, a fluorescence filter for EGFP, and a confocal scanning system CSU21 (Yokogawa Electronics, Japan) operated by IPLab software (Scanalytics, U.S.A.). Differential interference contrast (DIC) and EGFP fluorescence micrographs were taken through an Image-intensifier unit (Hamamatsu Photonics, Japan) and an AP imager camera (Hamamatsu photonics). 3D time-lapse images were captured using a confocal laser scanning microscope (CLSM). Sections encompassing the whole cell were scanned at intervals of 1.0μm, and 12 to 16 scanned images were used to construct the 3D images.

**Survival rate measurements against UV-irradiation and freeze-thaw treatment.** The A. oryzae RIB40, 128, 177, 609 and 647 strains were grown on potato dextrose agar (PDA) (Nissui, Japan) plates for 7 d. Conidia collected as described earlier were suspended in 0.02% Tween 80 solution, and about 100 conidia were spread on PDA medium containing 0.25% Triton X-100. Conidia on agar plates were irradiated with UV light for 3 min at about 40μW/cm² in a safety cabinet SV-CCHIA (Hitachi, Japan) equipped with a 13 W UV lamp (Toshiba, Japan). After further incubation for 2 d at 30°C, survival rates were calculated by averaging the number of colonies on the 3 plates. The number of colonies on 2 non-irradiated plates was averaged and represented as 100% viability. The values were depicted as mean ± standard deviation of three independent experiments. To determine the effect of freeze-thaw
treatment on conidial survival, the conidial suspension was adjusted to about $1 \times 10^3$ conidia/ml in 22.5% glycerol and spread on PDA medium containing 0.25% Triton X-100 after freezing in liquid nitrogen for 5 min and thawing at 30°C for 10 min. The number of colonies derived from untreated conidia was represented as 100% viability. The values were depicted as mean ± standard deviation of three independent experiments.

Fig. 1. Nuclear Migration during Uninucleate Conidiation in A. nidulans.

Mycelial mats of the A. nidulans HGR1 strain expressing histone H2B-EGFP were transferred onto a thin layer (<1 mm) of MM agar medium (without glucose) covered with a cover slip in a glass bottom dish and incubated at 30°C overnight. Nuclei in the developed conidiophores were observed by microscopy (A). Nuclear migration during conidiation was observed using CLSM, as described in “Materials and Methods” (B). Scale bar, 5 μm.
Results

Time-lapse microscopy of nuclear migration during uninucleate conidiation of A. nidulans

In order to clearly observe nuclear migration during conidiopore formation and conidiation in the aerial hyphae of the A. nidulans HGR1 strain, a suitable method was designed as follows: Mycelial mats were inoculated on the surface of a thin layer of agar medium between a coverslip and the glass bottom dish as illustrated in Fig. 1A, and soon after the conidiophores grew out on the surface of the thin medium layer they were subjected to microscopic analysis. Glucose was omitted from the agar medium to induce efficient conidiation. As shown in Fig. 1B, by 127 min after the start of microscopy, the nuclei that accumulated in the vesicle migrated into the metulae (refer to the DIC image captured at 137 min). Subsequently, the nuclei from the metulae migrated into the phialides between 137 to 327 min. The DIC image captured at 327 min confirmed the formation of phialides. Finally, formation of uninucleate conidia was evident from the DIC image (at 441 min) and the EGFP images (at 335, 395, and 448 min).

Multinucleate conidia formation occurs not by mitosis in newly formed conidia but by migration of plural nuclei into conidia in A. oryzae

The A. oryzae (NHG10 and niaD300) strains, in contrast to A. nidulans, do not form metulae but instead produce phialides directly upon the vesicle which then give rise to conidia. In order to observe the formation of conidiophores and multinucleate conidia in A. oryzae, the microscopic observation method mentioned in Fig. 1A was followed. The DIC image (at 0 min) and the fluorescence microscopic image (at 2 min) in Fig. 2A showed the young phialides (indicated by black arrows) originating from the vesicle, however, the nuclei accumulated within the vesicle started migrating into the phialides by 47 min (indicated by white arrows) from the start of observation. The complete migration of two or three nuclei (plural nuclei) from the vesicle into the newly formed phialides was noted in the DIC image at 93 min after the start of observation (Fig. 2A). In contrast to the migration of a single nucleus from the vesicle to each phalide via the metulae in A. nidulans (Fig. 1B), plural nuclei moved from the vesicle to the phialides in A. oryzae. Although we often observed both phialides with plural nuclei, anucleate phialides were also evident on a single vesicle, indicating an asynchronous nuclear translocation from the vesicle into the phialide. The most probable explanation for such an occurrence is the immature morphological status of the phialide. In some other instances all the nuclei accumulated within a vesicle migrated into the phialides (data not shown). Subsequently, conidia were produced soon after the phialide tips swelled and formed spherical conidial structures (Fig. 2B). Three nuclei from the phialide (indicated by white arrows) migrated into the conidial structure although the others (indicated by an arrow head) still remained within the phialide even at 47 min. This confirmed that the nuclear migration from the phialides into the conidia was also asynchronous. After the nuclei migrated, the base of the spherical structure was sealed, resulting in the formation of a multinucleate conidium (Fig. 2B). As indicated by an arrowhead in Fig. 2B, mitosis occurred by 87 min within the phialide. These results demonstrated that multinucleate conidium formation occurs by the migration of plural nuclei from a phialide into the conidium but not by a mitotic division in a newly formed conidium.

Multinucleate conidia germinated earlier than uninucleate conidia in A. oryzae

While previous reports on conidial germination have laid emphasis on the availability of nutrients and suitable external environments, several other factors that might influence germination formation are as yet unknown. To investigate whether the multinucleate status of a conidium also influences its germination efficacy in A. oryzae (NHG10 strain), we performed both time-lapse microscopic observation (Fig. 3) and statistical analysis (Table 1) on the emergence of germ tubes from conidia with varying nuclear numbers. Time-lapse observation revealed that mitosis occurred before germ tube formation in uninucleate conidia, but that multinucleate and binucleate conidia germinated without mitosis (Fig. 3). Additionally, it was observed that multinucleate conidia germinated earlier than uninucleate conidia. Most conidia (≥90%) including uninucleate conidia germinated within a 12-h time period. Uninucleate conidia of A. oryzae showed mitosis and germination resembling A. nidulans. The percentages of conidia with a germ tube were calculated at 1-h intervals for a total incubation period of 6-h. They were sorted according to the number of nuclei in the conidium as summarized in Table 1. The percentage of conidia with a germ tube in binucleate and trinucleate conidia increased after 3-h of incubation. Multinucleate conidia showed higher percentages of germination than did uninucleate conidia by 4- and 5-h growth period. In order to verify that germination in multinucleate conidia was not caused by an increase in nuclei through mitosis, the distribution of the number of nuclei in each conidium was determined at different times of incubation as indicated in Table 2. The distribution of the number of nuclei in each conidium was relatively constant until 5-h of incubation, and altered between 5- to 6-h. While the percentage of uninucleate and binucleate conidia decreased, the number of conidia with 4 nuclei increased. This revealed that the first cycle of mitosis occurred in uninucleate and binucleate conidia after germination in most of the multinucleate conidia at 5-h (Table 1 and Table 2), indicating that germination occurred earlier than mitosis in multinucleate conidia.
Multinucleation of conidia conferred greater viability and resistance to UV-irradiation and freeze-thaw treatment

Multinucleation of conidia is considered to attenuate the phenotypic effects of recessive mutations. To evaluate this hypothesis and verify the advantages of multinucleation, we investigated conidial viability after UV-irradiation in five strains of *A. oryzae* (RIB40, 128, 177, 609, and 647). All these strains, except for the RIB40 strain, were isolated from koji for industrial production of sake and miso. An examination into the number of nuclei in the conidium of each of these strains showed that the strains exhibited a varied nature of nuclear distribution in their conidia even under similar culture conditions (Fig. 4A). Importantly, in comparison to the RIB40 strain, other strains produced larger populations of multinucleate conidia. To determine whether these variations in the number of nuclei contribute to the viability or survival of these strains, the strains were subjected to UV-irradiation (Fig. 4B) and freeze-thaw treatment (Fig. 4C). Data obtained from the viability tests clearly demonstrated that the strains...
possessing a larger proportion of multinucleate conidia exhibited higher survival rates. In comparison to the RIB40 strain, the conidia of the other strains showed about 30% and about 20% higher survival rates against UV-irradiation and one cycle of freeze-thaw treatment, respectively (Fig. 4B and C). Supporting this observa-

Fig. 3. Multinucleate Conidia Germinated Earlier Than Uninucleate Conidia in A. oryzae.
Conidia of A. oryzae NHG10 strain were grown in DPY liquid medium at 30°C for 180 min, prior to observation. Observation started at time zero and the germinations of four conidia: A, B, uninucleate; C, binucleate; and D, trinucleate, were periodically monitored by CLSM. Note that the multinucleate conidia (C and D) germinated earlier without mitosis, and that in uninucleate conidia (B) a single cycle of mitosis occurred before germination. Scale bar, 5 μm.

Table 1. Percentage of Conidia with Germ Tubes Sorted by Conidial Nuclear Number and Growth Period in A. oryzae

<table>
<thead>
<tr>
<th>Number of nuclei</th>
<th>Incubation time (h)</th>
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<td></td>
<td>0 1 2 3 4 5 6</td>
</tr>
<tr>
<td>1</td>
<td>0.0 0.0 0.0 0.7 1.3 4.5 16.9</td>
</tr>
<tr>
<td>2</td>
<td>0.0 0.0 0.0 1.0 7.3 35.3 65.4</td>
</tr>
<tr>
<td>3</td>
<td>0.0 0.0 0.0 3.2 25.7 80.2 89.7</td>
</tr>
<tr>
<td>≥4</td>
<td>0.0 0.0 1.0 0.0 32.2 90.2 93.8</td>
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</tbody>
</table>

*Conidia were inoculated into DPY liquid medium and incubated at 30°C from 0 to 6 h. The numbers of nuclei were counted in more than 100 conidia with or without a germ tube. The number of nuclei in each conidium versus the formation of germ tube as a function of time was determined. Each percentage of conidia with germ tubes represents the average of 4 independent experiments.

Table 2. Percentage of the Number of Nuclei in Each Conidium A. oryzae during Germination

<table>
<thead>
<tr>
<th>Number of nuclei</th>
<th>Incubation time (h)</th>
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<tbody>
<tr>
<td></td>
<td>0 1 2 3 4 5 6</td>
</tr>
<tr>
<td>1</td>
<td>23.5 22.8 25.0 23.4 22.0 22.1 10.3</td>
</tr>
<tr>
<td>2</td>
<td>61.8 63.6 61.2 62.1 60.4 56.2 42.8</td>
</tr>
<tr>
<td>3</td>
<td>10.5 9.1 10.4 11.1 12.6 10.7 11.2</td>
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<tr>
<td>4</td>
<td>3.2 3.1 2.8 3.0 4.5 8.1 27.3</td>
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<tr>
<td>≥5</td>
<td>0.4 0.3 0.5 0.3 0.5 2.7 2.7</td>
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</table>

*Conidia were inoculated into DPY liquid medium and incubated at 30°C from 0 to 6 h. The numbers of nuclei were counted in over 100 conidia. The experiment was repeated 4 times. Each percentage of the number of nuclei in each conidium at indicated incubation times represents the average of 4 independent experiments.

Fig. 4. Multinucleate Conidiation Conferred Higher Survival Rate after UV-Irradiation and Freeze-Thaw Treatment in A. oryzae.
(A) Conidia of A. oryzae strains were stained with DAPI. The number of nuclei in 200 conidia were counted for each strain and are shown as percentages. (B) The conidia were spread on a PDA plate, irradiated with UV, and incubated at 30°C for 2 days. The number of viable colonies was counted, and their survival rates determined. Results depicted are mean ± standard deviation of three independent experiments. (C) The conidia were spread on PDA with 0.25% Triton X-100 after freezing in liquid nitrogen for 5 min and thawing at 30°C for 10 min. The number of viable colonies was counted, and their survival rates determined. Values represent mean ± standard deviation of three independent experiments.
tion, a survival rate of about 80% was reported during one freeze-thaw cycle in the macroconidia of *Tricho-phyton mentagrophytes*. In an effort to reconfirm the higher survival rate of multinucleate conidia, an enriched uninucleate conidial suspension (about 70%) prepared by filtration\(^3\) and a non-enriched (without filtration) conidial suspension (about 37%) of the NHG10 strain were UV-irradiated and compared for their survival rates. The conidial suspension rich in uninucleate conidia showed a lower survival rate (16.3 ± 1.9%) than the non-enriched conidial suspension (22.3 ± 3.8%). From the above results it seems reasonable to consider the suitability of strains with larger populations of multinucleate conidia for conidial preservation and industrial use.

**Discussion**

Nuclear migration is a vital aspect of cellular differentiation and development, and has been reported to have several implications for the cell.\(^{18,19}\) In fungi, its role in hyphal branch initiation\(^{20}\) and an aberrant conidiophore morphology due to defective nuclear migration\(^4\) have been reported. Recently, Lin and Momany suggested that nuclear number correlates with branching frequency linking nuclear division and branching.\(^{21}\) Although the participation of motor proteins in nuclear migration\(^{22}\) and maintenance of nuclear number\(^{23}\) and the morphological and physiological implications of nuclear migration have received much attention, several questions on the formation and consequence of multinucleate conidiation in fungi remain unanswered. While there have been very few studies based on visual evidence for the movement of nuclei in living hyphae,\(^{24}\) nuclear migration during the formation of conidiophore, an essential structure giving rise to conidia with either a single nucleus or multinuclei, has not yet been examined in living cells.

In order to follow and distinguish the mechanism of nuclear movement during conidiophore formation and conidiation in *A. nidulans* (known to produce uninucleate conidia), and *A. oryzae* (a multinucleate conidia forming fungus), we carried out time-lapse confocal laser scanning microscopy in the respective strains expressing H2B-EGFP fusion protein. For this purpose, a suitable method of sample preparation for confocal microscopy was designed to conveniently observe the aerial conidiophore development (Fig. 1A). Time-lapse microscopy in *A. nidulans* revealed that nuclear migration during conidiation (Fig. 1B) coincided with nuclear distributions in fixed conidiophores as described earlier.\(^{6,7}\) Interestingly, in contrast to *A. nidulans*, plural nuclei migrated from the vesicle into the phialides and finally into the conidia, resulting in multinucleate conidia formation in *A. oryzae* (Fig. 2A and B). Surprisingly, it was not mitotic division in a newly formed conidium but instead the migration of plural nuclei into the conidium that led to its multinucleate status. Some smaller phialides and conidia remained anucleated probably due to lack of maturation which is perhaps a prerequisite for the migration of nuclei into them. While the results obtained from time-lapse imaging might contribute to a better understanding of multinucleate conidiation in other filamentous fungi, the microscopic observation method in this study can be adopted as a tool for visualizing organelle and protein transport during conidiation in some mutants of *A. oryzae*(\(^{23}\)) and *A. nidulans*(\(^{25,26}\)) showing increased nuclear numbers\(^{23,25}\) or defects in nuclear movement.\(^{26}\)

After obtaining clear visual evidence on the mechanism of multinucleate conidiation in *A. oryzae*, we sought to examine the significance of this phenomenon in comparison to uninucleate conidiation. Studies on the frequency of germination in relation to the conidial nuclear number revealed that multinucleate conidia germinated earlier than uninucleate conidia (Table 1 and Fig. 3). Although *A. nidulans* uninucleate conidia germinated after one cycle of mitosis in rich media,\(^{14,15}\) almost all the multinucleate conidia in *A. oryzae* germinated without mitosis in DPY medium. While it was previously reported that multinucleate conidia possess larger diameters than uninucleate conidia,\(^{9,17}\) this study for the first time demonstrated that multinucleate conidiation conferred higher germination efficiency. Considering the spreading of conidia on steamed rice in open air during koji preparation for sake making, it may be reasoned that early germination efficiency is useful in minimizing the chances of contamination. Supporting this assumption, the examined *A. oryzae* strains, used for sake making produced more multinucleate conidia than the RIB40 wild type strain (Fig. 4A).

In addition to possessing higher germination efficiency, multinucleation of conidia conferred greater viability and resistance to UV-irradiation and freeze-thaw treatment (Fig. 4B and C). It is worth noting that the RIB128 strain, in spite of its similarity to the RIB40 strain in conidial nuclear number, exhibited a higher survival rate. This might be due to the stronger pigmentation of conidia observed in the RIB128 strain. It has been reported that disruption of the gene encoding the fungal pigment, melanin, increased sensitivity to UV-irradiation.\(^{27}\) Since multinucleate conidia exhibited a higher survival rate after UV-irradiation, it is reasonable to assume that the presence of multinucleate conidia attenuated the phenotypic effects of recessive mutations. Furthermore, in the context of greater viability of multinucleate conidia during freeze-thaw treatments, it is considered that the production of multinucleate conidia is a favorable strategy for adaptation to adverse environmental conditions and conidial preservation.

The multinucleate characteristic of *A. oryzae* conidia not only provides survival efficiency under stressful environmental conditions, but also might contribute to the genetic stability required during the fermentation and enzymatic production processes. While this study
provides a first visual demonstration on the mechanism of multinucleation of conidia and evidences for greater germination efficiency and viability of multinucleate conidia in A. oryzae, future studies addressing the molecular mechanisms underlying these processes will contribute not only to advancing our knowledge on multinucleation of conidia formation, but also to a better utilization of this industrially important fungus.

Acknowledgments

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