Short Communication

Spirulina nitrate-assimilating enzymes (NR, NiR, GS) have higher specific activities and are more stable than those of rice

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ABSTRACT

Spirulina platensis, a cyanobacterium whose N-metabolic pathway is similar to that of higher plants like rice (*Oryza sativa*), produces tenfold more protein, indicating a higher capacity for nitrate utilization/removal. Our *in vitro* analyses in crude extracts revealed that this can be attributed, at least in part, to the higher specific activities (3-6 fold) and half lives (1.2-4.4 fold) of the N-assimilating enzymes, nitrate reductase (NR), nitrite reductase (NiR) and glutamine synthetase (GS) in *Spirulina.* **[Physiol. Mol. Biol. Plants 2008; 14(3) : 179-182]** *E-mail : raghuram98@hotmail.com*

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INTRODUCTION

Spirulina platensis has tremendous importance in nutritional, industrial and environmental biotechnology (Vonshak, 1997) and is best known for its high protein content (60-70 % by dry wt.). But it is not clear how the organism steers its nitrogen metabolism to produce so much protein, except that it is a nitrate-utilizing, non-nitrogen fixing, photosynthetic organism, comparable to higher plants in this respect. The nitrate-utilizing ability of *Spirulina* has also been exploited in the decontamination of nitrate-polluted waters and effluents (Kim *et al.,* 2000; Lodi *et al.,* 2003), though the biochemical basis for this is not well understood.

Nitrate assimilation involves its uptake and sequential reduction by nitrate reductase (NR) and nitrite reductase (NiR) into ammonium ions, which are then incorporated into amino acids mainly by the glutamine synthetase/glutamate synthase (GS/GOGAT) pathway. In higher plants, these enzymes are induced by nitrate and regulated by light, hormones, sugars and carbon and nitrogen metabolites (Stitt *et al.,* 2002; Raghuram *et al.,* 2006). Their genes have been cloned from many plants and mutants and transgenic lines are also available (Andrews *et al.,* 2004; Lam *et al.,* 1996; Lochab *et al.,* 2007). Cereals like rice are poor in their nitrogen use efficiency (25-30 %) and grain protein content (~6 %). Radiotracer studies suggested that *indica* rice utilizes nitrate better than ammonia, the predominant form of N in flooded fields fertilized with urea (Kronzucker *et al.,* 2000). The enzymes and genes of N-metabolism in rice are known (Choi *et al.,* 1989; Sakamoto *et al.,* 1989; Goto *et al.,* 1998) and their regulation by N metabolites and signaling processes have also begun to be understood (Ali *et al.,* 2007a,b).

Spirulina contains at least 10-fold higher protein than rice, indicating a correspondingly high capacity for nitrate utilization. However, its biochemical basis is not known (Vonshak, 1997), except nitrate induction of NR and its inhibition by nitrite and ammonium ions (Jha *et al.,* 2007), characterization of NiR (Yabuki *et al.,* 1985), and regulation of nitrate assimilation by calcium and phosphate (Singh and Singh, 2000). Therefore, we carried out simultaneous comparison of NR, NiR and GS in *Spirulina* with those of rice as a basis for further studies.

MATERIALS AND METHODS

Crude extracts were prepared from exponential shake flask cultures of axenic *S. platensis* (strain ARM 729) in

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Zarouk's medium and NR and NiR activities in *Spirulina* were assayed as nitrite formation and consumption respectively (Jha *et al*., 2007). Rice crude extracts were prepared from excised leaves of 11-day old seedlings of *Oryza sativa* var. Panvel 1 and assayed for NR and NiR (Ali *et al.,* 2007a and b). GS was assayed in both organisms (Shapiro and Stadman, 1970) as phosphate released. The concentration of protein in the crude extracts of rice and *Spirulina* was maintained at ~5 mg/ml for effective comparison. NR and NiR specific activities were defined as nmoles of nitrite produced or consumed mg protein^{-1} min^{-1} respectively. GS specific activity was defined as mg of phosphate released mg protein^{-1} min^{-1}.

RESULTS

To compare the specific activities of N-metabolizing enzymes in *Spirulina* and rice, crude extracts were prepared and assayed for NR, NiR and GS activities. For stability studies, the extracts were kept at RT and 4 ºC and monitored for the activities of above enzymes at different intervals (2-24 hrs).

Specific activities of NR, NiR and GS

In freshly prepared extracts, *Spirulina* NR, NiR and GS had mean specific activities of 3.48, 31.2 and 6.6 units respectively, as compared 1.08, 5.11 and 1.21 units in those of rice (Fig. 1). Clearly, the specific activities were higher in *Spirulina* by at least 3, 6 and 5 fold for NR, NiR and GS respectively, as compared to those of rice.

Stabilities of NR, NiR and GS

NR: *Spirulina* NR activity remained stable for 8 hrs at 4 ºC as well as at RT and decreased slightly at 10 hrs. The enzyme retained 76 % of its initial activity even after 24 hrs at 4 ºC, whereas at RT it fell to 44 % with a half–life of 22 hrs (Fig. 2 A). In case of rice, the NR activity in rice decreased continuously at RT with a half-life of 5 hrs and reached to 2.24 % of the initial level by 10 hrs, whereas at 4 °C the enzyme retained 61 % of its original activity even after 24 hrs (Fig. 2 B).

NiR: With time, *Spirulina* NiR retained 71 % of its activity after 24 hrs at 4 ºC whereas at RT, it retained only 62.19 % activity after 12 hrs and the activity decreased to almost 27 % by 24 hrs, with a half-life of 13 hrs (Fig. 2 C). In case of rice, NiR was very stable at 4 ºC even after 24 hrs, whereas at RT, the enzyme lost half of its activity by 12 hrs and retained only 23 % of its original activity by 24 hrs (Fig. 2D).

GS: GS in both organisms lost about half of its activity in 24 hrs at 4 °C, whereas at RT, *Spirulina* GS retained only 24 % of its activity after 24 hrs, with a half-life of 12 hrs (Fig. 2 E). In case of rice, GS activity was lost by 91 % in 24 hrs at RT, with a half-life of 5 hrs (Fig. 2 F).

DISCUSSION

The protein content of an organism depends critically on the availability of all the amino acids in requisite proportions, which in turn depends on the balance between assimilation of nitrate into ammonia and the incorporation of the latter into amino acids. Under situations in which photosynthetic carbon fixation is not limiting, the utilization of organic acids towards amino acid synthesis in plants and cyanobacteria is limited by the availability of nitrate in the environment and its assimilation into ammonium ions inside the cell (Stitt *et al.,* 2002). Our results based on *in vitro* analysis of enzymes in crude extracts suggest that the relatively higher specific activities of NR, NiR and GS contribute to the far higher nitrate utilizing capacity of *Spirulina* as compared to that of rice leaves. This is further enhanced by the higher stabilities of NR and GS in *Spirulina* at RT, assuming that the *in vitro* stabilities of these enzymes in crude extracts are a reflection of their longer half-lives *in vivo*, pending further validation. Unlike in *Spirulina*, the activities of NR and GS declined in rice extracts despite the presence of protease inhibitors, ruling out any role of proteases. Moreover, our data from another strain of *S. platensis* indicate that the higher stabilities of NR, NiR and GS are also true at

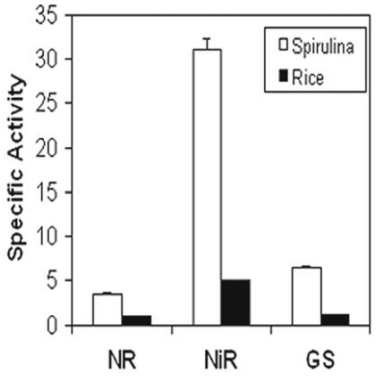

Fig. 1. Comparative chart of the specific activities NR, NiR and GS in *Spirulina* and rice. Crude extracts from exponential phase *Spirulina* culture and nitrate-treated excised leaves from 11 days-old rice seedlings were used to assay for Nitrate reductase (NR), Nitrite reductase (NiR) and Glutamine synthetase (GS) specific activities. The mean data from three different experiments are shown along with std. error bars.

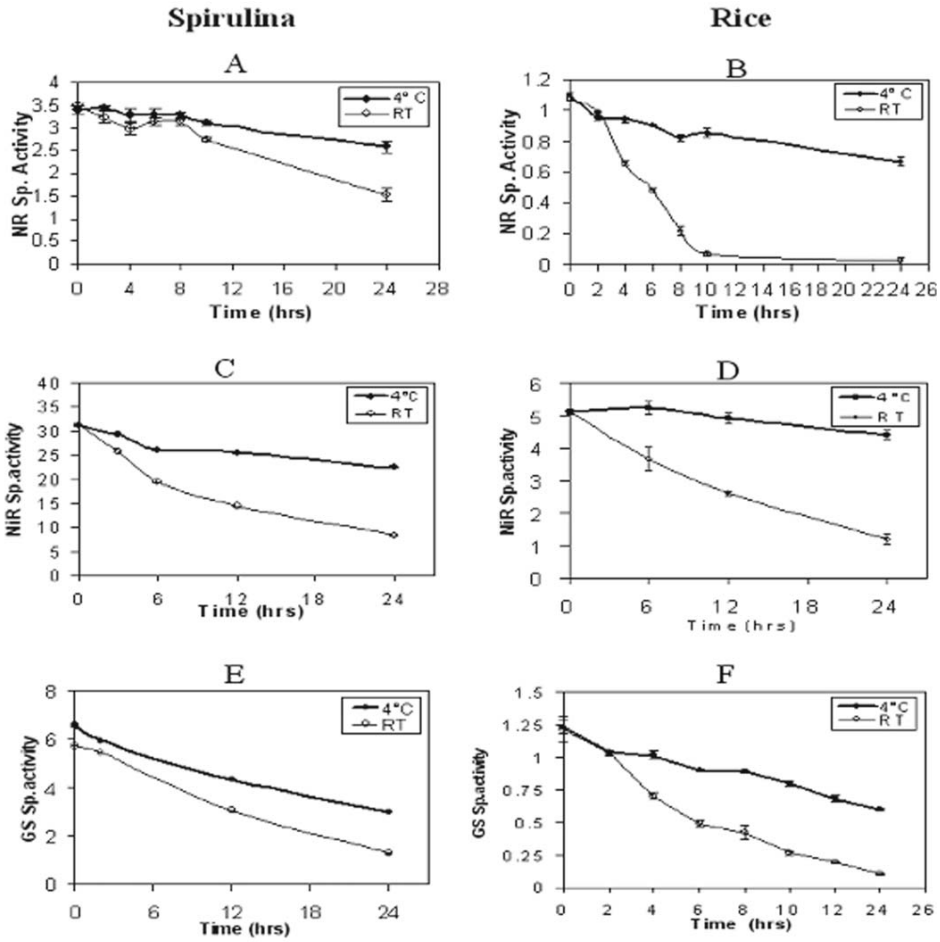

Fig. 2. Effect of temperature on the specific activities of N-assimilatory enzymes in *Spirulina* and Rice. A&B represent NR, C&D represent NiR and E&F represent GS in both the organisms. Crude extracts were prepared from exponential phase *Spirulina* cultures and nitrate-treated excised leaves from 11 days-old rice seedlings, which were then kept at RT or 4 °C and enzyme activities were measured at different time intervals. The mean data from 2 different experiments are shown (±SE).

higher temperatures, and NR stability remains unaffected by partial purification (Lochab, unpublished). Further studies on peptide sequences or purified enzymes may reveal whether the differences in stabilities are inherent to the enzymes of rice and *Spirulina*, or other factors.

NR is known to be a low abundance, unstable enzyme (Campbell, 1999), as corroborated by our rice data. Our results show that *Spirulina* could be an attractive natural source of NR for environmental, diagnostic and other applications (Glazier *et al.,* 1998; Kim *et al.,* 2000; Chuntapa *et al.,* 2003; Lodi *et al.,* 2003), considering that it has over 3 fold higher specific activity and almost 5 fold higher stability than that of rice, with a half-life of over 22 hrs at room temperature *in vitro*. The specific activity of NiR in *Spirulina* is not only six-fold higher than that of rice, but the fold difference between NiR and NR is far higher in *Spirulina* (9 fold) than in rice (5 fold). This may reflect the ability of *Spirulina* to effectively prevent the accumulation of nitrite, which is toxic to the cell.

GS is relatively more abundant in higher plants, with a plastidic isoform (GS2) playing a predominant role in primary nitrate assimilation in leaves and a cytosolic isoform (GS1) being more involved in secondary assimilation (Miflin and Habash, 2002). Recent QTL studies strongly suggest a role for GS1 in determining grain protein content in cereals like rice (Raghuram *et al.,* 2006; Lochab *et al.,* 2007). What is striking is that GS specific activity in *Spirulina* is over five fold higher than the combined activity of GS1 and GS2 in rice, and also has a higher stability, with a half-life of over 12 hrs as compared to 5 hrs in rice. This may contribute

significantly to the higher protein content in *Spirulina*, especially due to the lack of organelles and intracellular transport constraints that separate primary and secondary assimilation in higher plants. The prokaryotic, cellular architecture of *Spirulina* may also provide other advantages such as better coordination between transcription and translation, as well as between carbon and nitrogen assimilation.

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