TITLE

Electrophysiological properties of the temperature sensitive *cot–1* mutant of *Neurospora crassa*.[1]

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ABSTRACT

The *cot–1* mutant of *Neurospora crassa* exhibits normal growth at permissive temperatures (*ca* 25ºC) but grows as a colonial mutant at restrictive temperatures (greater than 32ºC). We confirmed the phenotype by microscopic observation of tip growth at the colony edge. When temperature was ramped from 25º to 32º in 4 minutes, a significant decline in growth rate was observed in the *cot–1* mutant but not in wildtype. Under the same conditions, we examined the effect of the temperature ramp on the membrane potential and resistance of hyphal trunks behind the colony edge of the *cot–1* mutant and wildtype strains. Membrane potentials and resistances were similar at the permissive temperature. Both wildtype and the *cot–1* mutant exhibited a similar hyperpolarization of the membrane potential (and resistance tended to decline slightly) when temperature was elevated to restrictive temperatures. The magnitude of the hyperpolarization was unaffected by temperature increases up to 40ºC. We conclude that electrogenic ion transport at the plasma membrane does not contribute to the phenotypic changes in the *cot–1* mutant, but cannot rule out a contribution from ion transport in the endomembranes.

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OBJECTIVE

To determine whether ion transport is involved in the morphological phenotype of the temperature sensitive *cot–1* mutant of *Neurospora crassa*.

INTRODUCTION

The temperature sensitive colonial mutant (*cot–1*) of *Neurospora crassa* is characterized by colonial growth at restrictive temperatures of 32º C or above, but normal growth at permissive temperatures of 25 º C or below (Mitchell et al., 1954). When cultured under restrictive temperatures, the mutant colonies grow slowly and their hyphae branch profusely accompanied by cessation of conidia-formation. When shifted back to permissive temperatures, the mutant grows normally through formation of wild-type-like spreading radial colonies (Yarden et al., 1992). The *cot–1* gene underlying this mutation has been isolated and characterized, and is predicted to code for a Ser/Thr-specific protein kinase based on amino acid sequences (Yarden et al., 1992). Other members of this kinase family are encoded by the *Drosophila warts/lats* gene and the human *DM* gene, which are important in normal cell differentiation. The *warts/lats* gene is a requirement for normal cell proliferation and morphogenesis in *Drosophila* (Justice et al., 1995), while mutations in *DM* result in myotonic dystrophy in humans (Mahadevan et al., 1993). The distribution of COT–1 has been examined. It was found in the cytoplasm but was mainly associated with the cytoplasmic membranes (Gorovits et al., 2000). Two proteins of sizes 73 kDa and 67 kDa were observed through the use of antibodies against COT–1. The membrane associated COT–1 protein and the 67 kDa isoform were not observed under restrictive temperatures in the *cot–1* mutant (Gorovits et al., 2000).

In order to further analyze the *cot–1* mutation, the purpose of this project was to determine the role of ion transport, if any, in the hyperbranching phenotype observed at elevated temperatures in the *cot–1* mutant. The mutant phenotype has been reported to be associated with ion effluxes through H^+-ATPase and Na^+ transporters (Gorovits et al., 2003). Hyperosmotic stress imposed on growing hyphae results in suppression of the *cot–1* phenotype (Gorovits et al., 2003). Since turgor recovery due to hyperosmotic stress in *N. crassa* has been shown to be associated with H^+, K^+, Cl^-, and Ca^{2+} fluxes (Lew et al., 2006), it is possible that ion transport has a role in the *cot–1* phenotype. To resolve this question, we used short-term elevation to the restrictive temperature, confirmed the *cot–1* phenotype, and examined the membrane potential and resistance of the *cot–1* mutant strain compared to wild-type.

EXPERIMENTAL AND RESULTS

First, morphological studies were carried out to confirm the *cot–1* phenotype. A perfusion system was used to control temperature in the petri dish using a temperature controller (Model TC-3248 Warner Instruments Inc., Hamden CT). The inflow and outflow tubes were positioned about 2-3 cm apart (the hypha was centered between the inflow and outflow). Temperature was monitored with a thermistor located at the outflow tube. Growth rates were measured before and after elevation of temperature from 24 to 31.5ºC. Hyphae embedded in the agar did not display profuse hyperbranching after temperature was increased, however, the hyphae on the surface of the agar normally formed two to three branches within a short period of time following the increase in temperature. Growth rates of these *cot–1* hyphae, before and after the temperature ramp, were

measured and compared with the growth rates observed in the wild-type strain of *N. crassa* under similar changes in temperature (Figure 1).

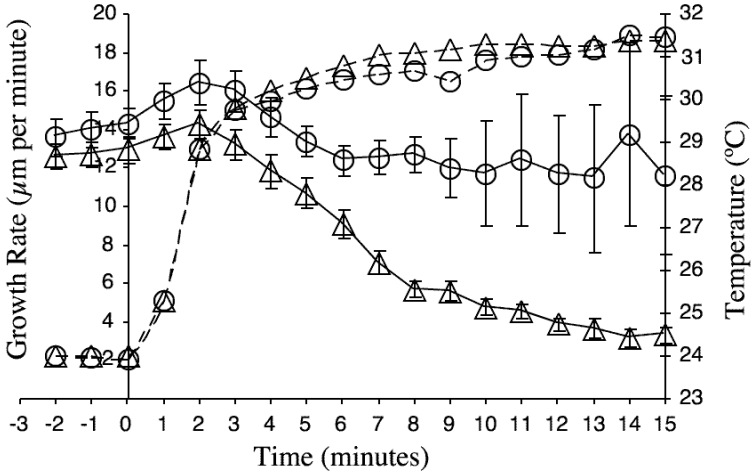

Figure 1 - Effect of temperature on average growth rates in wild-type and the *cot–1* mutant of *Neurospora crassa*: Time dependence. Mean ± SE (n= 5-41) are shown. Average growth rates are shown for wt (circles) and the *cot–1* mutant (triangles) in response to temperature (dashed lines).

Initially, when temperature is increased, both wild-type and *cot–1* growth rates increase, but as temperature reaches about 31-32ºC, *cot–1* growth rate declines markedly, while wild-type growth is relatively unaffected.

The temperature-dependent changes in growth rates before and after temperature elevation from all the experiments carried out on *both cot–1* and wild-type *N. crassa* are summarized in Figure 2.

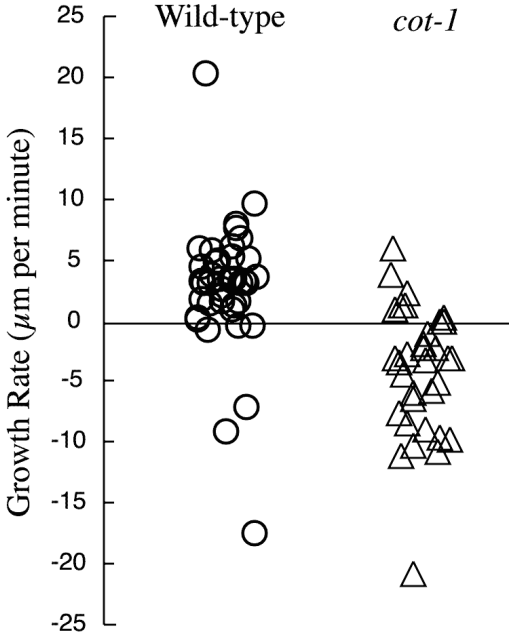

Figure 2 - Changes in growth rate in response to elevated temperatures for wild-type and the *cot–1* mutant of *Neurospora crassa*. Overall growth rate changes in wt (circles) and *cot–1* (triangles) are shown. These were obtained by subtracting the first measured growth rate from the last measured growth rate in each experiment. The experiments on cot–1 lasted an average of 12 minutes following the temperature increase while the experiments on wild-type lasted an average of 8 minutes after a similar increase in temperature.

Growth rate declined in the *cot–1* mutant, while in the wild-type the growth rate increased (Figure 2). A t-test comparison (two tail) indicated a significant difference in

growth rate changes between the *cot–1* and wild-type strains due to elevated temperatures ($P < 10^{-7}$).

The difference in growth rates between *cot–1* and wild-type was evident within 5-10 minutes following a temperature increase. Thus, the *cot–1* mutant responds rapidly to the change to the restrictive temperature. Since membrane potential can be easily observed over the period of time during which growth rate is affected in the *cot–1* mutant, we could monitor any changes in the electrical properties that could be affected by the transition from wild-type to *cot–1* phenotype.

Double barrel micropipettes (Lew, 2006) were impaled into the hyphal trunks of both strains. Two electrical parameters were measured: Membrane potential and resistance. The potential was monitored continuously (along with temperature); the resistance was measured by injecting a 5 nA current through one barrel of the double barrel micropipette and measuring the voltage change in the other barrel. Due to the difficulty in impaling hyphal tips because of vibrations during heating and perfusion system, tips were not impaled to record membrane potentials. Instead, hyphal trunks were chosen for electrophysiological readings. *Cot–1* phenotypic changes have been reported for hyphal trunks up to 750 μm behind the colony edge (Steele et al., 1977), so we expected *cot–1* to have an effect on ion transport as most of the experiments were carried out on regions of hyphal trunks about 0.5 - 1 mm from the colony edge. Furthermore, the ultrastructure of the *cot–1* mutant has been examined under restrictive temperatures and extremely disorganized cytoplasmic organelles were observed in the hyphal compartments behind the growing tips (Gorovits et al., 2000), indicating the appearance of the mutant phenotype in the trunks.

Examples of wildtype and *cot–1* membrane potential measurements are shown in Figure 3. An typical impalement was followed by stabilization of the membrane potential.

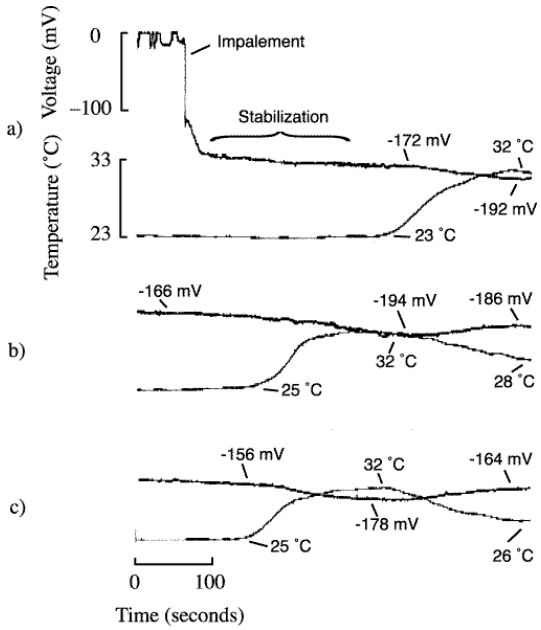

Figure 3 - Changes in membrane potential seen over time and temperature changes. a) Typical membrane potential reading obtained after impalement of wild-type *Neurospora crassa*. Measurements of the *cot–1* mutant were obtained similarly. b) Membrane potential reading observed in the wild-type strain of *Neurospora crassa*. Reversibility of the hyperpolarization is observed, indicating temperature dependence of the membrane potential. c) Membrane potential reading observed in the *cot–1* mutant of *Neurospora crassa*. Membrane potential behavior in response to temperature fluctuations indicates reversibility in the mutant as well.

The figure also shows the trace for a typical temperature ramp during these experiments. As shown, the membrane potential in the wild-type strain hyperpolarized in response to elevated temperatures. Figure3b shows the response of the membrane potential in the

wild-type strain to an increase, then decrease in temperature to indicate reversibility of the hyperpolarizing behavior. As seen in figure 3c, the *cot–1* mutant behaved similarly to the wild-type since it hyperpolarized in response to elevated temperatures. The membrane potential in the *cot–1* mutant showed reversibility with a return to 25ºC as well.

In a few experiments, membrane potentials of *cot–1* showed sudden changes and the experiments were deemed unreliable. Figure 4 illustrates the changes in membrane potential with respect to the changes in resistance for all experiments, including the ones with unreliable results.

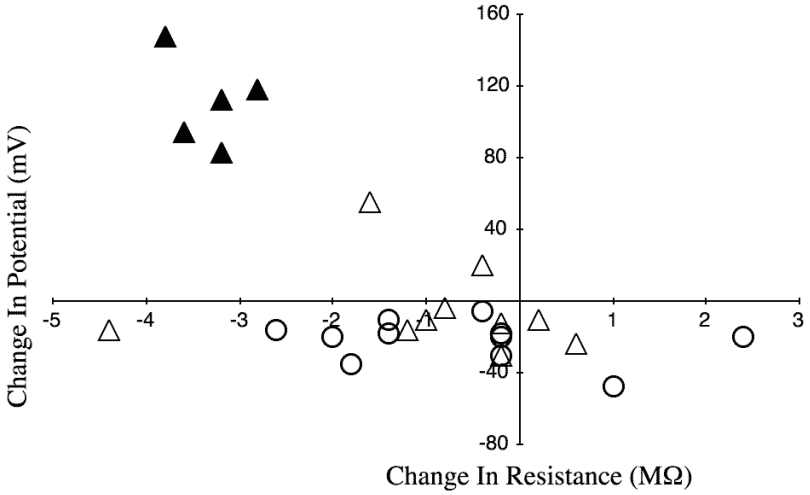

Figure 4 - Changes in membrane potential according to changes in resistance in wild-type (circles) and the *cot–1* mutant (unfilled triangles) of *Neurospora crassa*. Unreliable results with the *cot–1* mutant (filled triangles) are also shown.

As seen in Figure 4, not only did the unreliable *cot–1* experiments have sudden and large depolarizations, these depolarizations invariably occurred in tandem with a large decrease in membrane resistance. These tandem occurrences suggested that the depolarizations were most probably caused by damage to the membrane at the site of impalement. Thus, the unreliable results from experiments on *cot–1* were discarded as outliers. Only experiments in which there were no sudden changes in potential were retained for analysis.

At the permissive temperature, the membrane potentials were –147±39 mV (n=23) for wildtype, and –144±34 mV (n=21) for *cot–1*. Figure 5 illustrates the changes in membrane potential due to elevated temperatures in experiments on both *cot–1* and wild-type strains. Both hyperpolarized (–24±18 mV (n=23) in wildtype and –16±25 mV (n=21) in *cot*–1). A t-test comparison (two tail) indicated there was no significant difference in membrane potential at elevated temperatures between *cot–1* and wild-type strains (P= 0.218).

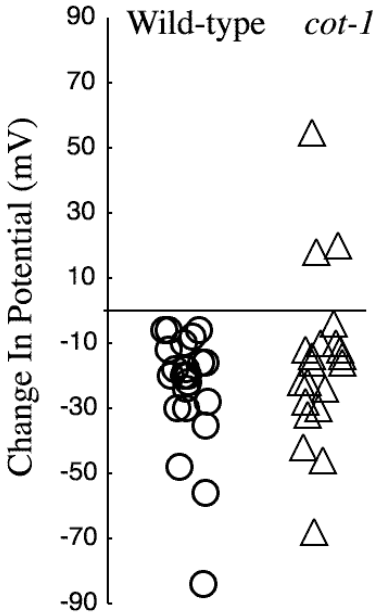

Figure 5 - Changes in membrane potential in response to elevated temperatures in wild-type (circles) and the *cot–1* mutant (triangles) of *Neurospora crassa*.

The resistance at the permissive temperature were very similar for wild-type (8.3±2.8 MΩ, n=11) and *cot–1* (7.0±3.1 MΩ, n=10). Resistances declined slightly in both wild-type and *cot–1* at elevated restrictive temperatures (–0.62 and –0.9 MΩ, respectively) (Figure 6).

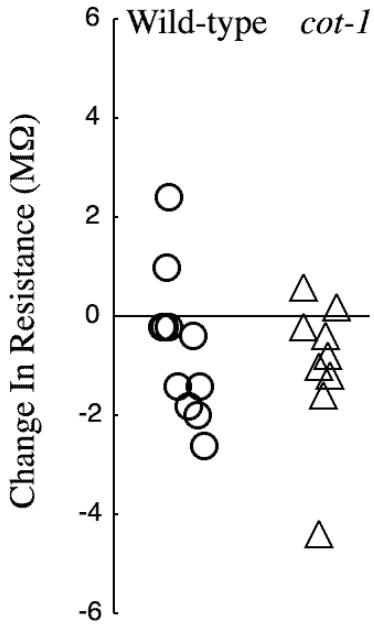

Figure 6 - Changes in resistance in response to elevated temperatures in wild-type (circles) and the *cot–1* mutant (triangles) of *Neurospora crassa*.

A t-test comparison (two tail) indicated there was no significant difference in resistance at elevated temperatures between *cot–1* and wild-type strains (P= 0.654).

Figure 7 illustrates changes in membrane potential according to changes in temperature in both *cot–1* and wild-type.

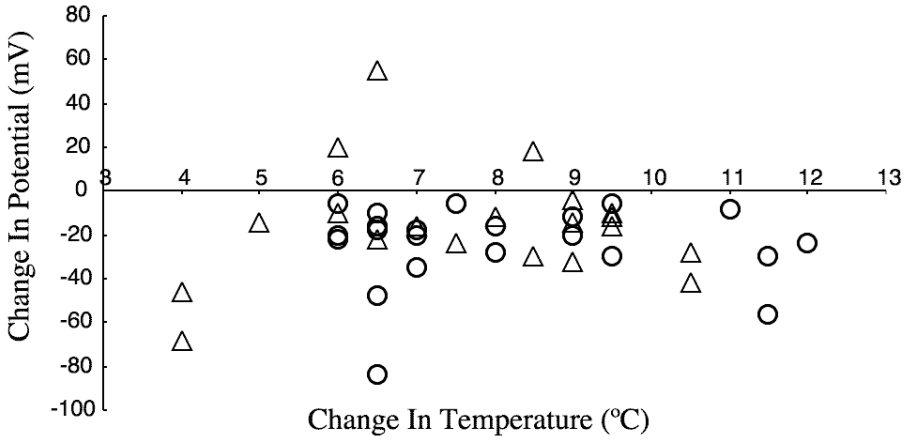

Figure 7 - Changes in membrane potential according to temperature changes in wild-type (circles) and the *cot–1* mutant (triangles) of *Neurospora crassa*

Figure 8 illustrates changes in resistance according to changes in temperature in both *cot–1* and wild-type.

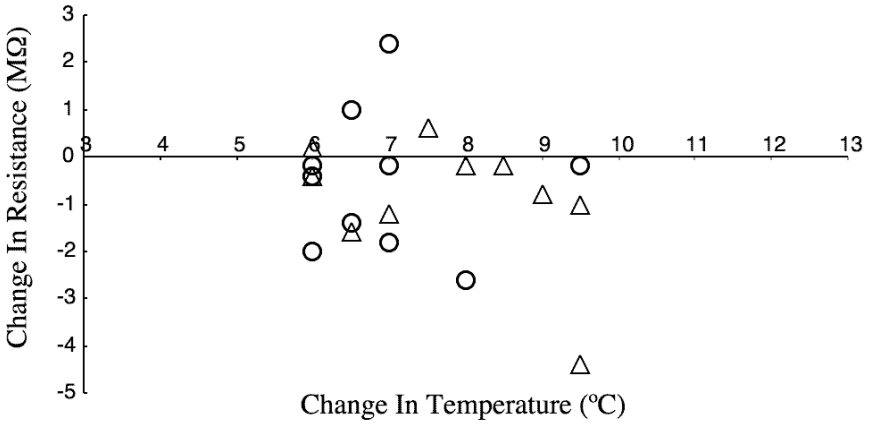

Figure 8 - Changes in resistance according to temperature changes in wild-type (circles) and the *cot–1* mutant (triangles) of *Neurospora crassa*.

In order to verify that the magnitude of temperature change had no linear relationship with the magnitude of membrane potential change, a few experiments were carried out where the temperatures were increased up to heat-shock range for the hyphae under observation. Figure 9 illustrates the membrane potential readings observed at different times throughout all heat-shock experiment.

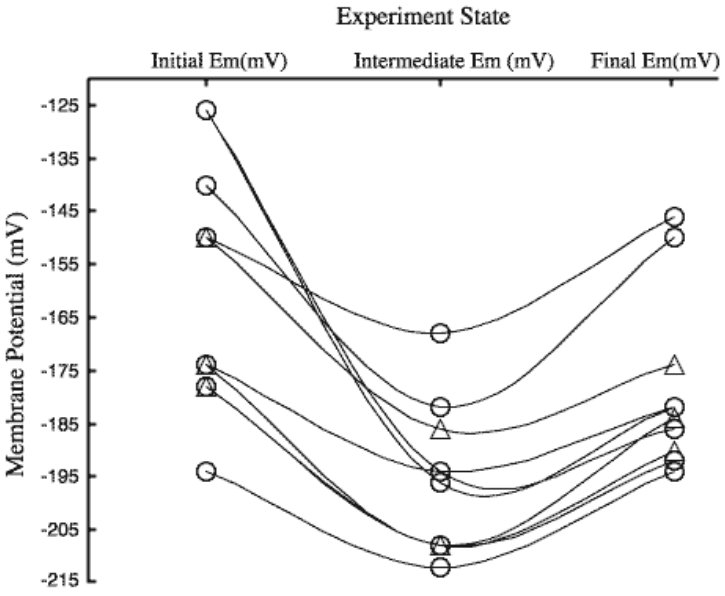

Figure 9 - Changes in membrane potential in response to temperatures inducing heat shock (39 to 41°C) in wild-type (circles) and the *cot–1* mutant (triangles) of *Neurospora crassa*. Initial potential was recorded as soon as the potential stabilized following impalement. The intermediate potential was the maximal hyperpolarization recorded in the middle of the experiment, while final potential was recorded at the end of the experiment.

Figure 10 illustrates changes in membrane potential according to changes in temperature in both *cot–1* and wild-type, including changes seen in the heat-shock experiments.

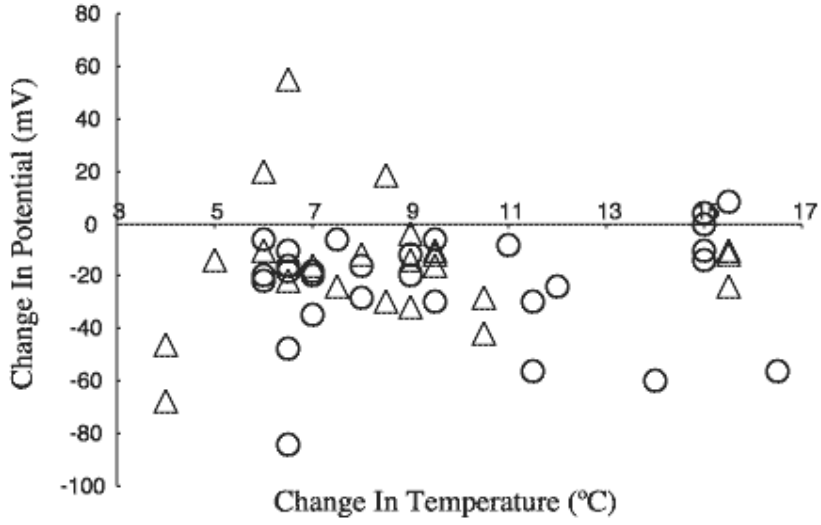

Figure 10 - Changes in membrane potential according to temperature changes in wild-type (circles) and the *cot–1* mutant (triangles) of *Neurospora crassa*. Results from heat-shock experiments are also shown.

The change in membrane potential for either wild-type or *cot–1* unexpectedly did not increase exponentially with increasing change in temperature, as shown in Figures 6 and Figure 10. Proton efflux through H^+-ATPase is a key regulatory mechanism for the maintenance of membrane potential in fungi (Benito et al., 2000). A higher temperature would normally increase metabolism and ATP turnover. This would increase H^+-ATPase activity, and thus cause increase in hyperpolarization with increasing temperature. Instead, the average hyperpolarization plateaus quickly and no relationship is observed

between increasing temperature change and hyperpolarization. This might be due to regulatory feedback, perhaps due to the the influx of other ions like K^+ and even due to influx of H^+ through other transporters which would compensate for the increased H^+-ATPase activity with increasing temperature.

DISCUSSION

There was no difference in the electrical properties of wild-type and the *cot–1* mutant at permissive or restrictive temperatures.

Since the changes in growth rate were observed at the tips, it could be argued that impalement of the tips would have been a better option than at the trunks. However, this was technically unachievable. All attempts resulted in damage to the hyphal tip, typically cytoplasm extrusion at the impalement site, within a few minutes of the impalement. The hyperbranching phenotype of the mutant has been observed at the trunks (Steele et al., 1977). Structural changes in hyphal compartments behind the tip were also detected in the mutant at restrictive temperatures (Gorovits et al., 2000). Thus, similar behavior is expected at either tips or trunks. If the tips and the trunks differ in ion-channel abundance, impalement of the tips could be a useful step in determining whether ion transport is involved in *cot–1*'s mutation of hyperbranching at high temperatures.

Regardless, the wild-type and *cot–1* behaves similarly with respect to membrane potential and resistance change at elevated temperatures. Thus, plasma membrane ion transport parameters are not significantly different between wild-type and *cot–1* at permissive or restrictive temperatures.

CONCLUSION

Although the growth rate slows down considerably in the *cot–1* mutant due to elevated temperatures as compared to in the wild-type strain of *Neurospora crassa*, values of membrane potential and resistance are quite similar in both strains. Thus, the *cot–1* mutation probably does not involve electrogenic ion transport. If ion transport is important, it may be related to ion fluxes at the endomembranes. Ca^{2+} is one possibility.

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