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Induction of sporulation by sulphate limitation in *Nostoc ANTH*, a symbiotic strain capable of colonizing roots of rice plants

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Nostoc ANTH is a symbiotically compatible cyanobacterium that associates with rice plants and carries out associative N_2-fixation. Investigations were carried out to induce profuse sporulation in the cyanobacterium for use as inocula in rice paddies. Impacts of *p*H and temperature changes, addition of various carbon sources and limitation of phosphate and sulphate on akinete formation were studied. Among these, only phosphate and sulphate limitation induced akinete formation in *Nostoc* ANTH. Under both the conditions all vegetative cells eventually became akinete. However, induction of akinete differentiation was quicker and resulted in more profuse akinetes differentiation in response to sulphate limitation than to phosphate limitation. These akinetes showed long-term viability (upto 5 years) and excellent germination frequency (90-95 %). This is the first report on induction of akinete formation by sulphate limitation.

Keywords: *Nostoc* ANTH, cyanobacteria, akinetes, akinete differentiation, sulphate limitation
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Introduction

Cyanobacteria are an ancient and diverse group of gram-negative eubacteria characterised by higher-plant type oxygenic photosynthesis[1]. They occupy a wide range of habitats as free-living as well in symbiosis with other organisms[2-6]. The potential role of diazotrophic cyanobacteria as biofertiliser in rice paddies has long been recognised[7-8]. In recent years the use of diazotrophic cyanobacteria as biofertiliser is being popularised in several countries including India[9-10]. A major concern in distributing cyanobacterial inocula to farmers is the high mortality of cyanobacterial cultures during storage and transport under field conditions. Akinetes are likely to serve as ideal inocula since they can withstand adverse conditions, can be stored and transported in dry form, and shall not require special storage conditions or packaging. It is, therefore, of great interest to devise strategies for triggering of akinete differentiation in selected diazotrophic cyanobacteria that may lead to quick and profuse formation of akinetes endowed with high viability and germination efficiency. Environmental and nutritional factors which include limitation of nitrogen, carbon, iron, trace elements, and light have been reported as trigger for sporulation in cyanobacteria. In the past, several authors have reported that phosphate limitation is a major trigger for akinete formation in *Anabaena variabilis, A. cylindrica, A. circinalis and Nostoc linckia*[11-17]. However, cyanobacterial strains maintained in laboratory tend to accumulate polyphosphate reserve (polyphosphate bodies) that takes long to deplete. This makes it difficult to create phosphate limitation and trigger akinete formation quickly.

In the present communication we report for the first time that sulphate-limitation triggers profuse akinete formation in *Nostoc* ANTH, a symbiotically compatible heterocystous N_2-fixing cyanobacterium known to associate with rice plants and carry out associative N_2-fixation[18]. Such cyanobacterial strains have high potential as biofertiliser in rice cultivation and can be applied in the form of akinetes having high viability and efficient germination. In addition, this study demonstrates that sulphate limitation is a better trigger than phosphate limitation for akinete formation in *Nostoc* ANTH.

Materials and Methods

Organism and Culture Conditions

Nostoc ANTH was grown from axenic stock culture in N_2-medium (BG-11$_0$ medium[19]) at 25° C with a light intensity (photon fluence rate) of 50 µmol photons.$m^{-2}.s^{-1}$. As and when required, the N_2-

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medium was supplemented with 5 m*M* KNO_3 (nitrate-medium) or 2 m*M* NH_4Cl (ammonium-medium) as sources of combined nitrogen. The medium was always buffered with equimolar concentration of HEPES [4-(2-Hydroxyethyl)-1-piperazine ethane sulphonic acid]. The *p*H of the medium was adjusted to 7.5 before autoclaving.

Culture Condition for Akinete Differentiation

Cultures of Nostoc ANTH were washed three times with BG-11_0 medium minus $MgSO_4$ and allowed to sporulate in the same medium lacking $MgSO_4$. The medium was supplemented with equimolar concentration of $MgCl_2$ to counter the effect of reducing the concentration of $MgSO_4$ so that the combined cation and anion concentration remained the same in all the cultures. As and when required, the medium was supplemented with 5 m*M* potassium nitrate or 2 m*M* ammonium chloride and buffered with equimolar concentration of HEPES. Sporulation experiments were also conducted at different temperature (20°, 25°, 30° 35°, 45° or 50° C) and *p*H (5, 7.5, 9 or 11). The start of akinete differentiation was taken to be the time when akinetes first appeared. The end of akinete differentiation was taken to be when maximum number of cells became akinetes and no further akinete differentiation occurred.

Storage Condition for Akinete

Akinete population was centrifuged and the pellet containing akinetes washed. The pellet was air dried and stored at room temperature.

Culture Condition for Akinete Germination

Akinete population was washed twice and resuspended in fresh BG-11_0 medium at a concentration of 2×10^6 akinetes mL^{-1} and incubated in light at 50 µmol $photons.m^{-2}.s^{-1}$. As and when required, KNO_3 (5 m*M*) or NH_4Cl (2 m*M*) was added as sources of combined N.

Akinete and Heterocyst Frequency and Akinete Viability

Heterocyst and akinete frequency was calculated as percentage of total cell population by light microscopically. The percentage of germinate akinete was determined by examination of at least 1000 akinetes under light microscope. Akinetes that failed to produce germling and remained as single cell, were considered non-viable.

Chlorophyll *a* and Protein Estimation

Chlorophyll *a* was extracted in 90% methanol in darkness at 4°C. The absorbance at 663 nm was measured using a Beckman DU-530 Spectrophotometer and chlorophyll *a* concentration calculated according to Mackinney[20]. Protein was measured according to Lowry *et al.*[21]

Oxygen Exchange

Oxygen evolution/consumption was measured polarographically using a Clark-type oxygen electrode installed in a 3 mL Plexiglass container with magnetic stirrer (Rank Brothers, England). Three mL cyanobacterial culture was added to the sample chamber of the non-polarised electrode and allowed to equilibrate for 5 min while stirring. The electrode was then polarised and the linear rate of oxygen evolution was obtained in light (100 W tungsten).The light intensity at the surface of the sample chamber was 50 µmol $photons.m^{-2}s^{-1}$ Oxygen consumption was measured in dark with the chamber wrapped with aluminium foil. The rate of oxygen evolution and consumption is expressed as nmol O_2 $evolved/consumed.min^{-1}.mg^{-1}$ protein.

Enzyme Activities

Nitrogenase activity was measured as ethylene production using acetylene reduction assay[22]. Glutamine synthetase (transferase) assay was essentially as described by Sampio *et al*[23] except that CTAB (alkyltrimethylammoniumbromide) permeabilised cells were used. Nitrate reductase activity was measured *in situ*[24] using CTAB-permeabilised cells.

Microscopy

Olympus microscope fitted with a JVC digital video camera was used. Microphotographs were taken using excitation filter (BP 545-580 nm). Heterocysts and akinetes did not fluoresce due to lack of phycobiliproteins while vegetative cells gave strong fluorescence. Akinetes were distinguishable from heterocysts since the latter contained distinct polar nodules (Fig.1).

Results and Discussion

Various factors that may trigger akinete formation in *Nostoc* ANTH were tested in the present investigation. When *Nostoc* ANTH was grown in BG-11_0 medium (N_2-medium), no akinete formation was evident at any stage of the growth. Akinete differentiation was not triggered even after altering the *p*H of the medium (from *p*H 7.5 to 5, 9, 11) or temperature at which the cells were cultured (20°, 25°, 30°, 35°, 45° or 50° C). Even when *Nostoc*

ANTH, grown in NH_4^+- or NO_3^-- supplemented BG-11_0 medium (NH_4^+- and NO_3^-- media, respectively), was transferred to BG-11_0 medium (N_2-medium), akinete formation was not triggered. Thus, limitation of combined nitrogen did not cause akinete differentiation (Table 1). Similarly, addition of glucose, fructose or sucrose (each at concentration of 10, 20, 30, and 50 m*M*) to the growth medium was ineffective in triggering akinete formation although such additions prolonged the exponential growth phase (Table 1). However, when N_2-grown cultures of *Nostoc* ANTH were transferred to fresh N_2-medium from which sulphate was omitted (BG-11_0 minus $MgSO_4$), there was no growth and akinete differentiation started within 3 d of the transfer and by day 24, all vegetative cells became akinetes (Table 1, Fig. 2).

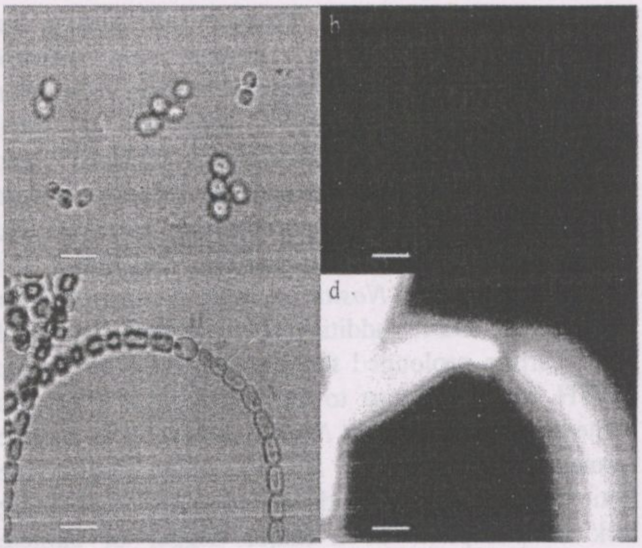

Fig. 1—Light micrograph of *Nostoc* ANTH. a, akinetes; b, same as 'a' under fluorescence (excitation 545-580 nm); c, a filaments showing heterocyst (H) and vegetative cells (V); d, same as 'c' under fluorescence. The absence of flourescence indicates lack of phycobiliprotein in akinetes and heterocysts. Bar = 100µm

Akinete differentiation also occurred when *Nostoc* ANTH from N_2-medium was transferred to N_2-medium lacking phosphate (K_2HPO_4). However, the akinete differentiation was delayed compared to that in the medium lacking sulphate (akinete differentiation started after 8 d of transfer instead of just 3 d). In addition, the akinetes continued to differentiate for a longer period than that in the medium lacking sulphate (35 d instead of 24 d). In the medium lacking phosphate, *Nostoc* ANTH also showed significant level of growth during the initial 7-8 d in contrast to the sulphate lacking medium where no growth was observed (Fig. 2). The initial growth of *Nostoc* ANTH in the medium lacking phosphate can be explained by the fact that repeated subculturing of cyanobacteria in laboratories leads to accumulation of phosphate (polyphosphate bodies) that can be mobilised under phosphate limiting conditions[2]. Therefore, under phosphate-limiting conditions they continue to grow as long as internal reserve of phosphate lasts. Thus, for the first 7-8 d the cells grew in phosphate-limiting medium using internal reserve phosphate, after which growth ceased and akinete formation started. No such reserve for sulphate are known in cyanobacteria, therefore, the effect of sulphate limitation is quicker on cessation of growth and triggering akinete formation. In fact *Nostoc* ANTH lost the ability to form akinetes in

Table 1—Factors affecting akinete differentiation in the cyanobacterium *Nostoc* ANTH

Growth conditions	Observations	[a] Start of akinete differentiation (d)	[b] End of akinete differentiation (d)
BG-11_0	No akinetes were observed during or at the end of exponential phase of growth	-	-
*p*H	No akinetes were observed in culture grown at *p*H 5, 7.5, 9 or 11	-	-
Temperature	No akinete were observed in culture grown at 20, 25, 30, 35, 45, or 50^0 C	-	-
BG-11_0 + exogenous C sources (glucose, fructose or sucrose; 10-50 m*M*	Exogenous C sources prolonged the exponential phase, but no akinete were observed during or at the end of exponential phase of growth	-	-
BG-11_0 minus sulphate	Cessation of growth followed by akinete differentiation	3±1	24±2
BG-11_0 minus phosphate	Akinete differentiation observed during the exponential growth phase	8±1	35±2

[a] Time when akinetes first appeared.

[b] Time when maximum numbers of cells became akinetes and no further akinete differentiation occurred

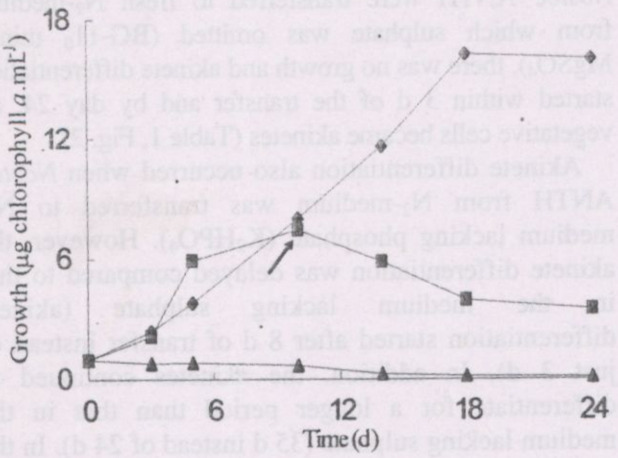

Fig. 2—Growth of *Nostoc* ANTH in $BG\text{-}11_0$ (♦); $BG\text{-}11_0$ minus K_2HPO_4 (■) and $BG\text{-}11_0$ minus $MgSO_4$ (▲) medium. Arrow indicates the start of akinete differentiation (i.e. when akinetes first appeared).

response to phosphate limitation after 3 years of repeated culturing in $BG\text{-}11_0$ medium in our laboratory. However, even after 5 years of repeated culturing in $BG\text{-}11_0$ medium, the same cyanobacterium, *Nostoc* ANTH retained the ability to differentiate akinetes in response to sulphate limitation. In medium lacking phosphate and sulphate, the growth and akinete formation response of *Nostoc* ANTH remained similar to that observed in the medium lacking only the sulphate.

An interesting phenomenon was noticed when these experiments were repeated using medium lacking sulphate that was buffered with 5 mM HEPES. As discussed above, there was no growth and akinete differentiated in $BG\text{-}11_0$ medium lacking sulphate. In contrast, *Nostoc* ANTH grew and no akinete differentiation occurred when the medium lacking sulphate was buffered with HEPES. Similarly, in nitrate-supplement $BG\text{-}11_0$ medium lacking sulphate, there was no growth and akinete differentiation occurred. However, when this medium was buffered with HEPES, there was growth and no akinete differentiation occurred. HEPES contains sulphonic acid and *Nostoc* ANTH may be using it as a source for S, at least under the sulphate limiting conditions, as indicated by the growth of *Nostoc* ANTH in HEPES-buffered media lacking sulphate. These results clearly show that the cessation of growth and triggering of akinete differentiation was due to sulphate limitation and that the addition of HEPES relieved this limitation. When in symbiosis with *Anthoceros*, *Nostoc* ANTH rarely forms akinetes although the growth rate is much slower in symbiosis than when free living. It is unclear what prevents akinete formation by the cyanobiont when in symbiosis and this aspect would merit further study.

Some of the above observations on akinete differentiation in *Nostoc* ANTH are consistent with the observations on akinete differentiation in other cyanobacteria by earlier workers. However, a number of features regarding akinete differentiation in *Nostoc* ANTH are unique and/or in contrast to the features of akinete differentiation in other cyanobacteria. Limitation of light due to the increase in culture density during growth that results in self shading, has been suggested as a trigger for akinete development[11,12,14,26-28]. Furthermore, there have been reports that iron limitation[29], limitation of fixed nitrogen[30, 31] increase in concentration of NaCl[32], and provision of amino acids trigger or increase akinete differentiation in various cyanobacteria. However, the results of the present study indicate that this is not true in the case of *Nostoc* ANTH. As reported for *Nostoc* PCC 7524[27], addition of exogenous sources of fixed carbon prolonged the growth phase of *Nostoc* ANTH, but in contrast to the *Nostoc* PCC 7524, no akinetes were formed in *Nostoc* ANTH. The akinete formation in *Nostoc* ANTH under phosphate limitation is consistent with earlier reports implicating lack of phosphate as a major trigger of akinete development[11,12,14,16].

The triggering of akinete differentiation in *Nostoc* ANTH under sulphate limitation reported in the present investigation is the first report of its kind, and is in contrast to the report by Sinclair and Whitton[29] that sulphate limitation has no effect on akinete differentiation in *Anabaena cylindrica*. It appears that the two cyanobacteria respond differently to sulphate limitation. Both under phosphate limitation and sulphate limitation, the akinete differentiation was associated with cessation of growth of *Nostoc* ANTH. This is consistent with similar observations on *A. cylindrica*[25,33,34], *Nostoc* PCC 7524[27] and *Anabaena doliolum*[35]. Overall, the data indicate that sulphate limitation is a powerful trigger, and better than phosphate limitation, for quicker and more profuse akinete formation in *Nostoc* ANTH.

Certain physiological properties of the akinete population was checked to compare with the earlier studies. The mature akinetes of *Nostoc* ANTH showed a respiratory O_2 consumption rate of 17.6% of that in N_2-grown filaments but lacked photosynthetic pigments and O_2 evolution. These observations are

Table 2—Activities of nitrogen-metabolising enzymes in whole filaments and akinetes of *Nostoc* ANTH

Enzyme	Activity (nmol product formed . min^{-1} mg^{-1} protein)	
	Akinetes	Whole filaments
Nitrogenase	0.0	12.7 ± 0.5*
Glutamine synthetase(transferase)	0.0	768 ± 9.1
Nitrate reductase	0.0	2.7 ± 0.08

*nmol C_2H_4 formed g^{-1} Chlorophyll $a.h^{-1}$

The values presented are means ± SE of two independent experiments

consistent with the findings regarding akinetes of *A. cylindrica*[25,36], *Nostoc* PCC 7524[27,37], *A. doliolum*[35,38] and *Nostoc spongiaeforme*[39]. The mature akinetes of *Nostoc* ANTH also lacked the primary enzymes of inorganic nitrogen metabolism such as nitrogenase, nitrate reductase and glutamine synthetase (Table 2). The lack of these enzymes in mature akinete of *Nostoc* ANTH is also in keeping with the finding on akinete of *A. doliolum*[38] and of other cyanobacteria[40].

To check that these akinetes were viable, we harvested akinetes from sulphate-limiting medium ($BG\text{-}11_0$ lacking sulphate), resuspended them in fresh N_2-medium ($BG\text{-}11_0$) and incubated in light at 25°C. About 25% of the akinetes, in suspension, germinated within 24 h and a germination frequency of 95% was achieved after 96 h. The akinetes showed prolonged viability. Prolonged storage of akinetes in dry state at room temperature did not lead to any significant loss of viability. Over 90% of the akinetes germinated even after 4-5 years of storage at room temperature. When stored at higher temperatures (upto 35°C) or at 4°C, there was still no loss in viability of the akinetes and > 90% of akinetes germinated, even after 5 years of storage.

In summary, the results presented in this investigation clearly showed that sulphate limitation can be used as a trigger to induce quick and profuse akinete formation in *Nostoc* ANTH. The excellent germination rate, even after long-term storage at room temperature, makes these akinetes a good candidate for use as inoculum if *Nostoc* ANTH is used as biofertiliser in rice paddies.

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