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Differential Carbon Monoxide Sensitivity of Cytochrome Oxidase in High and Low Nitrogen Use Efficient Wheat (*Triticum aestivum* L.) Genotypes

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ABSTRACT

A small increase in cytosolic NADH can influence the nitrate reduction and thus appears to be critical for improving nitrogen use efficiency. The differential carbon monoxide (CO) sensitivity of high and low nitrogen use efficient wheat genotypes and relatively higher induction of CO response in low nitrogen use efficient wheat genotypes by prior infiltration with 2,4 dintro phenol before CO exposure suggest differences in redox state of cytochrome oxidase during in situ respiration. The process of oxidative phosphorylation appears to tightly coupled in mitochondria of nitrogen use inefficient wheat genotypes due to low NADH/ NAD+ ratio as evident from oxidized state of cytochrome oxidase which does not form complex with CO, whereas in high nitrogen use efficient wheat genotypes the non-energy conserving alternative dehydrogenases appears to be more active due high matrix reduction level with cytochrome oxidase in the more reduced form.

Key words: *CO sensitivity, Cytochrome oxidase, NADH, Nitrate reductase, Nitrogen use efficiency*

Introduction

Identification of new traits for improvement of nitrogen use efficiency (NUE) of cereals is an important aspect both from economic and environmental considerations. The process of nitrate reduction catalyzed by nitrate reductase (NR) limits nitrate assimilation and thus nitrogen use efficiency. The ability of mitochondria or chloroplasts to transfer the reductant to the cytosol seems to be a crucial factor in regulating the rate of nitrogen assimilation, as the Km NADH of NR is significantly higher than the cytosolic NADH level. Nitrate was significantly reduced under aerobic conditions when the mitochondrial electron transport chain was inhibited by carbon monoxide, leading to increased in vivo nitrate reductase activity in response to TCA intermediates (Ramrao *et al*., 1981; Sawhney *et al*., 1978). The work with Nicotianasylvestris CMS mutant which respires through low- affinity NADH-dehydrogenases suggest that modulation of NADH use by the mitochondrial electron transport chain exert influence on nitrate assimilation and integration of carbon and nitrogen metabolism. The coordinated increase in AOX, cellular NADH/NAD+ ratio, N Dex and nitrogen-rich amino acids in complex I defective CMS mutants is an example of communication between nitrogen and redox metabolism (Dutilleul *et al*., 2005). Plant mitochondrial oxidative phosphorylation is characterized by alternate electron transport pathways that can dissipate reductants partially even under high energy levels. Non-

energy linked enzymes serve to maintain the cellular reduction state in the independent of ATP generation (Rasmusson *et al*., 2020).

Carbon monoxide (CO) is an inhibitor of cytochrome oxidase as it forms a binary complex with cytochrome a 3 only when it is in the reduced state. In tightly coupled mitochondrial cytochrome a3 is most likely in the oxidized form by donating electrons to O_2 and thus unable form a complex with cytochrome a3. When CO forms adduct with cytochrome a3 (reduced) the NADH are exported to cytosol for reduction of nitrate to nitrite. Thus accumulation of nitrite under CO-aerobic condition is indication of redox state and thus tightness of coupling (Naik *et al*., 1992, 1998).

Complex I in plant mitochondria has a 10 fold lower Km for NADH than NDA mediated N Din activity, implying that complex I is responsible for the basal rate of NADH oxidation, particularly at low NADH/NAD+ levels, whereas NDA proteins are only active when the matrix reduction level is very high (Senkler, 2017). Plant responses to nitrogen form and availability can thus be influenced by changes in mitochondrial redox processes. It has been recently reported that nitrate assimilation limits nitrogen use efficiency in maize (Loussaert *et al*., 2018). They reported increase in NADH level by selective co-suppression of ubiquinone oxidoreductase 51 k Da subunit of complex I which is associated with increase in vivo nitrate reductase activity. Thus, mitochondrial impacts on NAD status have a significant impact on the rate of nitrate assimilation. The present study is an attempt to understand basic differences in mitochondrial electron transport and nitrogen use efficiency.

Materials and Methods

Plant material

The seeds of high-nitrogen-use efficient wheat genotypes (HD-2781 and NIAW-1994) and low-nitrogen-use efficient wheat genotypes (C-306 and HD-2932) were obtained from the Division of Plant Physiology, IARI, New Delhi, and wheat breeder Niphad. The soil was taken from a field where the previous crop was *Zea mays* L., and the N, P, K, pH, and EC levels were analyzed.

Experiment details

The experiment was conducted using three nitrogen levels (N+, 0.537 g urea/pot; N-, 0.270 g urea/pot; and N0, 0 g urea/pot); 0.619 g SSP/pot; and 0.110 g M.O.P./pot. The plants were raised in small pots in medium-black soil in full sunlight and were irrigated daily with 15 mM KNO_3 to achieve sufficient nitrate accumulation in the leaves. The carbon monoxide (CO) reaction was examined using leaves from 15-day-old seedlings.

Enzyme analysis methods

The in vivo nitrate reductase assay was performed as described by Jaworski (Jaworski, 1971), and the in vitro assay was performed as described by Hageman and Huckles (Hageman and Huckles, 1971). The NADH/NAD^+ ratio were analyzed by Matsumura and Miyachl method (Matsumura and Miyachl, 1980). For carbon monoxide sensitivity, the leaf discs were exposed to a CO: O_2 ratio of 30 and incubated in the dark for 30 min (Naik and Nicholas 1986). To examine the effect of 2,4-dinitrophenol (DNP), whole leaves were dipped in 2 mM DNP and vacuum infiltrated for 10 min. The leaf seg-

Table 1. Nitrate reductase activity in high and low NUE wheat genotypes at different nitrogen levels

Treatment	*In vivo* NRA (µmol NO2- g^{-1} fr wt h^{-1})		*In vitro* NRA (µmol NO2- g^{-1} fr wt h^{-1})	
	HD-2781	C-306	HD-2781	C-306
N+	2.30	2.08	6.28	3.70
N-	2.15	1.79	4.92	3.42
N0	1.02	0.70	1.83	1.37
Mean	1.82	1.52	4.34	2.83
Comparison	SEm (+)	CD@5%	SEm (+)	CD@ 5%
Genotype	0.02	0.07	0.01	0.04
Treatment	0.03	0.08	0.02	0.05
G x T	0.04	0.01	0.02	0.07

ments were then exposed to a CO/O_2 ratio of 30 for 30 min, and the nitrite content was then estimated.

Results and Discussion

In vivo NR activity at optimum level of nitrate in the incubation medium was significantly less than the in vitro NR activity where both NADH and nitrate were optimum does suggest that NADH availability is a critical factor for nitrate assimilation at suboptimal or optimum level of nitrogen (Table 1). The in vivo NR activity and the NADH level were much higher in high nitrogen use efficient wheat genotypes (Table 2). In spinach leaves, NO3- reduction in situ is primarily limited by cytosolic NADH in a nitrate-sufficient environment (Pollok *et al*. 1989). The high and low nitrogen use efficient wheat genotypes showed differential response to carbon monoxide in absence and presence of 2,4 dinotrophenol (2,4 DNP) an uncoupler of oxidative phosphorylation (Table 3).

The cytochrome a3 of low nitrogen use efficient wheat genotypes (C-306 and HD-2932) was in the oxidized state due to tightly coupled mitochondria and could not form complex with CO. It was insensitive to CO as nitrite was not accumulated under co-aerobic incubation. However, pretreatment with 2,4DNP before CO exposure caused significant accumulation of nitrite thus confirming relative tightness of coupling. Thus, at low matrix NADH/NAD+ ratio the basal rate of NADH oxidation is mainly through complex I. The cytochrome a3 of the high nitrogen use efficient wheat genotypes (HD-2781 and NIAW-1994) was more in the reduced state as evident from significant accumulation of nitrite under CO-aerobic incubation. Thus at higher NADH/NAD+ ratio alternative dehydrogenase(s) are more active and can dissipate reductants.

From the results it appears that cytosolic NADH availability and the basal rate of NADH oxidation is critical for nitrate assimilation in high nitrogen use efficient wheat genotypes. The mitochondria of high nitrogen use efficient wheat genotypes were insensitive to carbon monoxide thus indicating relatively less tightness of coupling due to high matrix reduction level NADH/NAD+. The alternative low affinity NADH dehydrogenases rather than complex I thus are active under high matrix reduction level in high nitrogen use efficient wheat genotypes. The findings of the present investigations thus suggest that modulation of NADH use or manipulation of complex I, a major sink for NADH/partitioning of electrons through low affinity alternative dehydrogenases through genome editing can improve nitrogen use efficiency.

Table 2. $NADH/NAD^+$ ratio in high and low NUE wheat genotypes at different nitrogen levels

Sr. No.	Treatment	HD-2781 NADH / NAD^+ ratio	C-306 NADH/ NAD^+ ratio
1	N+	0.84	0.77
2	N-	0.73	0.62
3	N0	0.46	0.38
Mean		0.67	0.59
Comparison		SEm (+)	CD @ 5%
Genotype (G)		0.01	0.01
Treatment (T)		0.01	0.02
G x T	0.01	0.03	

Abbreviations

CO- Carbon monoxide, AOX- Alternative oxidase, NUE- Nitrogen use efficiency, NR-Nitrate reductase, TCA- Tricarboxylic acid, CMS- Cytoplasmic male sterility, N Dex-NADH dehydrogenase.

Table 3. Carbon monoxide sensitivity of cytochrome oxidase in high and low NUE wheat genotypes

Treatment	*In vivo* NRA (µmol NO2- g^{-1} fr. wt h^{-1})			
	HD-2781	C-306	NIAW-1994	HD-2932
Anaerobic assay	2.08	1.84	1.90	1.67
CO aerobic assay	0.49	NIL	0.42	NIL
DNP CO aerobic assay	0.81	1.20	0.57	0.96
Mean	1.12	1.01	0.96	0.87
Comparison	SEm (+)	CD @ 5%	SEm (+)	CD @ 5%
Treatment (T)	0.01	0.02	0.01	0.02
Genotype (G)	0.01	0.03	0.01	0.02
G x T	0.01	0.04	0.01	0.03

Declarations

Conflict of interests - The authors declares that there is no conflict of interests.

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