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Effect of Vitamin E on Autolysis and Sporulation of *Aspergillus nidulans*

Tamás Emri,*[,1] Zsolt Molnár,[1] Tünde Pusztahelyi,[1] Stefan Rosén,[2] and István Pócsi[1]

[1]Department of Microbiology and Biotechnology, Faculty of Sciences, University of Debrecen, PO Box 63, H-4010 Debrecen, Hungary, E-mail: emri@freemail.hu; and [2]Department of Microbial Ecology, University of Lund, Ecology Building, Sölvegatan 37, S-223 62 Lund, Sweden

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Abstract

The morphologic and physiologic effects of vitamin E, a powerful antioxidant, on the autolysis and sporulation of *Aspergillus nidulans* FGSC26 were studied. In carbon-depleted submerged cultures, reactive oxygen species (ROS) accumulated in the cells and, concomitantly, progressing autolysis was observed, which was characterized by decreasing dry cell masses and pellet diameters as well as by increasing extracellular chitinase activities. Vitamin E supplemented at a concentration of 1 g/L hindered effectively the intracellular accumulation of ROS, the autolytic loss of biomass, the disintegration of pellets, and the release of chitinase activities. In surface cultures, vitamin E inhibited autolysis of both *A. nidulans* FGSC26 and a loss-of-function *FlbA* autolytic phenotype mutant. In addition, supplementation of the culture medium with this antioxidant also had a negative effect on the sporulation of strain FGSC26 and the *FadA*^{G203R} hypersporulating phenotype mutant. These results suggest that accumulation of ROS was involved in the initiation of both sporulation and autolysis in this filamentous fungus, but that FadA/FlbA signaling was not involved in this vitamin E-dependent regulation. Vitamin E can be recommended as a supplement in fermentations in which the disintegration of pellets and gross autolysis should be avoided.

Index Entries: *Aspergillus nidulans*; vitamin E; menadione; sporulation; autolysis; *fadA*; *flbA*.

*Author to whom all correspondence and reprint requests should be addressed.

Introduction

Fungal autolysis is a physiologic process that usually should be avoided or, at least, controlled tightly in bioprocessing industries to increase secondary metabolite yields or to prevent degradation of heterologous protein products *(1)*. Under certain circumstances, autolysis might be advantageous, such as when it promotes intracellular product recovery *(1)*. Articles published in this field are mainly either physiology or morphology oriented aiming at the same phenomenon using different experimental tools *(1,2)*. Currently, the area most lacking in the study of fungal cell death is at the molecular level *(1)*. More efforts and more holistic approaches are definitely needed to elucidate the underlying mechanism of this very important part of the filamentous fungus's life cycle. In addition to the benefits that can be taken advantage of by the bioprocessing industry, the deeper understanding of fungal autolysis may lead to the development of a new type of antifungal agents *(1–3)*.

The genetically tractable filamentous fungus *Aspergillus nidulans* serves as a model organism for the study of multicellular development in fungi. The asexual phase in the life cycle of this fungus involves the formation of the multicellular conidiophores that produce conidia *(4)*. The initiation of conidiation is a genetically determined event that occurs at a precisely scheduled time *(5)*. The primary activator of conidiation-specific genes is the product of the *brlA* gene *(6,7)*. The isolation of conidiation mutants has facilitated the identification of several genes required for *brlA* expression. Among these genes, *fadA* and *flbA* control the balance between cell growth and sporulation *(6,8,9)*. The RGS (regulator of G protein signaling) domain protein FlbA is required to suppress growth signaling via FadA, the α-subunit of a G protein. Concomitantly, this FlbA-dependent inhibition of FadA signaling is a prerequisite for the onset of conidiogenesis *(6,8,9)*.

In another widely used filamentous fungus model organism, *Neurospora crassa*, Hansberg and Aguirre *(10)* put forward a hypothesis on the importance of free radicals in the initiation of sporulation. Meanwhile, the redox regulation of the conidiogenesis of *A. nidulans* was questioned *(6)*. Although the abrupt formation of an air/water interface at the hyphal surface seems to be important to initiate conidiation of developmentally competent liquid-grown hyphae, conidiation is not blocked in submerged cultures *(6,11)*.

In a previous work, we demonstrated that the involvement of reactive oxygen species (ROS) or antioxidant enzymes in FadA/FlbA signaling was unlikely in *A. nidulans* shake-flask cultures *(11)*. However, we had recorded high levels of ROS in submerged carbon-starved autolytic- and post-autolytic-phase *Penicillium chrysogenum* and *A. nidulans* cultures *(11–13)* and, therefore, the redox regulation of sporulation and autolysis through a FadA/FlbA-independent signaling pathway could not be excluded *(11)*.

In this article, we present data on the effect of DL-α-tocopherol (vitamin E, a lipid peroxyl radical scavenger) *(14–16)* on the autolysis and sporulation of *A. nidulans* in both surface and liquid cultures. The results suggest the involvement of ROS in the regulation of fungal autolysis and conidiogenesis.

Materials and Methods

Chemicals

Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich (Budapest, Hungary).

Organism, Growth Conditions, Sample Preparation, and Analytical Procedures

A. nidulans FGSC26 (*biA1*, *veA1*), RJH046 (*argB2*, *biA1*, *pyroA4*, *veA1*, Δ*flba*::*argB*) and FGSC1035 (*yA2 fadA*^{G203R}) strains were purchased from the Fungal Genetic Stock Center (University of Kansas Medical Center, Kansas City, KS).

For submerged cultivation, the FGSC26 strain was grown in shake flasks (500 mL) containing 100 mL of minimal-nitrate medium (pH 6.5) supplemented with 0.5% yeast extract *(17)*, 25 μg/L of biotin, and 1 μg/L of pyridoxine. Culture media were inoculated with 5×10^7 spores and incubated at 37°C, 200 rpm for 168 h. Vitamin E (1 g/L), paraffin oil (1 mL/L), or menadione (MQ; 0.3 or 1 mmol/L) was added at 24 h of cultivation. In all cases, the lowest effective concentrations of vitamin E and MQ were selected for the physiologic studies. The minimal inhibitory concentration value for MQ was 1.5 mmol/L.

To study the effect of vitamin E on dry cell mass (DCM) and glucose consumption in growing cultures, mycelia from 18-h submerged cultures were separated by filtration on sintered glass, washed and transferred immediately into fresh culture media, and incubated at 37°C and 200 rpm for 24 h.

In surface cultivations, strains were inoculated onto 2% agar plates containing the same components as the liquid medium just described and also supplemented with 10 g/L of vitamin E or 10 mL/L of paraffin oil. Both vitamin E and paraffin oil formed small drops on the surface of the agar plates. Surface cultures were incubated at 37°C for 120 h.

The surface tensions of the liquid media supplemented with either vitamin E or paraffin oil were determined on a Krüss instrument (Hamburg, Germany) using the du Nouy technique with a 20 mm Pt/Ir ring at 37°C.

The intracellular peroxide and superoxide levels were determined by the formation of 2',7'-dichlorofluorescein (DCF) from 2',7'-dichlorofluorescin diacetate and ethidium (Et) from dihydroethidium, respectively, as described previously *(12)*.

Changes in the specific activities of superoxide dismutase (SOD) were followed in separate experiments. In these cases, mycelia were harvested

by filtration on sintered glass, washed with distilled water, and resuspended in ice-cold 0.1 *M* K-phosphate buffer (pH 7.5). Cell-free extracts were prepared by disrupting frozen cells with a Type X25 X-press (AB Biox, Göteborg, Sweden; *[18–20]*). Specific SOD activities were measured according to Oberley and Spitz *(21)*.

Extracellular chitinase activities and glucose consumption were measured from the filtrates of the culture using Carboxy Methyl-chitin-Remayol Brilliant Violet (Loewe Biochimica GmbH, Sauerlach, Germany) as substrate and by the rate assay of Leary et al. *(22)*, respectively.

DCM was determined as described in previous publications *(23,24)*. Protein content of the cell-free extract was measured using a modification of the Lowry method *(25)*.

Microscopy

Cell morphology was examined under an Olympus BH-2 microscope equipped with an SPlan 20NH phase contrast objective *(26)*.

Statistical Analyses

All experimental data presented herein are expressed as the mean ± SD. The statistical significance of changes in physiologic parameters was estimated using the student's *t*-test. Only probability levels of $p \leq 5\%$ were regarded as indicative of statistical significance.

Results and Discussion

Accumulation of ROS is a typical event in carbon-limited and/or aging cultures of different fungi. Elevated ROS levels were observed in aging *N. crassa (27)*, *Saccharomyces cerevisiae (28)*, *P. chrysogenum (12)*, and *A. nidulans (11,13)* cultures. Accumulation of ROS triggers the induction of different antioxidant enzymes *(11–13,28)* and is tightly connected to complex physiologic processes including autolysis, aging, and programmed cell death *(12,29,30)*. In *N. crassa*, temporary hyperoxidant states preceded all morphologic steps observable during conidiogenesis in mycelia exposed to air *(31,32)*.

Vitamin E is a widely used antioxidant in basic research and human therapy *(14–16,33)*. Most of its effects are owing to its free-radical scavenger properties, but it may also show some side effects *(34)*. DL-α-Tocopherol has been demonstrated to suppress both intracellular peroxide levels and cyanide-resistant respiration in the cephalosporin C producer *Acremonium chrysogenum (35)* and to protect *Aspergillus niger* cells against ROS *(36)*.

In submerged *A. nidulans* cultures, vitamin E (1 g/L) did not alter the vegetative growth and glucose consumption of exponential growth phase mycelia (data not shown) but decreased the accumulation of ROS and, as a consequence, the induction of SOD when it was added after glucose depletion (Fig. 1). The autolysis markers monitored in this study included loss of biomass, disintegration of pellets, and induction of extracellular

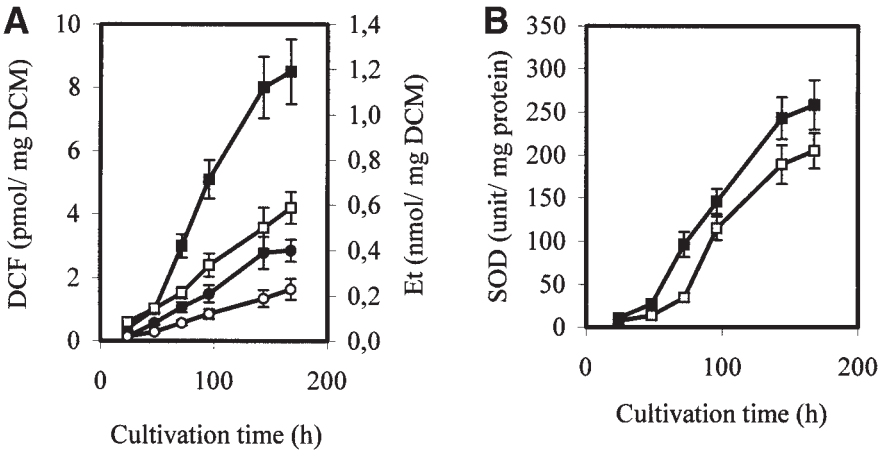

Fig. 1. Physiologic effects of vitamin E on autolysing *A. nidulans* cultures. **(A)** Changes in DCF (■, □) and Et (●, ○) production in control cultures (closed symbols) and in presence of vitamin E (open symbols). The specific DCF and Et productions are indicative of the intracellular peroxide and superoxide levels. Symbols represent the mean ± SD calculated from four independent experiments. **(B)** Specific SOD activities in presence of vitamin E (□) and in control (■) cultures. Symbols represent the mean ± SD calculated from four independent experiments.

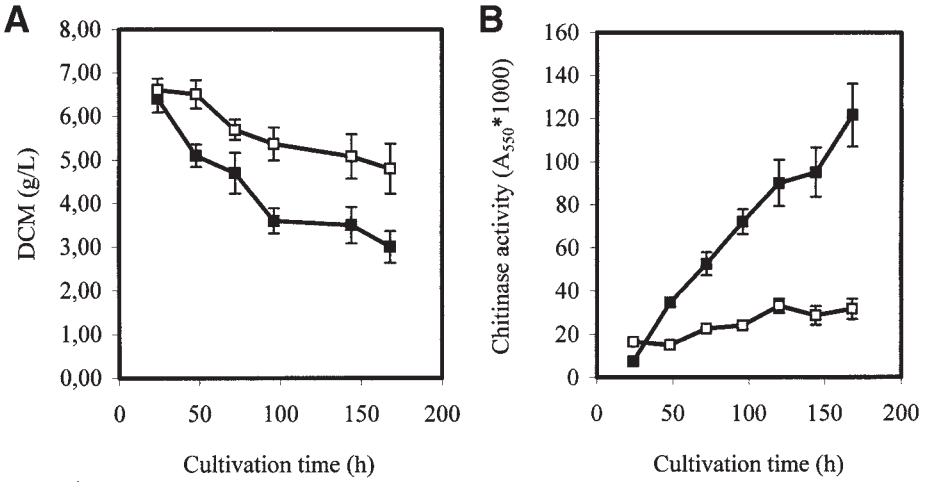

Fig. 2. Changes in DCM **(A)** and extracellular chitinase activities **(B)** of *A. nidulans* cultures in presence of vitamin E (□) and in control cultures (■). Symbols represent the mean ± SD calculated from four independent experiments.

chitinases *(11,13)*. The addition of vitamin E negatively affected all these processes and, hence, hindered the progress of autolysis itself (Figs. 2 and 3). It is remarkable that cultures treated with vitamin E preserved their pelleted morphology even 1 wk after vitamin supplementation.

Vitamin E is an oily compound and could change the aeration of the cultures and may provoke physiologic and morphologic changes solely

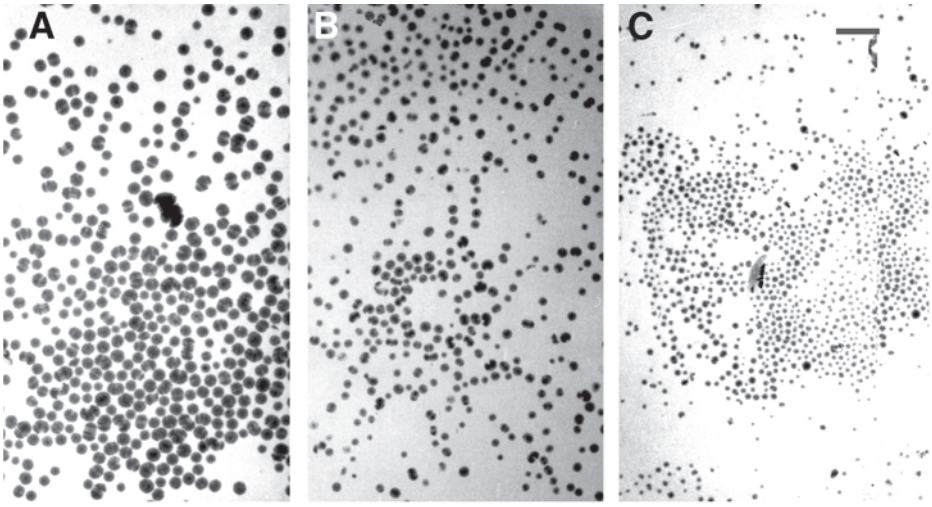

Fig. 3. Pellet sizes in 96-h *A. nidulans* cultures in presence of vitamin E **(A)**, in control cultures **(B)**, and with paraffin oil **(C)** (bar = 10 mm). Average pellet diameters and SDs were 3.1 ± 0.4 (n = 30), 1.9 ± 0.3 (n = 30), and 1.2 ± 0.2 (n = 30), respectively.

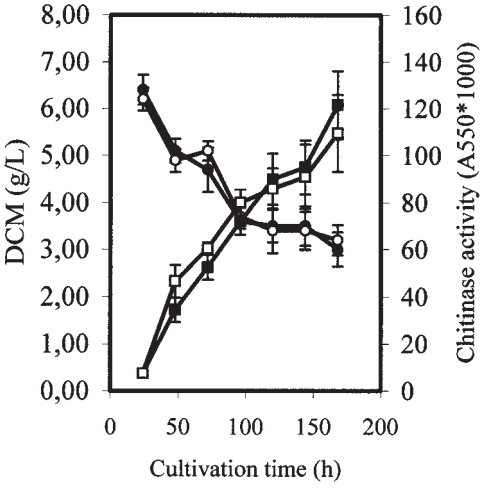

Fig. 4. Physiologic effects of paraffin oil on autolysing *A. nidulans* cultures. **(A)** Changes in DCM (●, ○) and extracellular chitinase activities (■, □) in control cultures (closed symbols) and in presence of paraffin oil (open symbols). Symbols represent the mean ± SD calculated from three independent experiments.

owing to this fact and independently of its antioxidant properties. Therefore, we also tested the effect of paraffin oil (1 mL/L) on the autolysis of *A. nidulans*, which is known to be inert against the majority of ROS observable in biologic systems. As shown in Fig. 4, paraffin oil did not cause any significant changes in either the DCM or the chitinase activities in comparison to the controls (Fig. 4) and even decreased the average pellet diameter

(Fig. 3). Paraffin oil had no effect on the intracellular accumulation of ROS and the specific SOD activities either (data not shown). More important, both paraffin oil and vitamin E decreased the surface tension of the medium (from 54.9 ± 0.002 to 50.1 ± 0.002 and 47.8 ± 0.002 mN/cm, respectively) and, hence, their profoundly different effects on the pellet morphology and autolysis most likely were attributed solely to the ROS quenching properties of vitamin E. These results suggest that age-dependent accumulation of ROS and/or free-radical-initiated oxidative modifications of cellular biopolymers could be important autolysis signals in submerged *A. nidulans* cultures.

In agreement with this hypothesis, MQ, which is a superoxide-generating agent *(20)*, accelerated the autolysis when it was supplemented in a concentration of 0.3 mmol/L. In addition, MQ treatments resulted in significant decreases in DCMs and pellet diameters as well as increased extracellular chitinase activities (Table 1, Fig. 5). On the other hand, when MQ was supplemented in a much higher concentration, 1.0 mmol/L, autolysis was inhibited (Table 1, Fig. 5). The oxidative damage caused by MQ blocked the disintegration of hyphae, the autolytic loss of biomass, and the release of chitinase. These findings are supportive of the view that fungal autolysis is not a self-evident consequence of accumulating oxidative cell damage; instead, it seems to be a well-regulated, energy-requiring process *(2,12,37)*. It is worth mentioning that fungal apoptosis, another energy-dependent, well-regulated process of cell death, is clearly inducible by ROS *(29,30,38)*.

In contrast to paraffin oil, vitamin E inhibited both the sporulation and autolysis processes and resulted in the appearance of white, thick, "sprawling" colonies in surface *A. nidulans* cultures (Fig. 6). This is indirect evidence for the hypothesis that ROS are also essential in the initialization of sporulation, an important morphologic event in the asexual reproductive cycle of fungi *(31,32)*.

Among the genes involved in the regulation of sporulation in *A. nidulans*, *fadA*, and *flbA* regulate the balance between growth (autolysis) and sporulation *(6)*. The phenotype of the dominant interfering mutant of *fadA* (*fadA*^{G203R}) is characterized by reduced growth and hypersporulation *(6)*. Vitamin E treatment inhibited both the autolysis and sporulation of the *fadA*^{G203R} mutant and resulted in thick, nonsporulating colonies very similar to those observed in the wild-type strain (Fig. 7). The loss-of-function mutant of *flbA* possesses an autolytic phenotype characterized by uncontrolled growth and extensive autolysis in mature colonies *(6)*. Similar to the wild-type strain, vitamin E blocked the autolysis in this mutant too and gave rise to thick, leathery, crowded, longevous colonies (Fig. 8). The majority of the biomass in these colonies consisted of rounded cells, which had broken away from the hyphae (Fig. 8). After transferring these round cells onto vitamin E-free nutrient agar plates, they reverted immediately to normal hyphal growth (data not shown).

The inhibitory effect of vitamin E on both the autolysis and sporulation of *A. nidulans* suggests that ROS can be important elements of the

Table 1
Effect of MQ on ROS Formation and Autolysis of *A. nidulans*[a]

	DCF (pmol/mg of DW)	Et (nmol/mg of DW)	DCM (mg/mL)	Chitinase activity ($A_{550} \times 1000$)	Growth (mg/mg)[b]
Control	2.2 ± 0.2	0.16 ± 0.02	3.8 ± 0.2	1 ± 0.05	2.1 ± 0.2
0.3 mmol/L of MQ	4.5 ± 0.5[c]	0.3 ± 0.03[c]	2.1 ± 0.2[c]	1.2 ± 0.06[e]	2.0 ± 0.2
1 mmol/L of MQ	16 ± 2.3[d]	0.44 ± 0.05[d]	4.0 ± 0.3	0.5 ± 0.03[c]	0.5 ± 0.1[d]

[a]Figures represent the mean ± SD calculated from five independent experiments. *p* Values were calculated using the student's *t*-test.
[b]Mycelia were collected and transferred into fresh, MQ-free medium. The specific increase in the DCM/mg of DCM mycelia during 24 h of cultivation was calculated.
[c]$p < 1\%$.
[d]$p < 0.1\%$.
[e]$p < 5\%$.

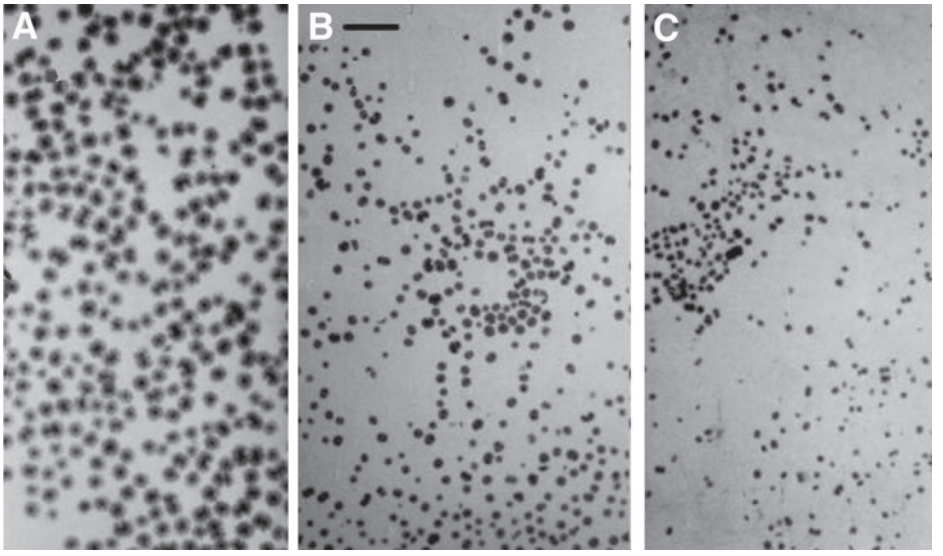

Fig. 5. Pellet sizes in 96-h-old cultures in presence of 1 mmol/L of MQ **(A)**, in control cultures **(B)**, and with 0.3 mmol/L of MQ **(C)** (bar = 10 mm). Average pellet diameters and SDs were 2.6 ± 0.3 mm (n = 30), 1.6 ± 0.3 mm (n = 30), and 1 ± 0.2 mm (n = 30), respectively.

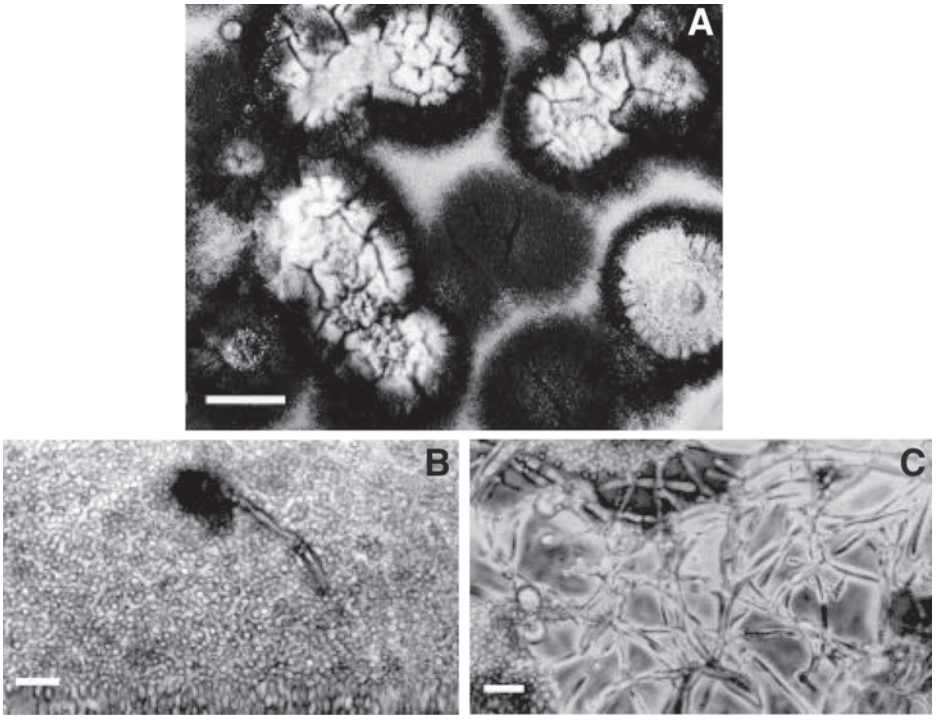

Fig. 6. Effect of vitamin E on sporulation of *A. nidulans* FGSC26 in surface culture. **(A)** Formation of white, nonsporulating, thick, "sprawling" colonies around and on vitamin E droplets. The dark colonies showed intensive sporulation and autolysis (bar = 10 mm). **(B)** Microscopic picture of dark colonies containing mainly conidia and conidiophores (bar = 10 μm). **(C)** Microscopic picture of white colonies containing mainly mycelia (bar = 10 μm).

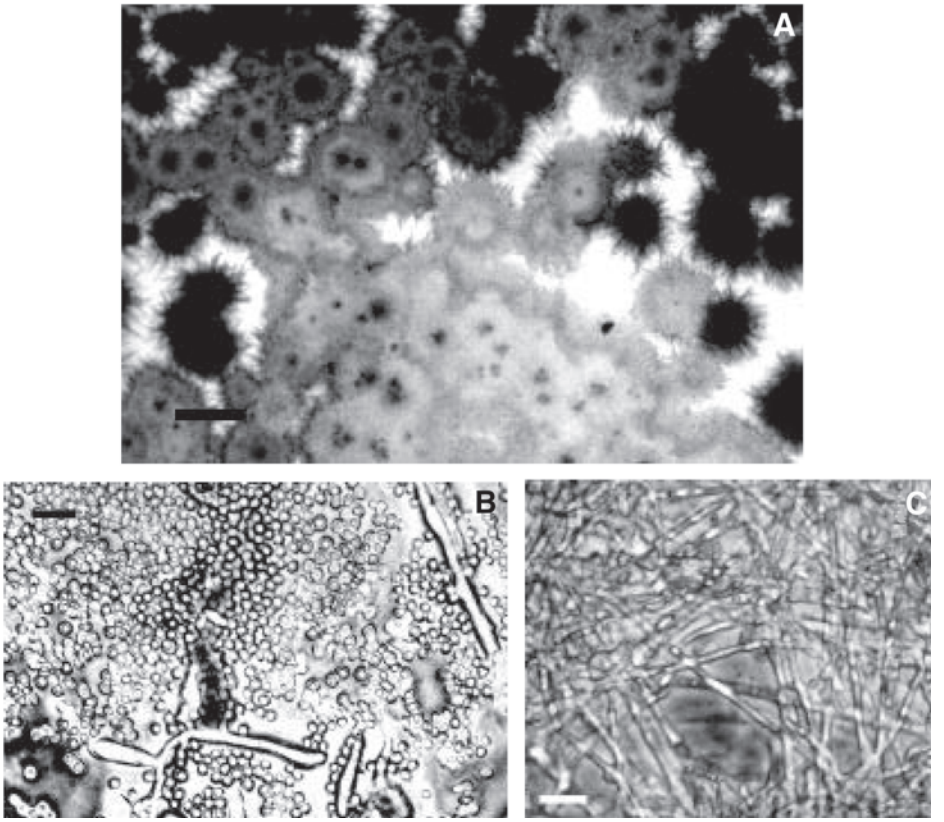

Fig. 7. Effect of vitamin E on sporulation of *A. nidulans* FGSC1035 (*fadA*^{G203R}) in surface culture. **(A)** Formation of white, nonsporulating colonies around and on vitamin E droplets. The dark colonies showed intensive sporulation and autolysis (bar = 5 mm). **(B)** Microscopic picture of dark colonies containing mainly conidia (bar = 5 µm). **(C)** Microscopic picture of white colonies containing mainly mycelia (bar = 5 µm).

signal transduction pathway activating the genomic expression programs governing both the sporulation and autolysis. These experiments further strengthened the view *(11)* that FadA/FlbA signaling most likely does not involve ROS-dependent transduction elements. More recent experimental data published by Shimizu and Keller *(39)* indicated that FadA/FlbA signaling, at least partially, is mediated through cyclic adenosine monophosphate (cAMP)-dependent protein kinase. Global gene expression profiling experiments in *A. nidulans* indicated that the transcription of the cAMP-dependent protein kinase catalytic subunit was profoundly downregulated by ROS under MQ stress *(40)*. This means that a ROS-dependent regulation of this important protein kinase may operate and govern the morphologic and physiologic changes observable in the presence of MQ and vitamin E.

Although the molecular background of vitamin E-dependent signaling has yet to be elucidated, the hindrance of the disintegration of pellets

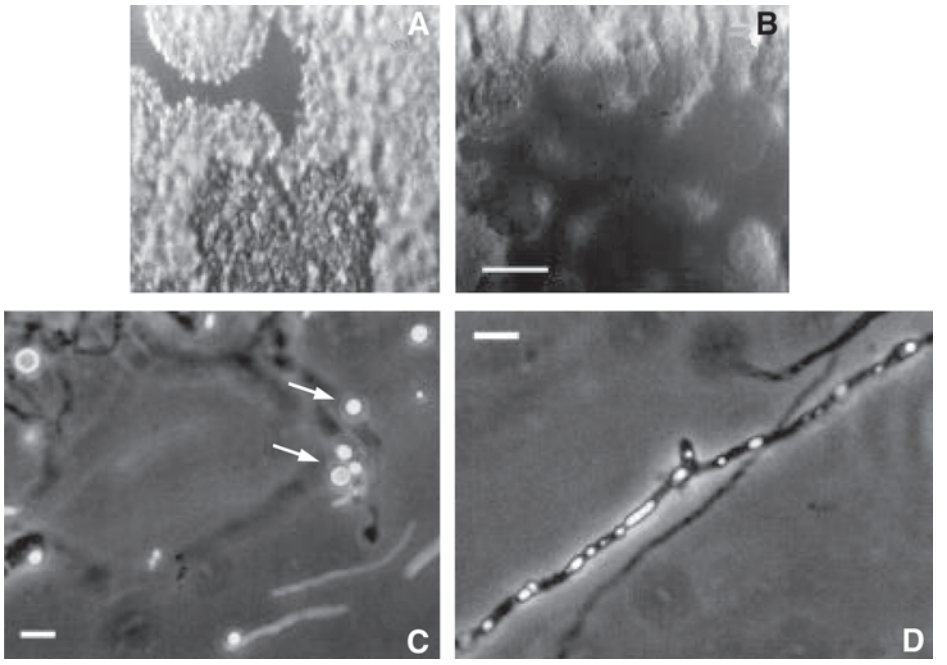

Fig. 8. Effect of vitamin E on autolysis of *A. nidulans* RJH046 (Δ*flbA*) in surface culture. **(A)** Formation of thick, leathery, crowded, longevous colonies in presence of vitamin E. **(B)** In the absence of vitamin E, intensive autolysis was observed (bar = 10 mm). **(C)** Rounded cells (indicated by arrows) from vitamin E-treated colonies (bar = 5 μm). **(D)** Formation of rounded cells from vitamin E-treated hyphae (bar = 5 μm).

and gross autolysis by the addition of vitamin E may be exploitable in the bioprocess industry. In addition to the specific inhibition of age-related chitinases by allosamidin *(3,41)*, researchers now have a second tool to tailor the cell morphology in autolysing/aging cultures of filamentous fungi.

Acknowledgments

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