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Effect of 7-azatryptophan on heterocyst differentiation in *Anabaena doliolum* Bharadwaja

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Abstract. The effect of DL-7-azatryptophan, an analogue of tryptophan, has been studied on the heterocyst spacing pattern and the probability of proheterocyst regression in *Anabaena doliolum*. 7-azatryptophan suppressed growth and induced heterocyst differentiation in nitrogen-free medium. In ammonium (1 mM), nitrite (2 mM) and nitrate (2 mM) supplemented media, it caused proheterocyst regression with a frequency of 100%, 35% and 10% respectively. The role of azatryptophan in nitrogen metabolism has been discussed in relation to ammonia-uptake study.

Keywords. 7-azatryptophan; heterocyst; differentiation; *Anabaena doliolum*.

1. Introduction

Several filamentous blue-green algae generally show a characteristic pattern of heterocysts oriented in one-dimensional array. Treatment with certain substances or agents can produce deviation from this normal pattern of heterocysts; such abnormalities may include the production of heterocysts in chains or a reduction in the interheterocyst distance. Mitchison and Wilcox (1973) discovered that the tryptophan analogue 7-azatryptophan disturbed or upset the heterocyst pattern in *Anabaena catenula* by both the above methods. They suggested that 7-azatryptophan inhibits the formation of the heterocyst-inhibitory factor responsible for the heterocyst spacing pattern. An even more pronounced effect on heterocyst pattern formation in *Anabaena cylindrica* was observed by Wolk (1975) with rifampicin, an inhibitor of DNA dependent RNA polymerase. He proposed that rifampicin can effect the differentiation of the adjacent vegetative cells both by immature heterocysts (because of formation of short string of heterocysts) and mature heterocysts (because of reduced spacing pattern at a concentration having very little effect on growth). A similar effect on heterocyst spacing pattern was found by Ladha and Kumar (unpublished) using L-methionine-DL-sulfoximine, an analogue of both glutamate and methionine.

The process of heterocyst differentiation generally proceeds in two phases, viz., from vegetative cell to proheterocyst and from the latter to the heterocyst (Tyagi 1975). In the normally differentiating alga two adjacent proheterocysts are inhibited from differentiating into mature heterocysts by competitive interaction between them. As a result, only one differentiates into heterocyst whereas the other assumes the appearance of vegetative cell and undergoes heterocyst regression (Wilcox *et al* 1973a, b). Wilcox *et al* (1973b, 1975) have further studied the competitive process of heterocyst regression by breaking the filaments at regular intervals and treating them with

certain chemicals e.g. 7-azatryptophan and rifampicin. They showed that 7-azatryptophan reduced the frequency of proheterocyst regression and produced paired heterocysts indicating that proheterocyst regression constitutes an integral part of the heterocyst pattern formation.

Proheterocyst regression has thus far been studied in a nitrogen-free medium in the presence of azatryptophan. The present paper reports the studies of proheterocyst regression in media supplemented with inorganic nitrogen sources in the presence of azatryptophan in *A. doliolum.*

2. Materials and methods

The heterocystous, nitrogen fixing and sporulating blue-green alga *A. doliolum* raised in unialgal and clonal culture served as the experimental organism (figure 1A). The stock as well as the experimental cultures were maintained in Allen and Arnon's nitrogen-free medium as per the method described by Ladha and Kumar (1975).

Allen and Arnon's nitrogen-free medium, supplemented with a combined nitrogen source was used with ammonium as ammonium chloride, nitrate as potassium nitrate and nitrite as sodium nitrite at 1 mM, 2 mM and 2 mM concentrations respectively.

To study the differentiation of heterocysts in control and treated populations, a non-heterocystous culture was obtained by growing the alga in medium supplemented with 20 mM of potassium nitrate. Filaments from an exponential culture in nitrogen-containing medium were thoroughly washed with nitrogen-free medium by centrifugation before inoculation. Per cent heterocyst frequency (per hundred vegetative cells) was calculated by taking the average of at least 50 filaments.

Growth was recorded in terms of optical density. The culture was dispensed in 10 ml of 80% aqueous acetone. The tubes were kept overnight in the dark in a refrigerator at 10°C to ensure complete extraction of pigments. Absorbance of the cultures was measured in a Bausch and Lomb 'Spectronic 20' spectrocolorimeter at 663 nm.

The uptake of ammoniacal nitrogen from the culture medium was estimated using Nessler's reagent (Sigma grade).

DL-7-azatryptophan was obtained from Sigma Chemical Company, Missouri, USA. Its solution was sterilized by membrane filtration before use.

3. Results

3.1. *Effect on growth*

Table 1 shows the effect of azatryptophan on growth. Higher concentrations (10^{-3}M and 10^{-4} M) were inhibitory both in nitrogen-free and nitrogen-containing media. However, different inorganic nitrogen sources partially relieved such inhibition.

3.2. *Effect on uptake of ammoniacal nitrogen*

Figure 2 shows the effect of azatryptophan on growth in the presence of ammoniacal nitrogen and also the uptake of ammonia from the culture medium. At 10^{-3} M

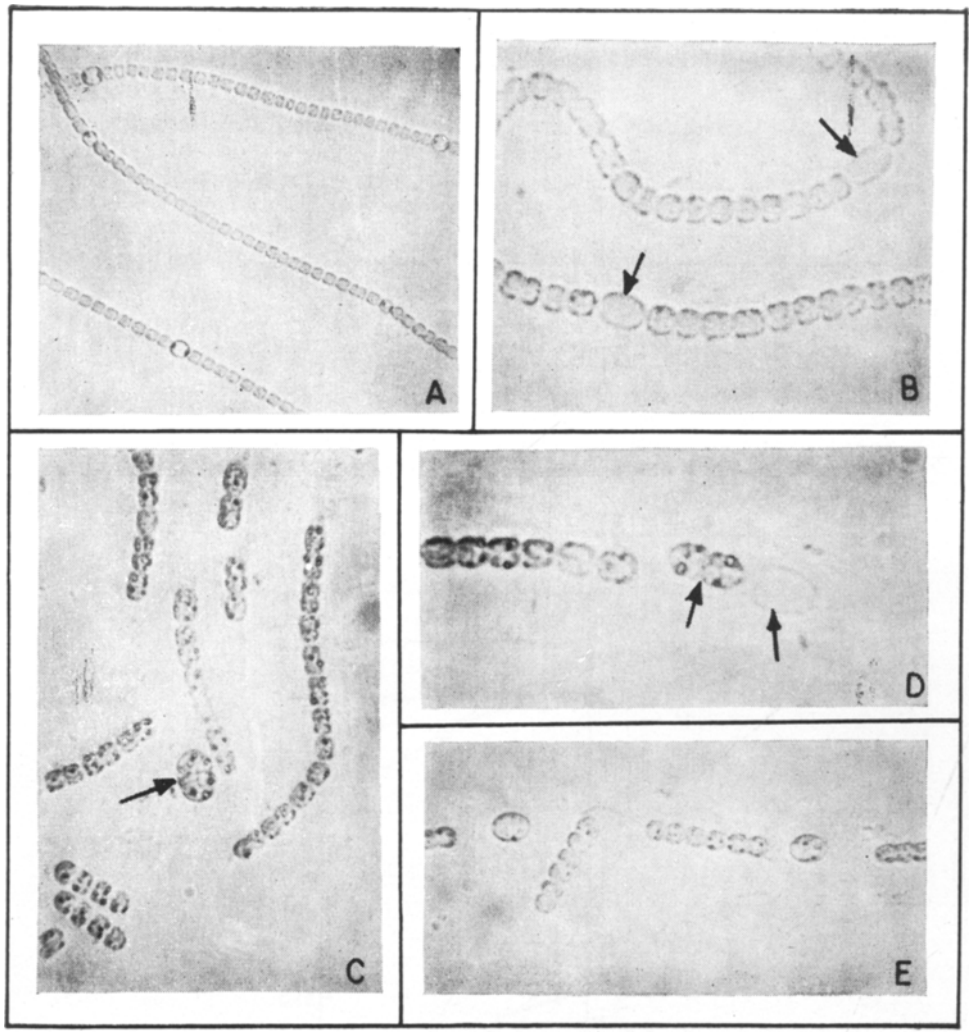

Figure 1

A *Anabaena doliolum* in nitrogen-free medium showing normal heterocyst pattern (X 250).

B Filaments with differentiated proheterocysts in the presence of 10^{-3} M azatryptophan in ammonium-medium (X 600).

C Detached proheterocyst showing transverse division in ammonium-medium with 10^{-3} M azatryptophan (X 450).

D & E Germination of proheterocysts with filaments coming out of the prohetrocystous wall in ammonium-medium containing 10^{-3} M azatryptophan (X 750 & 450).

azatryptophan enhanced the uptake of ammoniacal nitrogen. Almost all the ammonia from the medium was exhausted after the third day and then the level remained constant. After the sixth day, the level of ammonia in the medium again increased and was maximum on the twelfth day following treatment. In 10^{-4} M azatryptophan, the uptake of ammonia from the medium was equal to that in the control upto the sixth day, but thereafter the level of ammonia in the medium was comparatively higher

Table 1. Growth (Absorbance at 663 nm) and per cent proheterocyst germination of *A. doliolum* in varying concentrations of DL-7-azatryptophan in different inorganic nitrogen sources.

Concentration of 7-azatryptophan (M)	Control Nitrogen-free medium	Nitrogen sources		
		NH_4^+	NO_2^-	NO_3^-
—	0·43	0·31	0·31	0·32
10^{-3}	0·05	0·15	0·21	0·24
10^{-4}	0·14	0·21	0·25	0·25
10^{-5}	0·43	0·29	0·29	0·27
10^{-6}	0·45	0·31	0·31	0·30
% proheterocyst germination	—	100	35	10

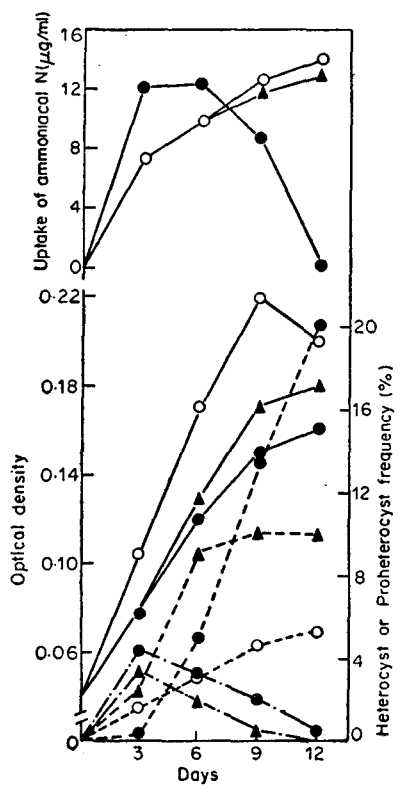

Figure 2. Uptake of ammoniacal nitrogen, Growth (——), heterocyst (- - - -) and proheterocyst (—·—·—) frequency in control (o), 10^{-3} M (●) and 10^{-4} M (▲) 7-azatryptophan in *A. doliolum*.

than in the control. In the control, on the other hand, there was continued uptake leading to complete exhaustion of ammonia from the medium.

3.3. *Effect on heterocyst formation*

In a nitrogen-free medium, azatryptophan induced frequent heterocyst formation (upto 30% as compared to 5-6% in control) by forming series of heterocysts which

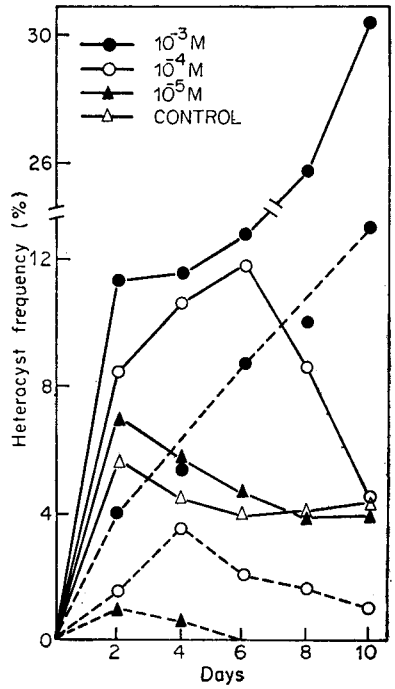

Figure 3. Effect of azatryptophan on the frequency of heterocyst (——) and double heterocyst (- - - -) formation in nitrogen-free medium.

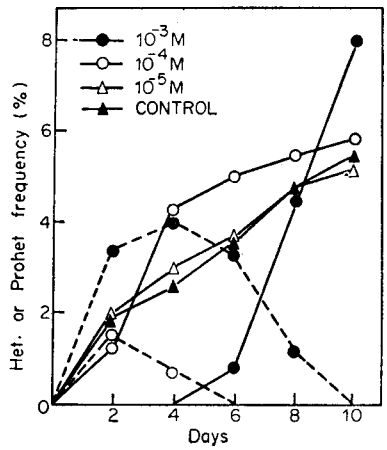

Figure 4. Effect of azatryptophan on heterocyst (——) and proheterocyst (- - - -) frequency in nitrite supplemented medium.

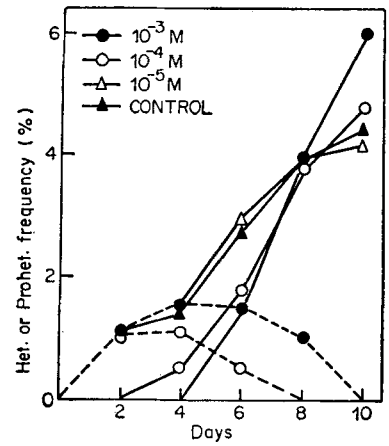

Figure 5. Effect of azatryptophan on heterocyst (——) and proheterocyst (- - - -) frequency in nitrate-medium.

Table 2. Effect of DL-7-azatryptophan (ATN) on *A. doliolum* in nitrogen-free (AA-N) ammonium (AA-NH_4), nitrite (AA-NO_2) and nitrate supplemented (AA-NO_3) media

Conditions	Control AA-N	AA-N+ 10^{-3}M ATN	AA-NH_4	AA-NH_4+ 10^{-3}M ATN	AA-NO_2	AA-NO_2+ 10^{-3}M ATN	AA-NO_3	AA-NO_3+ 10^{-3}M ATN
Growth	Normal	V. less	Normal	Less	Normal	Less	Normal	Less
Heterocyst differentiation	Normal	Normal	Normal	Prohet.	Normal	Het.+ prohet.	Normal	Het.+ prohet.
Het. or prohet. frequency	Normal	5-6 fold increase	Decrease	3-4 fold increase	Decrease	Slightly increased	Decrease	Slightly increased
Formation of joined or multiple heterocysts	No	Yes	No	No	No	No	No	No
Fragmentation	No	Yes	No	Yes	No	Yes	No	Yes
Germination of proheterocyst	No	No	No	100%	No	35%	No	10%

were closely spaced, on the average, than in medium lacking azatryptophan (figure 3). At a later stage, azatryptophan also caused fragmentation of filaments which generally occurred between two heterocysts (table 2).

Unlike in some other blue-green algae, in *A. doliolum* heterocysts differentiated in the presence of low concentrations of different inorganic nitrogen sources (in 1 mM of NH_4^+, and 2 mM of NO_2^- and NO_3^-) (figures 2, 4 and 5). Higher concentrations of azatryptophan (10^{-3} M) in ammonium medium resulted in the formation of characteristic larger cells having comparatively thicker walls than vegetative cells (figure 1B). These ' larger cells ' which seemed to be immature heterocysts (or proheterocysts) differentiated just after a period of 24 hr, at more or less regular intervals, in conformity with the known heterocyst spacing pattern. After formation, these proheterocysts got detached from the filaments and this was followed by their *in situ* division (figure 1C) resulting in the formation of 2-4 celled filaments which resembled spore-germlings

that had escaped out of the proheterocysts leaving their thick-walled envelopes behind (figure 1D and E). Sometimes, the whole content of proheterocyst emerged out as a single cell and then divided outside the proheterocystous wall. Those proheterocysts which failed to get detached from the mother filament differentiated further into mature heterocysts and they never showed regression.

In the presence of nitrate and nitrite similar results of proheterocyst division followed by germination were obtained though with a low frequency (table 2).

Figure 2 shows that as long as there was ammonium uptake from the medium containing 10^{-3} M azatryptophan, proheterocyst frequency was inversely proportional to the heterocyst frequency but when the ammonium concentration in the medium increased after the 6th day, the heterocyst frequency rose (20%) and proheterocyst frequency fell (4-5%) in comparison to control. In 10^{-4}M azatryptophan, heterocyst frequency also increased at a later stage when the ammonia level in the medium went up. Similar observations were made with nitrate and nitrite nitrogen (figures 4 and 5).

4. Discussion

On the basis of their observation of 7-azatryptophan effect on *A. catenula*, Mitchison and Wilcox (1973) concluded that azatryptophan substituted for tryptophan in the normal proteins and produced defective proteins which led to a change in the heterocyst pattern.

In *A. doliolum* in the present study, there was little growth in nitrogen-free medium containing high concentrations of azatryptophan but the heterocyst frequency was increased. The azatryptophan toxicity was partially reversed by different inorganic nitrogen sources. This indicated that azatryptophan might be responsible for the formation of defective proteins (Mitchison and Wilcox 1973) and perhaps, defective enzymes (Pardee and Prestridge 1958) of both nitrogen reduction and nitrogen assimilation pathways. The nitrogen assimilation pathway seemed to become completely blocked at later periods in the ammonium medium in the presence of 7-azatryptophan, thereby resulting in a high level of ammonium in the medium and a consequent steep rise in heterocyst frequency. Azatryptophan also seemed to block neither the enzymes of nitrogen reduction pathway nor ammonia assimilation pathway. If azatryptohan had impaired the enzymes of only the nitrogen reduction pathway then its toxicity to growth would be expected to be completely reversed by the inorganic nitrogen sources.

What might be the basis of formation of multiple heterocysts? Ladha and Kumar (unpublished) observed frequent production of multiple heterocysts both in nitrogen-free and ammonium-containing media when L-methionine-DL-sulfoximine (MSO) was added to the medium. MSO is an inhibitor of the primary amination pathway in blue-green algae (Stewart and Rowell 1975). This suggested that there could be an inhibitory substance (not ammonia) operating conversely to the process of heterocyst differentiation (Fogg 1949; Fritsch 1951; Wolk 1967). A fall in the level of this substance might be responsible for the formation of multiple heterocysts. Our present results seem to reinforce this idea because any decline in the concentration of the inhibitor of heterocyst spacing could be produced in two ways, viz. (i) by inhibiting nitrogenase activity, or (ii) by inhibiting the ammonia assimilatory pathway.

Wilcox *et al* (1973b) have suggested that a proheterocyst in an advanced stage of

development could be made to regress if completely isolated. Our results indicate that azatryptophan causes proheterocyst regression by germination in which azatryptophan seems to be responsible for the proheterocyst detachment whereas the combined nitrogen seems to bring about regression. However, the mechanism of how azatryptophan induces proheterocyst detachment remains unclear. Being the immediate product of nitrogen fixation, ammonia may cause heterocyst regression at a higher frequency than do nitrate or nitrite nitrogen in the presence of 7-azatryptophan.

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