Research Article

The alternative oxidase mediated respiration contributes to growth, resistance to hyperosmotic media and accumulation of secondary metabolites in three species

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ABSTRACT

Plant respiration, similar to respiration in animal mitochondria, exhibits both osmosensitive and insensitive components with the clear distinction that the insensitive respiration in plants is quantitatively better described as 'less' sensitive rather than 'insensitive'. Salicylic hydroxamic acid (SHAM)–sensitive respiration was compared with the respiration sensitive to other inhibitors in rice, yeast and *Dunaliella salina*. The influence of SHAM was largely in the osmotically less sensitive component and enhanced with external osmotic pressure unlike other inhibitors that inhibited the osmotically sensitive component. SHAM inhibited germination and root growth but not shoot growth. Osmotic remediation of respiration that developed in due course of time with rice seedlings was abolished by SHAM and was not due to water and ionic uptake mechanisms. Yeast and *Dunaliella* also showed susceptibility of growth and respiration to SHAM. Glycerol retention was influenced by all inhibitors, while growth was inhibited demonstrably by SHAM in *Dunaliella.* Respiration in plants needs to be seen as a positive contribution to overall growth and not merely for burning away of the biomass. **[Physiol. Mol. Biol. Plants 2008; 14(3) : 235-251]** *E-mail : sitaramamv@gmail.com*

Key words : *SHAM, inhibitors of respiration, yeast, rice, Dunaliella, osmotic sensitivity, voids.*

Abbreviations : *SHAM (salicylhydroxamic acid)*

INTRODUCTION

Previous studies from our laboratory demonstrated that electron transport, mitochondrial, photosynthetic and even bacterial, is inhibited by external osmotic pressure (Mathai *et al.*, 1993, Pan *et al.*, 1995, Natesan, *et al*., 2000). The pressures required are well beyond the range of simple water fluxes associated with gross changes in the volume of cells and organelles, bordering on physical compression and even dehydration (Sitaramam and Madhavarao, 1997; Sitaramam, 2006). The relevant conjugate 'volume' for this pressure was traced to be voids in the bilayer (Mathai and Sitaramam, 1994), through which the relevant quinone would diffuse, thereby controlling the rate of electron transport. This (Mathai *et al*., 1993) was the first ever demonstration that the rate limiting step in electron transport is not the concentration of a diffusible redox intermediate but the voids in which these intermediates could migrate in this exquisitely diffusion-controlled reaction. For instance, the osmotic sensitivity, measured as an activation volume (which relates to and, therefore, is an approximate measure of quinone diffusion) was ~ 3.4 L/mole for State III respiration for either glutamate/malate or succinate, while the corresponding State II and IV respiration were barely 0.41 and 0.5 L/mole respectively, i.e., osmotically insensitive. Osmotic responses were also nonlinear with sensitive and insensitive domains analyzed by spline regression techniques specially developed in our laboratory for this purpose (Shanubhogue *et al*., 1992), which yield an intersection point between two regressions of the two domains, termed the breakpoint. The insensitive respiration, expressed as activation volume, was ~10% of the sensitive respiration in animal mitochondria whereas, in respiring rice seeds, as in most other plant tissues, was ~ 22%. In 6 varieties of rice, the sensitive respiration was 1.71 $\pm$ 0.315 L/mole, while the insensitive respiration was 0.391 $\pm$ 0.156 L/mole (Bhate and Sitaramam,

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unpublished observations, Sitaramam *et al*., 2006). Since smaller voids require greater degree of pressure for compression, some residual respiration (up to 10 %) can be understood from a physical point of view (Sitaramam and Madhavarao, 1997; Mathai and Sitaramam, 1994; Rajagopal and Sitaramam. 1998). It was also confirmed that this activation volume is additive for the mitochondrial processes. While the ATPase/synthase itself is osmotically insensitive (0.82 L/mole) (Pan *et al*., 1995), the adenine nucleotide transporter was remarkably stretch sensitive, the first ever transporter shown to be so using a purified protein reconstituted into liposomes (Sambasivarao *et al*., 1988). Thus phosphorylation driven by state III respiration exhibited an activation volume > 10 L/mole, in fact more than the sum of respiration and the adenine nucleotide transporter (Mathai *et al*., 1993). Thus voids, which by themselves are capable of holding energy due to surface tension, would play a special role in mediating phosphorylation, serving excellent means for trans-molecular as well as intra-molecular transfer of energy. The thylakoid showed an osmotically insensitive ATPase and no enhancement of the activation volume of phosphorylation (2.1 L/mole) beyond that of electron transport per se (cf. Pan *et al*., 1995).

Plant mitochondria exhibit a cyanide-insensitive respiration via an alternative oxidase (AOX), an enzyme complex that accepts reducing equivalents directly from the quinone and is non-phosphorylating (Wagner and Krab, 1995). We examined whether the larger component of the relatively less osmo-sensitive respiration in plants arises from AOX. Since the need is to assess the 'capacity' and not 'engagement' of AOX, and also since the osmotic methodology developed by us requires very detailed quantitative and statistical analyses, we studied the problem using inhibitors of respiration rather than by methods of isotope discrimination (McDonald *et al*., 2002).

We investigated the role of alternative oxidase (defined as the SHAM-sensitive respiration) in osmotic stress in a monocot, rice, in yeast and in the halophilic *Dunaliella salina* and found that the alternative oxidase activity contributes to growth and also contributes to mitigation of the effects of drought and salinity. These statements are not only consistent with earlier studies (cf. McDonald *et al*., 2002 and references therein), but add further that SHAM-sensitive respiration contributes to growth and secondary metabolite accumulation as a competent ATP generating pathway. The effect of salinity/osmotic pressure is traced to a measurable aspect of respiration, the AOX mediated respiration, which is relatively less sensitive to external osmotic pressure. Further, the novelty here lies in that the effect of SHAM ***increases*** with osmotic pressure, consistent with the regulation of this pathway largely dependent on the physical forces and the consequent changes in the local *milieu* and not due to the induction of the enzyme. It appears that the evolution of this enzyme (inhibitor–sensitive activity present in yeast, well seen in the terrestrial monocot, rice and the even more in the halophile, *Dunaliella*) could have presaged the migration of plants from water to land.

MATERIALS AND METHODS

All the chemicals used for preparation of solutions were of analytical grade. Biochemicals including inihibitors and enzymes were from Sigma, USA. SHAM was initially a gift from Prof. Raghavendra. Seeds of commercially released rice (*Oryza sativa*) varieties were procured from Directorate of Rice Research, Hyderabad. Germination was $\geq$ 98% uniformly in these nuclear seed stocks. *Dunaliella salina* Strain 19/30 was from Culture Collection of Algae and Protozoa , U.K. (CCAP) and grown in the medium 2ASW defined by CCAP as described (http://www.ccap.ac.uk/cultures/cultures.htm).

Germination in rice seeds

% Germination was determined using seeds of variety Tulasi at various NaCl concentrations. Floating (empty) seeds (<1%) were removed, ensuring random sampling of seeds. Seeds were soaked under running tap water for 12h, surface dried by blotting and kept for germination in Petri plates at 25 $\pm$ 2 ^{o}C, (n = (193 $\pm$ 34) at each concentration) on blotting papers wetted with Arnon and Hoagland's nutrient solution (Hoagland and Arnon, 1950) with different concentrations of NaCl (from 0 to 300 mM). % Germination was determined by counting seeds at an interval of 24 $\pm$ 2 h up to 252 h, by which time germination was complete in seeds grown in media without added NaCl during germination. Germination was measured in the presence of SHAM (5mM) and cyanide (2mM). Petri plates were sealed with parafilm to prevent evaporation (checked gravimetrically). Root and shoot length measurements were carried out in 10 day old seedlings growing in the presence of varying NaCl concentrations in the media, after rapid (i.e., physiologically relevant) germination of the seeds without added NaCl. The length of the roots and shoots were measured at 7.2 x magnification of the image projected of each seedling with coordinate distortion less than 1%. Since SHAM is dissolved in dimethylsulfoxide, DMSO, separate controls were carried out also using DMSO only.

Measurements of respiration in rice

Respiration was uniformly measured as oxygen consumption in germinating seeds of rice (Tulasi) using constant pressure manometry (Slater, 1967) using Gilson, differential, single valve style respirometer (Model IGRP-14, USA). The seeds (soaked for germination for 48 ± 1h at 28 ± 2 °C) were weighed (1.02 ± 0.013 gm) and transferred to respiratory flasks containing 3mL solution of different NaCl molarities, (0.0M, 1.0 M, 1.5M and 2.0M) in 10 mM sodium phosphate buffer, pH 7.4. Crimped filter paper was placed in the central well of the flasks containing 0.2mL of 10% KOH solution, to enhance the CO_2 absorbing area. The germinating seeds in the Warburg flasks were pre-incubated with the surrounding osmolytes for 1h while equilibrating the flasks with the bath temperature (30°C). The rate of respiration (J_0) was expressed as nmoles of oxygen consumed. min^{-1}. g^{-1}, The measurements of respiration were continued till the rate stabilized and yielded regression better than r = 0.999 with manometric readings ≥ 5.

Respiration measurements with inhibitors in rice seedlings

The concentration required for inhibitors to inhibit the rate of respiration was initially determined. The alternate oxidase (AOX) inhibitor, SHAM (~5mM) was added to the flask, such that amount of the solvent DMSO, added did not exceed 0.25% of the total volume. Solvent control was also run simultaneously.

Water and ion fluxes in seeds

A known amount of seeds was soaked in several independent aliquots (~2 gm) with and without SHAM, in 0M and 1M NaCl. The seeds were surface dried with tissue paper to remove the residual water for gravimetric measurement. From these seeds ~500mg of seeds were kept for dry weight measurements. Remaining seeds were pre-weighed, washed 3 times in distilled water rapidly and pressure cooked in 25mL of water and the conductivity of the solutions was measured. Conductivity was expressed as NaCl equivalents in $molesL^{-1}$ of total water content of the seeds obtained as the difference in weight on drying the seeds to constant weight.

Growth experiment in wine yeast

Wine yeast (*Saccharomyces cerevisiae*, commercial variety) was grown in glucose-yeast extract (GYE) medium containing varying amounts of NaCl (0-0.5M). Conversion of optical density to log number of cells was calibrated by counting yeast cells using the optical imaging system of Hamilton Thorne Semen Analyzer (USA). Cells were counted in excess of 2000 at each dilution corresponding to an optical density range of 0.05 to 0.8. The optical density of cells was measured in a Kontron Spectrophotometer (UVIKON-810, Germany) at 540 nm. Growth, as growth rate constant, was also measured as a function of inhibitor concentration.

Respiration in yeast

Respiration of wine yeast was measured in 5/6 Model Gilson Oxygraph using 20mM Sodium Phosphate buffer (pH 6.8) and Saboraud's medium with 56mM glucose at 30 °C. Rate of oxygen consumption was expressed as µL of O_2 consumed. $min^{-1}.10^{-6}$ cells. Respiration was measured at different NaCl concentrations ((0–3) M) and also with respiratory inhibitors, Cyanide (500 µM), Antimycin A (50µM) and SHAM (5mM).

Growth, respiration and photosynthesis in Dunaliella

Effect of varying concentrations of respiratory inhibitors SHAM (0-10mM) (A), Antimycin (0-500 µM) (B) and an inhibitor of chlororespiration, Propyl gallate (0-5mM) (C), on respiration and photosynthesis was studied in *Dunaliella* cells. *Dunaliella salina* var. *Bardawill* was grown in 2ASW medium with 2M NaCl in presence of light for 30 days, cells were harvested using Sigma (USA) centrifuge and packed cells were used for the experiment. Packed cells had a chlorophyll content of 77.22mg/mL and were kept illuminated at an intensity of 6.84 µmole.$m^{-2}.s^{-1}$. Respiration and photosynthetic oxygen evolution were studied in Gilson Oxygraph at 30°C using 2M NaCl in 10mM sodium phosphate buffer (pH 7.4). Actinic light with intensity of 1953 µmole. $m^{-2}.s^{-1}$ was used for studying photosynthesis. Rate of oxygen consumption and evolution in *Dunaliella* cells was expressed as nmoles of O_2 consumed. min^{-1}. g $(chlorophyll)^{-1}$ and nmoles of O_2 evolved. min^{-1}. g $(chlorophyll)^{-1}$ respectively.

Fluorescence measurements in Dunaliella

a. Fluorescence in Dunaliella cells treated with respiratory inhibitors

Dunaliella was grown in 2ASW medium with 2M NaCl in presence of light for 30 days. Cells were harvested by centrifuging, packed and kept illuminated at 6.84 µmole.$m^{-2}.sec^{-1}$. The chlorophyll content of the packed cells was 0.257mg/mL. The volume of packed cells corresponding to 28µg of chlorophyll was used for the assay. Fluorescence was recorded as a function of time

in SFM-3 Fast Kinetics apparatus (Biologic, France) in the fluorescence mode at 30°C. Actinic light with intensity of 1953 μmole.m^{-2}.s^{-1} was used as illumination source and a band pass filter, which selectively allowed light of 700nm was used in the photomultiplier tube. The cells were incubated in dark for 5min in the water-jacketed reaction vessel before the actinic light was flashed. Respiratory inhibitor Antimycin A(50μM), inhibitor of chlororespiration, Propyl gallate (5mM), inhibitor of Alternate oxidase, SHAM (5mM) and control (DCMU) were added to the assay mixture (Cournac *et al.*, 2002). Inhibitor of the cytochrome b_6f, DBMIB (Izawa, 1980), was added to the assay mixture after the signal steadied to a constant value. The background signal was 4.84mV.

b. Fluorescence in Dunaliella cells at different NaCl concentrations

The chlorophyll content of the packed cells was 0.368mg/mL. The volume of packed cells corresponding to 28μg of chlorophyll was used for the assay. The background fluorescence was 150mV. The cells were incubated in 10mM sodium phosphate buffer with 10mM sodium bicarbonate at pH 7.4 and the respective NaCl concentration in dark for 5min prior to illumination. The signal was allowed to stabilize before DCMU (1μM) was added. Inhibitor of the cytochrome b_6f, DBMIB, was added to the assay mixture after the signal stabilized to a constant value.

c. Glycerol content at varying NaCl concentration

Cells were incubated in NaCl concentrations ((0 – 4) M) for 100h. The cell suspension containing 2.2 million cells mL^{-1} was filtered through Sartorius filtration assembly and the cells were resuspended in 1mL of sodium phosphate buffer (pH 7.4) with respective NaCl concentration and heated at 70°C for 5min to release the glycerol from cells. The supernatant of this extract was used for glycerol estimation. The amount of glycerol in the cells, expressed as nmoles. million $cells^{-1}$ was estimated using glycerol dehydrogenase assay, where fluorescence intensity of NADH was monitored. The glycerol content was previously calibrated using glycerol dehydrogenase by monitoring the fluorescence intensity of NADH at excitation 360nm and emission 450nm on a Kontron spectrofluorimeter.

d. Effect of temperature of incubation on the glycerol content in Dunaliella cells

Cells (of same concentration as mentioned above) were incubated at various temperatures for 100min. The amount of glycerol in the cells, expressed as nmoles. million $cells^{-1}$ was estimated as mentioned earlier.

e. Effect of respiratory inhibitors on the glycerol content in Dunaliella cells

Cells of concentration 2.2 million. mL^{-1} were incubated in absence and presence of respiratory inhibitors, Cyanide (2mM), SHAM (5mM) and Propyl gallate (3mM) for 100 min. The cell suspension was filtered, extracted by boiling and the extract used for glycerol estimation, as above.

f. Effect of light and dark cycle on the glycerol content in Dunaliella cells

Cells incubated in dark were subjected to light with intensity 1953 μmole.m^{-2}.s^{-1} and again incubated in dark. The cell suspension containing 2.2 million cells mL^{-1} was filtered through Sartorious filtration assembly and 1mL of the filtrate was directly used for glycerol estimation. The remaining cells were boiled and the extracted for glycerol estimation.

RESULTS

The studies reported here were aimed at a comparison of the effect of respiratory inhibitors on growth and respiration in three species: rice, yeast and *Dunaliella*. The SHAM-sensitive respiration was measured directly or as a component of cyanide-sensitive respiration in these organisms. The strategy was based on *a priori* surmise that was useful: if SHAM – sensitive respiration relates to stress in various organisms, the effect of SHAM itself could be stress-sensitive. If the alternative oxidase route plays a significant positive role in growth and energetics as well, SHAM should also affect the growth and the production of secondary metabolites. It should be noted that the consensus opinion is that ionic (salinity) and osmotic effects are indistinguishable in such studies (Munn, 2002, Sitaramam and Madhavarao, 1997).

Studies on rice

a. Germination

Germination in rice (determined by the appearance of radicle by visual means) is respiratory energy-intensive and is an excellent measure of osmotic sensitivity, hence a measure of the phenotype for drought tolerance, for the plant (Sitaramam *et al.*, 2006). Two findings with regard to seed respiration are important: faster respiring individual seeds germinate significantly faster and seeds

that germinate at a higher osmolality/salinity also continue to respire at higher osmolalities (data not given; cf. Sitaramam, *et al*., 2006). Fig 1 gives % germination profiles as a function of NaCl at 276 h, at which time 100% germination was achieved in media without added salt. Addition of SHAM led to inhibition of germination at higher osmolalities indicating that the influence of SHAM-sensitive respiration enhances with osmotic pressure of the growth medium and is not constant.

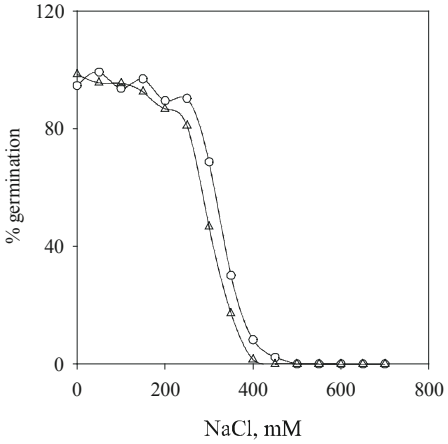

Fig. 1. % Germination of rice seeds of variety Tulasi as a function of varying concentrations of NaCl in presence and absence of SHAM. Rice seeds were allowed to germinate in Arnon-Hoagland medium containing NaCl of different osmolarities and SHAM (5mM) was added to the plates. The effect of DMSO (O.25% v/v) was indistinguishable from Control and hence omitted. Fig. 1 shows the % germination at 276 h for Control (circles) and SHAM (triangles).

Table 1. % Germination of rice seeds of variety Tulasi as a function of varying concentration of NaCl in presence and absence of SHAM. Rice seeds were allowed to germinate in Arnon-Hoagland medium containing NaCl of different osmolarities and SHAM (5mM) was added to the plates. The effect of DMSO was comparable with the Control. The data (n = 3) shows the NaCl values for 50% germination (mM, NaCl) for all the three treatments.

Treatment	Asymptote value for 50 % germination mM NaCl	S.D.
Control	320.9	2.76
DMSO	334.7	2.86
SHAM	304.9	2.58★

★Significantly different from DMSO treated control at $p<0.02$.

Table 1 shows that the effect of treatment with the solvent DMSO did not affect the sensitivity of germination to NaCl in a significant manner compared to the controls, SHAM produced a significant though small decrease in the NaCl concentration at which 50% germination occurs at this time interval. Detailed time studies from 24-400 hours at 6 hour intervals showed that the responses are comparable at all time points, while the osmotic influence on inhibition was best seen when germination was completed in the control group of seeds without exposure to added salt (data omitted for brevity, cf. Sitaramam, *et al*., 2006).

b. Growth of seedlings

The data in Fig 2 shows that increasing salt concentrations marginally inhibited the growth of apical shoot in the presence of SHAM, while the inhibition was large at all osmolalities in the root and additional

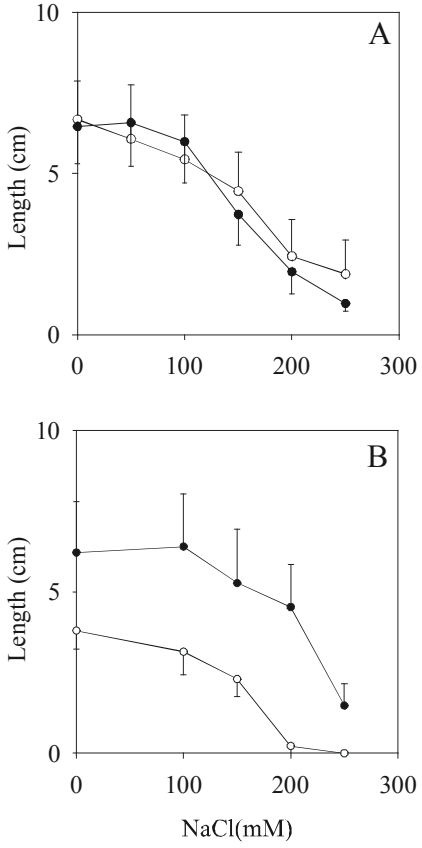

Fig. 2. Shoot length (A) and root length (B) in 10 day old rice seedlings (in cm) as a function of NaCl, mM. Shoot length in control (filled circles) and SHAM (open circles). In contrast, root length on addition of SHAM (open circles) as opposed to control (filled circles) indicates clear inhibition. Vertical bars represent S.D. (n=30, at each concentration).

influence of osmolality on inhibition by growth of roots could not be discerned. Since roots derive energy primarily from respiration unlike the photosynthetically active shoot, it was clear that one must choose the photosynthetically inactive phases of plant growth, i.e. germination and/or parts of the plant, i.e., the roots, if the role of SHAM-sensitive respiration is to be discerned. The SHAM-sensitive respiration does therefore contribute to the energetics of rice growth, i.e., biomass.

c. Effect of respiratory inhibitors on respiration in germinating seeds

Fig 2 shows systematic osmotic titrations of respiration on successive additions of SHAM and cyanide to the same set of flasks containing germinating seeds. Respiration was measured carefully such that the measurements were ensured to be in the linear phase initially and awaited to obtain linearity in the rates (linear regression of data points ≥ 5 yielding a regression better than 0.999) after addition of inhibitors. The design was necessitated by the limitation of flasks (n = 14) for simultaneous measurements. Dynamics of effects on respiration with NaCl (*vide infra*) required simultaneous measurements at varying NaCl concentrations. SHAM produced a small inhibition of respiration and hardly any difference in osmotic sensitivity in respiration (as measured by the discontinuity at which respiration profiles exhibit a break-point, vide Sitaramam, 2006, Sitaramam and Madhavarao, 1997) while cyanide caused a larger inhibition in respiration as also a smaller breakpoint. Direct interpretation in terms of changes in osmotic sensitivity was difficult due to relatively large

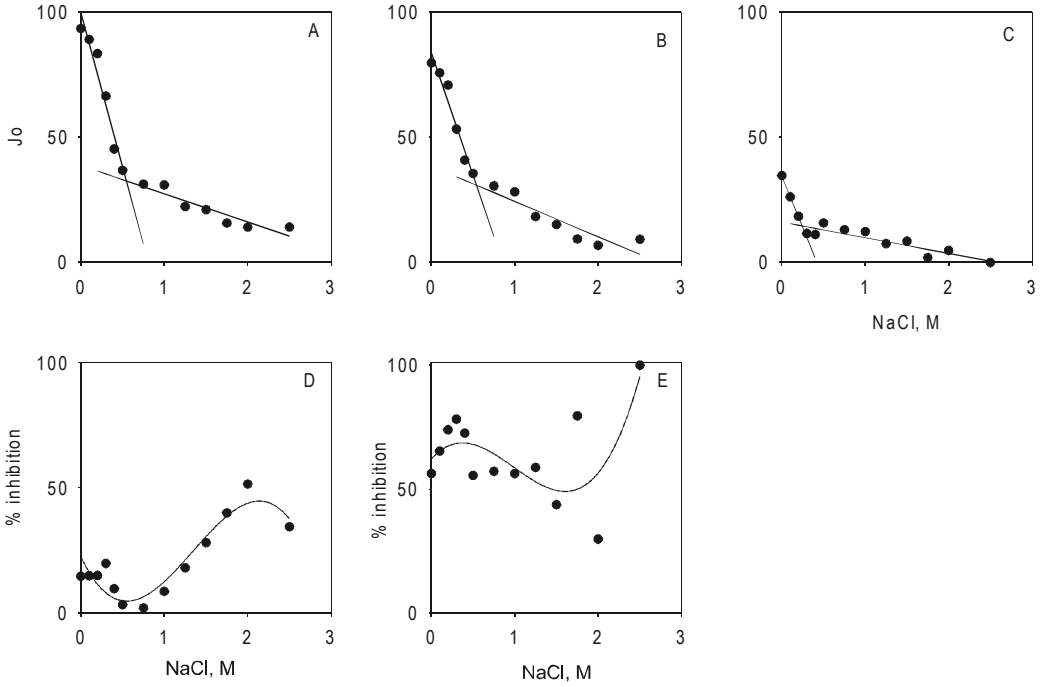

Fig. 3. Rate of oxygen consumption, as a function of NaCl, M in media (in all figures) in the presence of respiratory inhibitors. Oxygen consumption in germinating seeds was measured by constant pressure manometry and expressed as nmoles O_2 consumed.$min^{-1}g^{-1}$of tissue. Oxygen consumption was monitored in the germinating seeds without inhibitors (A) (the break point is (0.55±0.05) M NaCl), then the inhibitor of alternate oxidase, SHAM was added (5mM) (B) (break point (0.54±0.06) M NaCl) and then again oxygen consumption was monitored. Cyanide (2mM) (C) (break point (0.25±0.039) M NaCl) was added to the same flasks later (as SHAM did not induce major inhibition). The breakpoint for SHAM was not significantly different from the control where as the SHAM + cyanide was significantly different from the control ($P<0.02$). Fig 3D and 3E show the % inhibition in the rate of respiration due to addition of the inhibitors SHAM and cyanide respectively. % inhibition was calculated for SHAM from the difference between A and B, while that for CN was computed from the difference between B and C, fitted to the cubic equation for SHAM $y = 22.72-70.81\times(NaCl)+80.67\times(NaCl)^2-19.98\times(NaCl)^3$, (n=13; r=0.922; $P<0.001$ and each coefficient significant at $P<0.01$) and for SHAM+cyanide $y = 62.16+37.7\times(NaCl)-62.3\times(NaCl)^2 21.03\times(NaCl)^3$, (n=13; r=0.669, though significant at $P < 0.01$, none of the coefficients significant).

influence on respiration by these to inihibitors with possible scaling artifacts in the break-points. However, when % inhibition was plotted as a function of NaCl to yield a relatively scale-independent interpretation, the inhibitory effect of SHAM to the right of the break-point concentration of NaCl, i.e., specifically in the osmotically less sensitive domain, was distinctly progressive while that of cyanide was random and uncommentable (Fig 3).

d. Physiological remediation

An important aspect of responses to osmotic stress was the physiological remediation, often suggested to be production / accumulation of compatible osmolytes (Yancy *et al.*, 1982). The physiological expectation of the remediation is the restoration of the inhibited processes and an attempt return to physiological normalcy. We tested for response of rice seedling respiration to long term exposure to varying salt concentrations and found that this variety showed remediation of respiration between 4-10 hours in 1.0 M NaCl. The solvent, DMSO, was without effect while SHAM completely inhibited the remediation (Fig 4). The osmotically inhibited respiration continued to remain low at 1.0M NaCl and above, upto 16 hours tested.

We examined whether SHAM induced any demonstrable difference in water and ion uptake. Weight change incidental to germination was inhibited by 1.0 M NaCl compared to control with time of germination (Fig 5A,B) which was primarily due to water content, (Fig 5C,D). Ionic uptake was promoted by NaCl in the medium while hardly discernible in control seeds. SHAM had no influence on any of these parameters, consistent with the possibility that remediation of respiration was primarily due to some dynamics specific to the respiratory chain per se rather than peripheral effects in the cell such water and ion content.

Studies on yeast

a. Growth and respiration in yeast

Growth of yeast, expressed as growth rate constant, μ, d^{-1}, was inhibited in a concentration dependent manner in the presence of increasing salinity, Antimycin A and marginally but significantly with SHAM. The inhibitory effect of cyanide was barely discernible (Fig. 6). Respiration was inhibited in a concentration-dependent manner, SHAM being least effective, at much higher concentrations (Fig. 7).

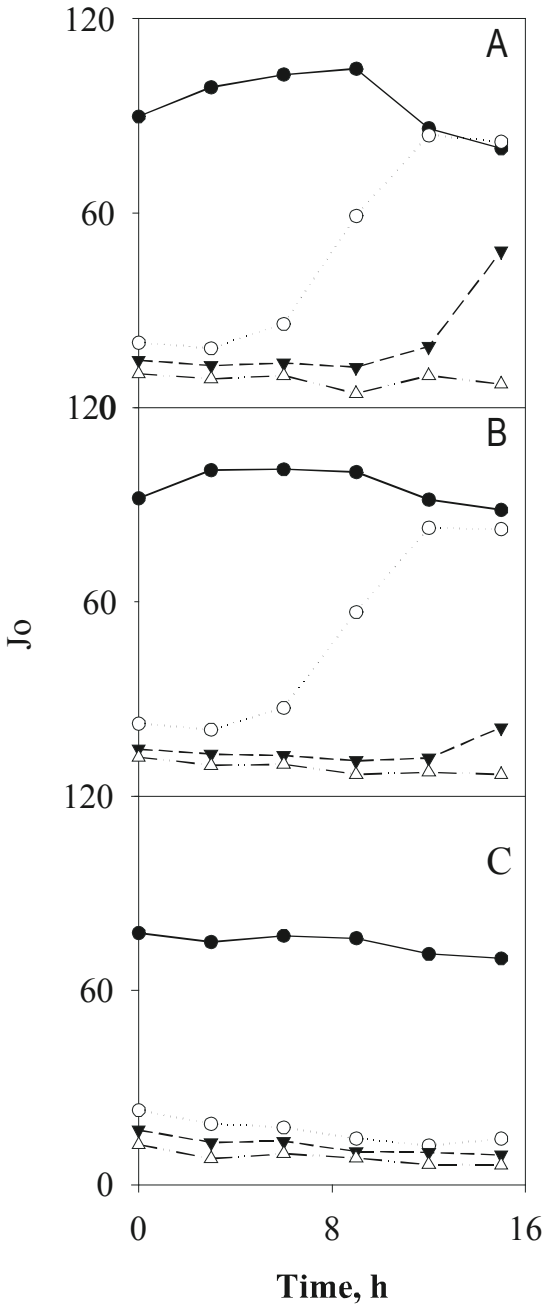

Fig. 4. Dynamic changes in respiration with time of incubation at varying NaCl**.** Rate of oxygen consumption expressed as nmoles O_2 consumed.$min^{-1}g^{-1}$, was plotted against time, h, for control (without inhibitor and without solvent) (A), only solvent i.e. DMSO (B) and solvent with alternate oxidase inhibitor, SHAM (C). Oxygen consumption was monitored at four different NaCl concentrations, 0.0M (filled circles), 1.0 M (open circles), 1.5M (filled triangles) and 2.0M (open triangles) as described in methods. Note that the dynamic changes in respiration in NaCl media (>1.0 M) were clearly inhibited by SHAM.

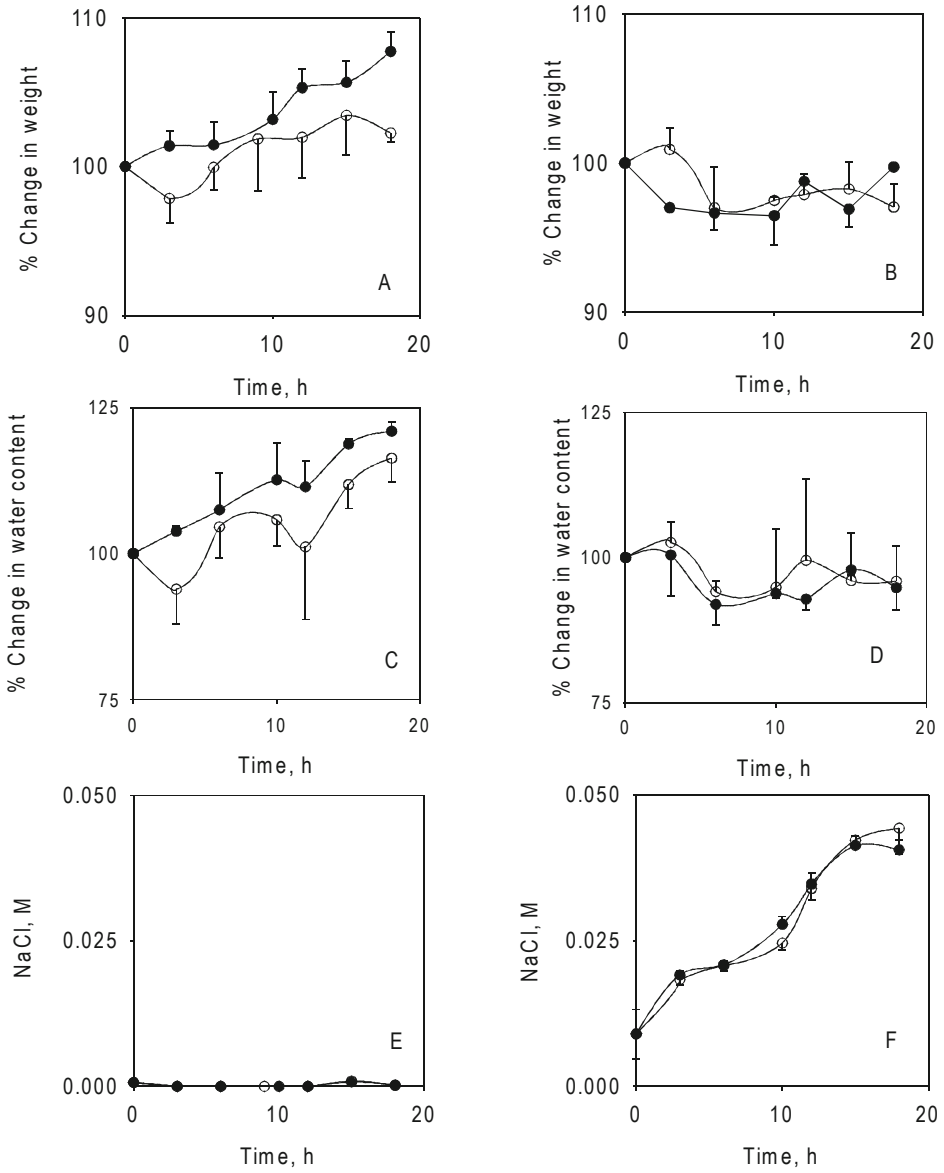

Fig. 5. Water uptake and ion fluxes in rice seedlings**.**
Top: Plot of time (h) vs % weight change in seeds. (A) Seeds grown in 0M NaCl (filled circles) and 0M NaCl with 5mM SHAM (unfilled circles). (B) Seeds grown in 1M NaCl (filled circles) and 1M NaCl with 5mM SHAM (unfilled circles). Vertical bars represent standard deviation (n = 3). The differences in slopes with and without SHAM were not significant in fig A or B.
Middle: Plot of time (h) vs. % change in water content (wet weight minus dry weight) in seeds. (A) Seeds grown in 0M NaCl (filled circles) and 0M NaCl with 5mM SHAM (unfilled circles). (B) Seeds grown in 1M NaCl (filled circles) and 1M NaCl with 5mM SHAM (unfilled circles). Vertical bars represent standard deviation (n = 3). The differences in slopes with and without SHAM were not significant in fig A or B.
Bottom: Plot of time (h) vs ion content (M) present in the seeds. (A) Seeds grown in 0M NaCl (continuous line with filled circles) and 0M NaCl with 5mM SHAM (discontinuous line with unfilled circles). (B) Seeds grown in 1M NaCl (continuous line with filled circles) and 1M NaCl with 5mM SHAM (discontinuous line with unfilled circles). Seeds were germinated in 0M NaCl, 0M NaCl with SHAM, 1M NaCl and 1M NaCl with 5mM SHAM. Conductivity was expressed as NaCl in moles per litre of total water content of the seeds obtained as the difference in weight on drying the seeds to constant weight. The differences in slopes with and without SHAM were not significant in either fig A or B, while the slopes *per se* were not significant in A but significant at $P<0.001$ ($r=0.98$, $n=7$ for both for data with and without SHAM) in B.

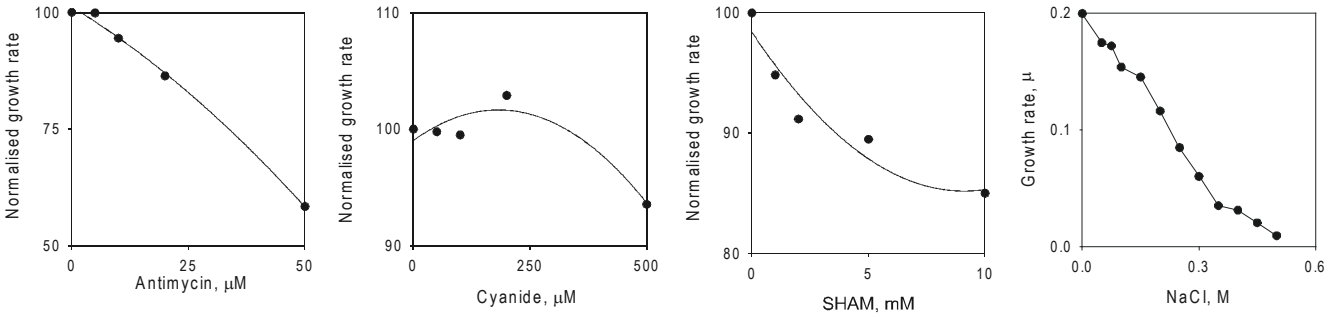

Fig. 6. Effect of respiratory inhibitors Antimycin (A), Cyanide (B), SHAM (C) on growth rate of wine yeast. Wine yeast was grown in GYE medium containing varying concentrations of Antimycin A(0-50 μM), Cyanide (0-500 μM) and SHAM (0-10 mM) and the growth rate was calculated by converting OD at 540nm to log number of cells. The calibration curve for number of cells needed to be obtained for each experiment separately. The growth rate of wine yeast in absence of inhibitor and NaCl measured at four different occasions was 0.22905±0.034. The growth rate were normalized and plotted as a function of inhibitor concentration and fitted to a lowest significant polynomial: Antimycin A, $y = -0.0049x^2 - 0.6115x + 101.1983$, $r = 0.9978$, $p<0.001$; Cyanide, $y = -0.0001x^2 + 0.0287x + 99.0238$, $r= 0.94$, $p<0.001$ and SHAM, $y = 0.1599x^2 – 2.9090x + 98.4115$, $r = 0.959$, $p<0.001$. (D) Growth of wine yeast at varying NaCl concentrations: Wine yeast was grown in GYE medium containing varying amounts of NaCl (0-0.5M). The calibration curve, log (n), y=1.0033 (O.D.) +0.8393 (e.g., as in D) was used to convert OD to log number of cells. The calibration curve for number of cells needed to be obtained for each experiment separately. The growth rate (μ) was calculated by plotting log number of cells as a function of time.

b. Combined influence of osmolality and respiratory inhibitors in yeast

Fig 8 shows the inhibition of respiration in yeast in the presence of salt in the presence of various inhibitors. The effect of the inhibitors, Antimycin and Cyanide, decreased with increasing concentration of NaCl in the media, while the effect of SHAM, in itself barely discernible as in Fig 7C, showed a clear pronouncement of its inhibitory effect with increase in NaCl concentrations, as seen with rice.

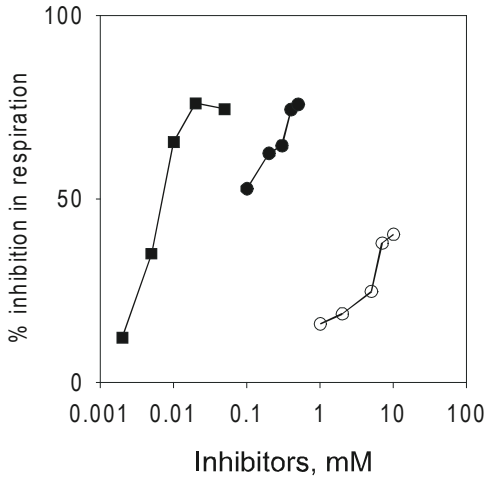

Fig. 7. Effect of varying concentrations of respiratory inhibitors on respiration of wine yeast. Antimycin (0-50 μM) (square), Cyanide (0-500 μM) (circle), SHAM (0-10mM) (open circle) Respiration was measured in Gilson Oxygraph at 30 °C using 20mM Sodium phosphate buffer (pH6.8) and 56mM glucose.

Since these effects are primarily dependent on the magnitude of the osmoticum deployed, the effect cyanide-insensitive, SHAM-sensitive respiration needed to be investigated in a halophile such as *Dunaliella*.

Studies on Dunaliella

a. Growth rate measurements

Dunaliella salina offers the means to study secondary metabolites such as glycerol production besides growth per se. The osmotolerant algal species showed a very slow growth rate with a doubling time of ~23 days in saline media (Table 2). The growth rate was inhibited by SHAM and unaffected by Antimycin A. The growth rate in Cyanide was initially slow, but however increased later to give a doubling time and growth rate similar to that of the control. Cyanide easily escapes as HCN which explains the surge in growth after all the cyanide was lost as HCN. Thus the concentration for cyanide for such long periods like 45 days would not be reliable.

b. Effect of respiratory inhibitors on respiration and photosynthesis in Dunaliella

It was advantageous to measure respiration (oxygen consumption) in dark followed by photosynthesis (oxygen evolution) on exposure to light in the same

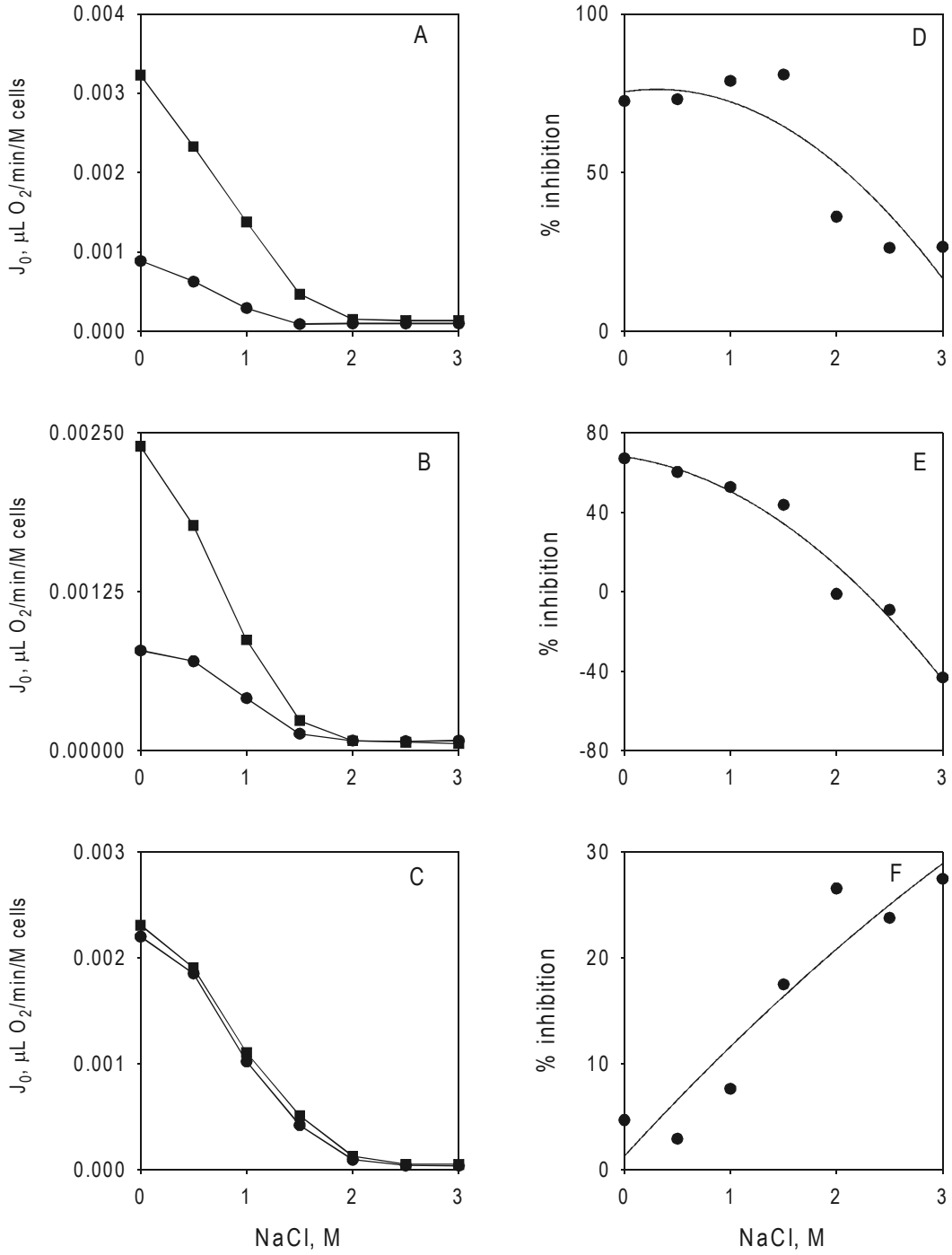

Fig. 8. Effect of inhibitors on the rate of respiration in wine yeast at different NaCl concentrations: Respiration of wine yeast was studied in Gilson Oxygraph using 20mM Sodium Phosphate buffer and 56mM glucose. Rate of oxygen consumption in wine yeast expressed as µL O_2 consumed.$min^{-1}.10^{-6}$ cells was plotted as a function of NaCl concentration, in the absence (square) and presence of respiratory inhibitors (circle) (Cyanide, 500 µM (A), Antimycin, 50 µM (B), and SHAM, 5mM (C). % Inhibition in the rate of respiration in presence of inhibitors (D, E, F) was plotted as a function of NaCl concentration, and were fitted to the lowest significant polynomial: Cyanide (D), y= 75.4090+ 5.0975x -8.2246 x^2, r = 0.8886, p<0.001; Antimycin, (E), y=67.9850-7.4315x-10.0015x^2; r = 0.985, p<0.001; SHAM (F) y= 1.2582+10.9145x -0.5664 x^2, r = 0.9399, p<0.001. % inhibition in respiration was defined '+ inhibitor' (presence) as percentage of '– inhibitor' (absence).

Table 2. Effect of respiratory inhibitors on growth rate and doubling time of *Dunaliella* cells. *Dunaliella* cells were grown in 2M NaCl in presence of the respiratory inhibitors, SHAM (5mM), Antimycin (50μM) and Cyanide (2mM). The cells were grown in presence of light (6.84 μmole.m^{-2}.s^{-1}) intensity. The cells were grown in boiling tubes with 10mL medium plus the respective inhibitor. Sampling was done every 72h and the cells were counted in a Hamilton Thorne Semen Analyzer. The optical imaging system of Hamilton Thorne Semen Analyzer was used for counting the cells in excess of 2000 at each time interval for each inhibitor in triplicates (p<0.05).

Inhibitor	Growth Rate μ, d^{-1}	Doubling time d
Control	0.0351	19.7
SHAM (5mM)	0.0134	51.7
Antimycin (50μM)	0.0277	25.0
Cyanide (2mM)★	0.0492	14.1

★The growth in cyanide was initially slow, but however increased later to give a doubling time and growth rate similar to that of the control. Cyanide easily escapes as HCN and it is difficult to maintain its concentration for such long periods like 45 days. The growth probably took off in the cyanide tubes after all cyanide was lost as HCN.

aliquot of cells with varying amounts of NaCl in the presence of inhibitors. Data in Fig. 9 shows that respiration and photosynthesis responded in an identical manner regardless of the experimental combination used indicating that their intrinsic regulation in these single cells cannot be dissected apart. SHAM and propylgallate were inhibitory, while Antimycin was hardly inhibitory.

Fig 10 further corroborates these observations when photosynthesis and respiration were measured as a function of the inhibitor concentration. Propyl gallate had a larger influence on photosynthesis than SHAM and Antimycin.

c. Effect of inhibitors on photosynthetic electron transport

A direct effect of an inhibitor on electron transport can be detected by measuring the fluorescence after addition of DCMU. Inhibition of electron transport would not allow DBMIB induced drop in electron transport after the stimulation of fluorescence by DCMU. Both n-propyl gallate and SHAM showed this reversal effect of DBMIB after DCMU treatment while Antimycin abolished the

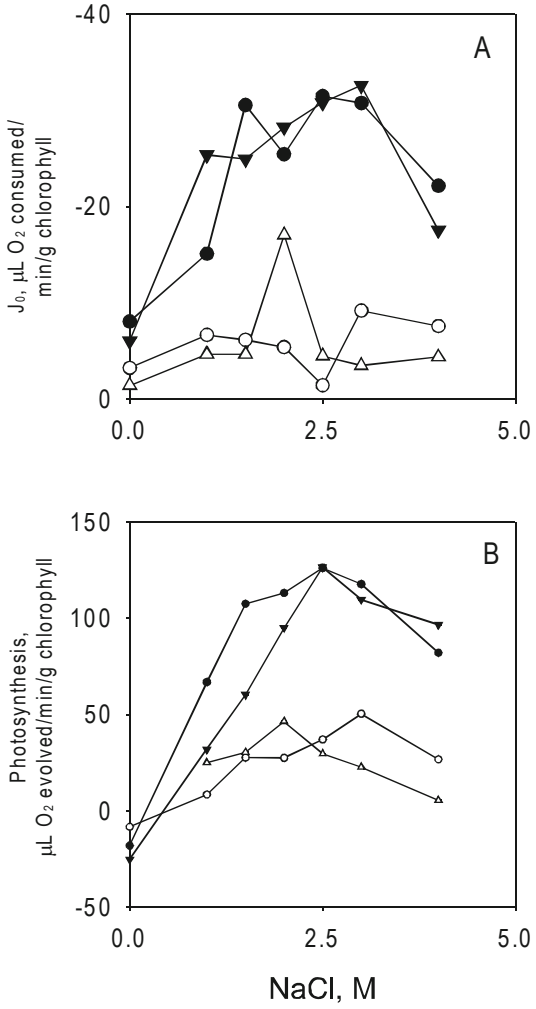

Fig. 9. Rate of respiration (A) and photosynthesis (B) in *Dunaliella* cells: *Dunaliella* was grown in 2ASW medium, with 2M NaCl in presence of light for 30 days after which cells were harvested. The packed cells had a chlorophyll content of 236.6 mg/mL and were incubated in presence of light (6.84 μmole.m^{-2}.s^{-1}) through out the experiment. Respiration and Photosynthesis (as O_2 evolution) of *Dunaliella* cells was studied in Gilson Oxygraph using 2M NaCl in 10mM Sodium Phosphate buffer pH 7.4 at 30°C. The oxygraph chamber was kept covered with black cloth during respiration measurements to avoid any stray light and while actinic light with intensity 1953 μmole.m^{-2}.s^{-1} was flashed on the chamber during photosynthesis measurements. Rate of oxygen consumption / evolved in *Dunaliella* cells expressed as μl of O_2 consumed (or evolved) per min per gram chlorophyll was plotted as a function of NaCl concentration, M in absence (filled circles) and presence of respiratory inhibitors such as SHAM, 5mM (empty circles), Antimycin, 300 μM, (filled inverted triangles), and Propylgallate, 5mM (empty inverted triangles). The error bars denote the standard deviation for three independent readings.

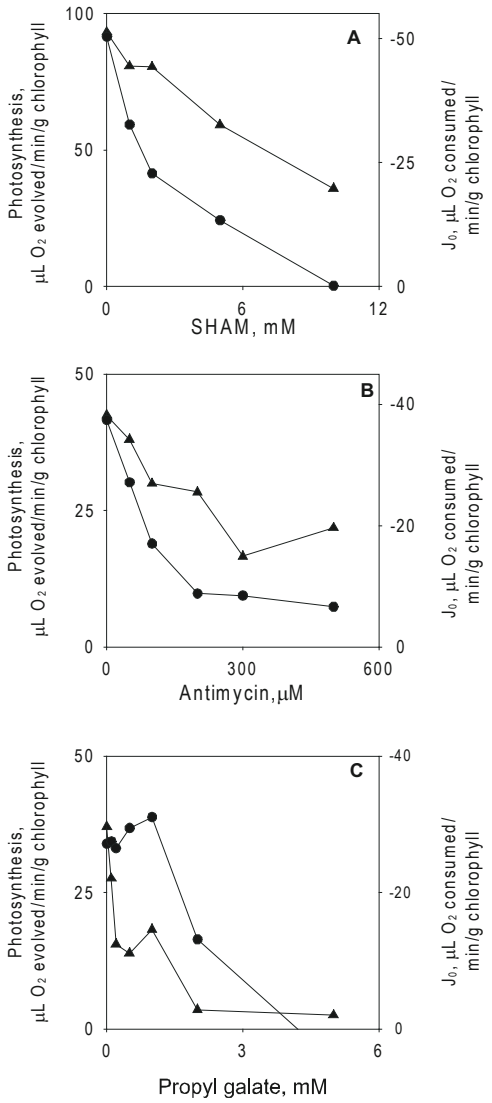

Fig. 10. Effect of varying concentrations of respiratory inhibitors SHAM (0-10mM) (A), Antimycin (0-500 µM) (B) and inhibitor of chlororespiration, Propyl gallate (0-5mM) (C), on respiration (filled triangles) and photosynthesis (filled circles) on *Dunaliella* cells. *Dunaliella* was grown in AAS medium with 2M NaCl in presence of light for 30 days, cells were harvested and packed cells were used for the experiment. Packed cells had a chlorophyll content of 77.22mg/mL and were kept illuminated at an intensity of 6.84 µmole.m^{-2}.s^{-1}. Respiration and Oxygen evolution were studied in Gilson Oxygraph at 30 ºC using 2M NaCl in 10mM Sodium phosphate buffer (pH 7.4). Actinic light with intensity of 1953 µmole.m^{-2}.s^{-1} was used for studying photosynthesis. Rate of oxygen consumption and evolution in *Dunaliella* cells expressed as nmoles of O_2 consumed per min per gram chlorophyll and nmoles of O_2 evolved per min per gram chlorophyll respectively was plotted as a function of inhibitor concentration. The error bars denote the standard deviation for three independent readings.

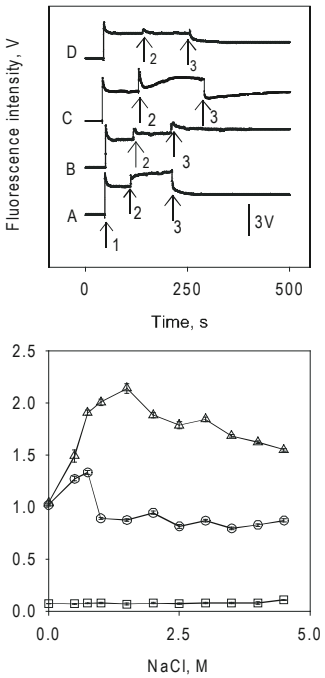

Fig. 11. I. Fluorescence in *Dunaliella* cells treated with respiratory inhibitors. *Dunaliella* was grown in 2AWS medium with 2M NaCl in presence of light for 30 days. Cells were harvested and illuminated at 6.84 µmole.m^{-2}.s^{-1}. The chlorophyll content of the packed cells was 0.257mg/mL. The volume of packed cells corresponding to 28µg of chlorophyll was used for the assay. Fluorescence was recorded as a function of time in Biologic Fast Kinetics at 30°C. Actinic light with intensity of 1953 µmole.m^{-2}.s^{-1} was used as illumination source and a band pass filter, which selectively allowed light of 700 nm was used in the photo multiplier tube. The cells were incubated in dark for 5 min in the water-jacketed reaction vessel before the actinic light was flashed (I). Respiratory inhibitor, Antimycin (50µM) (B); inhibitor of chlororespiration, Propyl gallate (5mM) (C); inhibitor of Alternate oxidase, SHAM (5mM) (D) and control (DCMU) (A) were added to the assay mixture (2). Inhibitor of the cytochrome b_6f, DBMIB was added to the assay mixture after the signal steadied to a constant value (3). The background signal was 4.84mV. II. Fluorescence in *Dunaliella* cells plotted as a function of NaCl concentration: The chlorophyll content of the packed cells was 0.368mg/mL. The volume of packed cells corresponding to 28µg of chlorophyll was used for the assay. The background fluorescence was 150mV. The cells were incubated in the 10mM sodium phosphate buffer with 10mM sodium bicarbonate at pH 7.4 with the respective NaCl concentration in dark for 5min before the light was shined. The signal was allowed to steady (filled circles) before DCMU (1µM) (filled triangles) was added. Inhibitor of the cytochrome b_6f, DBMIB was added to the assay mixture after the signal steadied to a constant value (filled squares). The data represented above is an average of the fluorescence intensity recorded over time.

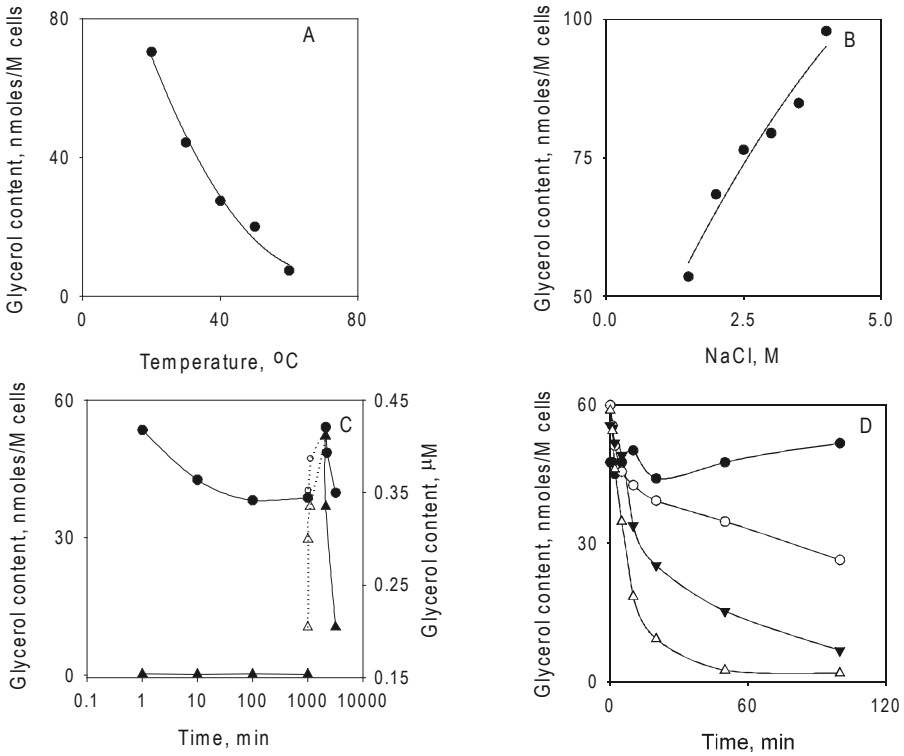

Fig. 12. A. Effect of temperature of incubation on the glycerol content in *Dunaliella* cells: Cells (of same concentration as mentioned above) were incubated at various temperatures for 100min. The glycerol content in the cells was plotted as a function of temperature and fitted to a lowest significant polynomial: $y=47.8754 -1.3138\ xs +0.0095x^2$, r = 0.9949.
B. Effect of external NaCl concentration on the glycerol content in *Dunaliella* cells: Cells were incubated in various NaCl concentrations for 100h. The cell suspension containing 2.2 million cells per mL was filtered through Sartorius filtration assembly and the cells were resuspended in 1mL of sodium phosphate buffer (pH 7.4) with respective NaCl concentration and boiled at 70°C for 5min to release the glycerol from the cells. This was used for glycerol estimation. The amount of glycerol in the cells expressed as nmoles per million cells was estimated using glycerol dehydrogenase assay where fluorescence intensity of NADH was monitored. The glycerol content was previously calibrated using glycerol dehydrogenase by monitoring the fluorescence intensity of NADH at excitation 360nm and emission 450nm on a Kontron spectrofluorimeter. The glycerol content in the cells was plotted as a function of NaCl concentration and fitted to a lowest significant polynomial: $y= 12.06 +11.69x-0.703x^2$, r =0.9776.
C. Effect of light and dark cycle on the glycerol content in *Dunaliella* cells: Cells incubated in dark (filled circled) were subjected to light (open circles) with intensity 1953 $\mu mole.m^{-2}.s^{-1}$ and again incubated in dark. The cell suspension containing 2.2 million cells per mL was filtered through Sartorious filtration assembly and 1mL of the filtrate was directly used for glycerol estimation. The cells were resuspended in 1mL of 2M NaCl in sodium phosphate buffer (pH 7.4) and boiled at 70°C for 5min to release the glycerol from the cells. This was used for glycerol estimation. The amount of glycerol in the cells expressed as nmoles per million cells was estimated using glycerol dehydrogenase assay where fluorescence intensity of NADH was monitored. The glycerol content was previously calibrated using glycerol dehydrogenase by monitoring the fluorescence intensity of NADH at excitation 360nm and emission 450nm on a Kontron spectrofluorimeter. The glycerol content of the cells (circles) and the filtrate (triangles) was estimated using the glycerol dehydrogenase assay where fluorescence intensity of NADH was monitored. The glycerol content was previously calibrated using glycerol dehydrogenase by monitoring the fluorescence intensity of NADH at excitation 360nm and emission 450nm on a Kontron spectrofluorimeter.
D. Effect of respiratory inhibitors on the glycrerol content in *Dunaliella* cells: Cells of concentration 2.2 million per mL were incubated in absence (filled circles) and presence of respiratory inhibitors, Cyanide (2mM) (empty circles); SHAM (5mM) (filled inverted triangles) and Propylgallate (3mM) (empty inverted triangles) for 100 min. The cell suspension was filtered through Sartorious filtration assembly and cells were resuspended in 1mL of sodium phosphate buffer (pH 7.4) and boiled at 70 °C for 5min to release the glycerol from the cells. This was used for glycerol estimation, by the glycerol dehydrogenase assay where fluorescence intensity of NADH was monitored. The glycerol content was previously calibrated using glycerol dehydrogenase by monitoring the fluorescence intensity of NADH at excitation 360nm and emission 450nm on a Kontron spectrofluorimeter.

DBMIB reversal (Fig. 11). The interfering effect of Antimycin was known on the photosynthetic electron transport (Joet *et al.*, 2001). Fig 11 also shows the inhibitory effect of NaCl on DCMU and control fluorescence while it had no effect after DBMIB bypass. This is consistent with our earlier studies on the role of osmotic pressure on the voids in the bilayer necessary for the migration of the diffusible redox intermediates (Mathai *et al.,* 1993).

d. Effect of SHAM on glycerol production

Since *Dunaliella* accumulates glycerol in response to osmotic stress/ salinity (Ben-Amotz *et al*., 1982, Brown *et al*., 1982), glycerol content of the cells was measured as a function of temperature (which promotes diffusion of glycerol) and NaCl, which promotes its synthesis. Data in Fig 12 show that glycerol content decreases with temperature, while it increases with NaCl in the medium as expected.

The effect of light was studied using a brief exposure to actinic light. The cell content of glycerol decreased in the dark and little was released to the medium. On exposure to light, the glycerol content in the cells as well as medium rapidly increased, which again decreased after removal of light. This could suggest that glycerol was synthesized more in light, which was also more permeable to the membrane while the uptake remarkably enhanced in the dark. Fig 12 D shows that the respiratory inhibitors affected the cell content, cyanide < SHAM < propyl gallate at their respective concentrations. Fig 13 shows that the intra- and extra-cellular content of glycerol in ambient light are exponentially related regardless of the compound used and the degree of inhibition, suggesting a common process that links them and the common effect of the inhibitors on the process.

DISCUSSION

Studies relating to the role of alternative oxidase in plant bioenergetics concentrated primarily on the capacity and engagement of the AOX system using inhibitors and/or isotope discrimination assays. Investigations into stress conditions invariably treated "stress" in a qualitative manner making no attempt to quantify the relative roles and performance of respiration, AOX-dependent and –independent. The role of AOX activity in plant growth is also limited by the methodology that did not take into account contribution to growth as explicitly as they attended to the question the relative contribution of AOX to respiration in growth phases (cf. Millar *et al.,* 1998; McDonald *et al.*, 2002).

a. Growth and respiration in a monocot, rice

The studies reported here clearly show that alternative oxidase is involved in the growth of rice in terms of % germination. The effect was differential in the seedlings in that the shoot growth was unaffected while the root growth was definitely inhibited. Respiration showed a rather curious phenomenon in that the inhibitory effect of SHAM increased with osmotic pressure in the less sensitive component of respiration. Besides being highly significant, inhibition at higher osmolality by SHAM increased while that with cyanide did not.

Studies on drought tolerance and salinity are typically based on replicate measurements of biological variables such as growth or respiration at a fixed external osmolality. Further, the justification for the fixed osmolality arises from water transit measurements or osmotic pressure of the soil/medium such as sea water. Assorted physiological measurements such as stomatal opening are assessed to justify the physiological relevance of the studies. Osmotic pressure itself is hardly varied systematically to assess responses nor were such measurements, rarely, if ever, carried out, interpreted quantitatively. This situation has been remedied in a recent set of publications exclusively from our laboratory in this journal and elsewhere, including a detailed analysis of the fallacies in current literature rampant in the published literature (Sitaramam, 2006).

Current experiments revealed that the effect of SHAM as a respiratory inhibitor varies with external osmotic pressure. What is curious is that both the site III (cytochrome *bc* complex) and AOX receive reducing equivalents from the quinone and yet, the CN dependent respiration is osmo- (stretch) sensitive while AOX-mediated respiration appears to be less so as it appears to dominate in the less sensitive domain. This conclusion does imply a judgement on AOX engagement, which needs to be corroborated independently as inhibitor studies are better, suited for capacity measurements. The fact that SHAM inhibition improved with osmotic compression could suggest the involvement of the kinetics of diffusion and binding of SHAM and quinol to AOX and the relative compressibility coefficients for the vicinal voids that permit the migration of the reduced quinone between these two pathways. It offers an interesting possibility that the presence and size of voids would differ for these pathways, which is a physical interpretation of preferential partitioning of the reduced quinone between these two pathways. This is particularly useful while interpreting the observed

remediation of respiration on prolonged incubation in NaCl media.

b. Physiological remediation and a plausible membrane mechanism via voids

The physiological basis of osmotic sensitivity and modulations thereof can only be *via* a relevant thermodynamic volume, which is compressible, i.e., voids. The rice seedlings exhibited a release phenomenon (remediation?) of inhibition in respiration at 1.0 M NaCl and higher concentrations. This 'release' was abolished by SHAM. The effect of salinity is to induce ion uptake, which was the case. But neither water imbibition nor salt accumulation were significantly affected by SHAM showing that neither the influence of SHAM on inhibition of restoration of respiration, nor even the restoration itself, can be explained by water and general ionic fluxes (since specific compartmentalized fluxes cannot be measured in intact seedlings). The mechanism that led to modulation of respiration could well be intramembranous in terms of the physical partitioning of the quinone, as indicated above. We have shown modified polymeric behaviour on dehydration in proteins, membranes and micelles leading to variable content and dynamics of voids *in vitro* (Pradhan *et al.*, 1999, 2003; Sauna *et al.*, 2001; Madhavarao *et al.,* 2001; Madhavarao *et al*., 2000; Rajagopal and Sitaramam, 1998) Induction of voids itself was confirmed by molecular dynamics *in silico* independently subsequent to our studies (Falck *et al.*, 2005 and references therein).

Since the hardshell of atoms cannot be compressed, the changes in the relevant thermodynamic volume could be identified to be the inter-lipidic, physically identifiable, volume. Voids are abundant in lipids and compression of these voids prevents diffusion of the relevant quinone, thereby inhibiting electron transport (Mathai *et al.*, 1993). Even in proteins, wherein, there exists variable degree of free space between the unstructured loops and structures such as helices etc., indeed a small to moderate osmotic susceptibility of activity could be measured, plausibly owing to the interference with the motion of side chains and the so-called random or unstructured loops. The osmotic effect on catalysis arises not from component molecules, but from a restricted diffusion, i.e., movement of parts of a protein inherent to catalysis (Somogyi *et al*., 1984). These so-called 'thermodynamic volume-dependent', relatively small changes in the proteins *per se (<1.0 L/ mole)* as suggested by Parsegian and others (Chong *et al*., 1985; Rand *et al.*, 1993; Parsegian *et al.*, 1986) do not account for the high osmotic sensitivity of the compression of the 'induced' voids in the bilayer itself incidental to energy transduction, which reflects much larger changes (~ 2-12 L/mole) (Rajagopal and Sitaramam 1998; Mathai and Sitaramam, 1989; Natesan *et al*., 2000).

We traced the phenotype for osmotolerance in plants, which includes both drought and salinity and thus measured in a common scale of external osmolality, to the limiting osmolality of sensitive respiration which is a measure of the ability of the plants to continue to breathe in the face of external osmolality. Since osmotic phenomena cannot be linear in bounded structures, it became possible to identify the phenotype as the switch-over point from the sensitive to the insensitive respiration (Sitaramam and Madhavarao, 1997, Sitaramam 2006). The method was found to yield experimental data for the phenotype in units of osmolality, consistent with the known preference of ecologically distinct plants (Sitaramam and Rao, 2006) as also with the known drought and salinity tolerance of traditional cultivars (Sitaramam, Rao and Bhate, 2007). The osmotolerance was shown to vary with the life cycle in *Arabidopsis*, the germinating seed being the most tolerant phase (Sitaramam and Atre, 2007). None of these quantitative studies envisaged a role for SHAM-sensitive respiration in osmotolerance thus far.

Besides the inhibitor sensitivity, SHAM-sensitive alternative oxidase accepts the reducing equivalents directly from the reduced quinone and passes it to oxygen in a pathway which is sensitive to rotenone but not Antimycin or Cyanide, the resultant bypass at site I yielding only one ATP molecule from NADH and not three. The alternative oxidase is often implicated in various stress responses as also in heat production during flowering, its major role being a relief valve for respiration without respiratory control (McDonald *et al.*, 2002). Thus, role of AOX in ATP production and growth was considered to be negative if any, not surprising since respiration itself was hardly considered to be a prerequisite for biomass production, which is primarily relegated to photosynthesis in plant energetics. The studies reported here indicate that the SHAM-sensitive respiration *via* AOX (which should not be confused with AOX itself) does contribute energy to growth and metabolism in the conventional sense. Our studies on *Arabidopsis* showed that budding, vegetative and floral, exhibit osmotic sensitivity (13 L/mole) closer to mitochondrial oxidative phosphorylation (10-12 L/ mole) (Sitaramam and Atre, 2007). The observed thermogenesis during flowering (Meeuse, 1975) is consistent with enhanced respiratory activity (since heat

is a major component of net flux of energy) in solely non-photosynthetic tissues with or without contribution from AOX. The conventional models of respiration as contributing to loss of biomass, which dominate thinking in plant physiology (Thornley, 1970, 1971, Thornley and Cannell, 2000), require a major revision since respiration is a major determinant of growth in specific stages and specific tissues such as the root, which in turn facilitate growth.

c. Glycerol as an osmolyte

Glycerol is highly permeable to cell membranes and its retention by *Dunaliella* cells as well as its leak into medium on inhibition of respiration indicates an active pump-leak mechanism. Data in Fig 13 pooling the inhibitor effects as well their dynamics on the partitioning of glycerol in ambient light shows a clear exponential relationship, consistent with a single process serving this mechanism. SHAM-sensitive respiration, as expected of respiration per se, contributes to retention of glycerol in the cell via utilization of energy (ATP) rather than its synthesis. SHAM as well as other inhibitors all merely aided in the loss of total glycerol to the medium than to the level of total glycerol per cell.

d. Evolutionary implications

The comparison between widely separated eukaryotes, yeast, *Dunaliella* and rice indicates directionality in the observed magnitudes of SHAM effects. The effects of SHAM-sensitive respiration, though small, could be seen in yeast, better in rice, and very dominantly in *Dunaliella*. It stands to reason that the evolution of the AOX pathway presaged the migration of plants from water to land due to its conspicuous role in growth and osmotolerance. In the case of animals, the ability to concentrate urine and the presence of a putative potassium channel in sperm that removes the osmotic inhibitory effects of hypertonic urea on sperms and hence reproduction appears to have facilitated the migration of animals from water to land (Sitaramam and Sauna, 2000). While the mechanisms are vastly different, the osmotic methodology used had much in common in these diverse studies.

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