Germination of conidia (Neurospora, Trichoderma) : evidence of an electrogenic sink of protons mitochondrially-extruded into emerging tubes

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Germination of conidia *(Neurospora, Trichoderma)*: evidence of an electrogenic sink of protons mitochondrially-extruded into emerging tubes

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It is now widely accepted that the universal process of oxidative phosphorylation implies, as a first step, proton extrusion from mitochondria into the cytosol (Mitchell, 1977). This then becomes relatively acidic compared to the mitochondria, at least by 0.5 pH unit according to recent NMR measurements (Shulman et al., 1979).

The exclusion zone in the apices of actively elongating hyphae is deprived of mitochondria along an average length of 5 μ. Such an essentially cytosolic plus vesicular zone (Bartnicki-Garcia, 1973; Gooday, 1978) has recently been found to be not only highly reductive, especially at the level of the "Spitzenkörper" (Turian, 1978), but more acidic (pH $\sim$ 5.5) and to contain less Ca^{+2} along acropetal gradients (Turian, 1979a). The subapically located mitochondria were observed to be not only pinkish-violet with alizarin and arsenazo III (Ca^{+2} sequestration) but also pinkish (pH $\sim$ 6.5) with bromcresol purple sublethally used.

We have extended our initial observations using a faster penetrating pH indicator, bromcresol green (Yamaha, 1935), providing a more contrasted staining between cytosol and mitochondria of germinating conidia of wild-type *Neurospora crassa* and *Trichoderma harzianum* (Fig. 1AB). The yellowish-green staining observed in the apical cytosol should correspond to a pH value closer to 5.0 than to 5.5, while the bluish to frankly blue staining of the subapical mitochondria should correspond to a pH value of at least 6.0.

The relatively high acidity in the emerging and polarly elongating apices was recently confirmed by the use of a new fluorescent pH indicator, 4-methylesculetin recently applied to intracellular pH measurements in the plasmodium of *Physarum* (Gerson and Burton, 1977). The fluorescence of this compound under ultraviolet light increases from pH 6.0 and reaches its high values in the range of pH 7.0-8.0; it fades below pH 6.0, becoming null around pH 5.0. Sprouting conidia bathed in a saturated water solution of 4-methylesculetin and observed with a Leitz microscope equipped for UV light showed a strong, general fluorescence in the conidial body contrasting with an apical darkening of the emergent tubes (Fig. 1C).

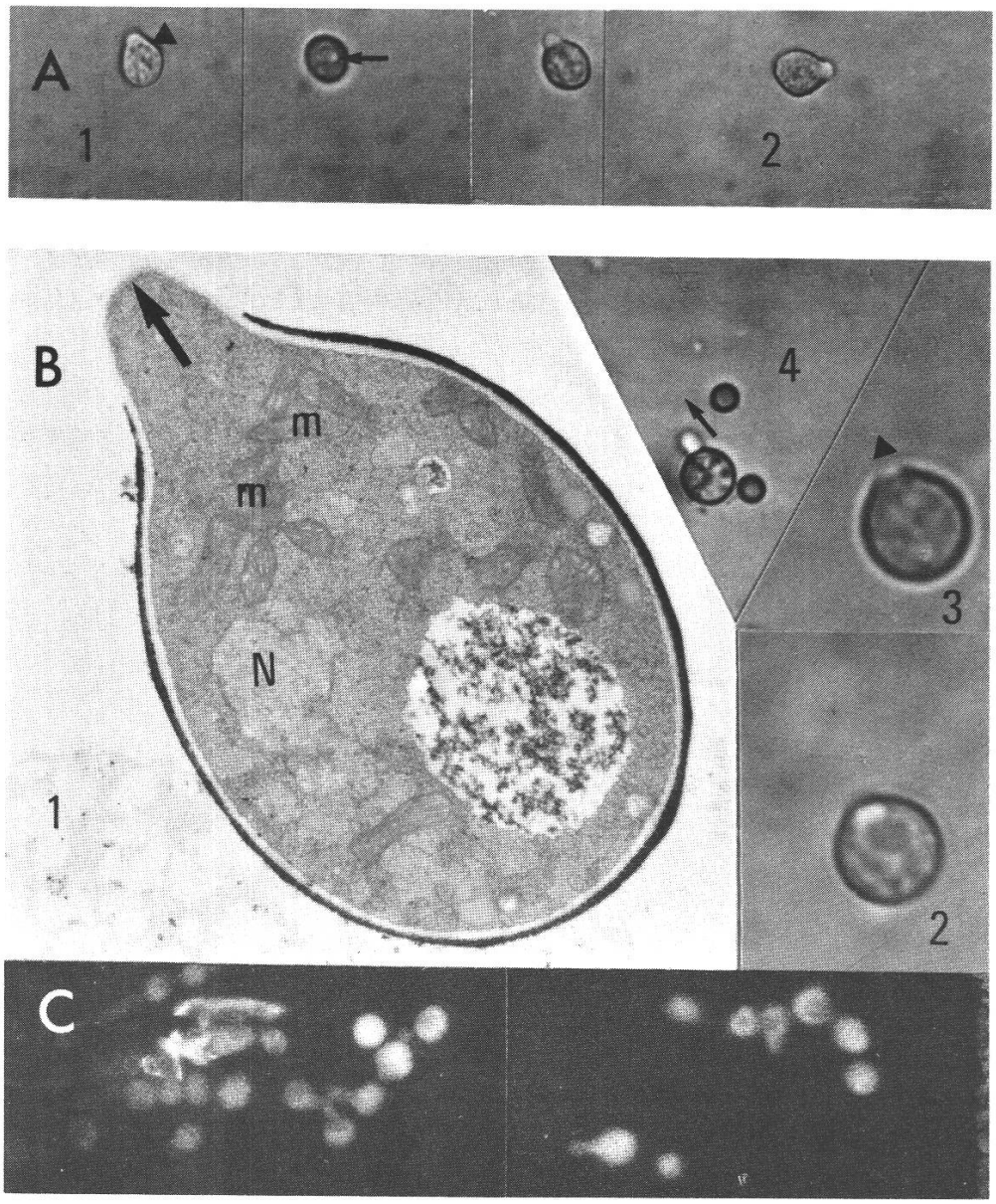

Fig. 1:

Stages of germ tube emergence in:
A) conidia of *Neurospora crassa* (x 500) incubated 3 h in Vogel's liquid medium before staining *in vivo* with the pH reagents (1) bromcresol green (10^{-4} in distilled H_2O) and (2) bromcresol purple (10^{-4} in distilled H_2O). 1. Short germ tubes homogeneously yellowish-green (pH $\sim$ 5.0) and mitochondrial granules (arrow) bluish-green to blue (pH $>$ 6.0); yellowish-green presumptive zone of germ tube emergence (arrow). 2. Short germ tube homogeneously orange-yellow (pH $\sim$ 5.5) and mitochondrial granules dark purple to violet (pH 6.5-7.0).
B) Conidia of *Trichoderma harzianum* incubated 9 h in 2% malt (1) or 12 h in Vogel's liquid medium (2,3,4). 1. Electron microscopy after fixation in 2,5% glutaraldehyde-2% 0_s0_4, uranylacetate staining, epoxy resin embedding and thin sectioning: emerging germ tube showing back positioning, in the sporal body, of "frontal mitochondria" (m) and nucleus (N) (x 11000). Optical microscopy after bromcresol green staining: 2. clear yellowish-green presumptive zone of germ tube emergence (x 2000); 3. yellowish-green emerging tube (x 2000); 4. longer germ tube bright greenish-yellow contrasting with the blue staining of mitochondrial granules in the sporal body (x 1000).
C) Fluorescence of conidia of *N. crassa* germinated for 3 h (20° C) in Vogel's liquid medium and then transferred for 15 min into a saturated water solution of 4-methylesculetin. Note fading fluorescence in the emerging germ tubes (x 500).

Of interest from the point of view of the origin of apical polarity was a cytoplasmic "spot" staining yellowish with bromcresol green, detected in a few swollen conidia at their pre-emergence stage (Fig. $1A_1B_2$). This acidic "spot" might be generated by an aleatory event (spatial dissipation principle of Prigogine ?) of anisotropic proton extrusion from mitochondria then kept in a back position (Fig. $1B_1$) by apical cytosolic gelation at low pH (Turian, 1979b).

In a first experimental attempt to understand the physiological basis of the decreasing pH gradient in the tips of the emergent germ tubes, we reasoned that the gradient might be fixed by a random event from *inside* the initially swollen germinating conidium rather than by some type of external, local influence impossible to explain when conidia are put to germinate in a homogeneous liquid medium. The staining pH tests showed that the mitochondria were less acidic and relatively alkaline compared to the cytosolic exclusion zone (Turian, 1979a) and led us to ascribe to them the leading role in the generation of an apical pH gradient implying anisotropic extrusion of protons (Turian, 1979b). The proton extrusion requires the functioning either of the respiratory electron chain or the hydrolytic activity of ATPase. As the emergence of the germ tube starts after $2^1/_2$ hours of incubation at 25^0 C in *Neurospora* (Schmit and Brody, 1976), a period insufficient for the conidium to develop its respiratory system (Brambl et al., 1978), we initiated experiments with known inhibitors of the respiratory chain to compare them with several effectors of ATPase.

As expected, we found that respiratory inhibitors, even when used at their high range of concentration (close to saturation point), could not prevent a large proportion of the conidia to sprout their germ tube (Table Ib).

The results with ATPase effectors were more interesting (Table Ic): those compounds known to prevent H^+-translocation across the internal membrane of the mitochondrion, namely N,N'-dicyclohexylcarbodiimide (DCCD), a compound known to bind irreversibly to the proton channel, and oligomycin to the stalk of the ATPase (Lehninger, 1975; Lloyd and Turner, 1980) were very effective in preventing emergence (Fig. 2C); oppositely, those known to uncouple oxidative phosphorylation by stimulating the activity of the normally latent hydrolytic ATPase, such as 2,4-dinitrophenol (DNP) and the toxic carbonylcyanide m-chlorophenylhydrazone (CCCP), significantly accelerated emergence of germ tubes; in the presence of DNP at 5 μM (Fig. 2B), a few sprouting conidia were already visible after $2^1/_2$ hours when almost all control conidia were still subspherical. Such DNP-stimulated rate of emergence of germ tubes was nullified in the presence of oligomycin (Table Ic). As oligomycin is known to inhibit DNP-stimulated ATPase activity (Lehninger, 1965), such finding strongly argues for a crucial function of mitochondrial H^+-ATPase at the "acidic initiation" of germ tube emergence. This is also supported by the fact that KCN, the classical oxidative inhibitor, additionally known to inhibit indirectly the ATPase activity of mitochondria (need for oxidized carriers, Lehninger, 1965), showed a stronger anti-germinative effect than other respiratory inhibitors (Table Ib).

Consideration of the above fact that respiratory inhibitors do not effectively prevent the emergence of the germ tubes in wild-type *N. crassa* led us to the prediction that conidia of respiratory mutants (cytochrome pathway) should sprout as fast as those of wild type. It was in fact found that under our experimental conditions (Table I), conidia of a "poky" (mi-1) mutant showed a nearly normal rate of germ tube emergence, namely 20% after 3 h at 20° C. However, while wild-type tubes elongate quickly after

Table I:

Germination tests of macroconidia harvested from *Neurospora crassa* (strain Lindegren A) grown for 10–12 days on solid Vogel's medium.

*Inhibitors**	*Germ tube emergence (average %)* after		
	$2^1/_2$ h	3 h	4 h
a) –	0–4	25	58
b) *Respiratory electron chain:*			
cyanide 5.0 mM	0	8	16
azide 7.5 mM	0	12	22
antimycin 1.0 mM	0	20	44
amytal 1.0 mM	0	17	35
rotenone 1.0 mM	0	15	34
c) *Oxidative phosphorylation (ATPase effectors):*			
DCCP 0,25 μM	0	0	2
oligomycin 1.0 μM	0	8	10
CCCP 1 μM	2-5	29	60
2,4-DNP 5 μM	*8-12*	*36*	*64*
2,4-DNP 5 μM +oligomycin 1,0 μM	0	5	12

* Dissolved in drops of conidial suspension in Vogel's minimal medium (1956) on slides incubated at 20° C in a multiplace wet chamber; microscopic observations – photographs – counting recordings from the drop center zone in a minimum of 3 separate experiments; KCN and NaN_3 dissolved in distilled H_2O, all other inhibitors in a minimum amount of ethanol checked to be innocuous to the controls.

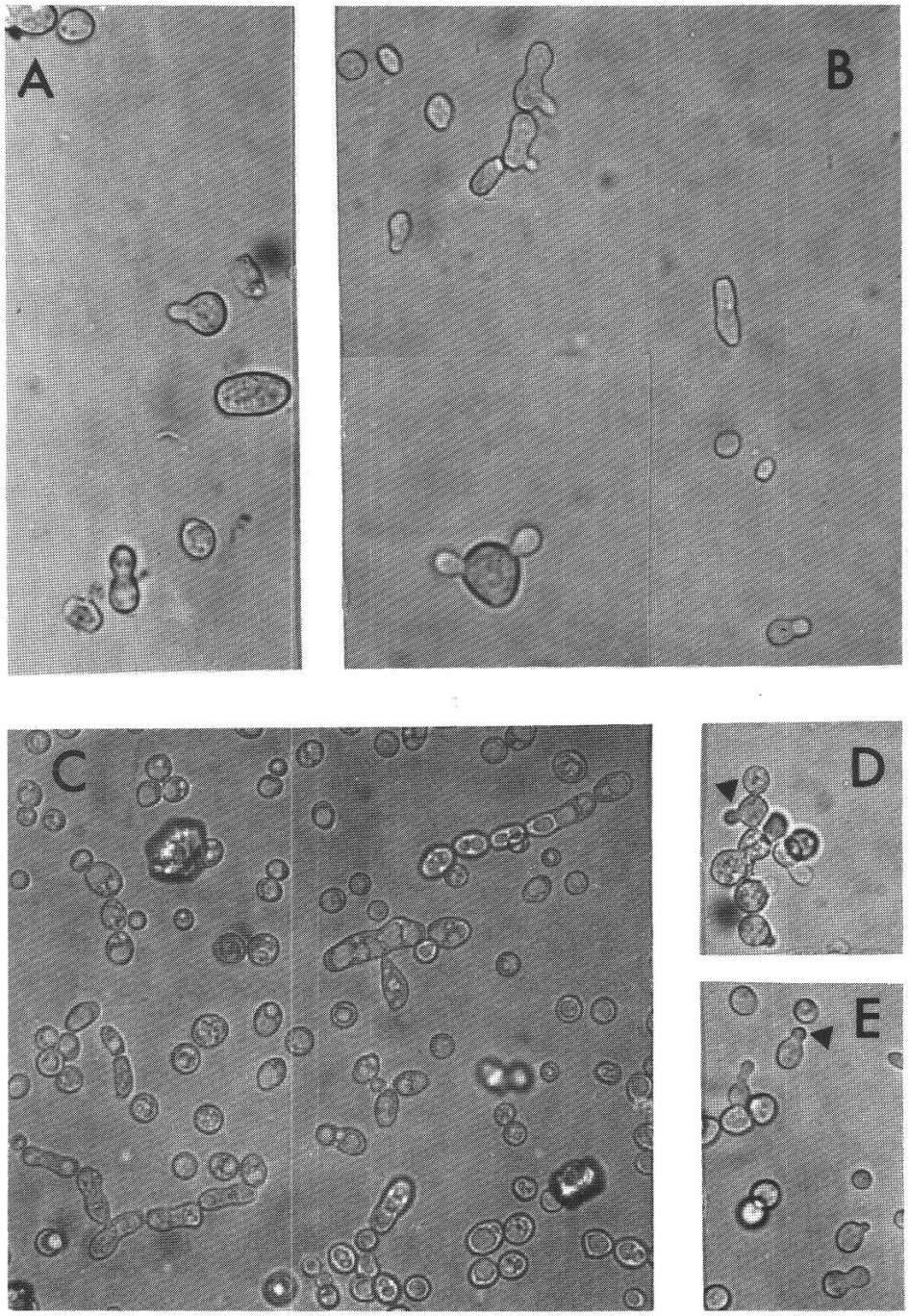

Fig. 2:
Germination of conidia of *N. crassa* in Vogel's minimal liquid medium (20° C):
A. controls, 3 h (x 500);
B. with 2,4-DNP 0.5 μM, 3 h (x 350); double budded conidium (x 500);
C. mixed with a few DCCD crystals, only ungerminated and vacuolated conidia (x 350);
D. "poky" mutant, 4 h (x 350);
E. wild type with antimycin 1.0 mM, 4 h (x 350).

their sprouting to 2-3 times the width of the conidial body after the third hour when 0_2-consumption starts to rise (Greenawalt et al., 1972), those of "poky" lag after their emergence (Fig. 2D). Interestingly, "poky" slowly-elongating germ tubes were phenocopied by antimycin-treated wild-type conidia: after a relatively rapid sprouting (Fig. 2E), the wide germ tubes elongate slowly before stopping at a concentration of antimycin (1.0 mM) known to inhibit cyano-sensitive respiration while inducing the alternative oxidative pathway (Lambowitz et al., 1972) which is normally predominant in "poky".

It can therefore tentatively be concluded that both wild-type and "poky" conidia first use their endogenous mitochondrial ATP as substrate for their H^+-ATPase initiating the polarized process of germ tube emergence. Paradoxically, "poky" contains even more ATPase than wild type (Mainzer and Slayman, 1978). Following depletion of the ATP available in the sprouting conidia, reactivated oxidative phosphorylation must replenish the ATP internal pool, a process inefficient in "poky" which depends mainly on the alternative pathway (Schmit and Brody, 1976). Therefore "poky" lacks the necessary power to pursue rapidly the elongation of its germ tube.

Another mutant of *N. crassa*, "crisp" (cr-1), has shown a marked delay in its emergence stage, with only around 10% germ sprouting after 7 h. "Crisp" is known to be deficient in adenylcyclase (Terenzi et al., 1976). This may be relevant to its slow sprouting and we have succeeded in activating emergence of tubes from the 4th hour with c-AMP (0.01 mM) and also 2,4 DNP (5 μM), both agents known to depolarize the plasma membrane (Pall, 1977). The positive action of 2,4–DNP is in agreement with our model according to which an anisotropic vectorial extrusion of protons from the "frontal mitochondria" into an apical sink should previsibly lead to a local, apical depolarization of the plasmalemma (inside less negative).

The generation of the proton sink should be followed, in an acropetal sequence, by the extrusion of protons through the apical plasmalemma ATPase driven K^+/H^+exchanges (Harold, 1977; Jennings, 1979). This creates the required conditions for secondary symport with $PO_4H_2^-$ and H^+ back into the apical cytosol (Seaston et al., 1976). Such symport provides a clue to the required self-entrained reinforcement of the acidic sink in the tips of germ tubes when they elongate further (Turian, 1979b). The importance of the apico-basal transport of phosphate (cytochemical apical localization, Turian, 1979a) in the entrainment of the polarized growth was further suggested by the following observations: a) slowing down of the elongation of widening germ tubes in Vogel's medium deprived of phosphate (K^+provided with KC1); b) directed orientation (average 70%) of tube emergence toward a gradient of phosphate provided by crystals of $Ca_3(PO_4)_2$ slightly dissolving on one side of a drop, squashed below a coverslip, of Vogel's medium deprived of phosphate (enriched in compensatory KC1 and $CaC1_2$ effectors).

Deficiency in Mg^{+2} ions in the Vogel's minimal medium (minus $MgSO_4$) was shown to delay and reduce the efficiency of germ tube emergence (Farach et al., 1979), a fact that we have confirmed ($\sim$ 20% of germ tubes after 4 h with Na_2SO_4 replacing equimolarly $MgSO_4$). Such results can be interpreted as support of the fundamental role of mitochondrial Mg^{+2}-ATPase in the vectorial, initial extrusion of protons toward a sink becoming the site of germ tube emergence. Considering Mg^{+2}-ATPase proton extrusion as the germination starter, we suggest that the aqueous imbibition required

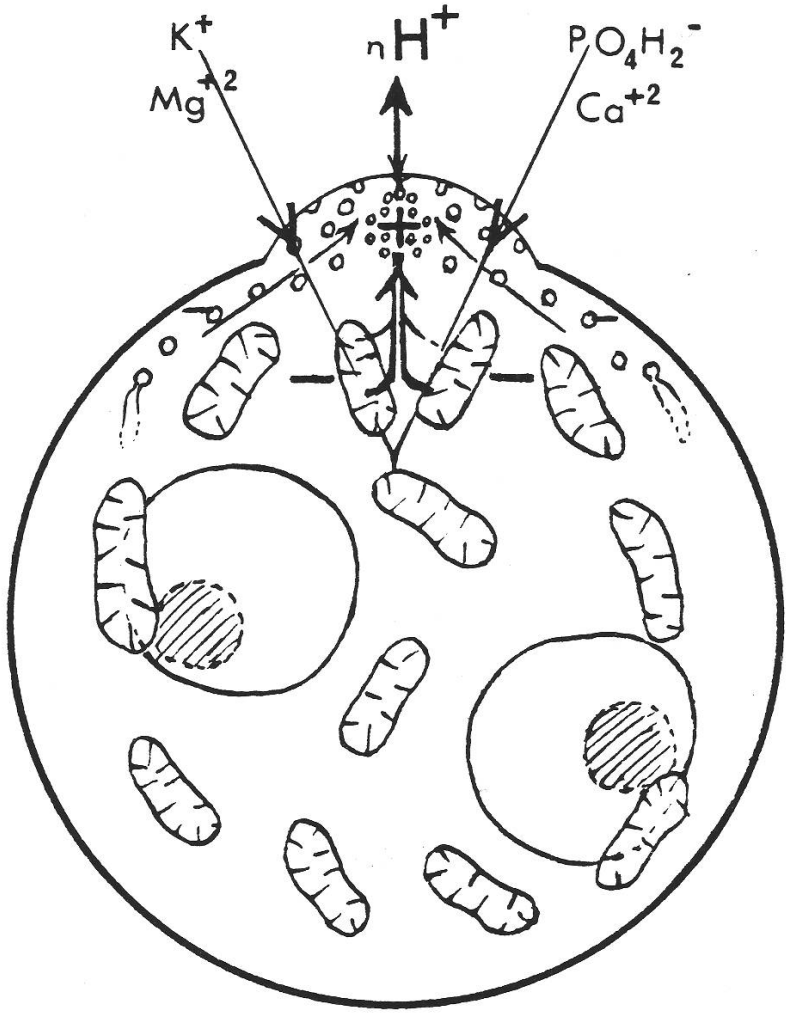

Fig. 3:

Schematic view summarizing data and hypotheses on the dynamics of conidium germination at its early emergence stage. Proton sink in the sprouting germ tube (+ in the "Spitzenkörper") ahead of the frontal mitochondria and attracting the presumably negatively-charged vesicles toward the plasmalemma and wall-forming tip.

Main arrow for the vectorial extrusion of H^+ from the frontal mitochondria into apical cytosol and H^+ efflux in antiport with K^+ and Mg^{+2} (required by mit.-ATPase). Return arrow for symport influx of $PO_4H_2^-$ and Ca^{+2}.

In a backward position, two nuclei with dashed nucleoli, as in a macroconidium of *Neurospora*.

for the awakening of conidia (Sussman and Halvorson, 1966) would in some way destabilize the mitochondrial ATPase by removing the F_1-ATPase inhibitor peptide found by Pullman and Monroy (1963).

All the above results and considerations led us to envisage that the vectorial extrusion of $^{+}$charged protons, and therefore the site of the germ tube emergence, could be enforced by submitting swelling conidia to an electrical field. Following such treatment of conidia put to germination in the median slit of agarose-covered slides maintained for 4 h at 110 V and 5 mA in a horizontal electrophoretic apparatus, we observed an average of two thirds of the conidial germ tubes emitted toward the negative pole; moreover their further elongation rate was almost twice as high toward that pole, an observation strikingly similar to those made on elongating nerve filaments (Jaffe and Nuccitelli, 1977).

As for the electropotential gradient linking the apical proton sink to the negatively charged, subapical mitochondria, it can be visualized (Fig. 3) as providing the driving force in the germ tube for the continuous acropetal transport of vesicles (containing $^{-}$ charged wall precursors and enzymes, Gooday, 1978) by a process akin to self-electrophoresis suggested by Jaffe (1968) to be at work in the elongating germ tubes of *Fucus* zygotes.

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Résumé

Un gradient décroissant de pH dans les tubes germinatifs de conidies de *Neurospora* et *Trichoderma* a été confirmé tant par le vert de bromocrésol (pH d'$\sim$ 5.0 dans les apex) que par l'extinction de la fluorescence de la 4-méthyl-aesculetine.

Une origine endogène de ce réservoir apical de protons est suggérée par la stimulation de l'émergence des tubes germinatifs par des agents chimiques tels que le 2,4-dinitrophénol et le CCCP, connus pour leur effet stimulant des activités hydrolytiques et excrétrices de protons de l'ATPase-Mg^{+2} mitochondrienne. Les mutants "poky" et "crisp" ont aidé à dissocier cet évènement vectoriel et électrogène, dirigeant les vésicules vers le point d'émergence du tube germinatif, de la suite de son élongation soutenue par la respiration.

Summary

A decreasing pH gradient in the germ tubes of conidia of *Neurospora* and *Trichoderma* has been confirmed both by bromcresol green (pH $\sim$ 5.0 in the tips) and by the quenching of the fluorescent 4-methylesculetin.

An endogenous origin of this apical proton sink is suggested by the stimulation of germ tube emergence by chemical agents such as 2,4-dinitrophenol and CCCP known to stimulate the hydrolytic and proton-extruding activities of mitochondrial Mg^{+2}-ATPase.

Mutants "poky" and "crisp" have been of help to dissociate the vectorial electrogenic event directing the vesicles toward the point of emergence of the germ tube from its respiratorily-sustained further elongation.

Zusammenfassung

Ein abnehmender pH-Gradient in den Keimschläuchen der Konidien von *Neurospora* und *Trichoderma* wurde durch Bromkresolgrün (pH ungefähr 5,0 in den Apices) und durch das Auslöschen der Fluoreszenz von 4-Methyl-Aesculetin bestätigt. Auf eine endogene Herkunft dieses apikalen Protonenreservoirs deutet die durch Stoffe wie 2, 4-Dinitrophenol und CCCP (bekannt für ihre stimulierende Wirkung auf die hydrolytischen und Protonen-abgebenden Aktivitäten der mitochondrialen Mg^{++}-ATPase) stimulierte Keimschlauchbildung. Die Mutanten "poky" und "crisp" ermöglichten die Trennung dieses vektoriellen und elektrogenen Phänomens, das die Vesikel gegen den Austrittspunkt des Keimschlauchs dirigiert, von der durch die Atmung stimulierte nachfolgenden Verlängerung des Keimschlauchs.

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