

**BIOCHEMICAL AND MOLECULAR BASIS OF DIFFERENTIAL  
NITROGEN USE EFFICIENCY IN CONTRASTING WHEAT  
(*Triticum aestivum* L.) GENOTYPES**

by

**Miss. Abuj Bhagyashree Bhaskarrao**  
(Reg. No. 018/49)

A Thesis submitted to the  
**MAHATMA PHULE KRISHI VIDYAPEETH,  
RAHURI - 413 722, DIST- AHMEDNAGAR,  
MAHARASHTRA, INDIA.**

In partial fulfilment of the requirements for the degree

of

**DOCTOR OF PHILOSOPHY (AGRICULTURE)**

in

**BIOCHEMISTRY**



**DEPARTMENT OF BIOCHEMISTRY  
POST GRADUATE INSTITUTE  
MAHATMA PHULE KRISHI VIDYAPEETH  
RAHURI- 413 722, DIST-AHMEDNAGAR  
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**2023**

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**2023**

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I hereby declare that this thesis or part  
there of has not been submitted  
by me or other person to any  
other University or Institute  
for a Degree or  
Diploma

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## CERTIFICATE

This is to certify that the thesis entitled, “**BIOCHEMICAL AND MOLECULAR BASIS OF DIFFERENTIAL NITROGEN USE EFFICIENCY IN CONTRASTING WHEAT (*Triticum aestivum* L.) GENOTYPES**”, submitted to the Faculty of Agriculture, Mahatma Phule Krishi Vidyapeeth, Rahuri, Dist. Ahmednagar (Maharashtra) in partial fulfilment of the requirement for the award of the degree of **DOCTOR OF PHILOSOPHY (AGRICULTURE)** in **BIOCHEMISTRY**, embodies the results of a piece of bonafide research work carried out by **Miss. ABUJ BHAGYASHREE BHASKARRAO** under my guidance and supervision and that no part of the thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of this investigation have been duly acknowledged.

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Date : / /2023

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(Bhagyashree B. Abuj)

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## LIST OF ABBREVIATIONS

%	Percent
/	per
/ gm	per gram
°C	Degree Celcius
2-βME	2- beta mercaptoethanol
μ	Micro
μg	Micro gram
μl	Microliter
μmol	Micro mole
Abs	Absorbance
Anon.	Anonymous
ANOVA	Analysis of Variance
AOAC	Association of Official Agricultural Chemists
APS	Ammonium Per Sulphate
AS	Asparagin synthetase
ATP	Adenosine trinucleotide phosphate
bp	Base Pairs
BPB	Bromo Phenol Blue
C. D.0.05	Critical Difference at 5% level of significance
C. V.	Coefficient of Variation
Ca	Calcium
c-DNA	Complementary Deoxyribo Nucleic Acid
Chl	Chlorophyll
cm	Centimeter
CRD	Completely Randomized Design
Ct	Threshold cycle
CTAB	Cetyl tri methyl ethyl ammonium bromide
CuSO <sub>4</sub>	Couper Sulfate
DAS	Days after sowing
DMSO	Di Methyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTPs	Deoxyribose Nucleotide Tri phosphate
ds	Double stranded
DW	Dry weight
EDTA	Ethylene Diamine Tetra Acetic Acid
eg.	For example
et al.,	Et alia
Et Br	Ethidium Bromide
etc.	Etcetera
ETR	Electron transport rate
FAO	Food and Agricultural Organization of United Nation

Fig.	Figure
FRBD	Factorial Randomized Block Design
g	Gram
G.S.	Glutamine synthetase
GC	Guanine cytosine
GOGAT	Glutamine 2-oxoglutarate amino transferase
H	Hour
H <sup>+</sup>	Hydrogen ions
H <sub>2</sub> O	Water
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
H <sub>2</sub> SO <sub>4</sub>	Sulfuric acid
HATS	High affinity transport system
HCl	Hydrogen Chloride
HNE	High nitrogen use efficient
HPLC	High performance liquid chromatography
hrs	Hours
i. e.	That is
K	Potassium
kDa	Kilo dalton
KNO <sub>3</sub>	Potassium nitrate
LATS	Low affinity transport system
LNE	Low nitrogen use efficient
M	Molar
M pa	Mega pascal
mA	Miliampere
max	Maximum
Mbp	Mega base pair
mg	Mili gram
MgCl	Magnesium Chloride
MI	Marker Index
min	Minutes
ml	Mili liter
mm	Mili meter
mM	Mili molar
mRNA	Messenger RNA
N <sup>-</sup>	Half nitrogen
N	Normal
N <sup>+</sup>	Full nitrogen
N <sub>0</sub>	No nitrogen
Na <sub>2</sub> CO <sub>3</sub>	Sodium Carbonate
NaCl	Sodium Chloride
NADH	Nicotinamide adenine dinucleotide
NaOH	Sodium Hydroxide
NBT	Nitroblue Tetrazolium

ND	Nano-Drop
NFW	Nuclease Free Water
ng	Nano gram
ng/l	Nano gram per liter
NH <sub>4</sub> <sup>+</sup>	Ammonium
NiR	Nitrite reductase
nm	Nano meter
nmol cm <sup>-1</sup>	Nano mole per centimeter
No.	Number
NO <sub>2</sub> <sup>-</sup>	Nitrite
NO <sub>3</sub> <sup>-</sup>	Nitrate
NPK	Nitrogen Phosphorous Potassium
NR	Nitrate reductase
NRE	Nitrogen remobilization efficiency
NS	Non-significant
NUE	Nitrogen use efficiency
OD	Optical Density
Pa.s	Pascal per second
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
Pg	Pico gram
pH	Reciprocal of the Hydrogen Ion Concentration
ppm	Parts Per Million
PVP	Polyvinyl Pyrrolidone
qRT-PCR	Quantitative real time polymerase chain reaction
QTL	Quantitative trait locus
RFU	Relative Fluorescence Units
Rm	Relative Mobility
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
rpm	Revolutions per Minute
RQ	Relative quantity
rRNA	Ribosomal Ribonucleic acid
RT	Room Temperature
RT-PCR	Real time polymerase chain reaction
RTs	Reverse Transcriptase
S	Swedberg
S <sup>-1</sup>	Per second
S. Em.	Standard Error of Mean
sd	Standard deviation
SDS	Sodium Dodecyl Sulphate
Sec.	Second
Sr.	Serial
SSR	Simple sequence repeat

T	Treatment
T	Temperature
t/ha	Tons Per Hectors
TA	Annealing temperature
TBE	Tris-Borate EDTA
TCA	Trichloroacetic acid
TE	Tris- EDTA
TEMED	N, N, N', N' – Tetra methyl ethylene diamine
Tm	Melting temperature
Tris	(Hydroxymethyl) aminomethane
TrisHCL	Hydroxymethyl aminomethane hydrochloride
tRNA	transfer Ribonuclic acid
TSS	Total Soluble Sugar
U	Unit
Ubq	Ubiquitin
UV	Ultraviolet
V	Volts
v/v	volume by volume
viz	Namely
w/v	Weight per volume
w/w	Weight per weight
wt	Weight
$\gamma$ -GHA	Glutamylhydroxamate

## ABSTRACT

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### BIOCHEMICAL AND MOLECULAR BASIS OF DIFFERENTIAL NITROGEN USE EFFICIENCY IN CONTRASTING WHEAT (*Triticum aestivum* L.) GENOTYPES

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**Miss. ABUJ BHAGYASHREE BHASKARRAO**

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**Department** : **Biochemistry**

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The investigations entitled "Biochemical and molecular basis of differential nitrogen use efficiency in contrasting wheat (*Triticum aestivum* L.) genotypes" were undertaken during *rabi* 2019–20 and *rabi* 2020–21 at the Department of Biochemistry and Phytotron facility at Department of Agricultural Botany, Post Graduate Institute M.P.K.V., Rahuri, Dist. Ahmednagar (MS) India.

This study was carried out in four different pot culture experiments. The experiments were laid out in Factorial Completely Randomized Design (FCRD) with four replications involving two contrasting wheat genotypes and three nitrogen levels, *viz.*, N+ (full nitrogen), N- (half nitrogen), and N0 (no nitrogen). The other experiment was carried out in growth chambers containing half-strength Hoagland solutions with and without omeprazole. Based on the results of the first two experiments, an experiment was planned with four genotypes (two nitrogen-use efficient and two nitrogen-use inefficient), which were screened for carbon monoxide sensitivity of cytochrome oxidase. In a separate experiment, thirteen promising wheat genotypes were grown in a completely randomized design with three replications under limiting nitrogen conditions to investigate NUE.

In the first experiment, two contrasting NUE wheat genotypes, high (HD-2781) and low (C-306), were undertaken to understand the basic difference in uptake, assimilation, remobilization of nitrate, and other physiological limiting factors. Even at half nitrogen level, the higher leaf nitrate content in low nitrate-tolerant, *i.e.*, high nitrogen use efficient (HD - 2781) wheat genotypes suggested that probably both high and low affinity nitrate transporters are actively operative. Chlorophyll content, leaf nitrate content, *in vivo* NR, *in vitro* NR, GS, Fd-GOGAT, NADH-GOGAT, and total grain protein were significantly high in the N-efficient genotype (HD-2781), even at half the dose of applied nitrogen both at pre- and post-anthesis stages. Glutamine synthetase, Fd-GOGAT, and NADH-GOGAT activities were higher at the post-anthesis stage. The higher activity of Fd-GOGAT at the post-anthesis stage signifies its importance in the remobilization of ammonia. The comparatively higher GS activity in the N-efficient genotype HD-2781 both at pre- and post-anthesis stages indicates higher assimilation efficiency due to the higher availability

of substrate. After native polyacrylamide gel electrophoreses (native-PAGE) and staining for transferase activity, GS produced distinct "upper" and "lower" activity bands. The expression of GS1 (cytosolic isoforms) was higher at post-anthesis, and the expression of GS2 (chloroplastic isoforms) was higher at pre-anthesis in the N-efficient genotype compared to the N-inefficient genotype. In both genotypes, the chloroplastic isoform (GS2) responsible for nitrate assimilation was highly expressed during pre-anthesis, the cytosolic (GS1) and chloroplastic (GS2) responsible for both assimilation and photorespiratory remobilization of  $\text{NH}_4^+$  were expressed, with differences in the level of expression.

In a separate experiment, thirteen wheat genotypes were compared to understand the basic differences in uptake, assimilation, remobilization of nitrate, and other physiological limiting factors with limiting nitrogen (N) supply. Chlorophyll content, leaf nitrate content, *in vivo* NR, *in vitro* NR, GS, Fd-GOGAT, NADH-GOGAT, and total grain protein were significantly higher in GW-322, MACS-6222, NIAW-301, NIAW-34, NIAW-3170, NIAW-1994, and HD 2781 even at a low dose of applied nitrogen suggesting that these genotypes are N-use efficient. The significantly higher *in vitro* NR activity compared to *in vivo* NR activity at saturating nitrate concentrations signifies that, beside nitrate, NADH appears to be a limiting factor. Glutamine synthetase, Fd-GOGAT, and NADH-GOGAT activities were higher at the post anthesis stage, which signifies their importance in the remobilization of photorespiratory ammonia. Isozymes profiling of GS1 and GS2 was done on Native-PAGE. At pre-anthesis, the N-efficient genotypes had higher relative levels of GS2 protein than the N-inefficient genotypes; indicating that GS2 is important in the vegetative growth stage and carbon-nitrogen metabolic balance. In the post-anthesis stage, there was an increased relative amount of the GS1 protein under the limiting N condition. The results revealed that the N-efficient genotypes exhibited increased assimilation ability during the pre-anthesis stage and remobilization ability during the post-anthesis stage. The relative contents of the GS1 and GS2 proteins at pre and post anthesis could be used as a marker for breeding NUE genotypes. A study of the activities of nitrate assimilatory enzymes in N-efficient and N-inefficient wheat genotypes showed that nitrate uptake (*in vivo*), nitrate reductase activity, glutamine synthetase (GS), and glutamate synthase (GOGAT) play important roles in N assimilation and remobilization under low-nitrogen conditions.

Four genotypes (HD-2781, C-306, HD-2932, and NIAW-1994) were selected to study nitrogen metabolism responsive genes, *viz.* GS1, GS2, and Fd-GOGAT. The expression level of the cytosolic isoform of GS1 was comparatively higher in high-NUE genotypes at the post-anthesis stage as compared to the pre-anthesis stage is supportive of better nitrogen remobilization efficiency. The expression of the chlorolastic isoforms of GS2 and Fd-GOGAT was higher in HD-2781 and NIAW-1994 during the pre-anthesis stage and post-anthesis stage, signifying better N assimilation efficiency. Under limiting nitrogen conditions, the GS2 gene was upregulated in N-efficient genotypes (HD-2781 and NIAW-1994) compared to N-inefficient genotypes (C-306 and HD-2496). Fd-GOGAT expression was higher at the post-anthesis stage as compared to the pre-anthesis stage.

Omeprazole (OP), a selective proton pump inhibitor that alleviates the growth limitation imposed by low-nitrogen, improved  $\text{NO}_3^-$  uptake and assimilation in C-306 (N-inefficient genotype) by altering its NR activation state and affinity for nitrate as a substrate. G6PDH activity in the root was higher in the N-efficient genotype during the nitrate assimilation, which provides reductants to the enzymes involved in the nitrate reduction and the assimilation of ammonium.

Four wheat genotypes: two nitrogen-use efficient (HD-2781 and NIAW-1994) and two nitrogen-use inefficient (C-306 and HD-2932), were screened for carbon monoxide sensitivity of cytochrome oxidase to understand the coordination of redox state in the integration of carbon and nitrogen metabolism. 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. Thus carbon monoxide sensitivity gives an idea regarding the redox state of cytochrome oxidase during steady state respiration. In tightly coupled mitochondria the cytochrome  $a_3$  is most likely in the oxidized form by donating electrons to  $\text{O}_2$ , and under the situation carbon monoxide cannot form a complex with cytochrome  $a_3$ . When CO forms adduct with cytochrome  $a_3$  (reduced), the NADH are exported to the cytosol for the reduction of nitrate to nitrite. Thus accumulation of nitrite under CO-aerobic condition is indication of redox state and thus tightness of coupling. In HD-2781 and NIAW-1994, the cytochrome  $a_3$  was found to be more in a reduced state, thereby indicating less extend of coupled mitochondria; however, in wheat genotype C-306 and HD-2932, the cytochrome  $a_3$  is more in an oxidised form, thereby indicating that mitochondria are tightly coupled. Infiltration with 2 mM DNP an uncoupler of the oxidative phosphor relation before CO exposure enhanced the CO aerobic response significantly in C-306 and HD-2932, as evident from the amount of nitrite formed, thereby confirming that mitochondrial electron transport and the oxidative phosphorylation are tightly coupled in the nitrogen use inefficient wheat genotype.

Nitrogen metabolising enzymes were found to be positively correlated with NUE and nitrogen content, suggesting that they are the rate-limiting enzymes in nitrogen metabolism. The changes in mitochondrial redox systems can affect plant responses to nitrogen as oxidized nitrate ( $\text{NO}_3^-$ ) or reduced ammonia ( $\text{NH}_4^+$ ). CO-sensitive genotypes are high nitrogen use efficient genotypes, which means CO forms complexes with reduced cytochrome  $a_3$ , matrix-generated NADH is exported to the cytosol and used for nitrate assimilation, whereas nitrogen use inefficient wheat genotypes are insensitive to CO, cytochrome  $a_3$  is more oxidized, indicating that mitochondria are tightly coupled. Thus, mitochondrial-driven effects on NAD status exert a major influence on the rate of nitrate assimilation. Due to the high activity of the nitrogen metabolising enzyme, nitrogen requirements will be reduced by up to 50% compared to the recommended amount. The wheat genotypes HD-2781 and NIAW-1994 are promising nitrogen-efficient genotypes that can be used for sustainable wheat crop yield even with less nitrogen, protecting the environment and lowering input costs.

## 1. INTRODUCTION

Wheat (*Triticum aestivum* L.) is the major staple food of the world and second significant staple food crop in the country. Nitrogen (N) is one of the key drivers of crop production. Given sufficient sunlight, water, and favorable temperatures, the growth yield and quality of crops are closely linked to N fertilizer availability and uptake (Hawkesford, 2012). It has been reported that approximately 50-60% of the applied N fertilizer is lost from the plant or soil system through leaching, volatilization and nitrification. One of the strategies to reduce N losses from the plant or soil system is to improve N uptake, assimilation and partitioning to sink by improving nitrogen use efficiency. Nitrogen use efficiency is defined as grain yield per unit of both applied fertilizer N and available soil N at sowing and is a product of N uptake efficiency and N utilization efficiency.

The sustainable agriculture production requires balanced and judicious, efficient, and eco-friendly management practices. To achieve agricultural sustainability and food security, judicious use of fertilizer inputs, particularly nitrogen, has a unique importance in modern agriculture (Kumar *et al.*, 2016). Genotypic variation for NUE traits has been reported in cereal crops which include N accumulation, N remobilization and yield under low N (Le Gouis *et al.*, 2000). However, identification of genetic and metabolic control linked to NUE phenotypes is difficult looking to the complexity of N metabolism.

NUE is considered to be a complex multigene trait, with no single gene contributing more than a small percentage of the phenotype. It was therefore unclear whether a single transgene could have a significant enough effect on the phenotype to make commercialization worthwhile. However, there are several examples where a single transgene had a significant phenotypic effect, although none has yet been commercialized (Kant *et al.*, 2011). The electron transport chain pathways are profoundly affected by soil nitrogen availability as oxidized nitrate or reduced ammonium (Rasmusson *et al.*, 2020). Nitrate reductase was considered to be pivotal in the growth response to nitrogen supply. However, numerous studies altering the expression of nitrate reductase encoding genes have resulted in no significant change in plant growth and yield (Crawford, 1995). Studies of maize (Masclaux *et al.*, 2001) and wheat (Kelly *et al.*, 1995) have also failed to find correlations between leaf nitrate reductase activity and yield. It appears, therefore, that nitrogen assimilation and yield are not generally limited by the level and activity of the nitrate reductase or nitrite reductase enzymes. Improving NUE through better nitrogen acquisition focus on enhancing nitrogen remobilization would be quite fruitful through the efficient degradation of proteins and other

organic nitrogen forms and transport during grain-filling. Maintenance of optimum nitrogen and carbon homeostasis is needed for maximum root and shoot development, photosynthetic rate, synthesis and mobilization of amino acids, organic acids, and lipids (Kant *et al.*, 2011).

In wheat, NRA (*in vitro*) does not reflect nitrate assimilation and is in excess of what is required, which suggests some other factor other than NRA (*in vitro*) that limits NA (Espen *et al.*, 2004). Both nitrate and NRA (*in vitro*) are in excess under conditions of high nitrate fertility and NiR (*in vivo*) is not limiting. The only other factor affecting NA would be the availability of reductant, NADH, to drive nitrate assimilation. The availability of cytoplasmic NADH as the limiting step in NA as proposed would be a logical alternative (Kaiser *et al.*, 2000; Foyer *et al.*, 2011). Increased cytoplasmic NADH using a transgenic co-suppression of complex I under the control of a tissue-specific promoter has been reported in maize. The increased kernel number plant<sup>-1</sup> (KNP) and grain yield under low NUE (high nitrogen fertility) conditions have been reported in maize. These results suggest that nitrate assimilation limits NUE in maize via reduced availability of cytoplasmic NADH (Loussaert *et al.*, 2018). A positive correlation between the total amount of N absorbed by the crop and the grain yield of wheat signifies that improvement of N uptake efficiency is more critical to improving grain yield and quality in wheat (Kong *et al.*, 2013).

Glutamine synthetase (GS) (EC 6.3.1.2) is a key enzyme in the first step of NH<sub>4</sub><sup>+</sup> assimilation, which is responsible for the synthesis of glutamine. GS isozymes have different metabolic roles, and their activities vary during plant development in different organs and cell types. Two GS isozymes, GS1 and GS2, are present in higher plants (Bernard and Habash, 2009). GS1 present in cytosol is primarily involved in N recycling in senescent leaves and N translocation during seed germination and GS2 is found primarily in the plastids of green tissues and is encoded by a single nuclear gene (Guan *et al.*, 2015). It is mainly involved in the photo respiratory release of NH<sub>3</sub>, and in NO<sub>3</sub><sup>-</sup> reduction during nitrate assimilation (Keys, 2006). Given that the released NH<sub>3</sub> by photorespiration is 10-fold higher than that of NO<sub>3</sub><sup>-</sup> reduction, the activity of GS2 plays an important role in the efficiency of N assimilation by preventing N loss and has different metabolic roles. The GOGAT enzyme catalyses the reductive transfer of the amide group of glutamine to 2-oxoglutarate to form two glutamate molecules. Together with GS, it maintains the flow of nitrogen from NH<sub>4</sub><sup>+</sup> into glutamine and glutamate, which are then used for several other aminotransferase reactions during the synthesis of amino acids.

Genetic studies in durum wheat and rice localized the GS genes using quantitative trait locus (QTL) analysis for grain protein content (GPC), cytosolic glutamine synthetase

content, and panicle number. Increased GS expression in transgenic rice also made the rice tolerant to N-deficiency. Principal component analysis (PCA) and correlation studies performed in several cereal species suggested the presence of a strong relationship between GS activity and total N, chlorophyll, soluble protein,  $\text{NH}_4^+$ , and amino acids. Using three independent alfalfa NADH-GOGAT over-expressing tobacco lines, Chichkova *et al.*, (2001) showed increased total C and N concentrations in over expressing plants just prior to flowering, in both shoots and roots. Rice plants over-expressing NADH-GOGAT obtained grain weights up to 80% greater than those seen in controls (Yamaya *et al.*, 2002; Tabuchi *et al.*, 2007). Studies in rice where both Fd-GOGAT and NADH-GOGAT have been suppressed show that tiller number, total shoot dry weight, and yield are decreased significantly compared with control plants (Lu *et al.*, 2011). Suppression of both GOGAT genes also affected plant growth in field conditions, by altering yield per plant and thousand-kernel weight, these being phenotypic indicators of N starvation (Lu *et al.*, 2011). Given these observed phenotypes and those observed for GS enzymes, interaction between isozymes of GOGAT with the GS isozymes and how this affects NUE, as well as post-transcriptional regulation of these enzymes appears to be critical factor for NUE. In developing transgenic improvements in NUE crops, linking biochemical or physiological information to the relative field performance is critical. The transcriptomic response of wheat to nitrogen starvation (Curci *et al.*, 2017). Attempts to improve NUE *via* transgenic approach by manipulation of overexpression of candidate genes, *viz.*, nitrate and ammonium transporters, resulted in no improvement in growth at low N and an enhanced NUE phenotype at adequate N in rice (Kumar *et al.*, 2003; Fan *et al.*, 2016).

Analyzing responses of crop plants to N limitation and identifying plants that show better growth under N-limiting conditions can provide a better understanding of how to improve NUE. The components of NUE involving increasing N influx, increased flux roots towards shoots, metabolic flux for assimilation into amino acids, and flux sequestration interact in multiple complex ways with other metabolic pathways. Nitrogen use efficiency technology is worth exploring to address the challenges of limited accessibility to nitrogen fertilizers among resource-poor farmers. Nitrogen use efficiency (NUE) technology produces crops with yields comparable to conventional varieties, which, on the contrary, require significantly less nitrogen. Even though breeders have sought to breed for N-efficient genotypes to improve yields among small-scale farmers, specific information on particular N-efficient genotypes is lacking.

Nitrogen use efficiency (NUE) is a complex trait involving uptake, assimilation and remobilization. The attempts to improve NUE either through manipulation of uptake using transporter (s) or through overexpression of key enzymes involved in nitrate assimilation, viz., NR, GS, or GOGAT, have not given expected improvement and the results have also given few contradictory outcomes concerning overexpression of GS. The plant mitochondrial metabolism appears to be a target to address these issues from a metabolic perspective concerning substrate limitation, end product inhibition, availability of reluctant (NADH), and C-skeleton. The contradictory result with GS overexpression also warrants looking at the past translational regulation and activity status of Fd- GOGAT and NADH - GOGAT, which provide substrate for GS.

Studies concerning the improvement of NUE have focused largely on the components of primary nitrogen assimilation and associated carbon metabolism. The exportation of the flexibility of respiratory pathways has the potential to enhance NUE. Several lines of evidence have elaborated on the role of organelle genome variation in plant adaptation.

The present investigation entitled, “Biochemical and molecular basis of differential nitrogen use efficiency in contrasting wheat (*Triticum aestivum* L.) genotypes” is therefore proposed with following major objectives

1. To evaluate the physio-biochemical parameters at pre and post anthesis stages concerning nitrogen use efficiency in contrasting wheat genotypes.
2. To investigate the correlation between carbohydrate metabolism, mitochondrial metabolism and nitrogen use efficiency at pre and post anthesis stages and
3. To study expression analysis of nitrogen use efficient genes and activity at pre and post anthesis stages of wheat.

## 2. REVIEW OF LITERATURE

This chapter provides general information about the topic of investigation by creating an insight and sense of integration about the subject as a whole and attempt was made to review the literature on the problem in respect “Biochemical and molecular basis of differential nitrogen use efficiencies in contrasting wheat (*Triticum aestivum* L.) genotypes”. The available literature in line with the objectives of the study is reviewed under following sub-headings.

2.1 Importance of nitrogen use efficiency in wheat

2.2 Agronomic approaches for improvement of NUE

2.3 Breeding efforts to improve NUE

2.4 Biochemical and molecular response to low nitrogen input

2.5 Mitochondrial target for improving NUE

2.6 Transgenic approach to manipulate NUE

2.7 Physio-biochemical parameters

2.8 Carbohydrate and mitochondrial metabolites

2.9 Molecular Parameters

### **2.1 Importance of Nitrogen Use Efficiency in Wheat**

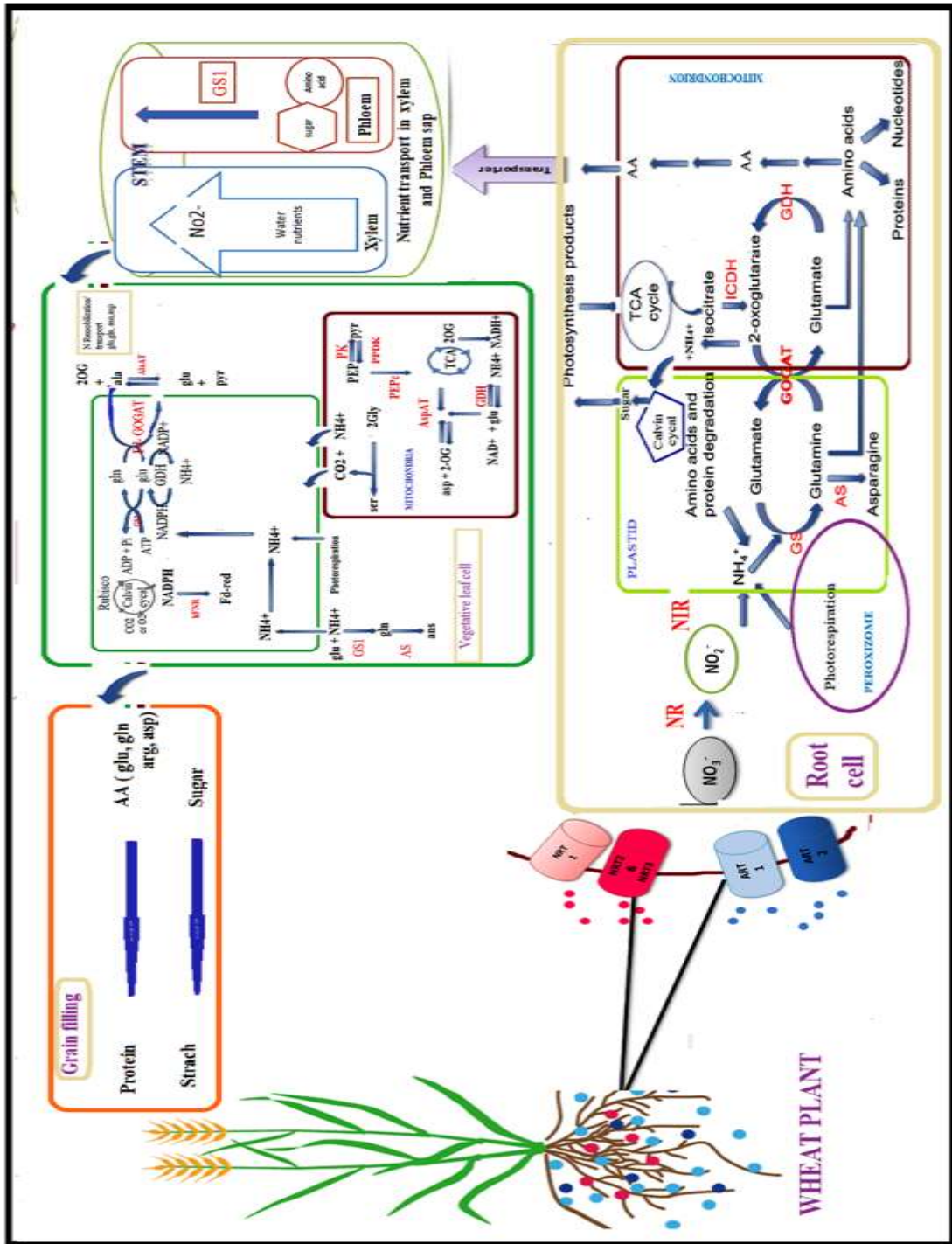
The green revolution resulted in increasing agriculture production around the world due to the selection of genotypes with higher yield potential and a simultaneous increase in fertilizer application, primarily nitrogen (Borlaug, 2007). The use of nitrogen fertilizers of crop plants has increased by more than 20 times in the previous 50 years over the world. The global usage of nitrogen fertilizer has continuously increased, as have agricultural emissions, primarily as nitrous oxide, into the environment (Hatfield *et al.*, 2012). Wheat alone uses 19% of the total nitrogen fertiliser used to all crops worldwide. The usage of nitrogen fertiliser is often inefficient, with only about 50% of the nitrogen applied to the environment, and is a potential cause of contamination and additional costs for farmers.

Improving the efficiency with which crops use nitrogen is a priority issue for assuring future cost effective and sustainable agriculture (Collins *et al.*, 2008). A major goal of crop development is to enhance nitrogen use efficiency, particularly in cereals. Such a crop would make greater use of the nitrogen fertiliser, resulting in bigger yields with increased protein content. Thus, increasing NUE increases farmer earnings while reducing the negative environmental impact of nitrogen losses in the ecosystem and its impact on food production.

Nitrogen is an important factor limiting plant growth and productivity worldwide. It is the most abundant molecule in the atmosphere as 78% of the atmosphere is composed of nitrogen. Nitrogen is provided to plant from both atmospheric air and soil minerals. The ability of plant to capture nitrogen naturally or that applied as nitrogen fertilizers, is one of the critical steps limiting the efficient use of N by plants. Plants absorb N from the soil in the form of  $\text{NO}_3^-$  and  $\text{NH}_4^+$  ions. Most N uptake is in the form of  $\text{NO}_3^-$  which moves from the soil solution into the plant root cell with absorbed water. Nitrate is either stored in the vacuoles or reduced in the cytosol and plastids eventually to  $\text{NH}_4^+$  through the action of nitrate and nitrite reductase (NR, NiR). ammonia is then assimilated to produce more complex N containing compounds. In wheat, studies published those wheat varieties grown with low nitrogen input levels can sustain profit margins even if yield are lower. Wheat genotypes that are specifically nitrogen stress tolerant still need to be developed through genetic variation for adaptation triad to nitrogen deficiency. These genotypes will have to maintain yield and grain protein content under moderate nitrogen deficiency as well as in the event of intense nitrogen stress under the low input cropping system (Laperche *et al.*, 2007). Hence, direct selection for yield under a low nitrogen supply would be more efficient than indirect selection conducted under higher N level.

Nitrogen use efficiency (NUE) is the part of applied nitrogen that is absorbed and utilized by the plant. Improving a plant's capacity to use nitrogen is a vital constituent in enhancing nitrogen environmental sustainability. The increased utilization of nitrogen fertilizer caused various issues, like high input costs for crop production, a decline in nitrogen use efficiency, nitrogen fertilizer loss, and environmental pollution (Xu *et al.*, 2012). The physiological and metabolic changes, such as soil nitrogen uptake, assimilation from roots to other parts (Fig. 2.1), source-sink tissues interaction for transportation, signaling and regulatory pathways that are responsible for N status within plants and growth, are all exploiting issues for discussion and research.

The ratio of yield to total N supplied is commonly referred to as NUE (Masclaux-Daubresse, 2010). Several techniques have been used to measure NUE, which can be divided into two categories: N uptake and N utilization efficiency. N uptake efficiency (NUpE) refers to the amount of nitrogen that a plant can absorb from nitrogen sources, whereas N utilization efficiency (NUtE) relates to the plant ability to absorb and remobilize nitrogen within the plant (Moll, 1982; Moose and Below, 2009). NUE, on the other hand, is the product of NUpE and NUtE.



**Figure 2.1: Nitrogen uptake, assimilation and remobilization in Wheat**

AA, amino acids; AAT, amino acid transporter; AMT, ammonium transporter; NRT, nitrate transporter; 2-OG, 2-oxoglutarate; PK, pyruvate kinase; CC, Calvin cycle.

The Food and Agribusiness Association of the Unified Countries assessed that N utilization would be around 119 million tons by 2020 with the expanded populace development and interest for food (Sharma *et al.*, 2017). Food security is firmly connected to nutrient accessibility for cropping, while its sustainability is connected to nutrient use efficiency. Specifically for nitrogen, which is quantitatively the most important component of all fertilizers (York *et al.*, 2016; Chen and Liao, 2017). Based on available data, N fertilizer demand is expected to increase by 1.2% per annum until 2022 (IFA, 2019). The crop plants are frequently not ready to get and utilizing the supplied nitrogen and take up under half of the nitrogen fertilizer (Zhu *et al.*, 2016). However, increasing nitrogen fertilization doesn't really bring about a relative increase in yield (Zhu *et al.*, 2016). Zhang *et al.* (2015) and Anas *et al.* (2020) detailed the increasing in yield and nitrogen use efficiency (NUE) related to a decrease in N application and N losses into the environment are squeezing objectives for a sustainable agriculture.

## **2.2 Agronomic Approaches for Improvement of NUE**

Agronomists can improve NUE both at an individual crop level and at cropping system level. At the crop level, the goal is to improve N management through different corresponding fertilizer strategies and soil management systems, for example, precision fertilization through split fertilization techniques (Hirel *et al.*, 2011) or fertilizer position methodologies. NUE is an ambitious objective as wheat grain yields are influenced by various factors, including genetic parameters and their variation among and within species, environmental effects such as location, soil conditions, and environment, and agronomic advances and the management practices (e.g., type, timing, amount, and place of N application, crop rotations, weed, conservation agriculture based cropping systems, partial retention of crop residues in soil, integrated nutrient management, green manuring, precision farming and pest control) (Martinez-Feria *et al.*, 2018 and Lemaire and Ciampitti, 2020).

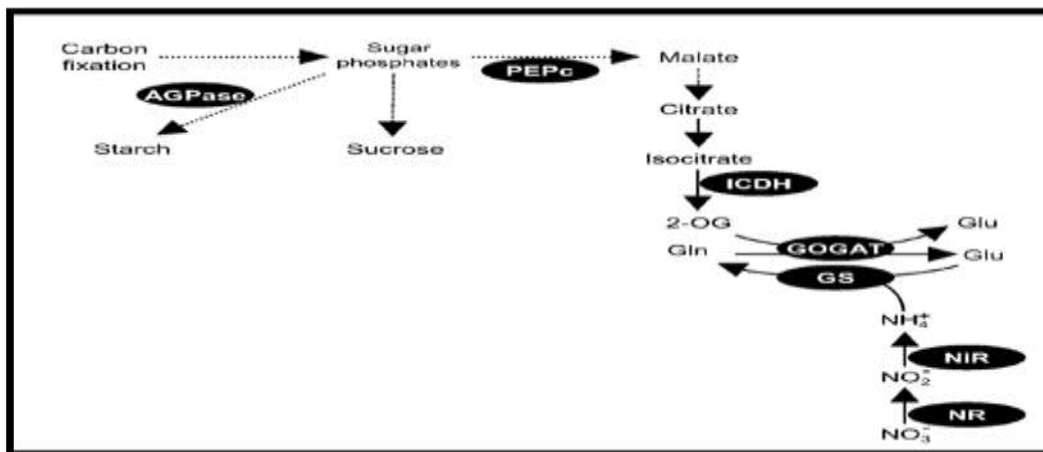
## **2.3 Breeding Efforts to Improve NUE**

Mostly selection in wheat breeding is carried out under non limiting N supply, and plant breeders select cultivars that perform well under optimum N supply conditions. From the above examinations, since genotype and N supply do interact, cultivars chose for high return under high N conditions may not be the highest yielding under lower N conditions. Plant breeding for NUE will require improved comprehension of the physiological characteristics determining higher NUE and response to N limitation. Promising traits to increased NUE in wheat for which there is reported genetic variation which include deeper roots for increased N uptake (Cormier *et al.*, 2016), increased leaf photosynthesis rate per

unit N (Gaju *et al.*, 2016), and stay-green characteristics related with optimized post anthesis N remobilization (Hawkesford, 2014) and additionally expanded post anthesis N uptake (Gaju *et al.*, 2014).

Today, improved plant breeding through the use of genetic engineering has the best potential to create plants that will use fertilizer more efficiently than conventional varieties (Sinha *et al.*, 2018 and Jensen *et al.*, 2020). Only the combination or interaction of these factors (genetic parameters x environment effect x agronomical management) will allow sustainable and secure crop production (Hawkesford and Riche, 2020 and Peng *et al.*, 2020). Different genes related to these developments and physiological cycles have been recognized and their significance for plant growth, function and seed yield has been reported.

Breeding efforts to enhance the NUE of crops need to be specifically targeted to improve NUE. However, owing to the complexity of phenotypic and physiological traits, there are no standard characters for assessing NUE. In this manner, several NUE-related traits are chosen and considered to influence NUE. The first QTL map of barley was developed and yield was the only trait relevant to NUE that was examined. Ongoing report in maize showed that grain yield and biomass are the two of significant characteristics related with NUE (Li *et al.*, 2015). Measuring NUE is a significant challenge because it is technically difficult to decide the N substance of soil, of various tissue types, and furthermore the N substance can be exceptionally factor among genotypes and conditions (Han *et al.*, 2015).



**Figure 2.2. Central reactions in the integration of nitrogen assimilation and carbon metabolism (Duttilleul *et al.*, 2005).**

Key enzymes are shown by black ellipses. Continuous arrows represent a single enzymatic step, while broken arrows indicate pathways involving more than one reaction. GOGAT, Glu synthase

## 2.4 Biochemical and Molecular Response to Low Nitrogen Input

Plant tissue glucose levels rise when N supply is limited (Sieger *et al.*, 2005). When wild-type (WT) tobacco cells are grown under N-limitation, they induce large amounts of

alternative oxidase (AOX), which constitutes a non-energy conserving branch of the respiratory electron transport chain. Under nutrient limitations, growth of wt cell cultures was dramatically reduced and carbon skeletal decreased by 42-63% (Sieger *et al.*, 2005). Mitochondrial respiration permits extra carbon to be consumed under N-limited circumstances, allowing the proper C/N ratio to be maintained. In response to low N circumstances, the activity of Krebs cycle enzymes NAD- and NADP-IDH, fumarase (Fum), citrate synthase (CS) and NAD-malic enzyme (NAD-ME) (Fig. 2.2) increased in diverse plant species (Noguchi and Terashima, 2006). The function of mtETC is similarly affected by low N availability. With a drop in type II NAD (P) H dehydrogenases (ND in/ex; Noguchi and Terashima 2006), there was a decrease in cytochrome pathway and an increase in alternative pathway activity. The activity of the cytochrome pathway is reduced under low N, when demands for ATP are low due to delayed protein turnover and decreased phloem loading, and up-regulation of alternative oxidase (AOX) facilitates efficient utilisation of surplus carbohydrates (Noguchi & Terashima 2006). On the other hand, the increased level of cytosolic GS1 seen under low N circumstances (Lothier *et al.*, 2011) might result in a significant demand for mitochondrial ATP, suggesting that the cytochrome route may play a role.

#### **2.4.1 Changes in Mitochondrial Metabolism Induced by Nitrate and Ammonia Supply**

The form in which N is uptake has a distinct impact on plant metabolism as a whole. The two-step reduction pathway from  $\text{NO}_3^-$  to  $\text{NH}_4^+$  takes eight electrons when plants grow on  $\text{NO}_3^-$ . The process of  $\text{NO}_3^-$  assimilation takes place mostly in the leaves. Because a portion of  $\text{NH}_4^+$  is immediately converted into amino acids in root plastids, N is transported to the leaves in the form of amino acid and ammonia *via* the xylem sap. The intake of various types of nitrogen changes the reductants and energy state of leaf cells. Plants must transfer excess NAD (P) H from the chloroplasts to avoid photo inhibition under  $\text{NH}_4^+$  feeding, according to Guo *et al.*, 2005. Different electron demands for  $\text{NO}_3^-$  reduction and  $\text{NH}_4^+$  absorption may have an impact on not just cytosolic and plastidic metabolism, but also mitochondrial pathways. Both the TCA cycle and mtETC activities are thought to increase during  $\text{NH}_4^+$  absorption (Weger and Turpin 1989). This rise is due to increased need for TCA cycle intermediates (2-OG or citrate) as C skeletons, as well as the need for simultaneous oxidation of surplus reductants not employed in NR and nitrite reductase processes (NiR). When nitrate feeding is required to support reduction processes, the TCA cycle activity is majorly increase, generating C skeletons for the GS-GOGAT cycle, but mtETC activity remains unchanged. The impact of varying amounts of N feeding on respiratory activity in

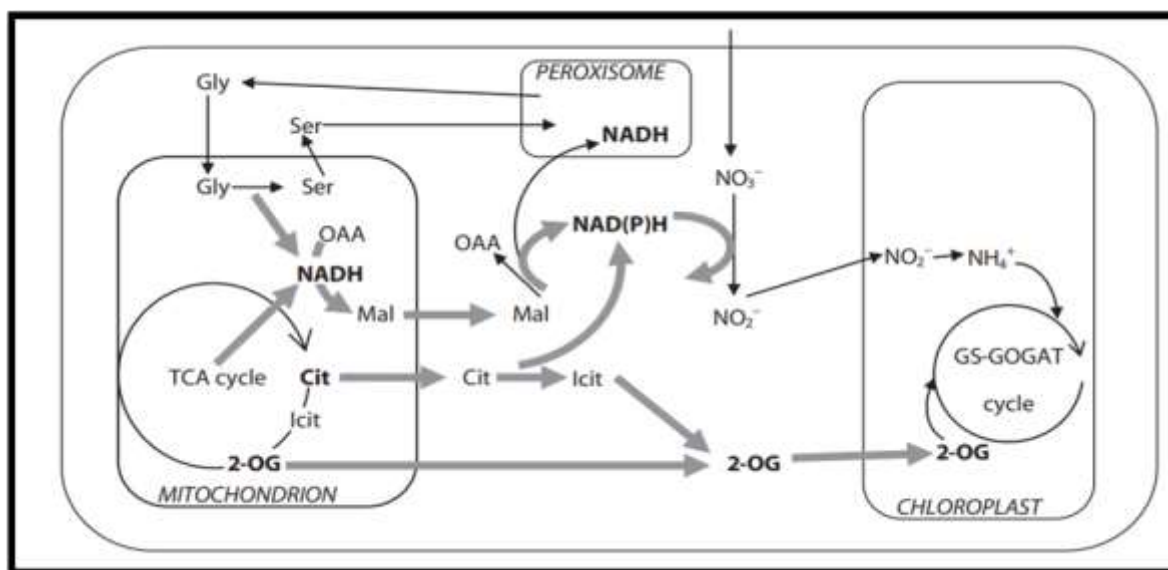
several plant species has been examined;  $\text{NH}_4^+$  has been shown to stimulate respiratory activity. (e.g. in barley, *Arabidopsis thaliana*, spinach, pea and French bean plants; Escobar, Geisler and Rasmusson 2006). Furthermore, it was postulated that the cytochrome *c* role in total respiration, as well as an alternate route, is dependent on the accessible (assimilated) form of N. The alternative pathway is known to reduce the efficiency of mitochondrial ATP production while allowing it to oxidise excess cellular reductants, allowing plants to grow under stress conditions (Rasmusson *et al.*, 2008); thus, AOX could play a key role in dissipating excess redox equivalents during  $\text{NH}_4^+$  supply. AOX capacity increases during  $\text{NH}_4^+$  feeding. In fact, in *Arabidopsis* plants fed with  $\text{NH}_4^+$ , greater AOX mRNA levels (primarily *aox1a*, *aox1d*, and *aox2*) were seen, as well as higher AOX capacity (Escobar *et al.*, 2006). Contrary to previous findings, Hachiya *et al.* (2010) revealed an increase in cytochrome *c* oxidase (COX) activity in  $\text{NH}_4^+$  grown plants, suggesting that this might be due to the increased energy requirement induced by greater  $\text{H}^+$  ATPase activity, which prevents cytosolic acidification. However, because cytosol acidification during  $\text{NH}_4^+$  supply has already been reported (Britto and Kronzucker 2005), the potential function of cytochrome oxidase must be investigated further. When comparing reductants and energy demand under  $\text{NO}_3^-$  and  $\text{NH}_4^+$  feeding, it's reasonable to assume that  $\text{NH}_4^+$  is the more beneficial N form for plants. Surprisingly, when  $\text{NH}_4^+$  is used as the only supply of nitrogen, it generates toxicity symptoms like as growth inhibition and chlorosis, which is known as the  $\text{NH}_4^+$  syndrome (Hoffmann *et al.*, 2007). Oxidative stress was thought to be the cause of  $\text{NH}_4^+$  syndrome. Increased respiratory activity in  $\text{NH}_4^+$  supplied plants may produce greater mitochondrial reactive oxygen species (mtROS) generation, leading to oxidative stress, according to Guo *et al.* (2005). During  $\text{NH}_4^+$  feeding, ROS buildup in mitochondrial membranes, which was identified cytochemically as cerium perhydroxide precipitates; however Guo *et al.* (2005) study has to be confirmed. mtROS are primarily engaged in conventional signal transduction under  $\text{NH}_4^+$  supply, which is comparable to what has been postulated under other stress situations (Noctor, De Paepe and Foyer 2007).

## **2.5 Mitochondrial Target for Improving NUE**

### **2.5.1 Mitochondria and Primary Assimilation of N**

The most prevalent N source for plants is Nitrate ( $\text{NO}_3^-$ ), proton ( $\text{H}^+$ ) and ammonia ( $\text{NH}_4^+$ ) are both transported through the plasma membrane at the same time, and pH gradients drive the symport across membranes. The plasma membrane  $\text{H}^+$  ATPase counteracts depolarization and is fueled by ATP produced mostly in the mitochondria. Dechorgnat *et al.* (2011) and Kraiser *et al.* (2011) have studied the processes of N transport through

membranes. When plants are supplied with  $\text{NO}_3^-$ , the initial stage in the reduction process occurs in the cytosol, and the conversion of nitrate to nitrite (catalysed by  $\text{NO}_3^-$  reductase, NR) necessitates the use of reducing equivalents. Even under photorespiratory conditions, the concentration of cytosolic NADH is around 0.3–0.7 mM (Heineke *et al.*, 1991), which is below the  $K_m$  of NR (7 mM) (Kaiser *et al.*, 2000). A large portion of the reductants is of chloroplast or mitochondrial origin, in addition to cytosolic-localized processes that decrease nicotinamide adenine dinucleotide ( $\text{NAD}^+$ ) to NADH (e.g. glycolysis). Up to 50% of NADH generated in mitochondria (primarily from the glycine (Gly) decarboxylase complex activity) may be transferred to the cytosol during steady-state photosynthesis (Fig. 2.3).



**Figure 2.3. Simplified scheme showing involvement of leaf mitochondria in  $\text{NO}_3^-$  assimilation** (Szal *et al.*, 2012).

Mitochondria provide 2-oxoglutarate (2-OG) for chloroplast-localized glutamine synthetase–glutamine: 2-OG aminotransferase (GS-GOGAT) cycle and reductants used by cytosolic  $\text{NO}_3^-$  reductase (NR).

The photorespiratory metabolism-driven mitochondrial malate shuttle' raises the NADH/NAD ratio in the cytosol, providing reductants for NR activity in  $\text{C}_3$  plants (Bloom *et al.*, 2010). There are little experimental data on cytosolic NADH concentration changes in photo respiratory and non photo respiratory situations (Igamberdiev *et al.*, 2001). However, because primary  $\text{NO}_3^-$  absorption is mostly dependent on photorespiration, eliminating the oxygenation reaction of ribulose 5-bisphosphate carboxylase/oxygenase (Rubisco) may result in N deficit in plants (Bloom *et al.*, 2010). The glutamine (Gln) synthetase Gln: 2-oxoglutarate (2-OG) aminotransferase (GS-GOGAT) cycle in chloroplasts requires continual availability to C skeletons (2-OG), and mitochondria are one among the sources of

those essential for this cycle (Tcherkez *et al.*, 2009 and Gauthier *et al.*, 2010). Remobilization of night-stored organic acids during light circumstances has been shown to play a substantial role in supplying 2-OG for Glu production (Gauthier *et al.*, 2010). Despite light-dependent inhibition of the mitochondrial pyruvate (Pyr) dehydrogenase complex and redox-dependent inhibition of other Krebs cycle dehydrogenases, partial operation of the Krebs cycle occurs under light circumstances. Which organic acid is exported from the mitochondria is still a point of contention (Foyer *et al.*, 2011). Through the di/tricarboxylate transporter, 2-OG may be removed from the TCA cycle and transported to the cytosol. Igamberdiev and Gardeström (2003) found that citrate or isocitrate may be exported from mitochondria (through the same transporter) and transformed to 2-OG in the cytosol. When 2-OG is removed from the mitochondria, mitochondrial NAD- or NADP-isocitrate dehydrogenase (-IDH) is engaged in 2-OG synthesis, but cytosolic NADP-IDH is implicated when citrate or isocitrate is transported from the mitochondria. Citrate has been identified as the main mitochondrial product in illuminated leaves by nuclear magnetic resonance (NMR) studies; however, recent results obtained using transgenic plants suggest that no specific IDH isoform is involved in the production of 2-OG for N assimilation, and both of the above reactions may occur (Foyer *et al.*, 2011).

### **2.5.2 Reassimilation of Ammonia Generated in the Photorespiratory Pathway**

Several enzyme processes in the chloroplasts, mitochondria, peroxisomes, and cytosol are involved in the photorespiratory pathway (Bauwe *et al.*, 2010; Peterhansel and Maurino 2011). NADH and ammonia (NH<sub>3</sub>) are released in mitochondria through processes catalysed by Gly decarboxylase complex (GDC) and Ser hydroxyl methyl transferase (SHMT). Ammonia is an important type of reduced nitrogen that must be reassimilated. By one order of magnitude, the reassimilation process out performs primary N assimilation (Keys *et al.*, 1978). Earlier, it was assumed that NH<sub>4</sub><sup>+</sup> simply diffuses into the chloroplast, where it is utilised in the GS-GOGAT cycle; however, current experimental findings suggest that this is not the case. GS2 is a dual-targeted peptide, according to Taira *et al.*, (2004). The fact that the GS2 isoform is found in both plastids and mitochondria shows that NH<sub>4</sub><sup>+</sup> is incorporated into Gln structure in both organelles. when GS activity in isolated chloroplasts and mitochondria was examined, the latter was around 4 to 10 fold greater than the former. Furthermore, photo respiratory circumstances increased organellar GS activity in both chloroplasts and mitochondria (Taira *et al.*, 2004). The fact that the second enzyme in the GS-GOGAT cycle (Fd-GOGAT) is likewise localized to both mitochondria and chloroplasts (Jamai *et al.*, 2009) suggests that the ammonia reassimilation process might be successful in both chloroplasts

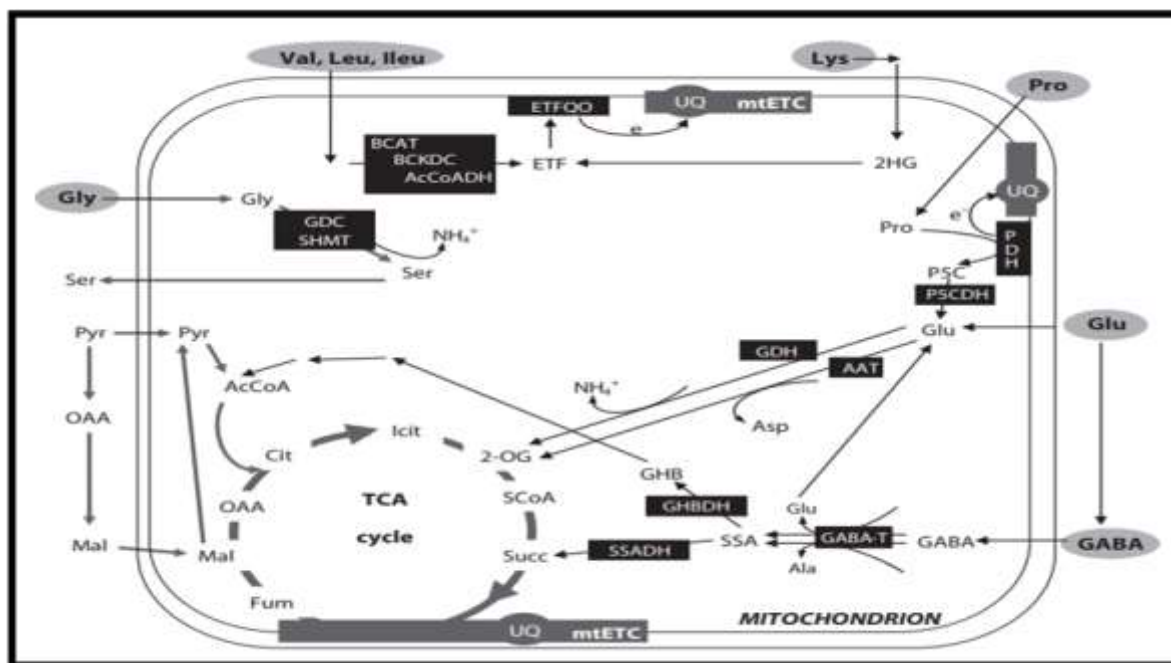
and mitochondria. There has yet to be discovered a source of electrons for mitochondrial Fd-GOGAT, and instead, a regulatory role for mt Fd-GOGAT has been hypothesised. Although mtFd-GOGAT is most likely necessary for increasing mitochondrial serine hydroxymethyl transferase function (Jamai *et al.*, 2009), Gln created in the matrix as a result of mtGS2 activity may be exported from the mitochondria at the same time as Glu import to preserve the reaction stoichiometry. Alternatively, it has been hypothesised that mitochondrial  $\text{NH}_3$  absorption is linked to the transport of carbon dioxide between chloroplasts and mitochondria *via* a citrulline–ornithine shuttle (Taira *et al.*, 2004). Because the mitochondrial carbamoyl phosphate synthetase (CPS) activity permits direct regeneration of the  $\text{NH}_3^+$  acceptor (Glu) inside the mitochondrial matrix, this process uses a lot of ATP. Taira *et al.* (2004) established the presence of this mechanism by demonstrating that isolated mitochondria can catalyse Gly-dependent ornithine to citrulline conversion. Further research on  $\text{NH}_3^+$  reassimilation in mitochondria is needed, and new results suggest that the photorespiratory route may be more sophisticated than previously thought.

### 2.5.3 Mitochondrially Localized Amino Acid Metabolism

Morot-Gaudry *et al.* (2001) and Sweetlove *et al.* (2007) have documented the anabolic routes of amino acids (AAs), including those mitochondrially localised such as cysteine, amino acids catabolism connected to the Krebs cycle or mtETC activity. Under photorespiratory circumstances, Gly decarboxylation is the most significant mitochondrial AAs catabolic process (Raghavendra and Padmasree 2003). The effect of GDC-produced NADH on the main N assimilation process, as well as the suggested routes for  $\text{NH}_4^+$  reassimilation. The process catalysed by Glu dehydrogenase is another major mitochondrial AA catabolism route (GDH). The amination reaction of 2-OG and the deamination of Glu might hypothetically be catalysed by GDH (Dubois *et al.*, 2003). MeloOliveira *et al.* (1996) demonstrated that GDH may be involved in N assimilation in the presence of inorganic N excess. The major function of GDH in plants, however, has been experimentally proven to be the deamination of Glu into 2-OG, which is subsequently absorbed into the Krebs cycle (Masclaux-Daubresse *et al.*, 2006). Under low C circumstances, the 2 OG flow from Glu oxidation is extremely essential (Masclaux-Daubresse *et al.*, 2006). Through the  $\gamma$ -aminobutyrate (GABA) shunt, mitochondria are also implicated in the metabolism of Glu (Fait *et al.*, 2008). GABA metabolism is linked to cytosolic pH control, insect defence against oxidative stress, and osmoregulation (Bouche and Fromm 2004). GABA is also thought to be a signalling molecule involved in the maintenance of C/N balance, and it is generated in both stress and non-stress situations (Tcherkez *et al.*, 2009). Glu in the cytosol is decarboxylated

to GABA within the GABA shunt. It is subsequently carried into the mitochondria and matrix *via* GABA transaminase (GABA-T) processes, and succinic semi aldehyde dehydrogenase (SSADH) metabolises it to succinate, which enters the Krebs cycle (Fig. 2.4). Mitochondrial GABA-T may receive Pyr or 2-OG as an amino acceptor and catalyse the conversion of GABA to SSA, resulting in alanine (Ala) or glutamine (Glu) (Fait *et al.*, 2008). SSA can be converted to 4-hydroxybutyrate (GHB) *via* GHB dehydrogenase and then metabolised to acetyl coenzyme A in addition to SSADH activity (CoA; Fait *et al.*, 2008). Because of the many routes of intermediate incorporation into the TCA cycle and the positive correlation of the expression of GABA shunt regulatory enzymes (e.g. SSADH) with genes associated with the TCA cycle, Fait *et al.* (2008) suggest that a GABA shunt may even be approved as an integral part of the TCA cycle. Proline (Pro) buildup is a well known plant response to stress, resulting from increased synthesis and slowed breakdown of the amino acid. During stress tolerance, stored Pro is quickly oxidised, providing N, reducing equivalents, and energy. Pro dehydrogenase (ProDH) and D1-pyrroline-5- carboxylate dehydrogenase work together in the mitochondria to convert Pro to Glu (P5CDH) (Fig. 2.4). P5CDH is found inside the mitochondrial matrix, whereas ProDH is linked to the inner mitochondrial membrane with its active site facing the matrix (Elthon and Stewart 1981). Plaxton and Podesta (2006) found that NADH generated by P5CDH is utilised in mtETC and that Glu following deamination to 2-OG may be absorbed into the Krebs cycle. The oxidation of Pro accounted for 20% of respiration in barley leaves recovering from stress. Branched chain AAs e.g. valine (Val), leucine (Leu), and isoleucine (Ile) can also be oxidised by particular dehydrogenase complexes found in plant mitochondria (Binder *et al.*, 2007). Val, Leu, and Ile are transaminated to their respective  $\alpha$ -keto acids by the branched chain AA transaminase in the first stage of the mitochondrial-localized branched-chain AA catabolic process (Maloney *et al.*, 2010). The  $\alpha$ -keto acids are further decarboxylated and esterified to CoA by the branched-chain keto acid dehydrogenase complex (BCKDC) (Taylor *et al.*, 2004). Mitochondrial localization of BCKDC was confirmed by Fujiki *et al.* (2000). The CoA esters generated by BCKDC are then oxidized by acyl-CoA dehydrogenases, and electrons are delivered through electron transfer flavoprotein/electron transfer flavoprotein:ubiquinone oxidoreductase (ETF/ ETFQO) system directly to mtETC (Ishizaki *et al.*, 2006). Arabidopsis mitochondria contain acyl-CoA dehydrogenase (isovaleryl-CoA dehydrogenase, IVD) showing activity towards both isovaleryl-CoA (from Leu) and isobutyryl-CoA (from Val) linked with the ETF/ ETFQO system (Araujo *et al.*, 2010). Subsequent steps along the catabolic pathways lead to the formation of propionyl-CoA (Val catabolism), propionyl-CoA

and acetyl-CoA (Ile), and acetyl-CoA and acetoacetate in Leu metabolism (Kochevenko and Fernie, 2011). All processes involved in the catabolic pathway are localised in the mitochondria solely in Leu (Taylor *et al.*, 2004). The ETF/ETFQO system also plays a role in the transfer of electrons from 2-hydroxyglutarate (through 2-hydroxyglutarate dehydrogenase), a lysine (Lys) metabolic intermediate (Araujo *et al.*, 2010).



**Figure 2.4. Mitochondrial pathways of amino acid (AA) catabolism and its relation with the tricarboxylic acid (TCA) cycle or mitochondrial electron transport chain (mtETC) (Szal *et al.*, 2012).**

Catabolism of branched-chain AAs is increased during C deficiency, when AAs can be employed as an alternate substrate for respiration (Fujiki *et al.*, 2002). The control of branched-chain AA oxidation, on the other hand, might be a detoxifying mechanism, enabling a pool of branched-chain AAs to be maintained for protein synthesis while preventing dangerous amounts from accumulating (Taylor *et al.*, 2004).

## 2.6 Transgenic Approach to Manipulate NUE

### 2.6.1 Manipulating Genes of Nitrogen Uptake

Root uptake of inorganic nitrogen forms dominates and requires the function of nitrate and ammonium transporters. N uptake is the first steps in nitrate assimilation, which can be manipulated to improve NUE. Tsay *et al.* (2007) classified transporters into low affinity transport systems (LATS) and high affinity transport systems (HATS), just as far as constitutive or inducible. LATS function at a comparatively higher concentration of N (>0.5 mM) and have larger Km values (5 mM). On the other hand, HATS function at low N concentration (0.2 mM–0.5 mM) and have smaller Km values (of around 50 μM). Also,

Miller *et al.* (2007) also classified the root low affinity transport system (LATS) shows linear kinetics, and its concentration to  $\text{NO}_3^-$  uptake becomes significant at external  $\text{NO}_3^-$  concentrations above 1 mM. The high affinity transport system (HATS) is able to absorb  $\text{NO}_3^-$  at low concentration (between 1  $\mu\text{M}$  and 1 mM).

#### 2.6.1.1 Genetic Manipulation of Nitrate Transporter to Improve NUE

Improving NUE under both high and low nitrogen have predominantly been accomplished when NR transporter were constitutively overexpressed (Feng *et al.*, 2017) and expressed in both root and leaf tissues (Chen *et al.*, 2016, 2017). Feng *et al.*, 2011 and Yan *et al.*, 2011 reported with respect to high affinity nitrate transporter in rice, four NRT2 and two NAR2 genes. OsNRT2.1, OsNRT2.2, and OsNRT2.3a need to involve with OsNAR2.1 for nitrate uptake, while OsNRT2.3b and OsNRT2.4 can work freely without NAR2. OsNRT2.3a is fundamentally communicated in xylem parenchyma cells of root steles, and knockdown of OsNRT2.3a debilitates xylem stacking of nitrate just as diminished plant development at low nitrate supply, showing a part in significant distance nitrate transport from root to shoot (Tang *et al.*, 2012), while the other joined structure, OsNRT2.3b, is primarily communicated in the phloem and perhaps works as a pH sensor (Fan *et al.*, 2016).

Wang *et al.* (2018) found that *OsNRT1.1A*, another rice *NRT1.1*, shows an unexpected expression pattern with strong induction by ammonium. However, functional characterization of the loss of function mutant and overexpression transgenic plants exhibited that *OsNRT1.1A* promoter the use of both ammonium and nitrate by up regulating the expression of N utilization related genes. In comparison with *AtNRT1.1* and *OsNRT1.1B* intervening the nitrate stimulated primary response, *OsNRT1.1A* seems to pay a more functional role in managing NUE a relative stable N supply with both ammonium and nitrate. Possibly, this functional divergence between *OsNRT1.1A* and *OsNRT1.1B* is associated with their sub cellular localization. The *HvNRT2* gene family in barley that encodes high affinity  $\text{NO}_3^-$  transporters was also identified as NUE applicants (Han *et al.*, 2016).

*OsNRT1.1a* and *OsNRT1.1B* overexpression lines increased total N accumulation in all N treatments, including 0.125 mM  $\text{NH}_4\text{NO}_3$  (Fan *et al.*, 2016). Overexpression of *OsNRT1.1* in rice under low nitrogen conditions in field increased grain yield per plant by 32%–50% and NUE by 38%–54% per plot. Its mutations decreased the panicle size; seed setting rate and grain yield (Hu *et al.*, 2019). Correspondingly, overexpression of *OsNRT2.1*, *OsNRT2.3b* and *OsPTR9* in rice expanded NUE, grain yield and plant growth (Wang *et al.*, 2018).

The balance of the outflow of N transporter has a beneficial impact on the overall plant NUE. Overexpression of OsNRT1 can accumulate more N in rice plants only under high N conditions, but expression of its grafting structure with six transmembrane domains can also accumulate more nitrogen in plants (Fan *et al.*, 2016).

### 2.6.1.2 Genetic manipulation of ammonium transporter to improve NUE

In add it on to nitrate; ammonium is another significant N source for plant growth. For the most part, the utilization of ammonium transporters as targets for improving N uptake and utilization problematic since changes in cellular ammonium pools or excess ammonium can be harmful for the plant cell (Bittsanszky *et al.*, 2015).

OsAMT1;1 is communicated in the two roots and shoots, and is actuated by ammonium. The mutants of OsAMT1;1 showed decreased ammonium uptake as well as repressed growth of roots and shoots under both low and high ammonium conditions (Li *et al.*, 2016). OsAMT1;2 and OsAMT1;3 is induced by N deficiency in roots. Considerable efforts have been made to improve rice ammonium transport efficiency by overexpressing different OsAMT genes, with very little achievement (Bao *et al.*, 2015). OsAMT1.1 is expressed in both roots and shoots and has an average of a 2.1fold increase in its expression in response to N deprivation, which improves ammonium uptake and expands grain yield (Bao, *et al.*, 2015). Li *et al.*, 2017 and Xuan *et al.*, 2017 reported that in *Arabidopsis*, there are 6 AMTs and rice genome has 12 AMTs which have been ordered into two subfamilies: OsAMT1 and OsAMT2. Numerous ammonium transporters (AMTs) have also been targeted to improve NUE by analyzing the phenotypic changes of specific overexpressing or mutant lines. AMTs have a place with the AMT/MEP/Rhesus transporter family, which are highly conserved in microorganisms, and plants with more than 700 homologs in bacterial organisms and plants. Duan *et al.*, 2018 observed AMT-type transporters are expressed in roots of *A. thaliana*, every one of them be coming up controlled under N-deficient conditions. In this species, the vast majority of the high affinity uptake capacity of  $\text{NH}_4^+$  is mediated by AMT1-1, AMT1-2, and AMT1-3 and, in the low-affinity range, different transporters, for example, AMT2-1 come into play to add to ammonium transport (Giehl *et al.*, 2017). Result shown that regulation of these transporter genes is strongly correlated with changes in N uptake action in roots and gives strong evidence to improving NUE in barley.

More recently, Chen *et al.*, 2020 worked in (*Arabidopsis thaliana*), tobacco (*Nicotiana tabacum*), and rice (*Oryza sativa*) showed that overexpression of the hyperactive chimeric nitrate transporter NC4N drive by the NRT1.7 promoter into the nrt1.7 mutant

nrt1.7p: : NC4N: :3 transgenic plants accumulated more nitrate in more younger leaves, and 15 NO<sub>3</sub><sup>-</sup> tracing analysis revealed that more 15N was remobilized into sink tissues brings about improved NUE under high and low N.

### 2.6.2 Genetic Manipulation of Nitrate Reduction to Improve NUE

Following uptake, nitrate is reduced in the cytosol to nitrite which is transported into the plastid for additional reduction to ammonium (Liu *et al.*, 2015). The two step reduction is accomplished by nitrate reductase (NR) and nitrite reductase (NiR) respectively. Nitrate reduction catalyzes the primary regulatory step in the nitrogen assimilation process and its movement is profoundly directed by components like nitrate, light, or water availability (Chamizo-Ampudia *et al.*, 2017; Mauceri *et al.*, 2020).

In 2013, Zhao *et al.* found that nitrate reductase (NR) is the first and rate-limiting enzyme in the nitrate assimilation process. Many studies have found that altering the expression of NR expressing genes has no effect on growth response or yield. Overexpression of the 35S NR gene significantly increased foliar NRA in more than 70% of T1 (kanamycin-resistant) offspring. Increased NADH levels generated by selective co-suppression of ubiquinone oxidoreductase (complex-I) resulted in increased NR activity, which led to an increase in grain yield.

Loussaert *et al.*, 2018 found that the positive event had significantly reduced leaf NADH-ubiquinone oxidoreductase 51 kDa subunit (Complex I) expression, higher extractable NADH, and higher NRA (*in vivo*), but no change in extractable NRA (*in vitro*). In root, a non-targeted tissue, there was no difference in NADH-ubiquinone oxidoreductase 51 kDa subunit (Complex I) expression between transgene and non-transgene. NA is limited by NADH rather than NRA, these results are to be expected (*in vitro*). Increases in kernel number plant<sup>-1</sup> linked with enhanced grain yields under high N fertility could have been brokered by improved NA during ear development, since the transgenic hybrid displayed higher NADH levels associated with improved NRA (*in vivo*) without an increase in NRA (*in vitro*).

The cytosolic enzyme nitrate reductase (NR) reduces nitrate to nitrite at the expense of NADH generated from malate shuttled from the mitochondrion or chloroplast during N assimilation (Foyer and Noctor, 2002). The presence of NADH influences the rate of nitrate assimilation, and NAD status is important for coordinating ammonia assimilation with the anaplerotic synthesis of carbon skeletons. Many researches are confirm that these genes are promising candidates for increasing yield in the presence of N deficit. Increased N absorption has frequently resulted from overexpression of NR or NiR, but no gains in seed yield, seed

protein level, or NUE have been seen, possibly due to negative feedback from N-containing metabolites and related transcriptional or post-transcriptional regulation (Li *et al.*, 2020). However, recent research in japonica rice found that constitutive expression of indica OsNR2, which encodes a NADH/NADPH-dependent NR, results in higher tiller number, grain yield, and NUE when N is abundant (Gao *et al.*, 2019a). These effects were increased when indica OsNR2 and OsNRT1.1B were both expressed, demonstrating that OsNR2 regulates nitrate absorption *via* positively regulating OsNRT1.1B. (Gao *et al.*, 2019a).

### 2.6.3 Genetic Manipulation of Nitrite Reductase to Improve NUE

Crete *et al.* (1997) made the first attempt to create plants with higher NiR activity, transforming tobacco with a tobacco NiR gene and obtaining a maximum of a 63% increase in leaf NiR activity in plants with no evident phenotype. Takahashi *et al.*, (2001) developed *Arabidopsis* plants with a spinach NiR gene that had an 82 % increase in NiR activity, concluding that NiR was considerably more essential than nitrate reductase and glutamine synthetase in the management of nitrate assimilation in plants.

May *et al.* (2012) cultivated tobacco plants with an overexpressed tobacco *nii2* gene, which showed a 30% increase in NiR activity but didn't show any phenotypic differences from WT plants. When the *Arabidopsis* gene (AtNiR), which encodes NiR, was overexpressed in tobacco, the older leaves of the transgenic NtAtNiR plants maintained a green color and appeared to senesce more slowly over a longer length of time than the WT. In an attempt to modify the level of residual nitrite in the leaf, which can act as a precursor to the synthesis of nitrosamines (Davenport *et al.*, 2015).

### 2.6.4 Manipulating Genes of Ammonia Assimilation and Remobilization

Ammonium is converted to glutamine and glutamate by the coordinated activities of glutamine synthetase (GS) and glutamate synthase (GOGAT), which serve as nitrogen donors for the production of other amino acids and amides (Lea and Miflin, 2011).

#### 2.6.4.1 Genetic manipulation of GS to improve NUE

The cytosolic GS1 and plastidic GS2 isoforms of GS, which have different activities in N assimilation, are encoded by multigenic or single gene families (Chardon *et al.*, 2012). Primary N assimilation (*i.e.*, *de novo* amino acid synthesis) and reassimilation of ammonium synthesis during photorespiration are both aided by GS2 (Ferreira *et al.*, 2019). With certain exceptions, such as AtGLN1; 2 in *Arabidopsis*, which is critical for N assimilation under high nitrate input (Lothier *et al.*, 2011) and OsGS1;1 in rice shoots and roots, GS1 isoforms are primarily involved in N reassimilation and remobilization (Kusano *et al.*, 2011). GS1 and GS2 genes have been overexpressed in a variety of species due to their important roles in N

metabolism and plant development and seed output. Furthermore, multiple investigations have emphasised the role of cytosolic GS1 genes in cereal grain filling. In rice (Obara *et al.*, 2004), maize (Martin *et al.*, 2006), and wheat (Hirel *et al.*, 2007), positive associations were found between grain number/size and cytosolic GS protein content/GS activity (Habash *et al.*, 2001).

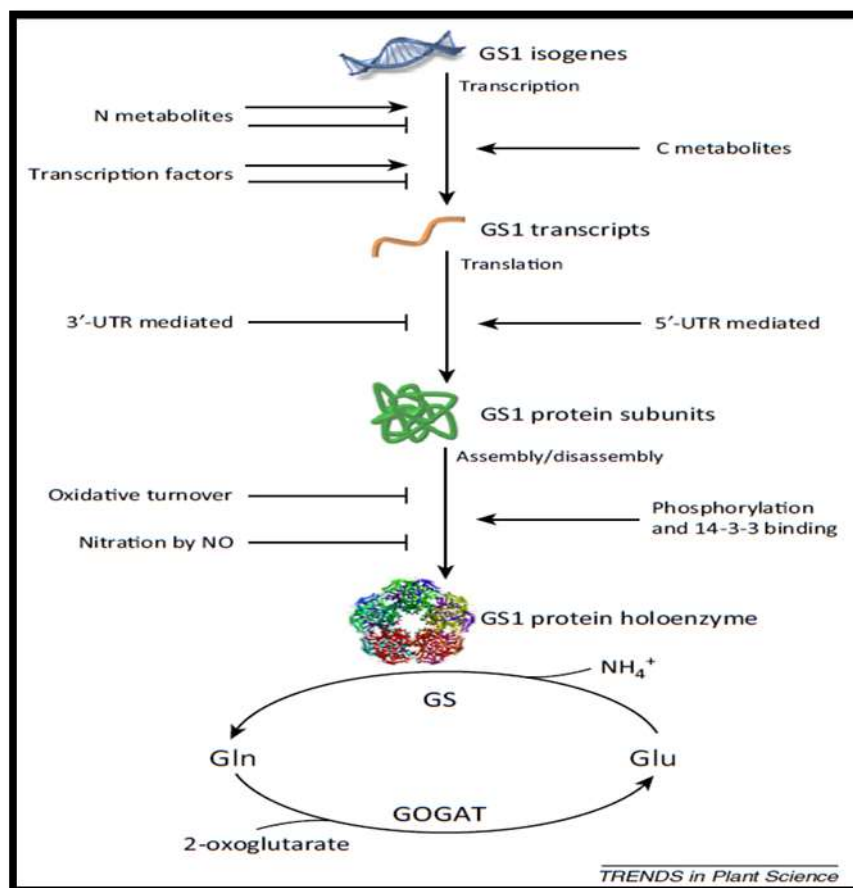
Cisgenic overexpression of cytosolic glutamine synthetase improves nitrogen utilisation efficiency, according to Gao *et al.* (2019b). They observed that increasing HvGS1-1 expression in barley under the direction of its own promoter increase grain yields and NUE under both high and low N conditions. In wheat, researchers have shown that overexpression of TaGS2-2Ab in wheat *via* its natural promoter resulted in higher seed yield and NUE when N availability was high and low (Cheng *et al.*, 2018). This is because the transgenic line had longer primary roots and a higher number of lateral roots.

Overexpression of GS1 in maize, wheat, and rice has resulted in increased grain yields Brauer *et al.*, 2011; Transgenic tobacco lines coexpressing Dof1 and GS grew better under low nitrogen conditions than Dof1-overexpressing or GS-overexpressing tobacco lines, according to Wang *et al.*, 2013, as evidenced by higher plant height and fresh and dry weights of shoots. In tobacco leaves, overproduction of chloroplastic AtGS2 or cytosolic AtGS1;4 re-assimilate ammonia produced by photorespiration. Overexpression of GS in leaves may lead the plant to reuse absorbed nitrogen, hence increasing NUE. Overexpression of Dof1 and GS from Arabidopsis boosted the NUE of tobacco plants significantly total N contents.

The two strategies, according to Thomsen *et al.* (2014), are critical for the successful use of TaGS22Ab in increasing N efficient wheat. The first was to enhancing GS activity in the leaves. The relationship between flag leaf GS activity and transgenic line grain yield revealed that high leaf GS activity had a detrimental impact on grain yield. They reported that transgenic lines with a lot of leaf GS activity stayed green for a long time. TaGS22Ab own promoter used to drive its expression. By optimizing GS activity and driving TaGS22Ab by its own promoter, metabolic imbalances avoided (Cheng *et al.*, 2018).

*Sorghum bicolor* growth and development are affected by overexpression of a glutamine synthetase gene. In comparison to non-transgenic controls Urriola and Rathore, (2015) the ubiquitin (Ubq) promoter offspring of the transformants had higher accumulation of Gln1 transcripts and up to 2.2-fold more GS activity. In GS1;1-, GS1;2-overexpressing plants, Bao *et al.*, 2014 found significant changes in individual sugars, organic acids and free amino acids, as well as gene expression patterns, indicating the distinct roles that these two

GS1 genes played in rice nitrogen metabolism, especially when sufficient nitrogen was applied in the environment. As a result, the unbalanced carbon-nitrogen metabolic status and poor ability of nitrogen transportation from stem to leaf in GS1;1-, GS1;2-overexpressing plants may be the main cause of lower growth and yield. The isogenes OsGS1;2 and OsGS1;3 are unable to compensate for the loss of OsGS1;1 in the OsGS1;1 knockout mutant, showing that OsGS1 isogenes are nonredundant and play nonoverlapping roles (Yamaya *et al.*, 2014).



**Figure 2. 5. Multilevel regulation of glutamine synthetase (GS)** (Thomsen *et al.*, 2014).

Nitrogen metabolites include  $\text{NH}_4^+$ , glutamate (Glu), and glutamine (Gln); carbon metabolites include sucrose and other sugar compounds; transcription factors include MYB and Dof as depicted.

Assimilation of inorganic N *via* the GS–glutamate synthase (GOGAT) pathway uses around 15% of the cell's ATP and is consequently tightly controlled at the gene, transcript, and protein levels (Harper *et al.*, 2010). Increased GS1 gene expression did not result in a same amount of rise in enzyme activity, as evidenced by research revealing that this multilayer control of GS can possibly interfere with overexpression strategies (Seger *et al.*, 2009). The variable results of GS1 overexpression could be explained in part by the fact that

the transformed lines were examined under diverse growing conditions, which could have confused the responsiveness to increased GS activity. The fact that most research only includes a few physiological and climatic factors useful for identifying potential growth limitations makes pass comparisons difficult. Because N assimilation requires the presence of carbon (C) skeletons, the relationship between N assimilation and C metabolism is complex (Schjoerring *et al.*, 2002). If N is otherwise sufficient, insufficient C availability may act as a limitation on N assimilation, reducing the consequences of GS1 overexpression.

#### **2.6.4.2 Genetic manipulation of GOGAT to improve NUE**

Fd-GOGAT is primarily involved in the assimilation of ammonium that originates either from nitrate reduction or leaf photorespiration, NADH-GOGAT is highly expressed in non-photosynthesizing cells and has a distinct function in non photo respiratory ammonium reduction (Lee *et al.*, 2020). Wang *et al.* (2018) investigated how genetic differences in ARE1 modulate rice grain production through nitrogen utilisation. *are1* is a nitrogen-assimilation-deficient rice Fd-GOGAT mutant's genetic suppressor. ARE1 is a chloroplast-localized protein encoded by a highly conserved gene. Loss of function mutations in ARE1 (*are 1-1*) delay senescence and improve crop yields by 10–20%, resulting in higher NUE under nitrogen-limiting conditions. Under nitrogen-limiting conditions, this results in a decrease in ARE1 expression and an increase in grain production.

Tamura *et al.* (2011) examined the function of NADH-GOGAT in rice plants using a reverse genetics technique. Rice genes encoding Fd-GOGAT, NADH- GOGAT1, and NADH- GOGAT2 were discovered in this investigation. OsNADH-GOGAT2, a newly discovered gene was found to be expressed mostly in fully expanded leaf blades and leaf sheaths. Disruption of this gene led to significant reductions in yield and whole plant biomass.

Rice Fd-GOGAT gene, which is encoded by the ABC1 gene, is critical for both N assimilation and carbon-nitrogen balance. The *abc1-1* mutation has no influence on ABC1 expression or protein accumulation, but it may cause a reduction in enzymatic activity, according to the findings. To see if this was true, researchers investigated at Fd-GOGAT activity in wild-type and *abc1-1* mutant seedlings. They revealed that *abc1-1* had a 45% decrease in Fd-GOGAT activity relative to wild-type, and that an ABC1-FLAG transgene could entirely restore this phenotype (Yang, *et al.*, 2016). Zeng *et al.*, 2017 studied contribution of a glutamate synthase to rice foliar nitrogen metabolism is unknown because there are no known knockdown lines of OsFd-GOGAT in rice (*Oryza sativa* L.). Under natural conditions, the *gogat1* mutant showed chlorosis, but under low light conditions, it

showed reduced premature leaf senescence. The *gogat1* mutant had a lower seed setting rate than the wild type, but its grain protein content was considerably higher. OsFd-GOGAT may be involved in nitrogen remobilization during leaf senescence, which could be a technique to increase rice nitrogen use efficiency.

Similarly, photorespiration does not limit glutamate biosynthesis, although glutamine and glycine are important amino acids. Consolidated overexpression of OsAMT1;2 and OsNADH-GOGAT1 in rice, enhanced NUE in both adequate and low N conditions. The transgenic lines achieved higher seed protein levels without changing seed yield when N was adequate, but both seed protein and yield were improved when N was low (Lee *et al.*, 2020).

OsGOGAT1 is crucial in the main absorption of  $\text{NH}_4^+$  in roots, according to previous reports by Bao *et al.*, in 2014. Tamura *et al.* (2010) found that knocking out OsGOGAT1 impaired seedling root growth and the formation of active tiller numbers, indicating the relevance of OsGOGAT1 in rice nitrogen uptake and management. In this investigation, looked at two OsGOGAT1 activation tagging lines that showed improved tolerance to N limitation. The OsGOGAT1 activation tagging mutants, such as the OsAMT1;2 activation lines, showed increased ammonium absorption and GS activity. The presence of a feedback regulation loop in the GS/GOGAT cycle for efficient  $\text{NH}_4^+$  assimilation has previously been reported in OsGS1;1 overexpressing transgenic rice, which led to the suggestion of a feedback regulation loop in the GS/GOGAT cycle for efficient  $\text{NH}_4^+$  assimilation.

The increased expression of OsGOGAT1 in activation tagging lines led to higher OsGS1;1 expression, which is consistent with the proposed feedback regulation mechanism. The expression of OsAMTs was then raised to fulfill ammonium substrate requirements for assimilation, regulating the GS/GOGAT cycle for N assimilation (Lea and Mifflin, 2003). Negative intrinsic effects that may occur when just AMT or GOGAT is overexpressed (e.g., through imbalanced pools of N metabolites), according to Lee *et al.*, 2020, may be avoided by increasing both N absorption and N assimilation. Yamaya and Kusano (2014) discovered that transgenic indica rice overexpressing the NADH-OsGOGAT1 gene from indica under the control of a promoter from japonica rice produced more grain. Irrespective of the increase in enzyme activity in the four types of transgenic plants, overexpression of NADH-GOGAT activity was only detrimental to shoot biomass production. An increase in shoot dry weight was only observed in tobacco plants overexpressing constitutively the enzyme from alfalfa, and only at the vegetative stage. Different results were observed for many genetic alterations

involving enzymes in the N assimilation pathway, which is likely due to the type of transgene involved (Plett *et al.*, 2017).

#### **2.6.4.3 Genetic manipulation of asparagine synthetase and alanine amino transferase**

In *Arabidopsis*, constitutive expression of *Arabidopsis* asparagine synthetase1 doubles soluble seed protein content and increases total seed protein content by up to 21% (Lam *et al.*, 2003), which is consistent with asparagine's primary role in supplying N for protein synthesis in developing seeds (Lam *et al.*, 2003). Alternatively, Good *et al.*, 2007 investigated the use of alanine aminotransferase to improve nitrogen utilisation efficiency. There have been multiple reports of transgenic overexpression of AS genes that are involved in amino acid remobilization and translocation, resulting in increased seed protein and total protein content. Recent findings of genetically modified plants overexpressing alanine aminotransferase have led to attempts at molecular manipulation of asparagine synthetase (encoded by the *Aln1* gene). AlaAT is involved in alanine synthesis and catabolism, and constitutive or root-specific expression of barley *HvAlaAT* resulted in improved seed yields in wheat (Pena *et al.*, 2017), and Shrawat *et al.* (2008) demonstrated that manipulating AlaAT increased both the percentage nitrogen and the plant biomass by improving the nitrogen uptake efficiency of the plant in rice (Shrawat *et al.*, 2008). They propose that rice engineering improves production and profitability while lowering input costs and reducing environmental damage. Even though, given the complexity of plant systems, different engineering approaches, such as novel genes and tissue-specific promoter selection to drive the expression of the gene of interest, will need to be investigated in order to produce transgenic plants with reliable field-based NUE improvements. Beatty *et al.* (2009) investigated the transcriptome of nitrogen-efficient rice that overexpressed alanine aminotransferase. Through root and shoot transcriptome research, they discovered that several potential genes were involved in transgenic rice overexpressing alanine aminotransferase (AlaAT) under the control of a tissue-specific promoter, resulting in a significant NUE phenotype. Under limiting and sufficient N treatments, constitutive overexpression of *OsASN1* resulted in enhanced seed N content and NUE, as well as significant improvements in shoot biomass and seed yield at low N. (Lee *et al.*, 2020). Furthermore, when *HvAlaAT* expression was localised to roots and altered plants were subjected to high N (Beatty *et al.*, 2013) and/or low N, NUE was raised in rice, canola, and *Saccharum officinarum* (sugarcane) (Snyman *et al.*, 2015). It's unclear how AlaAT function in roots influences seed yield and NUE, as it doesn't appear to be required for plant N metabolism or source-to-sink partitioning (McAllister and Good, 2015).

### 2.6.5 The Carbon-Nitrogen Balance

The main necessities for plant growth and development include sunlight, carbon dioxide, water, and mineral components. Nitrogen is essential because it is found in amino acids, which are the building blocks of protein, nucleotides, chlorophyll, and a variety of other metabolites and cellular components. Plants frequently require more nitrogen than any other mineral element, owing to the large quantity of nitrogen invested in the photosynthetic apparatus, particularly Rubisco and light-harvesting complexes (Zhu *et al.*, 2008). The light-dependent use of CO<sub>2</sub>, water, and inorganic N to produce sugars, organic acids, and amino acids, the basic building blocks of biomass growth, is facilitated by this invested N. The availability of nitrogen is definitely a significant influence of photosynthetic capability and crop output (Foyer *et al.*, 2006). In the effort to enhance NUE, it has long been understood that the relationship between C and N is critical, and that unless there is enough C available (carbon skeleton generated from non-photosynthetic processes), plants' ability to take up and use N may be compromised. Nitrogen levels in plants can have a big impact on C fixation (Reich *et al.*, 2006). Because photosynthetic proteins like Rubisco and PEP carboxylase contain high amounts of nitrogen. As a result, decreases in N absorption and storage will reduce the amount of C fixed by the plant overall (Nunes-Nesi *et al.*, 2010). Soil nitrogen deficiency has been shown to impact lateral root growth. This is supported by empirical evidence on the NO<sub>3</sub><sup>-</sup> transporter NRT2.1, which demonstrated that it works as a C/N sensor and signals when the ratios between these two elements are skewed; in control plants, high C/low N tends to induce lateral root growth (Zheng 2009).

The products of the GS / GOGAT pathway are also important for plant C/N ratios. Glutamate affects C and N metabolism in both C3 and C4 plants, acting as a signalling and N transport molecule as well as a substrate in the manufacture of other amino acids (Nunes-Nesi *et al.*, 2010). Increased production of the enzyme PEP carboxylase has been linked to higher glutamine levels and an increase in anaplerotic pathway components (Foyer *et al.*, 2003). Anaplerotic reactions are metabolic pathway intermediates that play a key role in both energy production and biosynthesis. In order to maintain cellular metabolism homeostasis, the cell should regulate the amounts of tricarboxylic acid cycle intermediates. Changes in C or N concentrations in sources or sinks must be coordinated with changing environmental inputs like as light conditions, nutrient and water availability (Coruzzi and Zhou 2001). While it has been demonstrated that the regulation of C/N ratios (different photosynthetic products as C and protein, amino acids as N) is critical for appropriate plant growth and development, it also acts as a constraint when determining how to improve NUE in crop plants (Mooshammer

*et al.*, 2014). Photosynthetic rates regulate and control nitrogen intake, assimilation, and remobilization, resulting in a plateau in NUE unless the photosynthetic rate is likewise enhanced (Zheng 2009). The difference in pool strength between the source and sink organs induced by the C-N metabolic substrate and status, resulting in a flow pressure between the source and sink organs, accelerating nutrient translocation to the grain and increasing NUE when nitrogen uptake reached a specific level. (Bao *et al.*, 2014).

#### **2.6.6 The Availability of 2-OG and Ammonia to the GS-Glu Synthase Pathway is Dependent on the NAD Status of the Leaves**

A radical redistribution of amines within the different amino acid pools accompanied the stimulation of nitrate reduction and an overall rise in amino acids in CMS leaves. Despite the discovery of a dual-targeted GS in the Arabidopsis (*Arabidopsis thaliana*) gene GLN2 (Taira *et al.*, 2004), most data suggests that net ammonia assimilation occurs mostly in the chloroplast, where Fd-GOGAT activity requires 2-OG (Hirel and Lea, 2002). Insufficient rates of 2-OG production to enable ammonia absorption may result in increased Gln:Glu ratios (Novitskaya *et al.*, 2002). Thus, an imbalance between 2-OG supply and ammonia synthesis is likely one mechanism driving the higher Gln:Glu in CMS leaves. Any limitation on 2-OG supply should become more significant under conditions when nitrate to ammonia reduction is promoted, resulting in the severe shift in reduced nitrogen partitioning that have seen in CMS. Even though anaplerotic pathway expression was up regulated, there was a decrease in 2-OG no induction of this activity. Indeed, using antisense technology to reduce this enzyme's capacity in potato (*Solanum tuberosum*) has relatively small effects on organic acid and amino acid levels (Kruse *et al.*, 1998). The increase of mitochondrial NAD-ICDH, on the other hand, suggests that this enzyme plays a critical role in anaplerosis in *N. sylvestris*. Changes in NAD and NADH levels are particularly sensitive to this isoform. Indeed, using antisense technology to reduce this enzyme's capacity in potato (*Solanum tuberosum*) has relatively small effects on organic acid and amino acid levels (Kruse *et al.*, 1998). The increase of mitochondrial NAD-ICDH, on the other hand, suggests that this enzyme plays a critical role in anaplerosis in *N. sylvestris*. Changes in NAD and NADH levels are particularly sensitive to this isoform. *i.e.* similar to the range of the whole leaf values. Although pyridine nucleotide extraction methods liberate both protein-bound and free pools, a 2-fold rise in both NAD and NADH is likely to increase NAD-ICDH control over 2-OG synthesis. Igamberdiev and Gardestrom (2003) found that mitochondrial NAD in pea leaves is likely above the NADICDH Km value, although NADH concentrations are likely below or close to the enzyme's Ki value. As a result, a two-fold rise in NAD should have a

minor impact compared to the higher limitation caused by a two fold increase in NADH. Increased restriction on NADICDH's *in vivo* action is likely one of the reasons why increases in enzyme quantity (and extractable activity, evaluated in the absence of NADH) are unable to restore 2-OG contents to wild-type levels (Dutilleul *et al.*, 2005). Changes in the cellular redox state are strongly associated to mitochondrial complex I malfunction (Juszczuk *et al.*, 2012). A increase in the cytosolic NADH/NAD ratio, for example, is a result of complex I impairment (Szal *et al.*, 2008). Because the availability of reductants is a major limiting factor in  $\text{NO}_3^-$  reduction (Kaiser *et al.*, 2000),  $\text{NO}_3^-$  reduction in mutants could be increased. The accumulation of AAs was found in CMSII and css1 plants (Nakagawa and Sakurai 2006).

## 2.7 Physio-Biochemical Parameters

### 2.7.1 Chlorophyll Content

Amiour *et al.*, 2012 investigated the use of metabolomics in combination with transcriptome and proteomic investigations to identify important processes in the control of nitrogen metabolism in maize. Plants were irrigated daily with a complete nutritional solution (N+) containing 10 mM  $\text{KNO}_3$  as the sole N source in maize (*Zea mays* L., genotype B73) cultivated in a glasshouse.  $\text{NO}_3^-$  was provided as 0.1 mM  $\text{KNO}_3$  for growing plants in low N-deficient circumstances (N-). The chlorophyll content in high N (N+) circumstances at the vegetative stag ( $1.87 \text{ mg/g DW}^{-1}$ ) and leaf maturity stage ( $2.51 \text{ mg/g DW}^{-1}$ ), as well as low (N-) conditions at the vegetative stag ( $0.69 \text{ mg/g DW}^{-1}$ ) and leaf maturity stage ( $0.22 \text{ mg/g DW}^{-1}$ ).

Optimization of nitrogen levels under different ages of seedlings transplanted on growth, chlorophyll content, yield, and economics of hybrid rice by Pramanik and Bera in 2013. Three levels of seedling age (10, 20 and 30 days) and five levels of nitrogen (N0, N50, N100, N150, and N200  $\text{kg ha}^{-1}$ ) were used in fifteen treatment combinations. Total chlorophyll content was significantly raised with increasing nitrogen levels from zero to 200  $\text{kg ha}^{-1}$ , and total chlorophyll content was gradually increased with rising nitrogen levels from zero to 200  $\text{kg ha}^{-1}$ , respectively.

Using proven low nitrogen susceptible and tolerant genotypes, Ranjitha *et al.* (2017) conducted media standardisation in hydroponic culture to screen wheat genotypes for nitrogen usage efficiency. Five wheat genotypes, UASBW 11328, GW 322 and PDW 291, NARBADA 4 and C 306, were grown in hydroponic culture for 30 days using four different modified Hoagland solutions ( $\text{NH}_4\text{NO}_3$  media,  $\text{NO}_3$  complete,  $\text{NO}_3$  half, and N minus media).

The results show that chlorophyll content was higher in the GW 322 and UAS323 genotypes (30.80 and 30.45 SPAD value) and lower in the K9107 genotype (28.65) respectively.

Zhang *et al.* (2017) studied two wheat cultivars' phenotypic and agronomic traits. The experiment used a criss-cross design with three N levels (N+, 3.02 g urea pot<sup>-1</sup>; N-, 1.61 g urea pot<sup>-1</sup>; N0, 0 g urea pot<sup>-1</sup>, and analytically pure) and two winter wheat cultivars, YM49 (N-efficient) and XN509 (N-inefficient). Under different N conditions, the functional leaves of the YM49 (N-efficient genotype) had a larger photosynthetic area than the XN509 (N-inefficient genotype), influencing not only leaf length and colour, but also the leaf area index (LAI). For example, under N level, the leaf lengths of YM49 (22.2 cm) were 17.46% larger than either XN509 (18.9). After anthesis, YM49 had a better stay-green ability than XN509 based on chlorophyll concentration under three N circumstances. Under N circumstances, YM49 had a 7.80 percent greater net photosynthetic rate (Pn) than XN509 during the anthesis stage, showing that YM49 has better assimilation ability.

### 2.7.2 Nitrate Content

Brouder *et al.* (2000) collected 428 observations from 13 N-response trials and evaluated stem nitrate and grain nitrogen assay for maize. The associations between yield and tissue-test values were studied using linear response and level (LRP) and binary logistic regression (BLR). The contents of LRP, stalk NO<sub>3</sub>N (0.42 g kg<sup>-1</sup>), and grain N (13.1 g kg<sup>-1</sup>), as well as the success rates of the two tests, were comparable (77 and 75 percent). Multiple BLR functions with yield (88%) and stalk or grain analysis (87 %) as parameters had similar success rates, although they were much higher than the LRP analysis. When it came to identifying adequacy from excess, stalk analysis beat grain analysis. At 2.9 g kg<sup>-1</sup>, fertiliser efficiency neared zero, with a consistent stalk NO<sub>3</sub> N content (1.67 g kg<sup>-1</sup>) separating sufficient from excessive cases. Premature sampling resulted in stalk NO<sub>3</sub>N levels that were 40 to 60% higher than those seen after the establishment of the black layer, with the biggest inaccuracy happening when N fertility was low.

Hirel *et al.* (2001) investigated into the genetic basis of maize (*Zea mays*) nitrogen usage efficiency and developed a quantitative genetic approach by associating metabolic processes, agronomic features, and DNA markers. Physiological characteristics such as nitrate concentration, nitrate reductase (NR), and glutamine synthetase (GS) activities were examined. They discovered that maize (*Zea mays* L.) had a high nitrate content (32.03 mg dry wt<sup>-1</sup>), as well as a nitrate reductase enzyme (204.95 mmol h<sup>-1</sup> mg<sup>-1</sup>), and activity of glutamine synthetase (525.20 nmol min<sup>-1</sup> mg protein<sup>-1</sup>) Regardless of the quantity of fertilization of mature plants, a substantial and positive association between leaf NO<sub>3</sub><sup>-</sup> concentration of

young vegetative plants and grain yield and grain nitrogen yield was always found.

Gallais and Hirel (2004) studied genetic variability and the genetic basis of nitrogen (N) use efficiency in maize using a set of recombinant inbred lines crossed with a tester for grain yield and its components, grain protein content, and post-anthesis nitrogen uptake and remobilization at low (N-) and high (N+) inputs. At the line level, several physiological features like nitrate concentration, nitrate reductase, glutamine synthetase (GS), and glutamate dehydrogenase activity were investigated. Nitrogen remobilization was adversely associated to leaf nitrate concentration in young plants under low N-input conditions, a study has shown. When soil nitrogen becomes scarce, efficient absorbing genotypes absorb a lot of nitrogen before flowering. The observed negative connection between N-remobilization and post-anthesis N-uptake observed. There was no association between leaf nitrate content or quantity and remobilization with high N input.

According to Jain *et al.* in 2011, they investigated nitrate assimilation in two wheat genotypes, HNR (HD 2285) and LNR (HD 1981), which were cultivated under hydroponics and used for nitrate uptake research. The nitrate content in the shoots of the LNR genotype induced seedlings was 2 times greater than the nitrate levels in the HNR genotype's shoots. The nitrate content of the roots of both genotypes' induced seedlings was identical. The LNR genotype had lower nitrate concentration in the shoots (49%) and roots (20%) of uninduced seedlings than the HNR genotype.

In three leafy vegetables, Chen *et al.* (2004) investigated the effects of nitrate input on plant development, nitrate accumulation, and metabolic nitrate concentration. To investigate the effects of nitrate supply on plant growth, nitrate accumulation, and nitrate reductase activity (NRA) 9 weeks after sowing, three leafy vegetables, rape (*Brassica campestris* L.), Chinese cabbage (*Brassica chinensis* var. *Oleifera* Makino et Nenoto), and spinach (*Spinacia oleracea* L.), were grown in plastic pots with 5 kg soil per pot at five nitrate supply rates, 0.00 (N1), 0.15 (N2), 0.30 (N3), 0.45 (N4), and 0.60 (N5) g N kg<sup>-1</sup> soil . Nitrate concentration increased with nitrate supply in the whole plant and different organs except in roots, where nitrate concentration at N5 decreased compared to N4. With increased nitrate input, the concentration of nitrate in leaf blades' metabolic pool (MP) and storage pool (SP) increased.

In 2012, Chandna *et al.* investigated at the nitrogen use efficiency (NUE) of ten distinct wheat genotypes as well as their genetic diversity in N-uptake. When compared to T1, nitrate concentration increased by 79 %, 76%, and 68 % in the HUW-468, HUW-206, and UP-262 (LNE genotypes) with 4 mM N treatment, and by 140 %, 129 %, and 106 % with

the supply of 10 mM N treatment, when compared with T1 (1 mM N). In medium nitrogen use efficient genotypes, there was no significant effect of supply of 10 mM N treatment on the nitrate content of the genotypes studied, when compared with T1. However, in PBW-154, 3077 Raj, HD-2207, HD-2307, and PBW-65, greater doses of N (25 mM) increased nitrate content by 83 %, 68 %, 97 %, 88 %, and 35 %, respectively, over T1. Higher levels of nitrogen input would have no effect on the nitrate content of HNE genotypes (VL-421 and HS-277), respectively.

Amiour *et al.*, 2012 determined the impact of N deficiency on plant N status, phenotype, and kernel production, plants were grown in a glasshouse until maturity under low (N<sup>-</sup>) and high N (N<sup>+</sup>) fertilization conditions. At the vegetative (V) stage and the leaf maturity (M) developmental stages, plants exhibited a similar pattern of development, including the flowering time. Although the N<sup>-</sup> plants were chlorotic at both the V and M stages, they were a similar height and had approximately the same number of leaves. They reported nitrate content at high N (N<sup>+</sup>) conditions 33.7  $\mu\text{mol g dry weight (DW)}^{-1}$  vegetative stage and 15.2  $\mu\text{mol g dry weight (DW)}^{-1}$  leaf maturity stage and low (N<sup>-</sup>) conditions 6.3  $\mu\text{mol g dry weight (DW)}^{-1}$  vegetative stage and 15.7  $\mu\text{mol g dry weight (DW)}^{-1}$  leaf maturity stage respectively.

Kaur *et al.*, 2015 analyzed six genotypes, with differential inherent genetic background for N metabolism (N assimilation and remobilization) at four N levels. Using urea as a source, nitrogen was applied at recommended doses (RDN), RDN-50 percent, RDN-25 percent, and RDN+25 %. During all four N dosages, genotype GLU 1356 demonstrated the highest NR activity at the anthesis and post-anthesis stages. GLU 1356 had higher NR activity at RDN-25 % and RDN and GLU 2001 had higher NR activity at RDN-50 % and RDN+25 % than other genotypes at the tillering stage, and activities of nitrogen assimilatory enzymes, NR, NIR, GS, GOGAT, GDH, and metabolite content increased significantly in response to high N fertilization during plant development.

### 2.7.3 Nitrate Reductase activity

In 2011, Jain *et al.*, investigated nitrate assimilation in different wheat genotypes. The two wheat genotypes, HNR (HD 2285) and LNR (HD 1981), were chosen and grown in pots. The fertilisers were applied at rates of 150 kg N ha<sup>-1</sup> (1.53 g urea pot<sup>-1</sup> divided into three equal splits), 60 kg P ha<sup>-1</sup> (1.7 g SSP pot<sup>-1</sup>), and 40 kg K ha<sup>-1</sup> (0.45 g MOP pot<sup>-1</sup>). NRA was found to be higher in seedlings of the HNR genotype compared to the activity of the enzyme in the leaves of the LNR genotype at different growth stages. The NRA in the leaves of the HNR genotype was 1.5 times higher and 69 % higher than the NRA in the leaves of the LNR

genotype, respectively.

Gupta *et al.*, 2012 investigated the nitrogen responsiveness of three-finger millet genotypes PRM-1 (brown), PRM-701 (golden), and PRM-801 (white) grown under various nitrogen doses, including high nitrogen dose (60 kg/ha), normal nitrogen dose (40 kg/ha), low nitrogen treatments (20 kg/ha), and farmyard, FYM (7.5 tonnes/hectare), as well as a control (no nitrogen added). Urea was used to apply nitrogen at three intervals: 50% at the time of sowing, 25% at the five leaf stage (30 days after sowing), and 25% at the time of flowering/post anthesis. In this study, nitrate reductase activity was found to be lowest in PRM-801 genotype (295 Units/mg protein), followed by PRM-701 genotype (736.6 Units/mg protein), and highest in PRM-1 genotype (1188.6 Units/mg protein), indicating that nitrate reductase activity was increased and thus PRM-1 was a high nitrogen use efficient genotype when compared to the other two.

Anjana *et al.*, (2011) used a sand culture experiment to investigate nitrogen use efficiency in wheat genotypes with different nitrate reductase activity, utilising the IC 321157 (HNR) and C 306 (LNR) genotypes of *Triticum aestivum* L.. The HNR genotype had a 2.39-fold higher nitrate concentration and considerably higher nitrate reductase activity than the LNR genotype. The distinctions in controlled conditions, the difference in NR activity between the two genotypes was 2.82, 2.33, and 2.74 times on a protein, dry weight, and per plant basis, respectively. After 15 days of development. The findings imply that a high NRA is advantageous. Higher NRA was positively related to differences in nitrate content in the whole plant, and the difference between two genotypes suggested that higher NRA was associated with a high nitrate content of the plant.

In 2012, Chandna *et al.*, found that nitrogen uptake and assimilation differed across N-efficient and N-inefficient wheat (*Triticum aestivum* L.) genotypes. The activity and expression levels of nitrate assimilatory enzymes in N-efficient and N-inefficient wheat genotypes also revealed that nitrate reductase is active in both N-efficient and N-inefficient wheat genotypes (NR). When compared to T1, NR activity of HUW468, HUW-206, and UP-262 (LNE genotypes) was raised by 22%, 47%, and 48% with the delivery of 4 mM N (T2), and by 95%, 81 percent, and 77 percent with the supply of 10 mM N (T3). There was a significant increase in NR activity in medium NUE genotypes (PBW-154, 3077 Raj, HD-2207, HD-2307, and PBW-65) with an increase in 4 mM N. There was no further increase in the activity of this enzyme. The increase in NR activity was, however, less than with the LNE genotype. With the supply of 4 mM N (T2), the rise in NR activity was 64 percent, 20 percent, 38 percent, 30 percent, and 42 percent, respectively, and with the supply

of 10 mM N (T3), it was 68 percent, 12 percent, 33 percent, 46 percent, and 36 percent, respectively (T1). When compared to 1 mM (T1), increasing the level of N treatment (4 and 10 mM) had no effect on the NR activity of HNE genotypes (VL-421 and HS- 277) significantly.

#### 2.7.4 Grain Protein

Gupta *et al.* (2012) investigated the nitrogen effect of three-finger millet genotypes PRM-1 (brown), PRM-701 (golden), and PRM-801 (white) grown under various nitrogen doses, including high nitrogen dose (60 kg/ha), normal nitrogen dose (40 kg/ha), low nitrogen treatments (20 kg/ha), and FYM (7.5 tonnes/hectare), as well as a control (no nitrogen added). Urea was used to apply nitrogen at three intervals: 50% at the time of sowing, 25% at the five leaf stage (30 days after sowing), and 25% at the time of flowering/post anthesis. They observed that the PRM- 801 genotype had the highest crude grain protein content (9.6%), followed by PRM-701 (9.0%), and PRM-1 (7.5%), demonstrating a negative association between nitrogen usage efficiency and seed protein content.

In 2015, Kaur *et al.*, revealed that nitrogen uptake, assimilation, and remobilization were studied in six wheat genotypes: GLU 1101, GLU 1356, GLU 2001, GLU 700, PH132-4836, and PH132-4840. Nitrogen was in the form of urea (46 % N), with doses of 120 kg N ha<sup>-1</sup> (recommended N dose, RDN), 90 kg N ha<sup>-1</sup> (RDN-25%), 60 kg N ha<sup>-1</sup> (RDN-50%), and 150 kg N ha<sup>-1</sup> (RDN+25%). At the tillering (30- 40 days after sowing; DAS), anthesis (about 100-140 DAS depending on genotype), and post-anthesis (15 days after anthesis) stages from the flag leaf, different biochemical parameters were examined. In tested wheat genotypes, days were taken for phenological events. At sub-optimal and recommended doses, genotype GLU 1356 had the maximum protein content, however at larger doses, genotypic behaviour changed at all three stages tested. RDN+25 % had the highest amino acid content, which was much greater than RDN. GLU 2001 and PH132-4836 showed increased accumulation of amino acids at RDN+25 %, while RDN-50 percent and RDN-25 % demonstrated consistent genotypic response as found for proteins at all three stages.

Taulemesse *et al.* 2016 found that grain protein concentration is associated to early post-flowering nitrate uptake under the putative regulation of plantlevel in bread wheat (*Triticum aestivum* L.). The cvs. Récital and Renan genotypes of winter wheat (*Triticum aestivum* L.) were utilised in this investigation. Semi-hydroponic culture containing 4 mM of NO<sub>3</sub><sup>-</sup> and 10 mM of NO<sub>3</sub><sup>-</sup>. It established four therapy combinations for each genotype: N4-LN, N4-HN, N10-LN, and N10-HN. By combining whole plant sampling and targeted gene expression techniques, researchers may investigate the genetic determinants of post-

flowering N uptake (PANU). These corresponding to high levels of grain protein concentration (GPC) (13.2% and 17.3% respectively).

## 2.8 Carbohydrate and Mitochondrial Metabolites

### 2.8.1 NAD<sup>+</sup> /NADH ratio

Abdin *et al.* (1992) investigated the biochemical basis of nitrate reductase variability. The endogenous NADH content of the HNR and LNR genotypes was compared. When compared to HNR genotypes, LNR genotypes were found to have lower levels of NADH. This option was subsequently investigated by infiltrating leaf discs with various amounts of NADH (from 0.0 to 0.8 mM). While the HNR genotype (HD 1925) revealed no increase in enzyme activity, the LNR genotype showed a 49 percent increase. The availability of NADH appeared to play a role in the enzyme's poor activity in genotypes classified as low nitrate reductase group (LNR) (Safed lerma).

The molecular basis of the maternally inherited heteroplasmic NCS2 maize mutant was studied by Marienfeld and Newton (1994). The NCS2 mitochondrial genome is remarkably similar to the progenitor cmsT mitochondrial genome, with the difference that the mutant genome has a fused nad4-nad7 gene and lacks the small fourth exon of the nad4 gene. A 16-bp repeat has been reorganized in the third intron of the nad4 gene and the second intron of the nad7 gene. Transcripts containing exon 4 of the nad4 gene are significantly decreased in mtRNA preparations from heteroplasmic NCS2 plants; larger transcripts are related with the first three nad4 exons. The 5' ends of the nad4 transcripts were found 396 and 247 bp upstream of the start codon in mtRNAs from NCS2 and non-NCS plants, respectively. Because the putative nad4 transcription termination signal is lacking in mutant DNA, separate longer transcripts are produced. The complex transcript sequence associated with nad7 is also altered in the mutant. Subunits of the mitochondrial electron transport chain complex I (NADH dehydrogenase) are nad4 and nad7. According to oxygen absorption experiments, complex I function is specifically impaired in mitochondria isolated from NCS2 mutant plants.

According to Dutilleul *et al.* (2005) CMS, a *Nicotiana sylvestris* mutant lacking the mitochondrial gene nad7 and functioning complex I, respire *via* low capacity NADH (alternative) mitochondrial dehydrogenases. CMS leaves have a metabolite profile that is abundant in amino acids and low in starch and 2-oxoglutarate, with low C/N ratios. 2-oxoglutarate deficit occurred despite increased citrate and malate levels and enhanced capability of important anaplerotic enzymes including the mitochondrial NAD-dependent isocitrate dehydrogenase.

Vega-Mas *et al.* (2019) Study monitored the incorporation of  $^{15}\text{N}$  or  $^{13}\text{C}$  into amino acids and organic acids in roots of plants cultivated under exclusive  $\text{NH}_4^+$  or nitrate ( $\text{NO}_3^-$ ) feeding. Roots of plants acclimated to ammonium media had a better capacity to incorporate both  $^{15}\text{NH}_4^+$  and  $^{15}\text{NO}_3^-$ . The provision of  $^{13}\text{C}$  pyruvate resulted in the accumulation of  $^{13}\text{C}$  citrate and  $^{13}\text{C}$  malate, as well as rapid  $^{13}\text{C}$  2-OG consumption for amino acid synthesis, underlining the importance of the TCA cycle's anaplerotic pathways. Our findings show that root adaptation to di ammonium lowered for efficient N assimilation by enhancing TCA cycle open flux modes in order to maintain C skeleton availability for successful  $\text{NH}_4^+$  detoxification into amino acids. Enzyme activities from primary N assimilation, TCA (tricarboxylic acid) cycle and its associated anaplerotic activities in wheat roots grown under nitrate and ammonium nutrition.

NA is a complex biochemical process that is both supported and inhibited by respiratory metabolism, according to Foyer *et al.* (2011). They discovered that while the tricarboxylic acid cycle supplies KG for NA, tricarboxylic acid cycle of its ability to create NADH within the mitochondria, resulting in decreased mitochondrial NADH concentrations. When mitochondrial NADH levels are restored by absorbing cytoplasmic malate, which limits the amount of reductant available to create NADH for nitrate reduction, NA may become self-regulatory.

Experiments on nitrate assimilation limits nitrogen use efficiency (NUE) in maize (*Zea mays* L.) were carried out by Loussaert *et al.* (2018). In a multi-environment experiment, they used transgenic hybrid to increase NADH levels by selective co-suppression of ubiquinone oxidoreductase 51 kDa subunit (complex I). They reported positive grain yield responses when cultivated in high N fertility (low NUE) for two years, one year. The positive event exhibited reduced leaf NADH-ubiquinone oxidoreductase 51 kDa subunit (complex I) expression, more extractable NADH, and more NRA (*in vitro*). The low frequency of good events is a concerning issue. There was no variation in NADH-ubiquinone oxidoreductase 51 kDa subunit (Complex I) expression between transgene and non-transgene. If NA is limited by NADH rather than NRA, these results would be expected (*in vitro*). Increases in KNP associated with increased grain yields under high N fertility could have been brokered by improved NA during ear development. It's possible that an increase in reductant (NADH) limits NA, which in turn inhibits NUE.

### 2.8.2 Glutamine Synthetase

The enzyme glutamine synthetase (GS) (EC 6.3.1.2) is responsible for the synthesis of glutamine in the first step of  $\text{NH}_4^+$  assimilation. GS isozymes play a number of metabolic

roles in plants, and their actions vary depending on the organ or cell type. Higher plants contain two GS isozymes, GS1 and GS2 (Bernard and Habash 2009).

In 2011, Jain *et al.* worked on two wheat genotypes, HNR (HD 2285) and LNR (HD 1981), which were grown in pots and supplied N in three splits (150 Kg N ha<sup>-1</sup> (1.53 g urea pot<sup>-1</sup> in three equal splits), 60 Kg P ha<sup>-1</sup> (1.7 g single superphosphate pot<sup>-1</sup>), and 40 Kg K ha<sup>-1</sup> (0.45 g muriate of potash pot<sup>-1</sup>). The activities of the other enzymes in the pathway, such as GS/GOGAT, were also higher in the HNR genotype's flag leaves than in the LNR genotype's flag leaves. At 60, 75, 90, and 110 DAS, the GS activity in the HNR genotype was 41%, 26%, and 2 times greater, respectively.

Gupta *et al.* (2012) determined that the GOGAT activity recorded at grain filling was highest for PRM-1 genotype (0.19 Units/mg protein), followed by PRM-801 (0.10 Units/mg protein), and lowest for PRM-701 genotype (0.07 Units/mg protein). Growth and yield parameters indicated that PRM-1 responds well at high nitrogen conditions while PRM-701 and PRM-801 respond well at normal and low nitrogen conditions respectively.

Overexpression of a glutamine synthetase gene affects sorghum growth and development, according to Urriola and Rathore (2015). A 1,071-bp coding sequence of a sorghum cytosolic GS gene (Gln1) under the control of the maize ubiquitin (Ubq) promoter and the CaMV 35S promoter, which was introduced into sorghum immature embryos using *Agrobacterium*. In comparison to non-transgenic controls, the ubiquitin (Ubq) promoter offspring of the transformants had higher accumulation of Gln1 transcripts and up to 2.2-fold more GS activity. These Gln1 transgenic lines demonstrated higher tillering and a 2.1-fold increase in shoot vegetative biomass when grown under optimal nitrogen conditions.

Bao *et al.* in 2015, revealed that the N-efficient cultivar had higher relative levels of GS2 protein before anthesis than the N-inefficient cultivar, suggesting that GS2 is essential in the vegetative growth stage and carbon-nitrogen metabolic balance, and that there were higher relative levels of the GS1 protein after anthesis under low (N) conditions. As a result, they concluded that increasing NUE requires boosting the remobilization of stored nitrogen from vegetative tissues to the grain.

Zhang *et al.* (2017) investigated the role of glutamine synthetase isozymes in improving nitrogen use efficiency in winter wheat that is N-efficient. They selected and compared two wheat cultivars, YM49 and XN509, identified as N-efficient and N-inefficient genotypes, respectively. In both the N- and N+ conditions, GS activity followed this pattern in both genotypes. The following was the order of GS activity in the different organs: functional leaf > outer glume > peduncle > kernel. The functional leaf's GS activity peaked at

7 days after anthesis, while the kernel's activity peaked at 14 days following anthesis.

### 2.8.3 GS Isozyme

Kichey *et al.* (2005) investigated the localization of plastidic (GS2) and cytosolic (GS1) glutamine synthetase isoenzymes in the flag leaf and stem during natural senescence. Large levels of GS1 were found in the connections between the mestome sheath cells and the vascular cells in mature flag leaves, suggesting an active transport of nitrogen organic compounds within the vascular system. In the mesophyll cytosol of senescing leaves, an increase of a GS1 polypeptide (GS1b) was detected, while the GS protein content represented by another polypeptide (GS1a) in the phloem companion cells remained nearly unchanged in both leaves and stems. The total amount of GS activity in the flag leaf remained high until 14 DAF, and then dropped drastically until 28 DAF. 28 DAF, only 25% of the remaining extractable GS activity was present. Protein gel blot analysis was used to estimate the amount of GS1 and GS2 isoenzymes in a plant protein extract, which reflects their relative activity, as described by Becker *et al.* (1992).

Purcino *et al.* in 2008 used five maize S5 inbred lines and five single cross hybrids previously chosen for contrasting NUE to examine probable systematic patterns of GS activity or isoenzyme expression response to nitrate. GS is encoded by a multigene family in maize, and its gene products are classified as cytosolic (GS1) and chloroplastic (GS2) isoforms. At the 4-leaf seedling and 2-day after silking stages, leaf GS biosynthetic activity was considerably affected by nitrate, however breeding for enhanced NUE did not result in the production of genotypes with increased GS activity. GS produced separate "upper" and "lower" activity bands after native polyacrylamide gel electrophoresis (native-PAGE) and staining for transferase activity. Although visual inspection of these bands showed that they were made up of multiple peaks, densitometric analysis using the peak height criterion for peak identification was unable to resolve them into more than one peak.

Wang *et al.* (2015) used native-PAGE and a transferase activity assay to study the glutamine synthase isozyme in wheat seedling leaf. In gels, the isoforms had different mobilities, with GSII>GSIII>GSI. The cytosolic GSI was made up of three subunits with the same molecular weight (39.2 kDa) but distinct pI values: GS1, GSr1, and GSr2. GSI first arose at the onset of leaf development and remained active throughout the leaf's lifespan. The chloroplast proteins GSII and GSIII were both made up of a single 42.1 kDa subunit with differing pI values. GSII was mostly active in green leaves, but GSIII was only active for a short time in green leaves cultivated in the field. Only two GS isoforms were found using a modified blue native electrophoresis (BNE) approach and in-gel catalytic activity analysis:

one cytosolic and one chloroplastic. The cytosolic GS1 holoenzyme was 490 kDa and presumably a dodecamer, while the chloroplastic GS2 holoenzyme was 240 kDa and likely a hexamer, as according mass calibrations on BNE gels.

According to Zhang *et al.* in 2017, a complicated carbon-nitrogen metabolic process may increase flow strength and enhance NUE by modulating GS isozymes. The relative activity or amount of the GS1 and GS2 isozymes could be used to predict and select wheat genotypes with improved NUE. They selected two wheat cultivars, YM49 and XN509, which were determined as the N-efficient and N-inefficient genotypes, respectively, and compared both. When comparing N-efficient genotypes to N-inefficient genotypes, the results showed that TaGS1 was highly expressed post-anthesis while TaGS2 was highly expressed pre-anthesis in the N-efficient genotype. The spatial and temporal distribution of GS isozymes, their expression of gene and protein subunits in source-sink-flow organs over development phases caused the pool strength and regulated the N flow, according to native-PAGE studies.

#### 2.8.4 GOGAT

Gupta *et al.* (2012) investigated the association between nitrogen use efficiency and enzyme activities involved in nitrogen uptake and assimilation in finger millet genotypes grown under various nitrogen input. Nitrogen use efficiency and nitrogen uptake efficiency have positive correlations with GS in the golden genotype at grain filling, but nitrogen utilization efficiency has a negative association with GS. Nitrogen use efficiency, nitrogen utilisation, and nitrogen uptake efficiency all have a positive connection with GS in the white genotype at grain filling. Correlation studies in brown genotype grain filling with another ammonium assimilating enzyme, GOGAT, show that in brown genotype nitrogen use, nitrogen utilisation, and nitrogen uptake efficiency have a negative correlation with GOGAT. GOGAT has a positive relation with nitrogen use, nitrogen utilisation, and nitrogen uptake efficiency in the golden genotype at grain filling. Except for nitrogen usage efficiency, GOGAT has a positive connection with nitrogen utilisation and nitrogen uptake efficiency in white cultivars at grain filling.

According to Jain *et al.*, an experiment was conducted in 2011 to study two wheat genotypes, HNR (HD 2285) and LNR (HD 1981), which were grown in (150 kg N ha<sup>-1</sup> (1.53 g urea pot<sup>-1</sup> in three equal splits), 60 kg P ha<sup>-1</sup> (1.7 g single superphosphate pot<sup>-1</sup>), and 40 kg K ha<sup>-1</sup> (0.45 g muriate of potash pot<sup>-1</sup>), and it was reported that among these genotypes, the differences in the Fd-GOGAT activity were more than 1 fold and 3 fold at 60 and 110 DAS, respectively.

The differential reaction in the leaves of tall and dwarf wheat to carbon monoxide (CO), a cytochrome oxidase inhibitor, and salicyl hydroxamic acid (SHAM), an inhibitor of alternative oxidase, appears to be linked with the presence of Rht dwarfing genes, according to Naik *et al.*, 1998. After CO treatment and direct O<sub>2</sub> uptake in the presence of SHAM, this was confirmed using an *in vivo* nitrate reductase assay. The CO response was significantly improved by pretreatment of the leaves with Triton X-100 at a dose that specifically inhibits the accessibility of exogenous NAD(P)H to alternative oxidase, as shown by an *in vivo* NR assay. This supports the theory that NADH-dehydrogenase, which is found on the outer surface of the inner mitochondrial membrane, controls the competition for NADH between NR and mitochondrial respiration.

Patil *et al.*, 1995 used an *in vivo* carbon monoxide-aerobic assay of nitrate reductase in the leaves of C3 plants (wheat, chickpea, and groundnut) to compare carbon monoxide - sensitivity of cytochrome-c oxidase in the leaves of C3 plants (wheat, chickpea, and groundnut) with a newer technique that used defined CO:O<sub>2</sub> ratios. CO:O<sub>2</sub> ratios of 40, 30, and 10 were shown to be optimal for inhibiting cytochrome c oxidase in wheat, chickpea, and groundnut, respectively. As the CO:O<sub>2</sub> ratio was gradually increased, nitrite production increased steadily in all C3 plants. The redox state of cytochrome-c oxidase was altered by pre-treating leaves of chickpea and groundnut cultivars with 2,4-dinitrophenol (2,4-DNP) (2mM), making them responsive to CO. Significant nitrite production was observed in 2,4-DNP-treated leaves at CO:O<sub>2</sub> ratios of 5 and even 2.5. As a result of this technique of using defined CO:O<sub>2</sub> ratios, it was possible to differentiate one C3 plant from another in terms of CO-sensitivity of cytochrome c oxidase.

In the leaves of the CMS II mutant of *Nicotiana glauca*, the loss of the main mitochondrial complex I subunit Nad 7 resulted in an increase in both *in vivo* and *in vitro* nitrate reductase activities, as well as a greater percent activation state of NR in CMS II compared to wild type. The variations point to a likelihood of mitochondrial NADH export due to a defect in complex I, a key and high affinity NADH sink. The absence of the complex I subunit resulted in constitutive production of alternative oxidase (Aox), which provided a mechanism for the constant supply of carbon skeletons needed for nitrate absorption. It is discussed whether there is a possibility of close synchronisation between mitochondrial redox disturbance and nitrate reduction and subsequent assimilation Naik, 2007.

### **2.8.5 Glucose 6 Phosphate Dehydrogenase**

The metabolic processes of GS and the production of reducing power by the OPPP are inextricably linked. The predominant root isoform was NADH GOGAT, whose activity

increased when the medium was supplemented with ammonia or nitrate. The predominant isoform in the leaves is Fd-GOGAT, which activity increased when nitrogen was added. The activity of G6PDH increased in nitrogen-supplied roots. They discovered that the pal isoform increased in the roots, with 13.6 percent G6PDH activity localised in the plastids under ammonium feeding conditions and 25.2 percent under nitrate feeding conditions. Nitrogen stimulated the plastid-G6PDH protein in the roots, according to Western blots. Changes in cytosolic G6PDH appear to be associated to larger cell growth processes rather than being specifically involved in glutamate synthesis (Esposito *et al.*, 2005).

## 2.9 Molecular Parameters

Chandna *et al.*, in 2012 studied expression profiling of GS gene families in wheat genotypes using real-time RT-PCR, the expression levels of GS(Gln1;1) were analyzed in the leaves of VL-421 and HUW- 468 at 1 and 10 mM nitrogen. Gln1;1(high-affinity form) of GS isoform is responsible for the primary assimilation of ammonium ions in plants. The expression of Gln1;1 was up-regulated in VL-421 by 1.0-fold under low-nitrogen levels (P50.05) in comparison with HUW-468, and no significant change in the expression level of Gln1;1 was observed with increase in N supply. However, HUW-468 showed an increase in the expression of Gln1;1 at 10 mM N by one-fold. Levels of expression of the GS gene family (Gln1;1) in leaves of VL-421 under low N levels show up-regulation in comparison with transcript expression of HUW-468 under high nitrogen conditions. These results show that a HNE wheat genotype is more efficient in N uptake and assimilation even at low nitrogen conditions.

The wheat GS gene codes for a protein found in cells of plants at anthesis. GS protein was found in parenchyma, phloem companion, and per fascicular sheath cells in plants at anthesis stage. GS2 and GS1 assimilated or recycled ammonia in leaves during grain development and filling, and expression of GS genes in leaves was developmentally controlled. GS1 transcripts were found in the per fascicular sheath cells, while GSr transcripts were only found in vascular cells. The ratio of leaf GS2 protein to GS1 protein was varying in three different wheat genotypes, according to early study. The cytosolic isozymes GS1 and GSr were the most prevalent forms during leaf senescence. These enzymes play important roles in absorbing ammonia during the critical stages of nitrogen remobilization to the grain (Bernard *et al.*, 2008).

GS1 was highly expressed at 14D under both N- and N+ conditions, indicating a significant difference between the two genotypes. TaGS1 expression in the peduncle and outer glume followed a similar pattern, with the maximum expression at 7D and then a

gradual decrease. The major GS isoform in leaves, TaGS2, showed a significant increase in expression with the rise in N, and it differed significantly between YM49 and XN509. No significant changes were observed at the N+ level or under the N- level. TaGS2 expression in the outer glume was significantly lower in YM49 than in XN509 at AS and 7D under N+. TaGS1 showed a significant difference in the kernel at 7D, but TaGS2 showed no change during the entire grain filling phases. TaGS2 gene expression was found to be nearly tenfold higher than TaGS1 gene expression in a cultivated cannabis leaf compared to a non-cultivated one. The findings suggest that distinct levels of gene expression could cause differences in assimilation and translocation ability among two cultivars (Zange *et al.*, 2017).

Li *et al.*, in 1993 investigated the differential expression of six glutamine synthetase genes in *Zea mays*. Overexpression of the OsGS1 gene increases N metabolites, biomass, and yield, but it is not an overall bottleneck that inhibits N assimilation at the whole-plant level. HvGS1 gene expression was enhanced by leaf senescence, according to Avila-Ospina (Gadaleta *et al.*, 2011). Throughout the growing phase of the wheat leaf, the TaGS1 gene showed the same pattern. TaGS1 expression was elevated in the peduncle and outer glume after flowering, peaked at 7D, and then gradually decreased. Although TaGS2 transcript was somewhat greater in the peduncle of N-efficient cultivar than in the peduncle of N-inefficient cultivar under N, it was higher in the functional leaves and outer glume of two cultivars in JS and AS. Thus, in wheat, TaGS1 may play a larger role in influencing NUE after anthesis, whereas TaGS2 may play a bigger role in influencing NUE before anthesis. The findings demonstrated that GS1 gene expression is important for N absorption efficiency in roots (Bowles *et al.*, 2015). Previous research has found that OsGS1 gene expression is important for carbon-nitrogen metabolism in rice at the latter stages of growth (Bao *et al.*, 2014). In maize, the ZmGS2 gene is primarily expressed during the vegetative growth stage (Keys, 2006). TaGS1 and TaGS2 genes have non-redundant roles and are potential targets during specific growth development stages for enhancement of NUE.

Wei *et al.* (2021) recently found that the localization, gene expression, and functions of GS isozymes varied in different grain development stages. TaGS1;1, TaGS1;3, and TaGS2 were all found to be expressed in different parts of the embryo using immunofluorescence. TaGS1;2 was found in the vascular bundle in grain carrying tissues, TaGS1;2 and TaGS1;1 in the chalaza and placentochalaza, TaGS1;1 and TaGS1;3 in endosperm transfer cells, and TaGS1;3 and TaGS2 in the aleurone layer. GS activity and expression reached a peak at 8 days after flowering (DAF), correlated with peak glutamine content in grains; after that,  $\text{NH}_4^+$  increased largely due to  $\text{NO}_3^-$  reduction, glutamate dehydrogenase (GDH) aminating activity

increased continuously, and the activities of GS and glutamate synthase (GOGAT) decreased, while only TaGS1;3 retained a stable expression in different TaGS isozymes. The GS-GOGAT cycle and GDH play different roles in grain ammonia absorption at different phases of grain development; TaGS1;3, which is found in the aleurone layer and endosperm transfer cells, is important for the assimilation of Gln into endosperm for gluten synthesis. At 30 DAF, grain amino acids are mainly transported from maternal phloem.

During grain filling stages, the expression of Fd-GOGAT and NADH-GOGAT genes in the flag leaves was up regulated at all N levels (Liu *et al.*, 2021). Under the N0, N1, and N2 levels, Fd-GOGAT gene expression in wheat flag leaves was 1.5–2.2 times, 1.5–1.8 times, and 1.2–1.6 times higher ( $P < 0.05$ ), respectively, while NADH-GOGAT gene expression was 1.4–6.7 times, 2.1–5.5 times, and 1.6–3.2 times higher, respectively ( $P < 0.05$ ). Fd-GOGAT and NADH-GOGAT gene expression in wheat flag leaves was 1.4 times and 2.3 times greater ( $P < 0.05$ ), respectively, regardless of N levels or growth stages.

### 3. MATERIAL AND METHODS

The present study entitled “**Biochemical and molecular basis of differential nitrogen use efficiency in contrasting wheat (*Triticum aestivum* L.) genotypes.**” was carried out at Department of Biochemistry and Phytotron facility, Department of Botany, Mahatma Phule Krishi Vidyapeeth, Rahuri, Dist- Ahmednagar during the year 2019- 2020 and 2020-2021.

The details of the material used and methods adopted in the present investigation were mentioned under following subheadings.

#### 3.1 Material

##### 3.1.1 Seeds

The following seed materials of wheat (*Triticum aestivum* L.) genotypes were obtained from the ARS, Niphad (Dist. Nashik) and the Division of Plant Physiology, IARI, Pusa Campus, New Delhi-110012. The wheat genotypes identified as high nitrogen use efficient HD-2781 and low nitrogen use efficient C-306 was grown with three fertilizer treatments. Further, 11 genotypes, including these two genotypes, were grown with a half dose of nitrogen treatment. These genotypes are contrasting in nature. The study of these genotypes will be helpful for breeders and researchers for further studies involved in nitrogen use efficiency in wheat. Detailed information about the wheat genotypes used in the present research is given in Table 3.1.

**Table 3.1 Information of wheat genotypes used in the present study**

GW 322	MACS 2496	MACS 6222	HD 2932	
NIAW 917	NIAW 301	NIAW34	NIAW 1415	
NIAW3170	NIAW 15	NIAW1994	HD 2781	C 306

##### 3.1.2 Soil

The soil (sandy loam) used for these pot culture experiments was collected from the botany field, MPKV, Rahuri. The soil was obtained from a field where the previous crop grown was *Zea mays* L. and the soil was analyzed for N, P, K, pH and Electric conductivity. The results of soil analysis were as follows:

**Table 3.2 Soil sample analysis results**

Sr. No.	Parameter	Result	
		1 <sup>st</sup> trail	2 <sup>nd</sup> trail
1	Available nitrogen	166.208 kg/ha	184.788 kg/ha
2	Available phosphorus	18.87 kg/ha	21.55 kg/ha
3	Available potash	504 kg/ha	494 kg/ha
4	pH	8.7	8.6
5	EC	0.43 dS.m <sup>-1</sup>	0.48 dS.m <sup>-1</sup>

### 3.1.3 Chemicals

Most of the chemicals used in this study were analytical grade (sd. fine chemical Ltd., SRL Mumbai, E. Merck, Qualigen, Fine Chemical Pvt., Ltd., Mumbai, Bangalore, Genei Pvt. Ltd., Bangalore).

## 3.2 Methods

### 3.2.1 Treatment Details

#### 3.2.1.1 Experiment No. 01

In this experiment, four replications and three fertilizer treatments were used: N+ (full nitrogen), N- (half nitrogen), and N0 (no nitrogen). Two contrasting wheat genotypes, HD-2781 and C306, were grown in pots containing 3.7 kg of soil.

#### 3.2.1.2 Experiment No.02

Three replications and thirteen wheat genotypes were grown with N-(half-nitrogen) fertiliser treatments in this experiment. A total of soil (air dried, weighed, and sieved through a 0.8 cm mesh) mixed with fertilizer was packed into pots at a density of 1.12 g/cm. The first experiment used a FCRD with three N levels (N+ 1.06 g urea/pot, N-0.53 g urea/pot, and N0 0.00 g urea/pot), while the second experiment used a completely randomized design with half the N levels (N- 0.53g urea/pot). In addition, 1.24 g of single super phosphate per pot and 1.10 g of muriate of potash per pot were added to both experimental pots. For each pot, 3.7 kg of soil was taken and the recommended dose of N, P, and K was calculated, which was as follows:

**Table 3.3 Treatment details**

Sr. No.	Treatment	Fertilizer quantity (g/pot)		
		Urea	S.S.P	M.O.P
1	N+ (full nitrogen)RDN+RDP+RDK	1.06	1.24	1.10
2	N- (half nitrogen)Half RDN+RDP+RDK	0.53	1.24	1.10
3	N0 (no nitrogen)No RDN+RDP+RDK	0.00	1.24	1.10

### Procedure

1. The first (half) dose of N was applied at the time of sowing with three N levels, and the remaining dose of N was applied at the jointing stage. The full dose of P and K was applied at the time of sowing.
2. The soil was kept at 70% of its water-holding capacity.
3. The flag leaf sample was used for experimental analysis at the pre-anthesis (during 45-60 days) and post-anthesis (during 65-80 days) growth stages.

4. A weighed quantity of leaf sample was used for the various experiments for the determination of nitrate content, chlorophyll content, and assay of *in vivo* and *in vitro* nitrate reductase, *in vitro* nitrite reductase, soluble protein, NADH/NAD<sup>+</sup> ratio, *in vitro* glutamine synthetase, Fd-GOGAT, NADH-GOGAT, glucose 6 phosphate dehydrogenase, and glutamine synthetase isozymes.

### 3.2.1.3 Experiment 03

HD-2781 (HNE) and C-306 (LNE) wheat genotype seeds were sterilised and germinated for 7 days before being placed in thermocol sheets made to fit 0.5L containers. All of the experiment was carried out in growth chamber having  $25\pm 2$  °C and 70 % relative humidity with fluorescent light giving 16 hours of light per day for fifteen days in an aerated half-strength Hoagland solution pH 6.2. (Hoagland and Arnon 1950). One set of seedlings received 1  $\mu$ M OP in a half strength Hoagland solution (+OP with omeprazole), whereas the other received a half-Hoagland solution (-OP without omeprazole as a control). In the nutrient solution, the roots were completely submerged. Each tray contained 20 seeds. The entire sheet of seedlings could be transferred to the fresh solution 3–4 days later without any disturbance.

### 3.2.1.4 Experiment 04

Four replications and seedlings of four wheat genotypes were grown in small pots in medium-black soil in normal sunlight. After germination, the seedlings were daily irrigated with 15 mM KNO<sub>3</sub> so that sufficient nitrate accumulated in the leaves. Leaves from 10-day-old seedlings were generally used for various experiments. The sufficiency of KNO<sub>3</sub> accumulation was judged by testing the *in vivo* NR activity dependent on endogenous nitrate in the leaf, which was not further enhanced by the addition of KNO<sub>3</sub> to the liquid medium. Thus, substrate KNO<sub>3</sub> was not a limiting factor for NR activity.

## 3.2.2 Total Chlorophyll

The total leaf chlorophyll content of wheat was determined by the method described by Arnon (1949).

### Protocol

The third leaf from the top of the tillers was cutted into small pieces of known weight (0.2 g). A fresh leaf sample was macerated in a mortar and pestle before being extracted with 10 ml of 80% acetone. The content was centrifuged at 5000 x g for 10 min, and the supernatant was collected. The above step was repeated until the residue became colorless. The final volume of the extract was made up of 50 ml. On a spectrophotometer, the extinction

of chlorophyll extract was measured at 645 and 663 nm, and 80 percent acetone blank were running.

### Calculation

The amount of total chlorophyll content was calculated by using the following formula and expressed in  $\text{mg g}^{-1}$  FW.

$$\text{Total chlorophyll, mg g}^{-1} \text{ FW} = 20.2 (A_{645}) + 8.02 (A_{663}) \times V / (1000 \times W)$$

$$\text{Chlorophyll a, mg g}^{-1} \text{ FW} = 12.7 (A_{663}) - 2.69 (A_{645}) \times V / (1000 \times W)$$

$$\text{Chlorophyll b, mg g}^{-1} \text{ FW} = 22.9 (A_{645}) - 4.68 (A_{663}) \times V / (1000 \times W)$$

Where,

A= absorbance at specific wavelength

V= Final volume of chlorophyll extract in 80 % acetone

W= Fresh weight of tissue extracted in g.

### 3.2.3 Leaf Nitrate Content

Estimation of nitrate content involves the reduction of nitrate to nitrite chemically using hydrazine sulphate in an alkaline medium, and then nitrite was estimated colorimetrically using sulphanilamide and N (1-naphthyl) ethylene diamine dihydrochloride. The method as described by Downes (1978) was used as follows.

#### Reagents

1. Catalyst solution
  - i. Stock solution of copper sulphate (8 mg  $\text{Cu}^{2+}$  per 100 ml)
  - ii. Working catalyst solution (8 mg  $\text{Cu}^{2+}$  and 204 mg  $\text{Zn}^{2+}$  per liter)
 

Dissolved 90 mg of zinc sulphate in 50 ml of double distilled water, added 10 ml of stock solution of copper sulphate to it, and the volume was made up to 100 ml with double distilled water. The working catalyst solution was further used for nitrate estimation.
2. NaOH : 0.1 N
3. Hydrazine sulphate (Reductant solution): Dissolved 152 mg of hydrazine sulphate in 100 ml of distilled water.
4. Acetone: 10 % (v/v)
5. Sulphanilamide solution (Reagent A): 1 % sulphanilamide in 1 N HCl
6. N (1-naphthyl) ethylene diamine dihydrochloride (Reagent B) : 0.02 % (w/v) in distilled water.
7.  $\text{KNO}_3$

### **Extraction of nitrate**

Depending upon the concentration of nitrate present in the plant material, 20 to 100 mg of oven-dried, ground plant material (20 mesh) was used for extraction. Plant material and an equivalent amount (not more than 100 mg) of activated charcoal (free from nitrate) were added to a 100 ml conical flask containing 20 ml of double distilled water. Boiled the content for 3-4 min. The extract was then filtered through Whatman No. 42 filter paper. The residue was re-extracted and made up to a suitable volume with glass-distilled water.

### **Estimation of nitrate in the plant extract (sample solution)**

Depending upon the concentration of nitrate, 1 ml of sample solution was taken in a 30 ml capacity culture tube. A tube containing distilled water instead of sample solution serves as a blank. Added 0.5 ml of working catalyst solution, followed by 0.25 ml each of hydrazine sulphate and sodium hydroxide (0.1 N) solution, respectively, to the tube. The total volume was made up of 3.5 ml of glass distilled water. All the tubes were kept in a water-bath maintained at 33°C for 10 min. Removed the tubes from the water bath and kept them in an ice bath. Added 0.5 ml of 10% acetone to each. Nitrite formed by reduction of nitrate was then estimated by the addition of 1 ml each of sulphanilamide solution and N (1-naphthyl) ethylene diamine dihydrochloride solution into the culture tube. The pink colour was allowed to develop for 20 min. The absorbance was then recorded at 540 nm using a colorimeter/spectrophotometer. The standard curve of nitrate content in the sample and then calculated. The results were expressed as  $\text{nmoles NO}_3^{-1} \text{ g}^{-1}$  dry weight of the sample.

### **Calibration of a standard curve for nitrate estimation**

In a series of test tubes, 0, 0.2, 0.4, 0.6, 0.8, and 1.0 ml of the working solution of  $\text{KNO}_3$  (100 mM) were pipetted into test tubes in triplicate, corresponding to the nitrate concentration. Each of these tubes was filled to 3.5 ml with 0.5 ml of working catalyst solution were added followed by 0.25 ml of NaOH solution, 0.25 ml of hydrazine sulphate solution, and 0.5 ml of acetone were added. One ml of 1% sulphanilamide prepared in 1 N HCl was added to each of these tubes and mixed well. Then one ml of 0.02 % n-(1-naphthyl ethylene diamine dihydrochloride was added to each tube and the tubes were incubated at room temperature for at least 20 min. The absorbance was read at 540 nm on a spectronic-20 spectrophotometer against the distilled water blank. Absorbance against the concentration of  $\text{KNO}_3$  was plotted.

#### **3.2.4 *In vivo* Assay of Nitrate Reductase in Wheat Leaves**

The *in vivo* method as described by Hageman and Hucklesby (1971) for assaying nitrate reductase activity from fresh leaf materials was employed with some modifications.

## Reagents

1. Phosphate buffer, 100 mM, pH 7.5.
  - A. Solution of monobasic sodium phosphate (0.2 M).
  - B. Solution of dibasic sodium phosphate (0.2 M). 16 ml of monobasic sodium phosphate solution were mixed with 84 ml of dibasic sodium phosphate solution and the pH was adjusted to 7.5. The final volume was made to 200 ml with distilled water.
2. n-Propanol
3. Sulphanilamide (1 %, w/v)

One g of sulphanilamide was weighed accurately and dissolved in 1 N HCl and the volume was made up to 100 ml 1 N HCl.
4. N – (1-naphthyl) ethylenediamine dihydrochloride (0.02 %, w/v)
5. Stock solution of sodium nitrite (10 mM)
6. Working solution of sodium nitrite (100  $\mu$ M)

## ***In vivo* assay of nitrate reductase assay under anaerobic condition**

### **Enzyme extraction**

Tissue from 10 day old plants (physiologically active leaves) was excised, washed with distilled water, and blotted dry in filter paper folds. The leaf extremities were removed, and the leaves was cutted into 2 mm squares. The sample was thoroughly mixed. 200 mg of sample was placed in the test tubes containing infiltration medium already maintained at 0–4°C.

### **Assay of enzyme activity**

The assay conditions were optimized for substrate (nitrate). The effect of the n-propanol (0–5%, v/v) concentration required in the infiltration media was also investigated. The standard infiltration medium used in the subsequent experiment contained a total volume of 5 ml: 100 mM sodium phosphate buffer (pH 7.5), 100 mM KNO<sub>3</sub> (for KNO<sub>3</sub> assays), and 1% (v/v) n-propanol. Some assays did not include nitrate in the infiltration medium to determine the actual (*in situ*) amount of NO<sub>3</sub><sup>-</sup> reduced. All the operations, starting from the harvesting of the leaf tissue to the transfer into the assay tubes, were carried out at 0–4°C. Then the tubes were transferred to a desiccator and evacuated by vacuum infiltration for 2 min. with the help of an oil pump. The tubes were then incubated at 30  $\pm$  1°C for 45 min. in the dark. The tubes were removed from the desiccator and kept in a boiling water bath for 2 min. to stop the reaction. The tubes were kept in boiling water for a further 10 min. to ensure the complete extraction of the NO<sub>2</sub><sup>-</sup> formed in the leaf tissue. One ml of the aliquot was withdrawn for the estimation of nitrite.

### **CO-aerobic assay of nitrate reductase at 4:1 CO:O<sub>2</sub> ratios**

Whole leaves from 10 days old seedlings containing sufficient KNO<sub>3</sub> were cut into 2.5 cm segments and approximately 0.2 g of leaf material was placed in tubes (1.5 x 15 cm), without any liquid medium or buffer, which were connected in a series via needles inserted in the septa. The varying rates of flow of the compressed air and high purity CO were controlled via gas flow meters supplied by Indian Oxygen Limited, Bombay. Thus, the leaves were exposed to CO and O<sub>2</sub> only in the gaseous phase. The tubes were incubated at 30°C in complete darkness for 50 min. The reaction was terminated by adding 5 ml H<sub>2</sub>O at 100°C to the leaves. To extract all the nitrite from the leaves, the tubes were kept at 100°C for 10 min. Nitrite was then determined in suitable aliquots as described earlier Sawhney *et al.* (1978).

### **Absorption of DNP**

Whole leaves of 10-d-old seedlings excised just above the soil level were placed with their bases immersed in liquid in vials containing 2 ml solution of 2 mM DNP. In the control experiments, 2 ml distilled H<sub>2</sub>O was used. The vials were placed in a vacuum desiccator and air was completely removed. The uncoupler was allowed to be absorbed by the leaves by vacuum infiltration for 5 min. At the end of this period, the desiccator was opened and the leaves were tested for NR *in vivo* activity under varying CO to O<sub>2</sub> ratios as described earlier.

### **3.2.5 *In vitro* Assay of Nitrate Reductase in Wheat Leaves**

The extraction and assay procedures of Hageman and Hucklesby (1971) and Sawheny and Naik (1972), respectively, were followed with slight modifications.

#### **Enzyme extraction**

Physiologically active leaves from 10 day old plants were excised, washed with distilled water and blotted dry in filter paper folds. The leaves were cut into small pieces and thoroughly mixed. A representative sample of 0.5 g was drawn and extracted with 5 ml of ice cold extraction medium using a precooled mortar and pestle. The extraction medium consisted of 0.1 M phosphate buffer (pH 7.5), 1 mM cysteine and 0.5 mM EDTA. Acid washed sand was used to facilitate the grinding. All the operations were performed at 0–4°C. The extracted homogenate was passed through four layers of cheese cloth and then transferred to the cold centrifuge tubes already maintained in the ice. The homogenate was centrifuged for 15 min. at 15,000 x g at 2°C using a refrigerated centrifuge. The supernatant was collected, which was used as a source of crude enzyme assay. The crude enzyme was stored in an ice bucket for subsequent experiments.

### Assay of enzyme activity

The enzyme activity was assayed by measuring the NADH-dependent production of nitrite (Hageman and Hucklesby, 1971). The reaction mixture at a total volume of 2.0 ml in the tubes contained:

- |                                     |   |             |
|-------------------------------------|---|-------------|
| 1. Sodium phosphate buffer (pH 7.5) | : | 100 $\mu$ M |
| 2. KNO <sub>3</sub>                 | : | 20 $\mu$ M  |
| 3. NADH                             | : | 0.75 mM     |
| 4. Enzyme                           | : | 0.2 ml      |

Minus NADH served as a blank.

The reaction was started by adding 0.2 ml of enzyme extract. The tubes were incubated in a water bath maintained at 30°C for 30 min. The reaction was terminated by adding 0.1 ml of 1 M zinc acetate solution, which precipitated the protein, followed by the addition of 1.9 ml of 70% (v/v) ethyl alcohol. The contents of the tubes were centrifuged at 3000 x g for 5 min, and one ml of the supernatant was withdrawn for the assay of nitrite.

### Color development

The colour development procedure was the same for both the *in vivo* and *in vitro* methods of nitrate reductase. In the case of the *in vivo* assay, one ml of the aliquot was withdrawn directly from the infiltration medium. For *in vitro* assay, one ml of the supernatant after centrifugation was taken for the assay of nitrite. To this, one ml each of 1% (w/v) sulphanilamide prepared in 1 N HCl and (0.02% w/v) N-(1-naphthyl) ethylenediamine dihydrochloride were added. The tubes were incubated at least for 20 min at room temperature. To each of these tubes, 7 ml distilled water was added and the colour was mixed thoroughly on a vortex mixer. The colour was read on a Spectronic-20 Spectrophotometer at 540 nm against the reagent blank. The amount of NO<sub>2</sub><sup>-</sup> formed was calculated from the standard curve of NO<sub>2</sub><sup>-</sup> and the NR activity was expressed as  $\mu$ mol of NO<sub>2</sub><sup>-</sup> formed g<sup>-1</sup> fr. wt. hr<sup>-1</sup>.

### Calibration of a standard curve for nitrite estimation

In a series of test tubes, 0, 0.2, 0.4, 0.6, 0.8, and 1.0 ml of the working solution of NaNO<sub>2</sub> (100  $\mu$ M) was pipetted into test tubes in triplicate. The volume in each of these tubes was made up of 1 ml of distilled water. One ml of 1% sulphanilamide prepared in 1 N HCl was added to each of these test tubes and mixed well. Then one ml of 0.02 % N-(1-naphthyl) ethylenediamine dihydrochloride was added to each tube and the tubes were incubated at room temperature for at least 20 min. A vortex mixer was used to mix in seven ml of distilled

water. The absorbance was read at 540 nm on a Spectronic-20 Spectrophotometer against the distilled water blank. Absorbance vs. the concentration of  $\text{NaNO}_2$  was plotted.

### 3.2.6 *In vitro* Assay of Nitrite Reductase in Wheat Genotypes

In general, the enzyme nitrite reductase (NiR) is generally assayed by an *in vitro* method. In the present studies, it was characterised from the leaf materials of these wheat genotypes by an *in vivo* method as described by Karadge *et al.* (1994).

#### Reagents

1. Tris-HCL buffer: 0.5 M (pH 7.5)
2. Sodium nitrite solution: 5 mM
3. Methyl viologen solution: 105 mM
4. Dithionite reagent: Dissolve 40 mg of sodium dithionite and 40 mg of sodium hydrogen carbonate in 10 ml of water just prior to use.
5. Sulphanilamide (1 %, w/v)

One g of sulphanilamide was weighed accurately and dissolved in 1 N HCl and the volume was made up to 100 ml 1 N HCl.

6. N – (1-naphthyl) ethylenediamine dihydrochloride (0.02 %, w/v)
7. Stock solution of sodium nitrite (10 mM)
8. Working solution of sodium nitrite (100  $\mu\text{M}$ )

#### Enzyme extraction

The leaves were cut into small pieces and thoroughly mixed. A representative sample of 0.5 g was drawn and extracted with 5 ml of Tris-Hcl buffer (pH 7.5) using a pre cooled mortar and pestle. Acid washed sand was used to facilitate the grinding. All the operations were performed at 0-4°C. The extracted homogenate was passed through four layers of cheese cloth and then transferred to the cold centrifuge tubes already maintained in the ice. The homogenate was centrifuged for 15 min. at 15,000 x g at 4°C using a refrigerated centrifuge. The supernatant was collected, which was used as a source of crude enzyme assay.

#### Assay of enzyme activity

Prepare the reaction mixture by mixing,

- |                             |   |          |
|-----------------------------|---|----------|
| 1. Tris-Hcl buffer          | : | 6.25 ml  |
| 2. Sodium nitrite solution  | : | 2 ml     |
| 3. Methyl viologen solution | : | 2 ml     |
| 4. Distilled water          | : | 14.75 ml |

Pipette out 1.5 ml of reaction mixture and 0.3 ml of enzyme preparation into a test tube. Run a blank without the enzyme. Start the reaction by adding 0.2 ml of recently

prepared dithionite-sodium bicarbonate solution. Incubate for 15 min at 30°C. Stop the reaction by vigorous shaking (vortex mixer) until the blue colour disappears.

### **Colour development**

Using a 20 µl aliquot from the reaction tube, the nitrite content was estimated by adding one ml of 1% sulphanilamide prepared in 1 N HCl, followed by one ml of N-(1-naphthyl) ethylenediamine dihydrochloride. Then seven ml of distilled water was added to each of these tubes and mixed well into a vortex mixture. The absorbance was measured at 540 nm on a Sepctronic-20 Spectrophotometer. The activity of nitrite reductase was expressed in terms of µmole of nitrite disappeared g<sup>-1</sup> fr. wt. hr<sup>-1</sup>.

### **3.2.7 Crude Protein**

The protein content was determined by Microkjeldhal method (A.O.A.C, 1990).

### **Reagents**

1. Concentrated sulphuric acid (Sp. gr. 1.84, purity 98.08 percent and nitrogen free)
2. Catalyst mixture: Potassium sulphate, mercuric oxide, and copper sulphate were weighed at 99, 4.1, and 0.8 g respectively. mixed and ground into a fine powder.
3. Sodium hydroxide (50 %): 50 g of sodium hydroxide and 5 g of sodium thiosulphate were dissolved in distilled water separately, then mixed and the volume was made up to 100 ml with distilled water.
4. Boric acid (4%): 4 g of boric acid was dissolved in distilled water and the volume was made up to 100 ml with distilled water.
5. Hydrochloric acid (0.02 N): 0.17 ml of hydrochloric acid (Sp. gr. 1.18, purity 35.4%) was dissolved in distilled water and the volume was made up to 100 ml with distilled water.
6. Hydrogen peroxide: 30% (v/v) is commercially available in the market.
7. Mixed indicator: To make the mixed indicator, separately dissolve 0.1 g of bromocresol green and 0.1 g of methyl red in 100 ml of 95 percent alcohol. Ten parts of bromocresol green and two parts of methyl red indicator were mixed together and transferred to a bottle provided with a stopper.

### **Procedure**

A powdered and defatted sample (200 mg) was accurately weighed and transferred to the digestion flask. One gram of the catalyst mixture was added and mixed thoroughly with the sample. Five ml of concentrated sulphuric acid and five ml of hydrogen peroxide were carefully added, and the sample was digested in the digestion chamber. Initially, the flasks were heated slowly for 10 to 15 min., and then the temperature was raised gradually so that

the contents boiled briskly. The digestion was continued until the sample became clear and colorless. Then the flasks were cooled to room temperature, and after cooling, the contents were transferred to volumetric flasks. The digestion flasks were washed 3 to 4 times with distilled water. All the washes were transferred to volumetric flasks, and the volume was made up to 50 ml. Ten ml of boric acid solution was pipetted into 100 ml beakers and 6 to 8 drops of mixed indicator solution were added. The beaker was placed under the condenser of the distillation unit. At the end of distillation, the tip of the condenser was washed to collect all the ammonia. The distillate was then titrated with a standard hydrochloric acid (0.02N) solution. Before distillation, the colour of boric acid plus indicator was pink, which changed to blue-green during distillation and finally to pink-red at the end of the titration. A blank titration without a sample was also carried out. The percentage of nitrogen content was calculated from the quantity of standard hydrochloric acid required for titration of the sample. The protein content was calculated by multiplying the nitrogen content by a factor of 6.25.

$$\% \text{ Nitrogen} = \frac{(S - B) \times N \times 14.007}{\text{Weight of sample (g)}} \times \frac{\text{Volume made}}{\text{Volume taken}} \times 100$$

Where,

S = ml of hydrochloric acid required for sample titration

B = ml of hydrochloric acid required for blank titration

N = Normality of HCl (0.02 N)

% Protein = % Nitrogen x 5.58

### 3.2.8 NADH/NAD<sup>+</sup> Ratio

The NADH/NAD<sup>+</sup> ratio of wheat genotypes was analyzed by the method described by Matsumura and Miyachi (1980). This method utilises a cycling mixture composed of MTT, phenazine ethosulfate (PES), ethanol, and alcohol dehydrogenase for the determination of NAD<sup>+</sup> (or NADH) or glucose-6-phosphate (G6P). NAD<sup>+</sup> is reduced by the respective dehydrogenase system, and the NADH formed reduces MTT (3-(4, 5, Dimethyl thiazoyl-2)-2,5 diphenyltetrazolium bromide) through the mediation of PES (Phenyl methyl sulfonate). The rate of reduction of MTT is proportional to the concentration of coenzyme.

#### Reagent

1. Bicine –NaOH Buffer :1 M pH 8.0
2. NaOH : 0.1 N
3. HCL :0.1 N
4. Ethanol
5. Glucose – 6phosphate (disodium salt) :50 mM

6. EDTA- di sodium salt :40 mM
7. Phenylmethylsulfonate :16.6 mM
8. Alcohol dehydrogenase : 500U/ml (0.1 M)
9. 3-(4,5,Dimethyl thiazoy-2) 2-5 diphenyltetrazolium bromide: 4.2 mM
10. NAD(H): 5 mM
11. Standard solution of nicotinamide adenine dinucleotides, 5 mM

### **Extraction of nicotinamide adenine dinucleotides from plants.**

The equal amount of frozen leaf samples were ground to a powder in liquid N<sub>2</sub> for the preheated NaOH and HCl solutions (final concentration, 0.1 N), respectively. The suspensions were kept at 100°C for 2 min, then cooled to 0°C and centrifuged. NADH was specifically extracted in the supernatant obtained after the alkaline treatment, while NAD<sup>+</sup> was extracted after acid treatment.

### **Procedure**

Reaction was carried out in the dark or in a brown test tube. The blank without enzyme addition was higher if the reaction was carried by light. The acid as well as alkaline extract (0.1-0.5 ml) from the plant material was placed in a small test tube containing 0.1 ml Bicine-NaOH buffer. The extract was neutralised by adding an equivalent amount of NaOH or HCl, followed by the addition of 0.1 ml each of EDTA, MTT, PES, and ethanol for the determination of NAD (H), The test tubes were kept at 37°C for 5 min. after the total volume is adjusted to 1 ml by adding H<sub>2</sub>O. The reaction was started by adding 0.02 ml of alcohol dehydrogenase (for NAD (H)). After a predetermined reaction time, the reaction medium was transferred to a microcuvette having a light path of 10 mm, and the absorbency at 570 nm was determined. The proper reaction time had be 30-60 min. With each extract, a blank measurement must also be carried out. This may be done by adding an equal amount of Bicine-NaOH buffer instead of the enzyme.

The concentration of the coenzyme in each extract can be determined according to the standard curves, which were determined by the cycling systems and known amounts of nicotinamide adenine dinucleotides. The presence of the compounds in the extract which might affect the rate of enzyme cycling must also be checked.

### **3.2.9 *In vitro* Assay of Glutamine Synthetase Activity**

This enzyme was assayed by measuring the formation of  $\gamma$ -glutamyl hydroxamate which was reacted with ferric chloride and the brown colour in the acid medium was

measured at 540 nm. The method as described by Mohanty and Fletcher (1980) was used as follows

### Reagents

Extraction medium contained

1. Tris. HCl : 25 mM
2. Cysteine. HCl : 2.5 mM
3. MgSO<sub>4</sub>.7H<sub>2</sub>O : 4.0 mM
4. EDTA : 0.25 mM

The pH was adjusted to 7.5 and the final volume was made to 250 ml.

Ferric chloride reagent: This reagent was prepared by mixing 10 % FeCl<sub>3</sub>.6H<sub>2</sub>O in 0.2 N HCl, 20 % TCA (w/v) and 50 % HCl (v/v) in the ratio of 1:1:1.

### Enzyme extraction

Physiological active leaves from plants were excised, washed with distilled water, and blotted dry in filter paper folds. The leaves were cut into small pieces and thoroughly mixed. A representative sample of 1 g was drawn and extracted with 5 ml of ice-cold extraction medium using a precooled mortar and pestle. The extraction medium consisted of Tris-HCl (25 mM), cysteine. HCl (2.5 mM), MgSO<sub>4</sub>.7H<sub>2</sub>O (4 mM) and EDTA (0.25 mM). Acid washed sand was used to facilitate the grinding. All the operations were performed at 0-4°C. The extracted homogenate was passed through four layers of cheese cloth and then transferred to the cold centrifuge tubes already on ice. The homogenate was centrifuged for 15 min. at 15,000 g at 2°C using a refrigerated centrifuge. The supernatant was collected, which was used as a source of crude enzyme for the assay. The crude enzyme was stored in an ice bucket for subsequent experiments.

### Assay of enzyme activity

The enzyme activity was assayed by measuring the formation of  $\gamma$ -glutamyl hydroxamate. The reaction mixture in a total volume of 3.0 ml in the control and experimental tubes contained. The reaction was initiated by the addition of hydroxyl amine hydrochloride as the last component which was omitted from the control tube. The tubes were incubated in a water bath maintained at 27°C for 15 min. After incubation, one ml of ferric chloride reagent was added to each tube. A brown coloured compound of ferric  $\gamma$ -glutamyl hydroxamate formed was calculated from the standard curve and the GS activity was expressed as  $\mu\text{mol}$  of  $\gamma$ -glutamyl hydroxamate formed  $\text{g}^{-1}$  fr. wt.  $\text{min}^{-1}$ .

Reagents	Conc. in RM	Control tube	Experimental tube
Tris-buffer, pH 7.5	0.1 M	1 ml	1 ml
ATP	10 $\mu$ mol	0.3 ml	0.3 ml
Sodium glutamate	250 $\mu$ mol	0.5 ml	0.5 ml
MgSO <sub>4</sub> .7H <sub>2</sub> O	100 $\mu$ mol	0.3 ml	0.3 ml
Cysteine.HCl	10 $\mu$ mol	0.3 ml	0.3 ml
Enzyme extract	-	0.3 ml	0.3 ml
H <sub>2</sub> O	-	0.3 ml	-
Hydroxylamine hydrochloride, pH 7.0 (freshly prepared)	10 $\mu$ mol	-	0.3 ml
	<b>Total volume</b>	3.0 ml	3.0 ml

### Preparation of a standard curve of $\gamma$ -glutamyl hydroxamate

In a series of test tubes, 0.5 to 2.5  $\mu$ mol of the standard stock solution of  $\gamma$ -glutamyl hydroxamate was pipetted in triplicate. The volume in each of these test tubes was made up to 3 ml with distilled water. One ml of ferric chloride reagent was added to each tube. A brown coloured compound of ferric  $\gamma$ -glutamyl hydroxamate was formed immediately and the tubes were incubated at room temperature for 20 min. The absorbance of brown coloured supernatant was measured at 540 nm in a Spectronic-20 Spectrophotometer. The readings obtained were plotted on a graph by taking  $\mu$ mol of  $\gamma$ -glutamyl hydroxamate concentration on the X-axis and the absorbance on the Y-axis.

### 3.2.10 *In vitro* Assay of Glutamate Synthase

Fd-GOGAT activity was determined using reduced methyl viologen as the electron donor. The method as described by Lea *et al.*, 1990 and Fd-GOGAT activity was determined by the quantitative measurement of glutamate using a sepctronic-20 spectrophotometer as described previously by Esposito *et al.*, 2003 method. The extraction and assay procedures of NADH-GOGAT described by Esposito *et al.*, 2005, respectively, were followed with slight modifications.

### Reagents

Extraction medium contained

1. KH<sub>2</sub>PO<sub>4</sub>-KOH : 50 mM
2. KCl : 100 mM
3. EDTA : 5 mM
4. 2-oxoglutarate : 2 mM
5. DTT : 1 mM
6. Phenyl methyl sulphonyl fluoride : 1 mM

The pH was adjusted to 7.5 and the final volume was made to 250 ml.

### Enzyme extraction

Glutamate synthase isoforms were extracted with buffer A (50 mM  $\text{KH}_2\text{PO}_4$ -KOH pH 7.5, 100 mM KCl, 5 mM EDTA, 2 mM 2-oxoglutarate, 1 mM DTT, 1 mM phenyl methyl sulphonyl fluoride, PMSF), and 0.1% PVPP was added to the extraction buffer for the preparation of the crude extract of leaves, in order to avoid interference due to polyphenolic compounds. The homogenate was then passed through four layers of cheese cloth. The homogenate was centrifuged for 30 min. at 13,000 x g at 40°C using a refrigerated centrifuge. The supernatant (fraction designated as crude extract) was desalted on a Sephadex G25 column (Amersham Biosciences PD-10) equilibrated in buffer A, which was used as a source of crude enzyme assay.

**Table 3.4 Assay of Fd-GOGAT activity**

Sr. No.	Reagents	Conc. in RM	Control tube	Experimental tube
1.	$\text{KH}_2\text{PO}_4$ -KOH pH 7.5	200 mM	0.5ml	0.5ml
2.	L-Glutamine	10mM	0.5ml	0.5ml
3.	2-ketoglutarate	10mM	0.5ml	0.5ml
4.	methyl viologen	15mM	0.2ml	0.2ml
5.	amino-oxyacetic acid	1mM-	MI	MI
6.	Enzyme extract	-	0.5ml	0.5ml

After the addition of reaction mixture tubes for 5 min of pre-incubation at 30°C, the reaction was started by the addition of a reductant solution (47 mg  $\text{Na}_2\text{S}_2\text{O}_4$ , 50 mg  $\text{NaHCO}_3$  in 1 ml of water). After 30 min of incubation at 30°C the reaction was terminated by adding 1 ml of ethanol and then shaking vigorously.

Fd-GOGAT activity was determined by the quantitative measurement of glutamate using measured at 540 nm on a Sepctronic-20 Spectrophotometer. The activity was expressed as nmol glutamate formed  $\text{min}^{-1} \text{mg}^{-1}$  protein.

**Table 3.5 Assay of NADH-GOGAT Activity**

Sr. No.	Reagents	Conc. in RM	Control tube	Experimental tube
1.	$\text{KH}_2\text{PO}_4$ -KOH pH 7.5	50mM	0.5 ml	0.5ml
2.	Glutamine	10mM	0.5 ml	0.5ml
3.	2-ketoglutarate	10mM	0.5 ml	0.5ml
4.	amino-oxyacetic acid	1mM	0.5ml	0.5ml
5.	Enzyme extract	-	0.5ml	0.5ml
6.	Water	-	0.5ml	-
7.	NADH	1mM	-	0.5ml

### 3.2.11 Estimation of Alanine

Alanine was estimated by the colorimetric method described by Moore and Stein (1948) using alanine as the standard.

#### Reagents

1. 80% Ethanol
2. 0.2M Citrate buffer (pH, 5.0)
3. Ninhydrin reagent : Dissolve 0.8 g of stannous chloride in 500 ml 0.2 M Citrate buffer (pH,5.0). Add 20 g ninhydrin in 500 ml methyl cellosolve
4. Diluentsovent : mix equal volume of water and n propanol
5. Stock solution 50 mg of alanine/50 ml water
6. Working standard: dilute 10 ml of stock in100 ml water

#### Protocol

The enzyme extract was taken for it and diluted to 1 ml with distilled water. Take 0.1ml of extract, add 1ml ninhydrin reagent and mix .make up the volume 2ml with water. Heat in a boiling water bath for 20min. Add 5ml of diluent while still on water bath and mix. After 15 min of boiling, cool the tube under running tp water absorbance of purple against bank at 570 nm.

#### Calibration of a standard curve

In a triplicate set of test tubes, the alanine solution was pipetted as 0, 0.2, 0.4,0.6.....1ml. The volume was made to 1 ml with distilled water. The colour was developed as described above. The absorbance was recorded at 570 nm and a standard graph of absorbance against concentration was plotted.

### 3.2.12 *In vitro* Assay of Glucose-6p Dehydrogenase Activity

The extraction procedures of Esposito *et al.* (2005), respectively were followed with slight modifications. G6PDH activity was assayed monitoring NADP<sup>+</sup> reduction at 340 nm (Esposito *et al.*, 1998).

#### Reagents

Extraction medium contained

- |                      |          |
|----------------------|----------|
| 1. TRIS–HCl          | : 100 mM |
| 2. MgCl <sub>2</sub> | : 10 mM  |
| 3. EDTA              | : 5 mM   |
| 4. PMSF              | : 1 mM   |
| 5. glycerol          | : 10%    |
| 6. NADP <sup>+</sup> | : 15 μM  |

7. DTT : 1 mM
8. Beta-mercaptoethanol : 1.5 mM

The pH was adjusted to 7.9 and the final volume was made to 250 ml.

### Enzyme extraction

Glucose-6P dehydrogenase (G6PDH) was extracted in 100 mM TRIS–HCl buffer pH 7.9 containing 10 mM MgCl<sub>2</sub>, 5 mM EDTA, 1 mM PMSF, 10% glycerol, 15 μM NADP<sup>+</sup>, 1 mM DTT, and 1.5 mM mercaptoethanol. The homogenate was filtered through four layers of muslin cloth and centrifuged at 20, 000 g for 20 min at 4°C. The supernatant (fraction designated as the crude extract) was used for glucose-6 P dehydrogenase assays. Alternatively, the supernatant (crude extract) was desalted on a Sephadex G25 column (Amersham Biosciences PD-10) equilibrated in extraction buffer without PMSF and mercaptoethanol, giving no appreciable change in measurable G6PDH activity with respect to the untreated samples.

### Assay of enzyme activity

G6PDH activity was assayed monitoring NADP<sup>+</sup> reduction at 340 nm (Esposito *et al.*, 1998). The assay mixture contained: 50 mM TRIS-HCl pH 8.0 buffer, 10 mM MgCl<sub>2</sub>, 0.15 mM NADP<sup>+</sup>, 3 mM glucose-6P, and extract. The activity was expressed as μmol reduced NADP<sup>+</sup> min<sup>-1</sup> mg<sup>-1</sup> protein.

**Table 3.6 Assay of G6PDH activity**

Sr. No.	Reagents	Conc. in RM	Control tube	Experimental tube
1	TRIS–HCl	50 mM	1ml	1ml
2	MgCl <sub>2</sub>	10 mM	0.5ml	0.5ml
3	NADP <sup>+</sup>	0.15 mM	0.5ml	0.5ml
4	Glucose- 6P	3 mM	0.5ml	0.5ml
5.	Distilled Water	-	0.5ml	-
6.	Enzyme extract	-	-	0.5ml

### 3.2.13 Native PAGE

The proteins were extracted from the flag leaf of wheat genotypes and the protein bands were resolved using ATTO, AE 8450 model of Japan make electrophoresis unit as per the method described by Laemmli (1970).

### Reagents

1. Stock solution of extraction buffer: Tris-HCl buffer, 0.5 M, pH 6.8.
2. Working solution of extraction buffers: Tris HCl buffer, 0.125 M, pH 6.8 containing 5 % (v/v) β-mercaptoethanol.
3. Stock solutions for stacking gel and seperating gel

**A. 0.5 M Tris-HCl buffer (pH 6.8)**

- i. Tris : 5.98 g
- ii. 1 M HCl : 49 ml
- iii. TEMED : 0.46 ml

Small quantity of distilled water was added in it and pH was adjusted to 6.8. The final volume was made to 100 ml. The solution was filtered and stored in brown bottle at 4°C.

**B. Acrylamide solution (30 %)**

- i. Acrylamide : 30 g
- ii. Bisacrylamide : 0.735 g  
(N, N' methylenebisacrylamide)
- iii. Dissolved in distilled water and final volume was made to 100 ml. The solution was filtered and stored in brown bottle at 4°C.

**C. 3 M Tris HCl buffer (pH 8.8)**

- i. Tris : 36.3 g
- ii. 1 M HCl : 48 ml
- iii. TEMED : 0.25 ml

Small quantity of distilled water was added to it and pH was adjusted to 8.8. The final volume was made to 100 ml. The solution was filtered and stored in brown bottle at 4°C.

**D. Ammonium persulphate 10 % (w/v):**

Add 1 ml of water to 0.1 g of ammonium persulfate. Prepare just before use.

**4. Working solution for Native-PAGE****a. Separation gel (5 %, pH (8.7))**

3M Tris HCl (pH- 8.8)	4.50 ml
Acrylamide Solution (30%)	3 ml
Distilled water	10 ml
Ammonium persulphate 10 %	0.07 ml (70µl)
TEMED	0.01 ml (10µl)

**b. Stacking gel (3 %, pH 6.7)**

0.5M Tris HCl (pH- 6.8)	1.50 ml
Acrylamide Solution (30%)	0.80 ml
Distilled water	4 ml
Ammonium persulphate 10 %	0.07 ml (70µl)
TEMED	0.01 ml (10µl)

## 5. Electrophoresis buffer

(Tris, Glycine, SDS, pH 8.3)

- i. Tris : 3 g
- ii. Glycine : 14.4 g

Dissolved in distilled water, the pH was adjusted to 8.3 and final volume was made to 1 liter. This buffer was used for 2-3 subsequent runs.

For native gel electrophoresis buffer was prepared as described above without SDS.

## 7. PAGE staining solutions

- i. Coomassie brilliant blue : 1.25 g
- ii. Methanol (50 %, v/v.) : 454 ml
- iii. Acetic acid : 46 ml

The dye was first dissolved in methanol. Then acetic acid and distilled water were added in it and filtered 2 to 3 times before use. Fresh solution was prepared and used every time.

## 8. Distaining solution

- i. Acetic acid : 75 ml
- ii. Methanol : 50 ml
- iii. Distilled water : 575 ml

## 9. Standard protein molecular weight marker

The protein molecular weight markers were obtained from the Bangalore Genei Pvt. Ltd., Bangalore.

**Table 3.7 Proteins with their molecular weight**

Analysis	Proteins used	Molecular weight (KDa)
For protein and isozyme profile	Medium ranged molecular marker	14.3 to 97.4

**3.2.13.1 Glutamine synthetase isozyme**

The GS isozyme was extracted from the flag leaf of wheat genotypes (Wang *et al.*, 2015) and enzyme protein was estimated by Lowry *et al.* (1951). The isozymes were resolved using ATTO, AE 8450 model of Japan. The staining solution was prepared as described by Sadasivam and Manickam (1996).

**Reagents**

1. Extraction buffer: Tris-HCL Buffer 100 mM, pH 7.6

2. Sample buffer: The sample buffer was prepared by mixing 1.5 ml (Tris-HCL Buffer 100 mM, EDTA, 1 mM MgCl<sub>2</sub>, and 10 mM β-mercaptoethanol, pH 7.6).
3. Staining solution: The staining solution was prepared by dissolving
  - i. Tris-Hcl Buffer 100 mM pH 7.4
  - ii. EDTA 1.3 mM
  - iii. Sodium arsenate 20 mM
  - iv. MgSO<sub>4</sub> 20 mM
  - v. ADP 1 mM
  - vi. Hydroxamine 50 mM
  - vii. L-glutamate 100 Mm
4. Stop solution
  - i. FeCl<sub>2</sub>.6H<sub>2</sub>O – 370 mM
  - ii. HCl – 700 mM
  - iii. TCA (w/v) – 200 mM

### **Preparation of leaf extract**

Physiological active leaves from plants were excised, washed with distilled water and blotted dry in filter paper folds. The leaves were cut into small pieces and thoroughly mixed. A representative sample of 0.5 g was ground into powder in a chilled mortar with liquid N<sub>2</sub> and mixed with 1.5 ml Extraction Buffer (Tris-HCL Buffer 100 mM , EDTA, 1 mM MgCl<sub>2</sub>, and 10 mM β-mercaptoethanol, pH 7.6). Acid washed sand was used to facilitate the grinding. All the operations were performed at 0-4°C. The extracted homogenate was transferred to the cold centrifuge tubes already maintained in ice. The homogenate was centrifuged for 30 min. at 13,000 g at 4°C using a refrigerated centrifuge. The supernatant was collected, which was used for native gel analysis.

Samples were generally to 50 µL from 0.5 g fresh weight (FW) of leaf in each lane, and electrophoresis was carried out at 80 V for the stacking gel and 120 V for the resolving gel.

### **3.2.13.2 Electrophoresis**

The native gel system employed a 1.5 mm ×170 mm ×100 mm gel, the analyzing gel was composed of 5% polyacrylamide (pH 8.7), and the stacking gel was 3% polyacrylamide (pH 6.7) (Zhang *et al.*, 2017).

### **Staining solution**

After electrophoresis, GS activity was detected in-gel by the conversion of L-glutamine to γ-glutamyl hydroxamate (Barratt, 1998). The gel was immersed in 100 ml of

reaction buffer (100 mM Tris-HCl Buffer, 1.3 mM EDTA, 20 mM sodium arsenate, 20 mM MgSO<sub>4</sub>, 1 mM ADP, 50 mM hydroxylamine, and 100 mM L-glutamate, pH 7.4) and incubated at 37°C for 45 min with slow shaking, after which the reaction buffer was removed.

#### **Stop solution**

The reaction was terminated by the addition of 50 ml of stop solution of Ferric chloride reagent (370 mM FeCl<sub>3</sub>, 200 mM trichloroacetic acid, and 700 mM HCl mix it in 1:1:1 ratio) for 3 min until GS activity appeared as a brownish band on the yellow background.

#### **Procedure**

The two glass plates with spacer between them was fixed on the gel platform of the electrophoresis unit with due care to maintain leak proof. The separation gel solution were mixed appropriate proportion and degassed. The solution was immediately poured in between the glass plates mounted on a gel platform by Pasteur pipette so that about 2/3 of the space was filled with gel with due care that no air bubble was trapped. Then small quantity of distilled water was carefully over layed on the top of the gel to get uniform gel surface and avoid contact of air. The plates with gel were kept for gel polymerization under fluorescent light for 60 min. After polymerization of gel, the water layer was removed with the filter paper strips. The stock solutions of stacking gel were mixed in appropriate proportions to get a working solution and the mixture was immediately poured over the stacking gel. The 13 well combs were immediately kept over the gel. The comb was removed carefully after 30 min and the wells were washed with the electrode buffer. The traces of electrode buffer from the well were removed with filter paper strips. The plates were removed from platform and fixed on the gel cassette. The cassette was placed in the buffer tank and electrode buffer was poured in such a way that all wells were completely submersed into buffer.

The samples of each 50 µl (150 µg protein) depends upon the concentration of protein in the sample were mixed with 3 µl of bromophenol blue (sample buffer) to function as tracking dye and loaded by syringe in the separate wells carefully. It was connected to power supply unit. Initially the current 1.5 mA/well was applied till the sample migrates into the spacer gel and then it was adjusted to 2 mA/well. The electrophoresis apparatus except power supply was kept in a refrigerator at constant temperature of 4°C. The electrophoresis was carried out until the bromophenol blue (tracking dye) migrated almost to the end of the gel and current was switched off.

The cassette was removed from the unit and the gel was taken out gently. It was placed in a staining tray and sufficient staining solution was poured to cover the gel uniformly. It was incubated for 3-8 hours and the stain was decanted. The destaining was carried by keeping the gel in the destaining solution for one day with interchanging of the destaining solution frequently. The band intensity was assessed visually by placing gel over transilluminator and recorded as faint and dark bands and as absence or presence of specific bands. The relative mobility ( $R_m$ ) of resolved protein bands were calculated by using formula

$$R_m = \frac{\text{Distance migrated by protein band (cm)}}{\text{Distance migrated by tracking dye from the top of the separation gel (cm)}}$$

### 3.2.14 Estimation of Protein

Soluble protein was estimated by the colorimetric method described by Lowry *et al.* (1951) using bovine serum albumin as the standard.

#### Reagents:

1. Solution A (2% w/v,  $\text{Na}_2\text{CO}_3$  in 0.1 M NaOH): The solution was prepared by dissolving 4 g of sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) and 0.8 g of sodium hydroxide (NaOH) in small amount of distilled water and the final volume made to 200 ml with distilled water.
2. Solution B (0.5% w/v,  $\text{CuSO}_4$  in 1% w/v, NaK tartarate):
  - (a) 1%  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  solution: The solution was prepared by dissolving 0.5 g of copper sulphate in small amount of distilled water and the final volume made to 50 ml with distilled water.
  - (b) 2% potassium sodium tartrate solution: The solution was prepared by dissolving 0.5 g of potassium sodium tartrate in small amount of distilled water and the final volume made to 25 ml with distilled water.

Working solution of B: Prepare fresh before use by mixing equal volume of solution B (a) and B (b).

3. Solution 'C' (Carbonate- $\text{Cu}^{++}$  solution): The solution was prepared fresh before use by mixing 50 ml of solution A and 1 ml of working solution of B.
4. Folin-Ciocalteu reagent (1 N): The solution was prepared fresh by mixing equal volume of 2 N Folin-Ciocalteu reagent and distilled water and stored in amber coloured bottle.

5. Bovine serum albumin (250µg/ml): The solution was prepared by dissolving 25 mg of bovine serum albumin in small amount of distilled water and the final volume made to 100 ml with distilled water.

### Protocol

The enzyme assay, 50 µl was pipetted into a test tube and diluted to 1 ml with distilled water. Alkaline reagent 5 ml was added in it and mixed well and kept room temperature for 10 min. Folin ciocalteau reagent 0.5 ml was added rapidly in it, vortexed and kept 30 min at room temperature for colour development. The intensity of the colour was measured at 660nm on spectronic-20.

### Calibration of a standard curve

In a triplicate set of test tubes, the BSA solution was pipetted as 0, 0.2, 0.4, 0.6.....1ml having the corresponding BSA concentration of 0, 50, 100, 150, 200,...250 µg. The volume was made to 1 ml with distilled water. The colour was developed as described above. The absorbance was recorded at 660 nm and a standard graph of absorbance against centration.

### 3.2.15 Total RNA Isolation

Total RNA was isolated from flag leaves of selected wheat genotypes using TRIZOL method with min. modifications.

#### 3.2.15.1 Stock solutions

The reagents and buffers for RNA isolation were prepared as per the method described by Sambrook *et al.* (1989). All this solutions were prepared in sterile environmental condition. The composition and procedure for preparation of various stock solutions and buffers are given in the table 3.7.

**Table 3.8 Preparation of chemicals / reagents for RNA isolation**

Sr. No.	Solution	Method of preparation
1	TRIZOL RNA reagent	Ready to use from INVITROGEN
2	Chloroform	Ready to use biotechnological grade.
3	Isopropyl alcohol	Ready to use, biotechnological grade.
4	70% Ethanol	500 ml 350 ml of ethanol mixed with 150 ml of DEPC treated H <sub>2</sub> O. Dispensed in to reagent bottle and store at 4°C
5	Nuclease free water	Ready to use from FERMENTAS

#### 3.2.15.2 RNA extraction procedure

Flag leaf samples stored in RNA later were used for RNA isolation. Total RNA was isolated from 100 mg of leaf tissues of high- and low-nitrogen-efficient genotypes at pre- and post-anthesis samples using the TRIZOL reagent. The TRIZOL reagent combines phenol and guanidine thiocyanate in an amonophase solution to facilitate the integrity of the RNA while

disrupting cells and dissolving cell components, and to also provide immediate inhibition of RNase activity. The stepwise procedure for the extraction of RNA from the flag leaf samples has been described as follows:

- i. In a pre-chilled DEPC treated mortar and pestle, a 100 mg leaf sample was homogenised to a fine powder in liquid nitrogen.
- ii. One ml of Trizol (Life Technologies, USA) was added to 100 mg of tissue sample and collected in a 1.5 ml microcentrifuge tube. The sample was homogenised by pipetting and mixed. Note: Lay the tube horizontally to maximise surface area during RNA extraction.
- iii. Add 200  $\mu$ l of chloroform (molecular grade) per 1 mL of TRIZOL was added to homogenized The tubes were capped securely and shaken vigorously by hand for 15 seconds.
- iv. The tubes were incubated for 2–3 min. at room temperature.
- v. The samples were centrifuged at 12,000 g for 15 min at 4°C.
- vi. The supernatant was removed from the tube, leaving only the RNA.
- vii. The pellet was washed with 1 ml of 75% ethanol.
- viii. The samples were briefly vortexed, and the tubes were then centrifuged at 12000 x g for 10 min at 4°C.
- ix. The wash was decanted carefully, keeping an RNA pellet at the tube bottom.
- x. The RNA pellet was air dried for 15 min and re-suspended in 40  $\mu$ l Nuclease Free Water.
- xi. RNA concentration and quality were assessed with a nanodrop and stored at -80°C.

### **3.2.15.3 Quantitative Assessment of Total RNA by Qubit Fluorometer**

Total RNA from all samples was quantified using the Qubit RNA BR (Broad-Range) Assay Kit with the Qubit 2.0 Fluorometer (Life Technologies USA), which provides an accurate and selective method for the quantitation of high-abundance RNA samples.

#### **Material**

1. 1.5 mL plastic tubes (supplied with the kit, for mixing the Qubit™ working solution)
2. Qubit assay tubes (500  $\mu$ l)
3. Qubit RNA reagent and buffer
4. RNA standards

#### **Method:**

- i. The required number of assay tubes were taken and labelled with the sample name.

- ii. The Qubit working solution was diluted for the preparation of Qubit RNA reagents by 1:200 in the Qubit RNA buffer supplied in the kit.
- iii. 198 µl Qubit working solution was distributed in 8 different tubes for test samples and 190 µl for the working standard solution.
- iv. 1 µl of RNA sample was added to the respective tubes, and 10 µl standards were distributed in two tubes labelled as Std1 and Std2.
- v. All tubes were incubated in the dark for 15 min and readings were recorded.

#### 3.2.15.4 Agarose gel electrophoresis

Total RNA was 1:10 diluted, and 5 uL of each were loaded on a 1% (w/v) Agarose gel. Electrophoresis was performed at 5 v/cm in 0.5x TBE buffer for 30 min. The gel image was captured using the BioRad Gel Documentation System. The reagents and chemicals required are listed in Table 3.14.

**Table 3.9 Reagents and chemicals for agarose gel electrophoresis**

5x TBE buffer (pH 8.0) 1 liter	54.5 g of tris base, 27.5 g of boric acid (biogene) were taken, 20 ml of 0.5 M EDTA (pH 8.0) was added. The final volume of 1 liter was adjusted by adding distilled water and the pH was adjusted to 8.0.
TE Buffer (pH 8.0) 100 ml	1.0 ml of 1 M TrisHCl (pH 8.0) and 200 µl of 0.5 EDTA were taken and final volume of 100 ml was adjusted using distilled water
Ethidium bromide (10mg/ml)	Ready to use from AMERESCO
100 bp RNA ladder	Ready to use from Bangalore Genei
6X Gel loading dye	Ready to use from Bangalore Genei

#### 3.2.15.5 c-DNA Synthesis

- i. cDNA synthesis was carried out using oligo (dT) primers and M-MLV reverse transcriptase (Invitrogen, USA) as per the manufacturer's instructions.
- ii. Reverse transcription was performed using 1 µg of total RNA. cDNA synthesis was performed using 200 units of Moloney murine leukaemia virus reverse transcriptase (MuMLV-RT, Invitrogen, USA), 5 mM oligo (dT)18, 1 mM dNTP solution, and 3 mM Mg<sup>2+</sup>.
- iii. The RNA was quantified using a Qubit fluorometer.
- iv. 1 µg of RNA was used for cDNA synthesis.
- v. 1<sup>st</sup> strand of cDNA synthesis reaction was initiated using oligo (dT) primer.
- vi. If the RNA was more concentrated, dilution was used or the volume of RNA was reduced as per its concentration, final reaction volume was adjusted to 11.0 µl with nuclease-free water, dNTP mix 1 µl and oligo primer (dT) 1 µl

- vii. The reaction was incubated at 65°C for 5 min. Snap chilled the tubes on ice for 2-3 min. The tubes were then spun briefly.
- viii. 4µl of 5X Buffer and 2 µl of 0.1MDTT was added as below. Incubated tubes were at 37°C, 2 min.
- ix. 1 µlM-MLV Reverse Transcriptase was added to the tube.
- x. The reaction tubes were gently mixed and spun briefly before being placed in a thermal cycler. cDNA synthesis was carried out at 37°C for 60 min and was followed by the inactivation of the reverse transcriptase enzyme at 70°C for 10 min. The cDNA was stored at -20°C.

### 3.2.15.6 Real time PCR primers

Primers for amplification of mice-specific gene exons were designed using reference sequences from the Genebank database, available at <https://www.ncbi.nlm.nih.gov/genbank>. online tool, Primer 3, available at <http://bioinfo.ut.ee/primer3-0.4.0/primer3/> (Tables 1 and 2), for primer design.

The primer design parameters were set to cover the exon region such that primer pairs with similar T<sub>m</sub> and average GC content of approximately 50% would be returned. Each of the primer pairs was further validated using the Primer BLAST service from NCBI (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). Primers were custom synthesised at Macrogen Inc. (South Korea), received at our lab in lyophilized form, and further diluted in nuclease-free water of a 10 mM concentration for PCR purposes (Untergasser *et al.*, (2012) and Koressaar and Remm, 2007).

**Table 3.10 List of primers for gene expression study**

Gene	Name of Primers	Primer Sequence(5'-3')	No. of bases
Glutamine synthetase-1	GS1F	5'GTGGATGCCGTGGAGAAG3'	18
	GS1R	5'GCTGAAGGTGTTGATGTCG3'	19
Glutamine synthetase 2	GS2F	5'CTCGTCCGCGTCCTTGTCGG3'	20
	GS2R	5'GCCGACCTGCCCCGCACG3'	18
Ferredoxin-dependent glutamate synthase	FdGOGATF	5'GCTGATGCTGCTGTGC3'	16
	FdGOGATR	5'CAAATGCTGGTGGATGGC3'	18
Wheat actin	W ActinF	5'ACCTTCAGTTGCCCAGCAAT3'	20
	W ActinR	5'CAGAGTCGAGCACAATACCAGTTG3'	24

- i. The real-time PCR reaction were performed using primers specific to the test gene for expression study with the wheat actin gene as one of the housekeeping genes.
- ii. 1 µl of prepared cDNA was used in the reaction.

- iii. In real-time PCR, all PCR reactions were performed with the Thermo Fisher Scientific QuantStudio-5-384 real-time PCR platform (Applied Biosystems) using DNA-binding SYBR Green dye for detection of the PCR products, and results were analysed by Quantstudio Design & Analysis Software v1.4.3 (Applied Biosystem).
- iv. In each reaction, the following were the reaction components (Table 3.12)
- v. The following PCR mix was prepared for both the control cDNA
- vi. The final volume of each reaction was 8.4  $\mu$ L. The reaction mix were prepared for all samples and added to 384-well plates. cDNA synthesis was added as a template for the amplification in each tube after the complete addition of the master reaction mix into each

**Table 3.11 Components of RT-PCR**

Materials	Volume ( $\mu$ l)
cDNA	1.0
2X SYBR (Takara, ExTaq SYBR Green PCR System)	5
Forward Primer	0.5
Reverse Primer	0.5
Nuclease Free water	2.4

**Table 3.12 PCR conditions for RT-PCR**

Sr. No.	Step	Temperature( $^{\circ}$ C)	Duration	No. of cycles
1	Initial Denaturation	95	1 min.	1
2	Denaturation	95	30 sec.	40
3	Annealing	60	30 sec.	
4	Extension	72	30 sec.	
* data collection step				
Melt Curve Stage				

Non-template controls (NTC) were included for each primer pair, and each PCR reaction was performed in triplicate. The melting-curve analysis was performed after 40 cycles of amplification by following a three step procedure. First all the components were denatured at 95 $^{\circ}$ C followed by annealing at a set temperature ( $T_m$  value of primer). After that temperature increased from 65 $^{\circ}$ C continuously (5 $^{\circ}$ C) up to 95 $^{\circ}$ C and fluorescence was measured in terms of  $-df/dt$  resulted into generation of melt or dissociation curve. Melt curve estimation analysis was carried out to identify the actual cycle for threshold amplification without primer dimer.

### 3.2.16 Data Analysis

For each of the reference genes, the cycle threshold (Ct) was determined, *i.e.* the number of PCR cycles after which the fluorescence from a sample crosses the threshold (automatically amplification based). These Ct values have been transformed to relative quantities by subtraction of the lowest Ct value, *i.e.* the Ct value from the tissue with the highest transcript abundance from the Ct values of all tissues for each transcript measured, resulting in delta Ct values for each transcript (according to the different samples). Subsequently each data point was transformed according to the formula  $2^{-\text{delta Ct}}$  resulting in raw reference gene quantities which are the required input data (Vandesompele *et al.*, 2002).

## 4. RESULTS AND DISCUSSION

The present investigation was conducted during *Rabi* 2019–2020 and *Rabi* 2020–2021. The physio-biochemical parameters related to uptake, assimilation, utilization, and remobilization of nitrate and the activity profile of key enzymes of nitrate assimilation, *viz.*, NR, NiR, GS, and GOGAT, at two stages of wheat development, *i.e.*, pre- and post anthesis were evaluated in two contrasting wheat genotypes, *viz.*, HD-2781 (high nitrogen use efficient) and C-306 (low nitrogen use efficient). The relation of mitochondrial metabolism to integration of carbon and nitrogen metabolism was also investigated to find out new targets for improving nitrogen use efficiency. The results obtained during the investigations were statistically analyzed and are discussed in this chapter in reference to available literature.

### 4.1 Experiment I

#### 4.1.1 Leaf Nitrate Content in High and Low NUE Genotypes

The data presented in Table 4.1 revealed the effect of nitrogen levels on the leaf nitrate content of two contrasting wheat genotypes. When compared to the N-inefficient wheat genotype C-306, the N-efficient wheat genotype HD-2781 had significantly higher leaf nitrate content at all three N levels (N+ (full nitrogen), N- (half nitrogen), and N0 (no nitrogen)), with treatment mean in the pre- and post-anthesis stages (1.97 to 5.15 mg/g fr. wt). The N-efficient wheat genotype HD-2781 appears to accumulate a high content of nitrate even with limiting nitrogen (6.05 to 1.80 mg/g fr. wt) and no nitrogen (3.10 to 0.92 mg/g fr. wt) levels in both the pre and post anthesis stages compared to C-306 N-inefficient wheat genotypes.

The high-N-efficient genotype HD-2781 has a high uptake rate and can mobilize and use more nitrate in the storage pool than the N-inefficient genotype C-306. The leaf nitrate content was significantly higher at pre anthesis stage than post anthesis stage of growth. The nitrogen-efficient genotype efficiently uptakes soil nitrogen through high-affinity transporters, which could help to minimise nitrogen losses from soil. The nitrogen-inefficient genotype cannot perform well at low soil nitrogen levels.

According to Chandna and Hakeem in 2013, they found that nitrate uptake was mediated by both low and high affinity nitrate transporter systems in high nitrogen use efficiency genotypes but only a low-affinity nitrate transporter system in low nitrogen use efficiency genotypes. The activity and expression of nitrate assimilatory enzymes in high- and low-nitrogen-use efficient genotypes revealed that NR plays an important role in nitrate assimilation under optimal N conditions. Similarly, Gallais and Hirel (2004) found that pre

anthesis leaf nitrate content was higher than post anthesis leaf nitrate content. When soil nitrogen is limited, N-efficient genotypes uptake the majority of the nitrate before flowering. Tabuchi *et al.* (2007) observed that at post-anthesis, nitrate is re-assimilated, and around 80% of total nitrogen in rice panicles comes from recycling by phloem from senescent tissue. Masclauxdaubresse *et al.* (2010) found that nitrate uptake is negatively affected or decreased during the post anthesis stage, the high N demand during grain filling should be supplied by N remobilization from leaves, stems, and roots.

**Table 4.1 Leaf nitrate content (mg/g fr.wt.) in high and low NUE wheat genotypes at different nitrogen levels**

Treatment	Pre anthesis stage		Post anthesis stage	
	HD-2781	C-306	HD-2781	C-306
N+	6.29	5.73	3.19	2.80
N-	6.05	4.85	1.80	1.61
N0	3.10	2.05	0.92	0.54
Mean	5.15	4.21	1.97	1.65
Comparison	SEm ( ± )	CD @ 5%	SEm ( ± )	CD @ 5%
Genotype	0.054	0.163	0.016	0.047
Treatment	0.067	0.200	0.019	0.058
G x T	0.094	0.282	0.027	0.081

#### 4.1.2 Chlorophyll Content in High and Low NUE Wheat Genotypes

The data pertaining to the effect of N level on chlorophyll content was statistically significant in treatment, genotype, and interaction. The chlorophyll content increased as nitrogen levels increased. The chlorophyll content was maximum during pre anthesis stage than post anthesis stage. The nitrogen-efficient (N-efficient) wheat genotype HD-2781 had significantly higher levels of chlorophyll a (1.33 to 1.70 mg/g fr. wt), chlorophyll b (0.56 to 0.91 mg/g fr. wt), and total chlorophyll (1.66 to 2.48 mg/g fr. wt) in the pre- and post-anthesis stages than the nitrogen inefficient wheat genotype C-306 (Table 4. 2) at all three nitrogen levels. The chlorophyll content was significantly higher in N-efficient wheat genotypes even limiting N fertiliser application, indicating that nitrogen-efficient genotypes (HD-2781) have better stay-green ability, and the chlorophyll content is low during the post-anthesis stage compared to the pre-anthesis stage.

These findings are similarly with those of a previous report by Amiour *et al.* (2014) and Kaur *et al.* (2015). The chlorophyll content is mostly reduced at the post anthesis stage due to the degradation of flag leaf cellular components in low carbon and nitrogen ratios, which transport and accumulate these molecules in developing seeds. Mehta *et al.* (2011)

revealed that an increase in chlorophyll content with increasing nitrogen fertilizer dose attributable to enhanced molecule synthesis under high nitrogen availability. Furthermore, the amount of chlorophyll in leaves increased as the nitrogen dose increased, resulting in a significant increase in their ability to stay green (Zhang *et al.*, 2017a).

**Table 4.2 Effect of nitrogen levels on chlorophyll a, chlorophyll b, and total chlorophyll in high and low NUE wheat genotypes (mg/g fr. wt.)**

Treatment	Pre anthesis stage						Post anthesis stage					
	Chlorophyll a		Chlorophyll b		Total chlorophyll		Chlorophyll a		Chlorophyll b		Total chlorophyll	
	HD-2781	C-306	HD-2781	C-306	HD-2781	C-306	HD-2781	C-306	HD-2781	C-306	HD-2781	C-306
N+	2.17	1.86	1.05	1.06	3.27	2.90	1.85	1.52	0.74	0.45	2.28	2.29
N-	2.06	1.55	1.07	1.10	3.21	2.63	1.73	1.63	0.43	0.26	2.05	1.97
N0	0.88	0.56	0.62	0.16	0.97	0.70	0.43	0.34	0.23	0.12	0.64	0.44
Mean	1.70	1.32	0.91	0.77	2.48	2.08	1.33	1.16	0.56	0.27	1.66	1.57
Comparison	S.Em±	CD @5%	S.Em±	CD@5%	S.Em±	CD @5%	S.Em±	CD @5%	S.Em±	CD @5%	S.Em±	CD @5%
Genotype	0.016	0.049	0.009	0.027	0.010	0.030	0.015	0.051	0.006	0.018	0.006	0.018
Treatment	0.020	0.060	0.011	0.033	0.012	0.036	0.019	0.059	0.007	0.022	0.007	0.022
G x T	0.029	0.086	0.015	0.046	0.017	0.051	0.028	0.089	0.010	0.031	0.011	0.032

#### 4.1.3 *In vivo* Nitrate Reductase Activity in High and Low NUE Genotypes

The activity of *in vivo* nitrate reductase was higher in the pre-anthesis stage of plant development than in the post-anthesis stage. In the pre anthesis stage, *in vivo* nitrate reductase activity was significantly high in HD-2781 wheat genotype (N-efficient) (1.02 to 2.30  $\mu\text{mol NO}_2^- \text{g}^{-1} \text{fr wt h}^{-1}$ ) compared to C-306 (N-inefficient) (0.70 to 2.08  $\mu\text{mol NO}_2^- \text{g}^{-1} \text{fr wt h}^{-1}$ ) from no nitrogen to full nitrogen dose wheat genotype (Table 4.3). The addition of  $\text{KNO}_3$  in the infiltration medium increased the *in vivo* nitrate reductase activity of the N-efficient and N-inefficient wheat genotypes, but there was not that much differences between these two genotypes. The significantly higher *in vitro* NR activity compared to *in vivo* NR activity at saturating nitrate concentrations signifies that, beside nitrate, NADH appears to be a limiting factor.

*In vivo* NR activity increased as nitrogen levels increased, and genotypes differed genetically. Genetic differences in NR levels have been found in wheat, sorghum, maize, and barley. There was a positive correlation between higher NR activity and higher grain yields in sorghum has been reported earlier (Abrol, 1990). The differences in *in vivo* nitrate reductase activity of the two contrasting wheat genotypes were much higher, probably observed that the nitrate was stored in storage as well as in metabolic pull and utilization of it more by NR. Similarly, a genotype with a high NUE has higher utilization efficiency. Nitrate assimilation

is a known positive regulator of nitrate reduction, and nitrate reductase is the primary limiting factor for uptake (Jain *et al.*, 2011).

**Table 4.3** *In vivo* nitrate reductase activity ( $\mu\text{moles of NO}_2^-$  formed/g fr. wt / h.) in high and low NUE wheat genotypes at different nitrogen levels

Sr. No.	Treatment	<i>In vivo</i> NRA ( $\mu\text{mol NO}_2^- \text{ g}^{-1} \text{ fr wt h}^{-1}$ )			
		Pre Anthesis		Post Anthesis	
		+ KNO <sub>3</sub>		+ KNO <sub>3</sub>	
		HD-2781	C-306	HD-2781	C-306
1	N+	2.30	2.08	0.94	0.76
2	N-	2.15	1.79	0.82	0.46
3	N0	1.02	0.70	0.25	0.25
<b>Mean</b>		1.82	1.52	0.70	0.49
<b>Comparison</b>		<b>SEm (<math>\pm</math>)</b>	<b>CD @ 5%</b>	<b>SEm (<math>\pm</math>)</b>	<b>CD @ 5%</b>
<b>Genotype</b>		0.023	0.068	0.008	0.025
<b>Treatment</b>		0.028	0.083	0.010	0.031
<b>G x T</b>		0.039	NS	0.015	0.044

The *in vivo* NR activity of N-efficient and N-inefficient wheat genotypes differed significantly in the presence of an appropriate amount of nitrate at pre-and post-anthesis stage. According to Reddy and Menary (1990), NR is a substrate-induced enzyme, which means that the more NO<sub>3</sub><sup>-</sup> there is in the plant during the pre anthesis stage, the more nitrate reductase activity there is, implying a positive correlation. The relationship between nitrate reductase activity and nitrate content is dependent on external nitrate, which could be related to cellular nitrate compartmentation; hence, assessing nitrate assimilation only on the basis of NRA is insufficient (Chen *et al.*, 2004). Additionally, Jain *et al.* (2011) found that the observed variations may be due to the availability of NADH for nitrate reduction. The nitrate reductase in the N-efficient cultivar also has stronger affinity for its substrates and enzymatic potential (as shown by its low K<sub>m</sub> and high V<sub>max</sub> values) than those in the N-inefficient cultivar. They revealed that the N-efficient cultivar has the ability to use nitrogen more efficiently than the N-inefficient cultivar (Anjana *et al.*, 2011).

#### 4.1.4 *In vitro* Nitrate Reductase Activity in High and Low NUE Genotypes

The data on the effect of nitrogen levels on *in vitro* nitrate reductase activity were statistically significant in terms of treatment, genotype, and interaction at both the pre and post anthesis stages of growth. The *in vitro* NR nitrate reductase activity (NRA) in the leaves of the N-efficient (HD-2781) genotype (1.83 to 4.34  $\mu\text{mol NO}_2^- \text{ g}^{-1} \text{ fr wt h}^{-1}$ ) was higher than the activity of the enzyme in the leaves of the N-inefficient (C-306) genotype (1.29 to 2.83  $\mu\text{mol NO}_2^- \text{ g}^{-1} \text{ fr wt h}^{-1}$ ) (Table 4.4). Even with limiting or no nitrogen fertilizer input,

N-efficient genotypes had much higher *in vitro* NR activity, implying that they produce more nitrite.

The potential nitrate reductase activity of an N-efficient genotype (HD-2781) when measured with optimum concentrations of nitrate and NADH (*in vitro* NR) revealed significant differences in potential nitrate reductase activity, with the highest *in vitro* NR activity at both pre and post anthesis stage. The nitrogen use inefficient genotype, *i.e.*, C-306, had the lowest *in vitro* NR activity pre and post anthesis. Even at sufficient levels of substrate,  $\text{NO}_3^-$ , and NADH, the low-NUE genotype C-306 appears to have the lowest NR activity.

**Table 4.4** *In vitro* nitrate reductase activity ( $\mu\text{moles of NO}_2^-$  formed/ g fr. wt/h.) in high and low NUE wheat genotypes at different nitrogen levels

Sr. No.	Treatment	<i>In vitro</i> NRA ( $\mu\text{mol NO}_2^- \text{ g}^{-1} \text{ fr wt h}^{-1}$ )			
		Pre anthesis stage		Post anthesis stage	
		HD-2781	C-306	HD-2781	C-306
1	N+	6.28	3.70	2.37	1.73
2	N-	4.92	3.42	2.07	1.44
3	N0	1.83	1.37	1.05	0.69
<b>Mean</b>		4.34	2.83	1.83	1.29
<b>Comparison</b>		<b>SEm (<math>\pm</math>)</b>	<b>CD@ 5%</b>	<b>SEm (<math>\pm</math>)</b>	<b>CD@ 5%</b>
<b>Genotype</b>		0.014	0.042	0.008	0.024
<b>Treatment</b>		0.017	0.052	0.010	0.029
<b>G x T</b>		0.024	0.073	0.014	0.042

These two contrasting genotypes differ significantly from one another. Wheat genotypes have been found to have a 2–3 fold difference in NR activity has been reported by Abrol (1990). The result of the present investigation appears due to differences in transcriptional levels of NR rather than the low availability of reductant (NADH). As per Jain and Abrol (2005) and Chandna *et al.* (2010), genotypic variation in NRA exists. One of the likely elements in nitrate reduction regulation appears to be genetic differences in nitrate uptake and assimilation. Similarly, in this study, it was observed that in the N-efficient genotype HD-2781 uptake more nitrate by their nitrate reductase enzyme showed strong affinity for nitrate, implying that the NR enzyme threshold is low.

#### 4.1.5 *In vitro* Nitrite Reductase Activity in High and Low NUE Wheat Genotypes

In flag leaves of wheat genotypes, *in vitro* nitrite reductase activity showed significant variation in both the genotypes at different levels of nitrogen supply. N-efficient wheat genotype HD-2781 demonstrated higher nitrite reductase activity at (N+) full nitrogen level (0.21 to 0.41  $\mu\text{moles of NO}_2^-$  disappeared/g fr. wt/min) than (N-)half nitrogen (0.20 to 0.35

$\mu\text{moles of NO}_2^-$  disappeared/g fr. wt/min) and (N0) no nitrogen level (0.17 to 0.22  $\mu\text{moles of NO}_2^-$  disappeared / g fr. wt/min) with a treatment mean (0.19 to 0.32  $\mu\text{moles of NO}_2^-$  disappeared / g fr. wt/min) at pre and post anthesis stage as compared to C-306 (N-inefficient) wheat genotype which indicates that the N-efficient genotype have better utilization efficiency (Table 4.5). Among these genotypes, the N-efficient genotype HD-2781 appears to accumulate high  $\text{NO}_2^-$  even with no nitrogen (N0) and half nitrogen (N-) levels, compared to the N-inefficient wheat genotype C-306, with lower nitrite reductase activity. Chandna *et al.* (2012) made a similar observation. NiR activity in wheat genotype leaves showed no significant difference in high nitrogen-efficient genotypes at different levels of nitrogen supply; with increased nitrogen supply, low nitrogen-efficient genotypes showed a high NiR activity.

**Table 4.5** Effect of different nitrogen levels on *in vitro* NiR ( $\mu\text{moles of NO}_2^-$  disappeared / g fr. wt/min ) in high and low NUE wheat genotypes

Sr. No.	Treatment	<i>In vitro</i> NiR ( $\mu\text{moles NO}_2^-$ disappeared/ g fr. Wt/min)			
		Pre anthesis stage		Post anthesis stage	
		HD-2781	C-306	HD-2781	C-306
1	N+	0.41	0.35	0.21	0.18
2	N-	0.35	0.25	0.20	0.16
3	N0	0.22	0.17	0.17	0.13
<b>Mean</b>		4.34	0.32	0.26	0.19
<b>Comparison</b>		<b>SEm (<math>\pm</math>)</b>	<b>CD@ 5%</b>	<b>SEm (<math>\pm</math>)</b>	<b>CD@ 5%</b>
<b>Genotype</b>		0.006	0.018	0.008	0.024
<b>Treatment</b>		0.007	0.022	0.010	0.030
<b>G x T</b>		0.010	NS	0.014	0.042

#### 4.1.6 *In vitro* Glutamine Synthetase Activity in High and Low NUE Genotypes

When compared to the nitrogen inefficient (C-306) wheat genotype, the nitrogen efficient (HD-2781) wheat genotype recorded significantly higher (136.50 to 232.08  $\eta\text{moles of } \gamma$  glutamyl hydroxamate/mg protein/min) *in vitro* GS activity in all three nitrogen levels at pre anthesis and post anthesis stage (Table 4.6). Even limiting nitrogen supply, GS activity was to be maximum in HD-2781 N-efficient genotype (151.50 to 256.00  $\eta\text{moles of } \gamma$  glutamyl hydroxamate/mg protein/min) and not that much difference of increase in nitrogen levels was observed. It indicates that the N-efficient genotype was able to translocate and assimilate nitrate, and remobilized well at limiting nitrogen supply. In without fertiliser, *i.e.* N0 (no nitrogen) (96.25 to 180  $\eta\text{moles of } \gamma$  Glutamyl hydroxamate/mg protein/min), high NUE genotypes had considerably higher *in vitro* GS activity both pre and post anthesis stage,

indicating that high NUE genotypes have higher remobilization efficiency. In both the N-efficient and N-inefficient wheat genotypes, GS activity was significantly greater at the post anthesis stage (232.08 to 192.08  $\eta$ moles of  $\gamma$  Glutamyl hydroxmate /mg protein/min) than at the pre anthesis stage (136.50 to 122.33  $\eta$ moles of  $\gamma$  Glutamyl hydroxmate/mg protein/min).

**Table 4.6** *In vitro* GS ( $\eta$ moles of  $\gamma$  Glutamyl hydroxmate / mg protein /min) in high and low NUE wheat genotypes at different nitrogen levels

Sr. No.	Treatment	<i>In vitro</i> GS ( $\eta$ moles of $\gamma$ Glutamyl hydroxmate/mg protein/min)			
		Pre anthesis stage		Post anthesis stage	
		HD-2781	C-306	HD-2781	C-306
1	N+	161.75	156.00	260.25	225.00
2	N-	151.50	132.25	256.00	198.25
3	N0	96.25	78.75	180.00	153.00
<b>Mean</b>		136.50	122.33	232.08	192.08
<b>Comparison</b>		<b>SEm(±)</b>	<b>CD @5%</b>	<b>SEm(±)</b>	<b>CD @5%</b>
<b>Genotype</b>		0.530	1.600	0.378	1.135
<b>Treatment</b>		0.650	1.951	0.464	1.390
<b>G x T</b>		0.919	2.750	0.656	1.975

In case of N-inefficient wheat genotypes C-306, GS activity significantly higher in full dose of nitrogen (156 to 225  $\eta$ moles of  $\gamma$  glutamyl hydroxmate/ mg protein/ min.) compared to limiting nitrogen level at both growth stages. The results obtained in the present investigation were similar to an earlier report by Cao *et al.* (2008). It suggests that the N-inefficient genotype (C-306) needed more nitrogen to increase GS activity. The GS activity was sufficiently lower in low nitrogen-efficient genotypes to maintain a low level of ammonium in leaf tissue regardless of external supply, which explains the increase in GS activity with an increase in N supply. GS and GOGAT activities increased with increasing nitrogen doses but decreased with nitrogen starvation (Shah *et al.*, 2017). Kichey *et al.* (2006) revealed that N fertilisers significantly increase wheat GS activity, although the effects on GS gene expression in wheat have received little attention, particularly the relationship between GS gene expression and protein fraction content. This depicted that these enzymes are essential for acquiring a limiting dose of nitrogen for crops, reflecting the plants' overall physiological status, which can be improved by increasing N assimilation and NUE in the presence of a low N supply (Beatty *et al.*, 2010). However, Kaur *et al.* (2015) concluded a significant and strong relationship between GS-GOGAT and NUE, indicating crucial metabolic processes regulating N metabolism. These two enzymes appear to be useful in producing superior genotypes with NUE by limiting nitrogen.

#### 4.1.7 *In vitro* Fd- GOGAT Activity in High and Low NUE Wheat Genotypes

The nitrogen efficient wheat genotype (HD-2781) recorded a significantly higher level of Fd-GOGAT activity at full nitrogen (N+) (37.13 to 65.75  $\eta$ moles of glutamate formed/min/mg of protein) followed by half nitrogen (N-) (32.00 to 57.25  $\eta$ moles of glutamate formed/min/mg of protein) and no nitrogen (N0) (22.41 to 39.41  $\eta$ moles of glutamate formed/min/mg of protein) levels in the pre and post anthesis stage as compared to the nitrogen inefficient (C-306) genotype, which indicates that the N-efficient wheat genotype has better remobilization ability. In comparison to the N-inefficient (C-306) wheat genotype, the N-efficient (HD-2781) wheat genotype appears to form more glutamate even with a half dose of nitrogen fertiliser (Table 4.7). *In vivo* Fd-GOGAT activity was higher at post anthesis stage in both genotypes and it is more important for evaluation of NUE.

**Table 4.7** *In vitro* Fd-GOGAT ( $\eta$ moles of glutamate formed/min/mg of protein) in high and low NUE wheat genotypes at different nitrogen levels

Sr. No.	Treatment	<i>In vitro</i> Fd-GOGAT ( $\eta$ moles of glutamate formed/min/mg of protein)			
		Pre anthesis stage		Post anthesis stage	
		HD-2781	C-306	HD-2781	C-306
1	N+	37.13	33.25	65.75	56.38
2	N-	32.00	24.60	57.25	31.75
3	N0	22.41	17.21	39.41	29.75
<b>Mean</b>		30.51	25.02	54.14	39.29
<b>Comparison</b>		<b>SEm (<math>\pm</math>)</b>	<b>CD @5%</b>	<b>SEm (<math>\pm</math>)</b>	<b>CD @5%</b>
<b>Genotype</b>		0.19	0.58	0.28	0.85
<b>Treatment</b>		0.24	0.71	0.35	1.04
<b>G x T</b>		0.34	1.01	0.49	1.47

The amount of nitrogen availability did not significantly change the GOGAT activity in rice genotypes. Santos and Salema in 1992 reported that N-starved plants decreased GOGAT activity, and increasing N supply had little effect on GOGAT activity. According to an earlier finding, the rate of nitrate uptake also exceeds net flux through the GOGAT pathways by about 25%, causing an accumulation of reduced nitrogen in the photo respiratory metabolites glycine and serine as well as in the immediate downstream products ammonium and glutamine (Scheible *et al.*, 2004). In contrast, GS and GOGAT activity in source leaves increased with nitrogen input. Higher nitrogen doses resulted in a higher Fd-GOGAT activity in cereal flag leaves (Goodall *et al.*, 2013).

#### 4.1.8 *In vitro* NADH- GOGAT Activity in High and Low NUE Wheat Genotypes

In the present investigation, NADH-GOGAT activities were higher in the roots of the N-efficient (HD-2781) genotype (21.10 to 31.41  $\mu$ moles of NADH oxidized/min/mg of protein) than in the N-inefficient (C-306) genotype (15.49 to 26.60  $\mu$ moles of NADH oxidized/min/mg of protein) at pre and post-anthesis stages grown at different nitrogen levels (Table 4.8). When compared to full nitrogen (N+) and half nitrogen (N-) nitrogen levels, NADH-GOGAT activity was reduced in the roots of wheat genotypes grown without nitrogen (N0). NADH-GOGAT activities were significantly higher in the post-anthesis stage (31.41 to 26.60  $\mu$ moles of NADH oxidized/min/mg of protein) than in the pre-anthesis stage (21.10 to 15.49  $\mu$ moles of NADH oxidized/min/mg of protein) in both N-efficient and N-inefficient wheat genotypes.

In comparison to the C-306 wheat genotype, HD-2781 appears to oxidise more NADH, even with a half dose (N-) of nitrogen fertilizer. Our results are in accordance with the results of Diaz *et al.* (2005), who investigated the soluble amino acids in the leaves of different recombinant inbred lines of Arabidopsis. The roots are the primary locations for ammonium assimilation and detoxification, and glutamate synthase and NADH-GOGAT activities are induced to help in this process. According to Chandna *et al.* (2012), nitrogen-efficient genotypes differed in all aspects of N-metabolism, including the amount of rate of  $\text{NO}_3^-$  uptake, assimilation, and N harvest. The HD-2781 genotype had significantly higher GS and GOGAT activities than the C-306 genotypes, implying that N-efficient genotypes have higher enzyme activity at all stages of plant analysis, resulting in increased yield and N harvest.

**Table 4.8** *In vitro* NADH-GOGAT ( $\mu$ moles of NADH oxidized/min/mg of protein) in high and low NUE wheat genotypes at different nitrogen levels

Sr. No.	Treatment	<i>In vitro</i> NADH-GOGAT ( $\mu$ moles of NADH oxidized/min/mg of protein)			
		Pre asynthesis stage		Post anthesis stage	
		HD-2781	C-306	HD-2781	C-306
1	N+	23.01	19.31	34.60	31.20
2	N-	21.18	14.14	31.38	28.23
3	N0	19.11	13.01	28.26	20.36
<b>Mean</b>		21.10	15.49	31.41	26.60
<b>Comparison</b>		<b>SEm(<math>\pm</math>)</b>	<b>CD @5%</b>	<b>SEm (<math>\pm</math>)</b>	<b>CD @5%</b>
<b>Genotype</b>		0.03	0.10	0.05	0.15
<b>Treatment</b>		0.04	0.12	0.06	0.18
<b>G x T</b>		0.06	0.17	0.09	0.25

#### 4.1.9 NADH/NAD<sup>+</sup> Ratio in High and Low NUE Wheat Genotypes

The HD-2781 N-efficient wheat genotype had a considerably slightly higher NADH/NAD<sup>+</sup> ratio than the C-306 N-inefficient wheat genotype at full nitrogen (+N) (0.84), half nitrogen (N-) (0.73), and no nitrogen (N0) (0.46). In compared to the C-306 (N-inefficient) wheat genotype, the HD-2781 (N-efficient ) wheat genotype had a lower NADH/NAD<sup>+</sup> ratio, which means denominator is higher for nitrate assimilation (Table 4.9).

The result obtained for NADH/NAD<sup>+</sup> ratio of current study are in accordance with the findings with the Dutilleul *et al.* (2005) who reported that increased NADH in CMS involved in driving alternative dehydrogenase function, and the buildup of both NAD and NADH may be the result of redox-balancing activities. The cytosolic reduction of nitrate to nitrite in the leaves may use reductant exported from either the chloroplast or the mitochondria (Foyer *et al.*, 2011). The electron transport chain pathways are profoundly affected by soil nitrogen availability as oxidised nitrate or reduced ammonium. The flexibility of mitochondrial respiration in maintaining redox equilibrium and supplying reductant and the carbon skeleton appears to be critical during N assimilation (Rasmusson *et al.*, 2020).

**Table 4.9 NAD/ NADH ratio in high and low NUE wheat genotypes at different nitrogen levels**

Sr. No.	Treatment	HD-2781		C-306	
		NADH/NAD <sup>+</sup> ratio		NADH/NAD <sup>+</sup> 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%	SEm(±)	CD @ 5%
Genotype (G)		0.011	0.039	0.008	0.024
Treatment (T)		0.019	0.050	0.010	0.029
G x T		0.020	0.069	0.014	0.042

In plant mitochondria complex I has a 10 fold lower Km for NADH than the NDA mediated NDin activity thereby suggesting that the basal rate of NADH oxidation particularly at low NADH/NAD<sup>+</sup> is carried out by complex I, whereas NDA proteins are merely active under conditions where the matrix reduction level is very high (Senkler *et al.*, 2017). Thus changes in mitochondrial redox systems can affect plant responses to nitrogen as oxidized nitrate (NO<sub>3</sub><sup>-</sup>) or reduced ammonia (NH<sub>4</sub><sup>+</sup>). Thus mitochondria driven effects on NAD status exert a major influence on the rate of nitrate assimilation.

#### 4.1.10 Total Grain Protein Content in High and Low NUE Wheat Genotypes

The N-efficient HD-2781 genotype had the highest total grain protein content (12.34% to 12.50%), while the N-inefficient C-306 genotype had the lowest (10.71% to 11.86%) (Table 4.10). In comparison to the N-inefficient (C-306) genotype, the N-efficient (HD-2781) genotype had significantly higher levels of total grain protein content at full nitrogen (N+) (13.50%), half nitrogen (N-) (12.42%), and no nitrogen (N0) (12.34%), indicating that the high NUE wheat genotype has better utilization efficiency.

The proper amount of nitrogen in soil can have a large effect on wheat grain protein and amino acid content (Ercoli *et al.*, 2013), mainly through increasing the formation of gliadins and glutenins (Fuertes-Mendizabal *et al.*, 2013; Wan *et al.*, 2014). In *Brassica napus* L., total grain protein concentration was found to be positively related to nitrogen uptake efficiency, making it a useful grain quality feature, as reported earlier (Rathke *et al.*, 2005). Zhen *et al.* (2016) found that the high dose of nitrogen increased grain yield and protein quality, induced by the accumulation of metabolites involved in the TCA cycle and starch and storage protein synthesis.

**Table 4.10 Total grain proteins (%) in high and low NUE wheat genotypes at different nitrogen levels**

Sr. No.	Treatment	Total grain protein (%)	
		HD-2781	C-306
1	N+	13.50	11.86
2	N-	12.42	11.71
3	N0	12.34	10.71
<b>Mean</b>		12.75	11.43
<b>Comparison</b>		<b>SEm(+)</b>	<b>CD @5%</b>
<b>Genotype</b>		0.02	0.05
<b>Treatment</b>		0.02	0.06
<b>G x T</b>		0.03	0.08

#### 4.1.11 In Gel Detection of GS Isoforms by Native-PAGE in High and Low NUE Wheat Genotypes

The relative quantification of the GS 1 and GS 2 isoforms at pre and post-anthesis stages of two contrasting wheat genotypes at different nitrogen levels was analyzed using native-PAGE (Fig. 4.1). GS1 isozyme activity was not observed during the pre-anthesis stage in both wheat genotypes. At the post-anthesis stage, the activities of both GS1 and GS2 separated on gel electrophoresis. In all three nitrogen levels, the N-efficient wheat genotypes (HD-2781) showed significantly higher levels of both GS1 and GS2 isoforms than the

N-inefficient genotype (C-306). During pre anthesis, the chloroplastic isoforms (GS2) responsible for nitrate assimilation were significantly expressed in both genotypes, as were the cytosolic (GS1) and chloroplastic (GS2) isoforms responsible for both nitrate assimilation and photo-respiratory remobilization of ammonia. According to recent research, the relative amounts of the GS protein increased after anthesis under limiting N conditions. Before and after anthesis, the relative contents of the GS1 and GS2 proteins could be used as a marker for breeding cultivars with high NUE (Zhang *et al.*, 2017a).

## **4.2 Experiment II**

### **4.2.1 Leaf Nitrate Content in Different Wheat Genotypes**

The maximum leaf nitrate content was recorded in the pre-anthesis stage compared to the post-anthesis stage in different wheat genotypes (Table 4.11). The pre-anthesis (3.40 to 6.13 mg/g fr.wt) and post-anthesis (1.39 to 2.92 mg/g fr. wt) leaf nitrate contents of wheat genotypes differ significantly. Pre-anthesis leaf nitrate content was highest in NIAW-1994 (6.13 mg/g fr. wt), which was at par with NIAW-301 (6.10 mg/g fr. wt) and HD-2781 (6.02 mg/g fr. wt), whereas post-anthesis leaf nitrate content was highest in NIAW-1994 (2.92 mg/g fr. wt), which was at par with NIAW-15 (2.90 mg/g fr. wt) and NIAW-301 (2.88 mg/g fr. wt). The wheat genotypes with the lowest leaf nitrate content were HD-2932 (3.40 mg/g fr.wt) at the pre anthesis stage and NIAW-917 (1.39 mg/g fr.wt) at the post-anthesis stage.

This observation is in accordance with earlier findings of Harrison *et al.* in 2003, who found that higher leaf nitrate content was positively correlated with higher uptake in some studies. The decreasing content of nitrogen in leaves may be linked to low N uptake and assimilation at later stages of plant development. Variations in response to nitrogen supply among genotypes are related to differences in nitrate transporter gene regulation (Orsel *et al.*, 2002). Previous studies have indicated that high nitrogen use efficient genotypes are very desirable because they can be cultivated in environmentally friendly agricultural systems with optimum N supplies (Andrews *et al.*, 2004). According to Kaur *et al.* (2014), nitrate assimilation caused a net increase in the nitrate content of stems and leaves during the pre anthesis stage. These organs transform from source to sink in the post-anthesis stage as remobilization loads nitrate into the growing grain structure during grain felling. The developing grain serves as a significant nitrogen sink and dominates nutrient uptake from storage sources at the root in senescent plant organs.

**Table 4.11 Effect of limiting nitrogen levels on the leaf nitrate content (mg/g fr. wt) in different wheat genotypes**

Genotypes	Leaf nitrate content (mg / g fr. wt)	
	Pre anthesis stage	Post anthesis stage
<b>GW-322</b>	5.87	2.67
<b>MACS-2496</b>	3.63	1.91
<b>MACS-6222</b>	6.10	2.81
<b>HD-2932</b>	3.40	1.61
<b>NIAW-301</b>	5.70	2.88
<b>NIAW-917</b>	5.07	1.39
<b>NIAW-34</b>	4.93	2.83
<b>NIAW-1415</b>	3.93	1.66
<b>NIAW- 3170</b>	5.73	2.83
<b>NIAW- 15</b>	5.70	2.90
<b>NIAW-1994</b>	6.13	2.92
<b>HD-2781</b>	6.02	1.83
<b>C-306</b>	4.90	1.62
<b>Mean</b>	5.16	2.29
<b>SEm(±)</b>	0.046	0.040
<b>CD @5%</b>	0.134	0.116

#### 4.2.2 Chlorophyll Content in Different Wheat Genotypes

The chlorophyll content differed significantly in thirteen wheat genotypes at pre and post anthesis stages (Table 4.12). The highest chlorophyll content was found in NIAW-301 (3.33 mg/g fr. wt), which was at par with NIAW-1994 (3.30 mg/g fr. wt), HD-2781 (3.21 mg/g fr. wt), NIAW-34 (3.18 mg/g fr. wt), and GW-322 (3.08 mg/g fr. wt), while the lowest chlorophyll content was found in NIAW-1415 (1.88 mg/g fr. wt) in the pre anthesis stage. However, at the post-anthesis stage, the NIAW-1994 (2.67 mg/g fr. wt) genotype had the highest chlorophyll content, which was at par with NIAW-301 (2.63 mg/g fr. wt), GW-322 (2.57 mg/g fr. wt), NIAW-3170 (2.57 mg/g fr. wt), and MACS-6222 (2.56 mg/g fr. wt).

The findings presented above are similar to those of Amiour *et al.* (2012), who discovered that chlorophyll content was higher in the vegetative stage than in the plant maturity stage under low (N-) nitrogen levels. A significant reduction of chlorophyll and carotenoid content under low nitrogen conditions was reported in wheat genotypes (Nouriyani *et al.*, 2012). According to Mehta *et al.* (2011), an increase in chlorophyll content with increasing nitrogen fertiliser dose may be due to enhanced molecule synthesis under high nitrogen availability.

**Table 4.12 Effect of half nitrogen level on chlorophyll a, chlorophyll b and total chlorophyll in different wheat genotypes (mg/g fr. wt.)**

Genotypes	Pre anthesis stage			Post anthesis stage		
	Chlorophyll a	Chlorophyll b	Total chlorophyll	Chlorophyll a	Chlorophyll b	Total chlorophyll
<b>GW 322</b>	2.16	0.98	3.08	1.98	0.74	2.57
<b>MACS-2496</b>	1.68	0.74	2.48	1.43	0.35	1.90
<b>MACS-6222</b>	2.15	1.09	3.05	2.01	0.60	2.56
<b>HD-2932</b>	1.55	0.93	2.51	1.59	0.46	2.03
<b>NIAW 301</b>	2.23	1.11	3.33	1.98	0.56	2.63
<b>NIAW- 917</b>	1.76	0.22	2.51	1.76	0.23	1.98
<b>NIAW- 34</b>	2.10	1.07	3.18	1.79	0.64	2.45
<b>NIAW- 1415</b>	1.81	0.93	1.88	1.81	0.25	2.03
<b>NIAW- 3170</b>	2.18	1.08	3.05	2.00	0.58	2.57
<b>NIAW- 15</b>	1.90	0.86	2.22	1.74	0.50	2.19
<b>NIAW- 1994</b>	2.23	1.09	3.30	2.14	0.56	2.67
<b>HD-2781</b>	2.08	1.07	3.21	1.75	0.27	2.06
<b>C-306</b>	1.56	0.99	2.64	1.66	0.41	1.98
<b>Mean</b>	1.974	0.996	2.881	1.83	0.48	2.276
<b>SEm(±)</b>	0.026	0.018	0.088	0.041	0.026	0.060
<b>CD @5%</b>	0.075	0.052	0.256	0.120	0.074	0.173

#### 4.2.3 *In vivo* Nitrate Reductase Activity in Different Wheat Genotypes

The perusal of the data presented in table 4.13 shows significant differences in *in vivo* nitrate reductase activity in different wheat genotypes under limited nitrogen levels at pre-anthesis and post-anthesis stages.

The *in vivo* nitrate reductase activity was ranging from 0.96 to 1.99  $\mu\text{moles of NO}_2^-$  formed / g fr. wt / h. at pre anthesis stage and 0.181 to 0.565  $\mu\text{moles of NO}_2^-$  formed / g fr. wt / h. at post anthesis stage. The significantly highest *in vivo* nitrate reductase activity was found in NIAW-3170 (1.99  $\mu\text{moles of NO}_2^-$  formed / g fr. wt / h.) and lowest *in vivo* nitrate reductase activity was found in MACS-2496 (0.96  $\mu\text{moles of NO}_2^-$  formed / g fr. wt / h.) in the pre anthesis stage. In case of post anthesis stage wheat genotype NIAW-3170 (0.565  $\mu\text{moles of NO}_2^-$  formed / g fr. wt / h.) found higher and NIAW-1415 (0.181  $\mu\text{moles of NO}_2^-$  formed / g fr. wt / h.) lower *in vivo* nitrate reductase activity respectively.

The above description indicates that the N-efficient genotypes found higher nitrate content and nitrate reductase activity than the N-inefficient, possibly due to the substrate-inducible nature of the enzyme and the difference between the two genotypes in nitrate content. The N-metabolizing enzymes in N-efficient genotypes also had higher affinities for their respective substrates (low  $K_m$  values) and a greater potential for catalysing reactions

(high  $V_{max}$  and low  $K_m$  value) than in the N-inefficient genotypes. Anjana *et al.* (2011) revealed that the N-efficient genotype has the inherent property of utilising nitrogen more efficiently than the N-inefficient genotypes.

**Table 4.13** Effect of limiting nitrogen level on *in vivo* NRA in different wheat genotypes

Genotypes	<i>In vivo</i> NRA ( $\mu\text{moles of NO}_2^-$ formed / g fr. wt / h.)	
	Pre anthesis stage	Post anthesis stage
<b>GW-322</b>	1.47	0.359
<b>MACS-2496</b>	0.96	0.271
<b>MACS-6222</b>	1.60	0.418
<b>HD-2932</b>	1.16	0.314
<b>NIAW-301</b>	1.52	0.340
<b>NIAW-917</b>	1.51	0.378
<b>NIAW-34</b>	1.54	0.366
<b>NIAW-1415</b>	1.45	0.181
<b>NIAW-3170</b>	1.99	0.565
<b>NIAW-15</b>	1.31	0.315
<b>NIAW-1994</b>	1.71	0.428
<b>HD-2781</b>	1.63	0.472
<b>C-306</b>	1.32	0.236
<b>Mean</b>	1.47	0.35
<b>SEm(<math>\pm</math>)</b>	0.037	0.007
<b>CD @5%</b>	0.107	0.020

The result obtained in the present study for *in vivo* NRA is more similar to earlier reports by Kaur *et al.* (2015). They reported that high nitrogen use efficient genotypes (GLU-1356 and GLU-2001) maintained high activity of nitrogen assimilatory enzymes in relation to higher NUE at the limiting nitrogen level, and a positive relationship of nitrogen assimilatory enzymes (NR and GS) with NUE and nitrate content was also observed, indicating that they could be the rate-limiting enzymes in nitrogen metabolism. According to Chandna *et al.* (2010), genotypic variations in nitrate reductase activity occur. Among the most relevant factors in nitrate reduction regulation appear to be genetic differences in nitrate uptake and assimilation of nitrate ions (Rroco and Mengel, 2000). Similarly, in the current investigation, different genotypes showed significant variances in the modulation of NR activity with different nitrogen levels supplied.

#### 4.2.4 *In vitro* Nitrate Reductase Activity in Different Wheat Genotypes

The *in vitro* nitrate reductase activity (*in vitro* NRA) in different wheat genotypes was significantly different at pre anthesis stage (2.80 to 5.18  $\mu\text{moles of NO}_2^-$  formed/g fr. wt/h.) and the post anthesis stage (1.44 to 2.43  $\mu\text{moles of NO}_2^-$  formed/g fr. wt/h.), as shown in table 4.14. The highest *in vitro* NRA was recorded in NIAW-301 (5.186  $\mu\text{moles of NO}_2^-$  formed/g fr.wt/h), followed by NIAW-1994 (5.142  $\mu\text{moles of NO}_2^-$  formed/g fr. wt/h) and GW-322 (5.050  $\mu\text{moles of NO}_2^-$  formed/g fr.wt/h). The lowest *in vitro* NRA was recorded in MACS-2496 (2.80  $\mu\text{moles of NO}_2^-$  formed/g fr.wt/h) at pre anthesis stage. In the post anthesis stage, the highest *in vitro* NRA was observed in NIAW-301 (2.437  $\mu\text{moles of NO}_2^-$  formed/g fr. wt/h) and the lowest *in vitro* NRA was found in C-306 (1.447  $\mu\text{moles of NO}_2^-$  formed /g fr. wt/h), which was at par with HD-2932 (1.467  $\mu\text{moles of NO}_2^-$  formed/g fr. wt/h) respectively.

**Table 4.14** Effect of limiting nitrogen level on *in vitro* NRA in different wheat genotypes

Genotypes	<i>In vitro</i> NRA ( $\mu\text{moles of NO}_2^-$ formed / g fr. wt / h.)	
	Pre anthesis stage	Post anthesis stage
<b>GW-322</b>	5.050	2.133
<b>MACS-2496</b>	2.804	1.533
<b>MACS-6222</b>	4.199	2.360
<b>HD-2932</b>	3.177	1.467
<b>NIAW-301</b>	5.186	2.437
<b>NIAW-917</b>	4.093	2.087
<b>NIAW-34</b>	3.243	2.213
<b>NIAW-1415</b>	3.163	2.133
<b>NIAW-3170</b>	4.170	2.167
<b>NIAW-15</b>	3.168	2.250
<b>NIAW-1994</b>	5.142	2.340
<b>HD-2781</b>	4.927	2.071
<b>C-306</b>	3.427	1.447
<b>Mean</b>	3.98	2.04
<b>SEm(<math>\pm</math>)</b>	0.094	0.023
<b>CD @5%</b>	0.272	0.067

An earlier report showed that the higher nitrate content in the N-efficient genotypes might be due to their better nitrate uptake capacity. It is also important that the N-efficient genotype produced more biomass than the N-inefficient genotype, possibly because of its higher nitrate reduction rate, thus allowing a better allocation of nitrogen resources to

photosynthesis and carbon metabolism (Djennane *et al.*, 2004). These findings indicate that the plant capacity for biomass production and nitrogen uptake may be highly dependent on environmental conditions, including the nitrate supply (Lawlor, 2002). It has been shown that wheat genotypes with a high NR were able to mobilise a higher amount of N into the grains than the low NR genotypes (Jain *et al.*, 2011). During the post anthesis stage, active glutamine synthetase, rather than a substantial quantity of glutamine, is responsible for nitrate reductase suppression (Habash *et al.*, 2001). Nitrate reductase expression may be influenced by glutamine ability to reduce nitrate uptake (Dzuibany *et al.*, 1998), or glutamine (and asparagines) may directly influence nitrate reductase activity and expression without changing nitrite reductase (Sivasankar *et al.*, 1997).

#### 4.2.5 *In vitro* Nitrite Reductase Activity in Different Wheat Genotypes

*In vitro* nitrite reductase activity (*in vitro* NiR) differs significantly between thirteen promising wheat genotypes at pre-anthesis (0.230 to 0.427  $\mu\text{moles of NO}_2^-$  disappeared/g fr. wt/min) and post anthesis stage (0.154 to 0.203  $\mu\text{moles of NO}_2^-$  disappeared/g fr. wt/min), as shown in Table 4.15. The highest *in vitro* nitrite reductase activity was observed in NIAW-3170 (0.427  $\mu\text{moles of NO}_2^-$  formed/g fr. wt/ min) and NIAW-1994 (0.390  $\mu\text{moles of NO}_2^-$  disappeared /g fr. wt/min). The lowest *in vitro* NiR was found in MACS-2496 (0.230  $\mu\text{moles of NO}_2^-$  disappeared/g fr. wt/min). In the post-anthesis stage, the highest *in vitro* NiR was found in NIAW-1994 (0.203  $\mu\text{moles of NO}_2^-$  disappeared/g fr wt/min), and the lowest *in vitro* NiR was found in MACS-2496 (0.154  $\mu\text{moles of NO}_2^-$  disappeared/g fr. wt/min). The *in vitro* NiR activity was higher at pre anthesis than post anthesis stage.

The nitrite reductase enzyme interacts with nitrate reduction, accumulating nitrite produced by nitrate reductase activity and exhibiting a feedback mechanism on NiR activity, resulting in its suppression. This could be due to N-inefficient cultivars accumulating excess nitrate rather than utilizing it. In contrast, the amount of nitrate converted to nitrite in high-N-efficient cultivars is constant, permitting the NiR enzyme to synthesis ammonium without being impacted by the increase in levels of N (Ali *et al.*, 2007). Whereas in high nitrogen-efficient genotypes, the amount of nitrate converted to nitrite is constant, ammonia production by the NiR enzyme occurs immediately, and higher nitrogen levels have no effect on its activity (Ali *et al.*, 2008). Our results are in agreement with previous findings. Kaur *et al.* (2015) reported that decreasing nitrite reductase activity in leaves may be linked to low N uptake and assimilation at the post-anthesis stage of plant growth. In senescing leaves, the activity of nitrite reductase was reduced, indicating proteolysis and chloroplast breakdown.

**Table 4.15** Effect of half nitrogen dose on *in vitro* NiR ( $\mu\text{moles of NO}_2^-$  disappeared/ g fr. wt/min) in different wheat genotypes

Sr. No.	Genotypes	<i>In vitro</i> NiR ( $\mu\text{moles of NO}_2^-$ disappeared / g fr. wt/min)	
		Pre anthesis stage	Post anthesis stage
1	GW 322	0.343	0.199
2	MACS-2496	0.230	0.154
3	MACS-6222	0.373	0.199
4	HD-2932	0.260	0.172
5	NIAW-301	0.327	0.196
6	NIAW- 917	0.257	0.160
7	NIAW-34	0.360	0.196
8	NIAW-1415	0.243	0.190
9	NIAW-3170	0.427	0.201
10	NIAW-15	0.363	0.200
11	NIAW-1994	0.390	0.203
12	HD-2781	0.343	0.199
13	C-306	0.243	0.156
	Mean	0.320	0.187
	SEm( $\pm$ )	0.010	0.001
	CD @5%	0.029	0.004

#### 4.2.6 *In vitro* Glutamine Synthetase Activity in Different Wheat Genotypes

The data presented in table 4.16 revealed significant variations in *in vitro* glutamine synthetase activity (*in vitro* GS) in the pre anthesis (120.67 to 197.33  $\eta\text{moles of } \gamma$  glutamyl hydroxamate/mg protein/min.) stage and the post anthesis stage (190.33 to 299.67  $\eta\text{moles of } \gamma$  glutamyl hydroxamate/mg protein/min.) differ significantly in wheat genotypes. At the pre anthesis stage, NIAW-1994 had the highest *in vitro* GS activity (197.33  $\eta\text{moles of } \gamma$  glutamyl hydroxamate/mg protein/min.) and HD-2932 had the lowest activity (120.67  $\eta\text{moles of } \gamma$  glutamyl hydroxamate/mg protein/min.). The highest *in vitro* GS activity was found in NIAW-301 (289.67  $\eta\text{moles of } \gamma$  glutamyl hydroxamate/mg protein/min.) and the lowest *in vitro* GS activity was found in HD-2932 (190.33  $\eta\text{moles of } \gamma$  glutamyl  $\gamma$  glutamy hydroxamate /mg protein / min.) at post anthesis stage, respectively.

Likewise, in the present study, it was observed that the GS activity was increased in the post-anthesis stage in all wheat genotypes, and the wheat genotype with higher GS activities during the post-anthesis stage also showed increased grain protein content, in agreement with Zhang *et al.* (2017). According to Bernard *et al.* (2008), total GS activity increases during the grain filling stage. Wheat GS gene expression is developmental controlled, with GS1 and GS2 ammonia assimilation or recycling in the leaves during grain

filling. Increased activity of the GS (although not difference between GS1 and GS2), as well as higher activity of this enzyme in senescing leaves, was responsible for the transportation of plant N to the seeds in N-efficient genotypes. The results of the present investigation were found to be similar to the findings of Masclaux *et al.* (2001), which revealed that in the N-inefficient genotypes, a significant portion of N is not retranslocated to the harvested structures and GS activity in leaves is lower compared with N-efficient genotypes. Leaves of expression of the GS gene family (Gln1;1) in the leaves of VL616 (the N-efficient genotype) under low N levels show up regulation in comparison with transcript expression of UP-2382 (the N-inefficient genotype) under higher N supplies, indicating that the N-efficient genotype was able to utilize the absorbed nitrate and thus grow well at low N levels (Chandna *et al.*, 2012).

**Table 4.16** Effect of limiting nitrogen levels on *in vitro* GS activity in different wheat genotypes

Genotypes	<i>In vitro</i> GS ( $\eta$ moles of $\gamma$ Glutamyl hydroxamate / mg protein / min)	
	Pre anthesis stage	Post anthesis stage
<b>GW-322</b>	177.67	279.67
<b>MACS-2496</b>	139.33	195.67
<b>MACS-6222</b>	187.33	280.33
<b>HD-2932</b>	120.67	190.33
<b>NIAW-301</b>	168.33	299.67
<b>NIAW-917</b>	131.67	230.67
<b>NIAW-34</b>	163.00	212.00
<b>NIAW-1415</b>	160.33	236.67
<b>NIAW-3170</b>	175.33	247.00
<b>NIAW-15</b>	180.33	204.00
<b>NIAW-1994</b>	197.33	289.67
<b>HD-2781</b>	152.93	258.00
<b>C-306</b>	131.33	198.00
<b>Mean</b>	160.43	240.12
<b>SEm(<math>\pm</math>)</b>	0.546	0.381
<b>CD @5%</b>	1.587	1.108

#### 4.2.7 Fd-GOGAT Activity in Different Wheat Genotypes

The Fd-GOGAT activity of thirteen wheat genotypes varied significantly during pre anthesis (21.43 to 38.22  $\eta$ moles of glutamate formed/min/mg of protein) and post anthesis (31.92 to 63.48  $\eta$ moles of glutamate formed/min/mg of protein), as shown in table 4.17.

**Table 4.17 Effect of limiting nitrogen levels on *in vitro* Fd-GOGAT activity in different wheat genotypes**

Genotypes	<i>In vitro</i> Fd-GOGAT ( $\eta$ moles of glutamate formed/min/mg of protein)	
	Pre anthesis stage	Post anthesis stage
GW-322	34.77	61.77
MACS-2496	22.99	34.33
MACS-6222	34.39	63.48
HD-2932	21.43	33.54
NIAW-301	35.85	57.72
NIAW-917	22.51	31.92
NIAW-34	33.02	59.88
NIAW-1415	24.42	34.81
NIAW-3170	34.46	62.61
NIAW-15	34.10	62.45
NIAW-1994	38.22	61.93
HD-2781	31.99	59.87
C-306	23.89	32.00
Mean	30.15	50.48
SEm( $\pm$ )	0.560	0.723
CD @5%	1.628	2.100

The wheat genotype NIAW-1994 (38.22 $\eta$ moles of glutamate formed/min/mg of protein) had the highest Fd-GOGAT activity, and the lowest activity was found HD-2932 (21.43  $\eta$ moles of glutamate formed /min/mg of protein) genotypes at pre anthesis stage, while at post anthesis, the highest Fd-GOGAT activity was found in MACS-6222 (63.48  $\eta$ moles of glutamate formed/min/mg of protein) genotype, which was significantly at par with NIAW-3170 (62.61 $\eta$ moles of glutamate formed/ min/mg of protein) and NIAW-15 (62.45  $\eta$ moles of glutamate formed/ min/mg of protein). A similar result was found by Jain *et al.* and an experiment was conducted in 2011 to study two contrasting wheat genotypes. It was observed that, among these genotypes, the Fd-GOGAT activity was higher between 60 and 110 days after sowing, respectively.

#### 4.2.8 NADH-GOGAT Activity in Different Wheat Genotypes

The NADH-GOGAT activity in different wheat genotypes varied significantly (Table 4.18) in the pre-anthesis stage (14.17 to 31.11  $\mu$ moles of NADH oxidized/min/mg of protein) and even at the post-anthesis stage (24.54 to 35.42  $\mu$ moles of NADH oxidized/min/mg of protein). The NIAW-1994 (31.11 $\mu$ moles of NADH oxidized/min/mg of protein) had the highest NADH-GOGAT activity in the pre-anthesis stage. The lowest activity of

NADH-GOGAT was found in C-306 (14.17  $\mu$ moles of NADH oxidized/min/mg of protein) in the pre anthesis stage, followed by NIAW-1415 (14.24 $\mu$ moles of NADH oxidized/min/mg of protein) and MACS-2496 (15.17  $\mu$ moles of NADH oxidized/min/mg of protein), while in the case of post anthesis stage, the highest NADH-GOGAT activity was found in NIAW-1994 (35.42  $\mu$ moles of NADH oxidized/min/mg of protein) and MACS-6222 (34.17 $\mu$ moles of NADH oxidized/min/mg of protein), while the lowest NADH-GOGAT activity was found in MACS 2496 (24.54  $\mu$ moles of NADH oxidized/min/mg of protein), followed by HD-2932 (25.92 $\mu$ moles of NADH oxidized/min/mg of protein).

**Table 4.18** Effect of limiting nitrogen levels on *in vitro* NADH-GOGAT activity in different wheat genotypes

Genotypes	<i>In vitro</i> NADH-GOGAT ( $\mu$ moles of NADH oxidized/min/mg of protein)	
	Pre anthesis stage	Post anthesis stage
<b>GW-322</b>	28.40	32.82
<b>MACS-2496</b>	15.17	24.54
<b>MACS-6222</b>	24.44	34.17
<b>HD-2932</b>	21.32	25.92
<b>NIAW-301</b>	21.92	31.30
<b>NIAW-917</b>	17.11	29.27
<b>NIAW-34</b>	28.78	31.45
<b>NIAW-1415</b>	14.24	29.07
<b>NIAW-3170</b>	22.59	28.02
<b>NIAW-15</b>	20.75	31.17
<b>NIAW-1994</b>	31.11	35.42
<b>HD-2781</b>	21.42	31.35
<b>C-306</b>	14.17	28.15
<b>Mean</b>	21.6	30.20
<b>SEm(<math>\pm</math>)</b>	0.363	0.784
<b>CD @5%</b>	1.055	2.279

The results obtained in the present experiment are concomitant with Lothier *et al.* (2011), who stated that roots are the major site of primary ammonium assimilation and that GS and NADH-GOGAT activities are induced for ammonium assimilation and detoxification. In rice, the NADH-GOGAT activity is important in the utilisation of N in grain filling, and its activity in the developing grain is positively related to yield. NADH-GOGAT activity is critical for post-anthesis nitrogen consumption, and its activity in the growing grain is positively associated with yield (Forde and Lea, 2007). Similarly, in several transgenic lines, overproduction of OsNADH-GOGAT increased grain weight by up

to 80%, demonstrating that this enzyme is certainly a significant step in nitrogen reutilization (Yamaya *et al.*, 2002). Overexpression studies, together with age- and cell type specific expression, strongly suggest that NADH-GOGAT1 is important for the reutilization of transported Gln in developing plant organs (Tabuchi *et al.*, 2007).

#### 4.2.9 Total Grain Protein in Different Wheat Genotypes

The data presented in table 4.19 revealed significant variations in total grain protein content (11.70 % to 13.10%) in thirteen wheat genotypes. Total grain protein was highest in GW-322 (13.10%) followed by NIAW-1994 (12.92%), MACS-6222 (12.89%), NIAW- 3170 (12.88%), NIAW-15(12.86%) and NIAW-34(12.51%) and the lowest total grain protein was found in C-306 (11.70%) and MACS-2496 (11.90%).

Previously, Turner *et al.* (2004) discovered that the grain protein content should be greater than 12.5%, which is the level required for bread production and nutritional quality. Hence, the N application rate should be less than 120 kg/ha to ensure high grain quality and yield.

**Table 4.19 Effect of limiting nitrogen level on total grain protein in different wheat genotypes**

Genotypes	Total grain protein (%)
<b>GW-322</b>	13.10
<b>MACS-2496</b>	11.90
<b>MACS-6222</b>	12.89
<b>HD-2932</b>	12.14
<b>NIAW-301</b>	12.25
<b>NIAW-917</b>	12.29
<b>NIAW-34</b>	12.51
<b>NIAW-1415</b>	11.92
<b>NIAW-3170</b>	12.88
<b>NIAW-15</b>	12.86
<b>NIAW-1994</b>	12.92
<b>HD-2781</b>	12.43
<b>C-306</b>	11.70
<b>Mean</b>	12.46
<b>SEm(±)</b>	0.019
<b>CD @5%</b>	0.055

#### 4.2.10 In Gal, Detection of Isoforms by Native-PAGE in Different Wheat Genotypes

Native-PAGE analysis was used to quantify the GS1 and GS2 isoforms at pre and post anthesis stages of thirteen wheat genotypes at limiting nitrogen status (Fig. 4.2). Only GS2 activity was observed in all thirteen wheat genotypes during the pre-anthesis stage. The

activity of GS1 and GS2 during the post-anthesis stage was differing significantly. The N-efficient wheat genotypes (GW-322, MACS-6222, NIAW-301, NIAW-34, NIAW-3170, NIAW-1994, and HD-2781) had considerably higher levels of both (GS1 and GS2) isoforms than the N-inefficient (HD-2931, MACS-2496, NIAW-1415, NIAW-15, and C-306) genotypes.

N-efficient genotype has increased assimilation ability in the pre-anthesis stage and remobilization ability in the post-anthesis stage. The relative contents of the GS1 and GS2 proteins at pre- and post-anthesis stages could be used as a marker for breeding varieties with high NUE. With the progression of leaf senescence, GS2 activity decreases as chloroplasts disintegrate (Pageau *et al.*, 2006). Flag leaf GS1 activity was significantly greater than GS2 activity in this study (Fig. 1B), indicating that GS1 had a unique role in controlling N metabolic flux (Thomsen *et al.*, 2014). At pre anthesis, the N-efficient genotypes had higher relative levels of GS2 protein than the N-inefficient genotypes, showing that GS2 is important in the vegetative growth stage and carbon-nitrogen metabolic balance (Bao *et al.*, 2015). In the post-anthesis stage, there were increased relative amounts of the GS1 protein under the limiting N condition (Fig. 1B).

#### **4.2.11 Gene Expression Study of Contrasting Wheat Genotypes**

Four promising wheat genotypes were chosen out of thirteen for study of nitrogen metabolising responsible genes after screening of nitrogen use efficient and nitrogen use inefficient genotypes on the basis of uptake assimilation and remobilization.

According to the results of uptake assimilation and remobilization study, two wheat genotypes, HD-2781 and NIAW-1994, were nitrogen-use efficient genotypes, while two other genotypes, C-306 and HD-2932, were nitrogen-use inefficient genotypes. These four genotypes were sown in two sets of phytotron units in pots. The pre anthesis and post anthesis stages of the plant were observed in one set of genotypes sown 15 days before the second set. The flag leaf stage was then obtained from all four genotypes, both pre and post anthesis, for gene expression analysis.

In the present investigation, the quality, quantity, and integrity of total RNA extracted from flag leaves of wheat genotypes were analyzed through a Qubit Flurometer. The quantity of total RNA extracted was in the range of 283 ng/μl to 890 ng/μl.

**Table 4.20 Quantitative analysis of total RNA from flag leaves of wheat genotypes**

Sr. No.	Sample	Qubit Reading (ng/ $\mu$ l)	Total calculated concentration of stock ( $\mu$ g /mL)
1	HD-2781 Pre anthesis stage	283	56.5
2	C-306 Pre anthesis stage	510	100
3	HD-2932 Pre anthesis stage	427	85.3
4	NIAW-1994 Pre anthesis stage	419	83.8
5	HD-2781 Post anthesis stage	355	70.9
6	C-306 Post anthesis stage	429	85.8
7	HD-2932 Post anthesis stage	890	180
8	NIAW-1994 Post anthesis stage	364	72.8

All the samples were processed in triplicate analysis including negative control in each run. Using Quantstudio Design and Analysis Software v1.4.3 (Applied Biosystem), the real-time ddCT PCR data were examined. Actin gene expression served as a housekeeping gene for the purposes of this investigation. The table of Ct values that was derived from the experiment is shown below (Table 4.21).

**Table 4.21 Ct value of target genes obtained through qRT-PCR in flag leaf of wheat genotypes**

Genotypes	Stage	Threshold cycle (Ct) value			
		GS1	GS2	Fd-GOGAT	Wheat actin Selected Control
<b>HD-2781</b>	Pre	28.29	27.00	40.00	31.57
<b>HD-2781</b>	Post	24.91	40.00	27.00	31.96
<b>C-306</b>	Pre	26.89	26.01	40.00	31.68
<b>C-306</b>	Post	25.64	40.00	27.60	30.59
<b>HD-2932</b>	Pre	27.86	25.37	30.33	33.19
<b>HD-2932</b>	Post	27.85	26.13	28.18	30.63
<b>NIAW-1994</b>	Pre	30.29	28.63	40.00	33.00
<b>NIAW -1994</b>	Post	24.98	23.35	28.40	31.98

#### 4.2.11.1 Glutamine synthetase 1 gene expression

Glutamine synthetase plays a major role in the assimilation of  $\text{NH}_4^+$  into glutamine, which is then used for the biosynthesis of all amino acids. GS isozymes have different metabolic functions, and their activities vary during plant development in different organs and cell types (Bernard *et al.*, 2008). GS is an important enzyme because it catalyses the ATP-dependent fixation of ammonium to the  $\delta$ -carboxyl group of glutamate to form glutamine. Two GS isozymes, GS1 (cytosolic isoform) and GS2 (plastid isoform), are present in higher plants. The GS1 of GS is particularly important for assimilating ammonium from

different sources, for both primary nitrogen assimilation and reassimilation in senescent leaves.

The real-time PCR amplification of GS1 with all samples showed a Ct value (Table 4.21), which indicates the gene was strongly expressed. The relative quantification plot presented in Figure 4.3 revealed that the target gene GS1 was expressed in all the genotypes. The expression level was higher in HD-2781 and NIAW-1994 at the post-anthesis stage as compared to the pre-anthesis stage. In HD-2494, the GS1 gene expression was higher in the pre anthesis stage than in the post anthesis stage, whereas in the C-306 genotype, the gene expression was lower in both the pre anthesis and post anthesis stages with wheat actin as an endogenous control.

The glutamine synthetase expression in the four wheat genotypes in response limiting nitrogen supply was analyzed in Figure 4.3. The high protein subunit expression of GS1, which increased nitrogen translocation and remobilization efficiency, is probably due to the significantly higher levels of GS1 expression in the nitrogen-efficient genotypes than the nitrogen-inefficient genotypes at the post-anthesis stage under nitrogen-limiting conditions (Figure 4.3). According to earlier studies, over expression of the rice GS1 gene can significantly increase nitrogen metabolites, biomass, and yield, but it does not constitute an overall bottleneck that limits nitrogen assimilation (Li *et al.*, 1993). Gadaleta *et al.* (2011) examined whether leaf senescence induced the expression of the barley GS1 gene. Similar to this, the GS1 gene followed the same pattern during the course of the growth cycle in wheat flag leaves. The GS1 gene plays a major role in carbon and nitrogen metabolism in rice at the post-anthesis stage (Bao *et al.*, 2014).

Earlier Masclaux *et al.* (2001) observed that in low nitrogen efficient genotypes, a significant portion of N is not retranslocated to harvested structures, causing decreased GS activity in leaves when compared to high nitrogen efficient genotypes. In comparison to the transcript expression of UP2382 under high nitrogen conditions, the levels of expression of the GS gene family (Gln1;1) in the leaves of VL616 showed up regulation under low N levels. As an outcome, the high nitrogen-efficient genotypes' ability to harvest more nitrogen and redistribute it to grains is attributable to a well-coordinated system of N intake and assimilation (Shi *et al.* 2010).

#### **4.2.11.2 Glutamine synthetase 2 gene expression**

GS-2 (the plastid isoform) is found in green tissues and is primarily involved in photorespiratory release ammonium. GS2 plays an important role in the nitrogen use efficiency by preventing nitrogen loss.

The Ct value of the GS2 real-time PCR amplification plot for all samples indicated that the gene was highly expressive (Table 4.21); the target gene GS 2 was expressed in all genotypes, according to the RQ plot provided in Figure 4.4. In comparison to the other genotypes, HD-2781 expression levels were higher during the pre-anthesis stage and NIAW-1994 post-anthesis stages. Under limiting nitrogen conditions, the GS 2 gene was upregulated in N-efficient genotypes (HD-2781 and NIAW-1994) compared to N-inefficient genotypes (C-306 and HD-2932), with wheat actin acting as the endogenous control.

Our study revealed that GS1 and GS2 wheat genes have non-redundant functions and are potential targets during specific growth and development stages to improve NUE. In the N-efficient genotype compared to the N-inefficient genotype, GS1 (cytosolic isoforms) was highly expressed at post-anthesis, and GS2 (chloroplastic isoforms) was highly expressed at pre-anthesis. There is minimal evidence that increasing NR, GS, and the GOGAT enzyme could be a viable approach for increasing grain yield and NUE. There is significant evidence that GS1 is the major enzyme involved in the mobilisation of nitrogen from senescing cereal leaves and that its activity in leaves is linked to grain nitrogen (Fei, 2003).

Although the transcript of GS 2 was slightly higher in the flag leaf of nitrogen-efficient genotypes under nitrogen-limiting conditions, a similar trend was observed in the present investigation. In wheat, GS 1 may be more important in regulating NUE at post anthesis, while GS 2 more crucial at pre and post anthesis stage. In maize, the GS2 gene is predominantly expressed at the pre anthesis stage (Keys, 2006). Our research revealed that the GS1 and GS2 genes have non-redundant functions and are potential targets for improving nitrogen use efficiency during specific growth and developmental stages. However, the detection of rice GS2 expression in the flag leaf at the post-anthesis stage showed its mRNA accumulation at very low levels, as reported by Zeng *et al.* in 2017. According to Masclaux Daubresse *et al.* (2010), GS2 is found in chloroplasts, which display the first indicators of damage during leaf senescence.

#### **4.2.11.3 Fd-GOGAT gene expression**

Fd-GOGAT catalyses the conversion of glutamine and 2-oxoglutarate into two molecules of glutamate, thus providing glutamate for ammonium assimilation. The net outcome of the GS-GOGAT cycle is the production of glutamate, which can then be incorporated into other amino acids through the action of aminotransferases or transaminases (Forde and Lea, 2007).

The Ct value of the Fd-GOGAT real-time PCR amplification plot, which included all the samples that indicated the gene was significantly expressed (Table 4.21), The target gene

Fd-GOGAT was expressed in all genotypes, according to the relative quantification in Figure 4.5. When compared to the other genotypes, only the HD-2932 expression level was higher in the pre-anthesis stage. Gene expression was unregulated in the post-anthesis stage in N-efficient genotypes (HD-2781 and NIAW-1994) compared to N-inefficient genotypes (C-306 and HD-2932), but it was up-regulated in HD-2932 in the pre-anthesis stage compared to the post-anthesis stage with wheat actin as an endogenous control.

Previous studies by Kendall *et al.* (1986) revealed that GS2 and Fd-GOGAT are chloroplastic genes responsible for the reassimilation of ammonia produced during photorespiration. In addition, Lea (1993) investigated that ammonium-assimilating enzymes during the grain filling stage are important because they demonstrate how nitrogen assimilated during the active development stage is remobilized. In the case of maize, the main mechanism for converting ammonium into amino acids involves GS and GOGAT. Zeng *et al.* (2017) found that OsFd-GOGAT participates in nitrogen remobilization during leaf senescence, suggesting a potential way to increase NUE in rice. The Fd-GOGAT was mainly expressed in the leaf compared to other organs of the plant. NADH-GOGAT and Fd-GOGAT gene expression in wheat flag leaves was up regulated at all nitrogen levels during the post-anthesis stage, as reported recently by Liu *et al.* (2021).

### 4.3 Experiment III

#### 4.3.1 Effect of Omeprazole on Nitrate Uptake and Assimilation in High and Low NUE Genotypes

Omeprazole treated wheat seedlings of HD-2781 (N-efficient) and C-306 (N-inefficient) genotypes recorded the maximum leaf nitrate content, *in vivo* and *in vitro* NR activities as compared to untreated (Table 4.22). After OP supplementation in medium increase the activity of leaf nitrate content (2.46 mg/g fr. wt), *in vivo* NR ( $2.43 \mu\text{mol NO}_2^- \text{g}^{-1} \text{fr. wt. h}^{-1}$ ) *in vitro* NR activity ( $3.13 \mu\text{mol NO}_2^- \text{g}^{-1} \text{fr. wt. h}^{-1}$ ) in C-306 N- inefficient genotypes than the control. Because of the omerazole treatment, there may be a potential for both low and high affinity nitrate transporters to work in the N-inefficient (C-306) genotype. The rate of  $\text{NO}_3^-$  uptake was similar in both genotypes and the control. Both wheat genotypes had the highest *in vivo* and *in vitro* nitrate reductase activity when omeprazole was supplemented; however, the C-306 (N-inefficient) wheat genotype with omeprazole supplement had similar nitrate reductase activity to the HD-2781 (N-efficient) wheat genotype.

**Table 4.22 Effect of omeprazole on nitrate uptake and assimilation in high and low NUE wheat genotypes**

Treatment	Leaf nitrate content (mg / g fr. wt)		<i>In vivo</i> NRA ( $\mu\text{mol NO}_2^- \text{g}^{-1} \text{fr wt h}^{-1}$ )		<i>In vitro</i> NRA ( $\mu\text{mol NO}_2^- \text{g}^{-1} \text{fr wt h}^{-1}$ )	
	HD-2781	C-306	HD-2781	C-306	HD-2781	C-306
With Omeprazole	2.91	2.46	2.77	2.43	3.52	3.13
Without Omeprazole	2.12	1.83	2.29	1.32	2.85	1.52
Mean	2.52	2.14	2.53	1.88	3.18	2.32
Comparison	SEm ( $\pm$ )	CD @ 5%	SEm ( $\pm$ )	CD @ 5%	SEm ( $\pm$ )	CD @ 5%
Treatment (T)	0.021	0.065	0.018	0.055	0.016	0.050
Genotype (G)	0.007	0.023	0.006	0.019	0.006	0.018
G X T	0.030	0.092	0.025	0.078	0.023	0.071

According to Van Oosten *et al.* (2019), using OP (50 M) increased the catalytic efficiency of the enzyme and its specificity for nitrate (as a substrate), resulting in a higher  $V_{\text{max}}$  and a lower  $K_m$ . This shows that omeprazole helps nitrate reductase maintain its appropriate affinity for its substrate and its catalytic rate through a possible physical interaction. Similarly, previous research found that high and low nitrogen-efficient cultivars have high and low nitrate reductase activities, respectively (Jain *et al.*, 1997), and that nitrate reductase activity was higher in the N-efficient genotypes than in the N-inefficient genotypes under similar growing conditions (Jain and Abrol 2005). In tomato (Rouphael *et al.*, 2018), basil (Carillo *et al.*, 2019), and *Arabidopsis thaliana*, OP treatment improved nitrate assimilation. It was postulated that OP could influence nitrogen uptake and assimilation, resulting in enhanced growth. According to our findings, OP improves NUE by enhancing nitrate assimilation and changing several primary and secondary metabolic pathways. The use of OP to describe the primary phenotype under N stress, as well as a few of the metabolites that change in response to OP treatment, is in addition to evidence that OP interacts directly with NR, improving assimilation by increasing substrate affinity and activating the enzyme in a constitutive mechanism.

#### 4.3.2 Effect of Omeprazole on G6PDH Activity in High and Low NUE Wheat Genotypes

The G6PDH activity varied in response to omeprazole in the root as well as the shoot of wheat seedlings. Higher G6PDH activity was recorded in root tissues (108.56 to 130.23  $\mu\text{mol reduced NADP}^+ / \text{min} / \text{mg of protein}$ ) as compared to shoots (26.79 to 36.53  $\mu\text{mol reduced NADP}^+ / \text{min} / \text{mg of protein}$ ) of both genotypes and also omeprazole induced response was higher in C-306 (118.58  $\mu\text{mol reduced NADP}^+ / \text{min} / \text{mg of protein}$ ) rather than

untreated (98.61  $\mu\text{mol}$  reduced  $\text{NADP}^+$  /min/ mg of protein) in concern to G6PDH (Table 4.23). Total G6PDH activity in leaves remained unchanged, showing that OPPP and nitrogen uptake in higher plants' photosynthetic cells are unrelated. The increase in cytosol enzyme activity and the appearance of a plastidic glucose 6P dehydrogenase isoform after omeprazole treatment in the roots can be linked to the rise in G6PDH rate (Esposito *et al.*, 2001).

**Table 4.23 Effect of omeprazole on G6PDH activity ( $\mu\text{mol}$  reduced  $\text{NADP}^+$  /min/ mg of protein.) in high and low NUE wheat genotypes**

Treatment	G6PDH ( $\mu\text{mol}$ reduced $\text{NADP}^+$ /min/ mg of protein.)			
	Shoot		Root	
	HD-2781	C-306	HD-2781	C-306
<b>With Omeprazole</b>	39.36	28.48	147.76	118.58
<b>Without Omeprazole</b>	33.71	25.11	112.70	98.61
<b>Mean</b>	36.53	26.79	130.23	108.56
<b>Comparison</b>	<b>SEm (+)</b>	<b>CD @ 5%</b>	<b>SEm (+)</b>	<b>CD @ 5%</b>
<b>Treatment (T)</b>	0.111	0.343	0.100	0.309
<b>Genotype (G)</b>	0.039	0.121	0.035	0.109
<b>G x T</b>	0.158	0.486	0.142	0.437

#### 4.4 Experiment IV

##### 4.4.1 Differential Carbon Monoxide Sensitivity of Cytochrome Oxidase in Nitrogen Use Efficient and Inefficient Wheat Genotype

In nitrogen use efficient wheat genotypes HD-2781 and NIAW-1994 the cytochrome  $a_3$  appears to be more in reduced state thereby indicating less extend of coupled mitochondria however nitrogen use inefficient wheat genotype C-306 and HD-2932 cytochrome  $a_3$  more in oxidized form thereby indicating that mitochondria are tightly coupled. Infiltrated 2mM DNP uncoupling of oxidative phosphor elation before CO exposure enhance CO aerobic response significantly in C-306 ( $1.20 \mu\text{mole NO}_2^- \text{ g}^{-1} \text{ fr. wt h}^{-1}$ ) and HD- 2932 ( $0.35 \mu\text{mole NO}_2^- \text{ g}^{-1} \text{ fr. wt h}^{-1}$ ) at evident amount of nitrite formed thereby confirming that mitochondrial electron transport and oxidative phosphor relation are tightly coupled in nitrogen use inefficient wheat genotype (Table 4.24).

Plant mitochondria appears to be target for improving NUE has been documented in order to see differences in mitochondrial metabolism in nitrogen use efficient and inefficient wheat genotype were tested for CO sensitivity. Carbon monoxide is known inhibitor of cytochrome oxidase and it forms binary complex with cytochrome  $a_3$  only when it is in the reduced state. In tightly coupled mitochondria cytochrome  $a_3$  is highly to be more in oxidized form by donating electron to oxygen. CO-sensitive genotypes are high nitrogen use efficient genotypes, which means CO forms complexes with reduced cytochrome  $a_3$ , matrix-generated

NADH is exported to the cytosol and used for nitrate assimilation, whereas nitrogen use inefficient wheat genotypes are insensitive to CO, cytochrome  $a_3$  is more oxidized, indicating that mitochondria are tightly coupled.

**Table 4.24 Differential carbon monoxide sensitivity of cytochrome oxidase nitrogen use efficient and inefficient wheat genotype**

Treatment	<i>In vivo</i> NRA ( $\mu\text{mol NO}_2^- \text{g}^{-1} \text{fr. wt h}^{-1}$ )			
	HD-2781	C-306	NIAW-1994	HD-2932
Anaerobic assay	2.08	1.84	1.90	1.67
CO aerobic assay	0.49	0.00	0.42	0.00
DNP CO aerobic assay	0.81	1.20	0.57	0.96
Mean	1.13	1.52	1.03	1.31
Comparison	SEm ( + )	CD @ 5%	SEm ( + )	CD @ 5%
Treatment (T)	0.004	0.014	0.006	0.018
Genotype (G)	0.005	0.017	0.007	0.021
G x T	0.008	0.024	0.010	0.030

The result of current study are in agreement with the result obtained by Kumar, (2003) investigated the relationship between light and NR synthesis, determining that either the presence of NADH or the process of nitrate reduction itself plays a key role in NR induction. Because CO inhibited Cyt  $c$  oxidase in wheat leaves under aerobic conditions, cytoplasmic NADH was available for catalysed nitrate reduction. Thus, the accumulation of nitrate in leaves demonstrated the degree to which CO inhibited Cyt  $c$  oxidase, which in turn reflected the reduced status of Cyt  $a_3$  during steady-state respiration according to Sawhney *et al.* (1978).

Thus carbon monoxide sensitivity gives an idea regarding the redox state of cytochrome oxidase during the steady state respiration. In tightly coupled mitochondria the cytochrome  $a_3$  is most likely in the oxidized form by donating electrons to O<sub>2</sub> and under the situation carbon monoxide cannot form a complex with cytochrome  $a_3$ . When CO forms adduct with cytochrome  $a_3$  (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,1992,97 ).Previously, it was found that higher levels of alternative oxidase expression provide a mechanism for the continual supply of carbon skeletons required for continued nitrate assimilation, irrespective of the physiological limitations of electron transfer through the cytochrome pathway (Naik, 2007).

## 5. SUMMARY AND CONCLUSION

The present investigation, "Biochemical and molecular Basis of Differential Nitrogen use efficiency in contrasting wheat (*Triticum aestivum* L.) genotypes" was carried out at the Department of Biochemistry, PGI, MPKV, Rahuri, during 2019–22. The laboratory and pot culture experiments were laid down in FCRD and CRD in four and three replications for physiological and biochemical parameters, respectively. Biochemical and molecular study was undertaken to understand the processes of uptake, assimilation, and remobilization of nitrate. For better understanding about nitrogen metabolism contrasting wheat genotypes with high and low nitrogen use efficiency were evaluated under limiting nitrogen supply under following objectives:

1. To evaluate the physio-biochemical parameters concerning nitrogen use efficiency at pre- and post-anthesis stages
2. To investigate the correlation between carbohydrate metabolism, mitochondrial metabolism, and nitrogen use efficiency and
3. To study expression analysis of nitrogen use efficient genes and activity at pre and post anthesis stage of wheat.

The understanding of the mechanisms that determine NUE in cereals is essential, as the nitrogen use efficiency of cereals is around 40%. Identification of novel targets for improvement of NUE is desirable both from economic and environmental considerations.

Two contrasting wheat genotypes, HD-2781 and C-306, were obtained from the Division of Plant Physiology, IARI, Pusa Campus, New Delhi-110012, and were sown in pots with a factorial completely randomized design having four replications with three fertiliser treatments. During 45–60 days, considered the pre-anthesis stage, and 65–80 days, considered the post-anthesis stage, observations was recorded for both genotypes, and the results obtained are given below:

The leaf nitrate content in the N-efficient HD-2781 wheat genotype was higher at all three nitrogen levels in the pre- and post-anthesis stages than in the nitrogen-inefficient C-306 wheat genotype. The leaf nitrate content of the N-efficient genotype HD-2781 was significantly higher, indicating that the high-NUE genotype has a better uptake efficiency and storage pool.

In pre- and post-anthesis, the N-efficient wheat genotype HD-2781 had significantly higher levels of chlorophyll a, chlorophyll b, and total chlorophyll content than the nitrogen-use inefficient wheat genotype C-306 at all three nitrogen levels, *i.e.*, N+ (full nitrogen),

N- (half nitrogen), and N0 (no nitrogen). Even without fertiliser, the chlorophyll content of the HD-2781 wheat genotype was significantly higher, indicating better stay-green ability.

The nitrogen-efficient wheat genotype HD-2781 exhibited significantly higher *in vivo* nitrate reductase activity at all three nitrogen levels at pre- and post anthesis stages than the nitrogen-inefficient wheat genotype C-306, which recorded less *in vivo* NR activity.

The *in vivo* nitrate reductase activity, with a saturating level of  $\text{NO}_3^-$  was much less than the *in vitro* NR activity in both the N-efficient and N-inefficient genotypes, suggesting that, beside nitrate, NADH is a major factor controlling the nitrate reductase activity and the process of nitrate assimilation.

The N-efficient wheat genotype HD-2781 recorded significantly higher nitrite reductase activity (NiR) at N+ (full nitrogen level) than N- (half nitrogen level) and N0 (no nitrogen level) at pre- and post-anthesis stages as compared to the N-inefficient genotype C-306, which indicates a better reduction state due to higher photosynthesis reflecting better utilisation efficiency.

The activity of GS, Fd-GOGAT, and NADH-GOGAT were higher in the N-efficient HD-2781 genotype than in the N-inefficient C-306 genotype at the post-anthesis stage than at the pre-anthesis stage. The higher activity of these nitrogen-metabolising enzymes at the post-anthesis stage signifies their importance in N assimilation and remobilization, suggesting that they are key enzymes in nitrogen metabolism.

Isozyme profiling of GS1 and GS2 was done on Native-PAGE, which revealed GS1 and GS2 isoforms at pre- and post-anthesis stages. GS1 (cytosolic isoform) activity was not detected during the pre-anthesis stage in either of the wheat genotypes. However, at the post-anthesis stage, the bands of both GS1 (cytosolic isoform) and GS2 (plastid isoform) were seen on the gel and clearly resolved. The N-efficient wheat genotype HD-2781 showed significantly higher band intensities of both GS1 and GS2 isoforms than the N-inefficient genotype C-306.

Total grain protein content increased as the level was higher in the N-efficient wheat genotype HD-2781, which showed better nutrient mobilisation to grain as compared to C-306.

Thirteen promising wheat genotypes were used for this experiment to evaluate the nitrogen use efficiency of these genotypes based on the results obtained with the contrasting wheat genotypes. Nitrate uptake and assimilation potential under limiting N-status in wheat genotypes were evaluated in pre- and post-anthesis stages by recording various physio-biochemical and molecular observations.

The higher chlorophyll content, leaf nitrate content, *in vivo* NR, *in vitro* NR, *in vitro* NiR, GS, Fd-GOGAT, NADH-GOGAT, and total grain protein were found in GW-322, MACS-6222, NIAW-301, NIAW-34, NIAW-3170, NIAW-1994, and HD 2781 low nitrogen tolerant genotypes, while the low were found in MACS-2496, HD-2932, NIAW-1415, and C-306 low nitrogen sensitive genotypes. The low-nitrate-tolerant wheat genotypes performance was better under conditions of limited nitrogen supply, indicating that both low- and high-affinity nitrate transporters are actively operating, as well as that the assimilation potential is better due to the higher activity of the N-assimilation enzyme.

The genotypes GW-322, NIAW-1994, and MACS-6222 reported the highest total grain protein content of the thirteen wheat genotypes, whereas C-306 and MACS-2496 recorded the lowest total grain protein.

The isozyme profiles of GS1 and GS2 in 13 wheat genotypes were analysed during the post-anthesis stage and differed significantly. The N-efficient wheat genotypes GW-322, MACS-6222, NIAW-301, NIAW-34, NIAW-3170, NIAW-1994, and HD-2781 had considerably higher levels of both GS1 and GS2 isoforms than the N-inefficient HD-2932, MACS-2496, NIAW-1415, NIAW-15, and C-306 genotypes. Only GS2 activity was observed in all thirteen wheat genotypes during the pre-anthesis stage.

Based on the results of the uptake and assimilation studies, four genotypes (HD-2781, C-306, NIAW-1994, and HD-2932) were selected for the investigation of nitrogen metabolising genes.

The expression level of GS 1 was significantly higher in HD-2781 and NIAW-1994 at the post-anthesis stage than at the pre-anthesis stage, supporting better nitrogen remobilization efficiency. The expression of the chlorolastic isoforms of GS2 and Fd-GOGAT was higher in HD-2781 and NIAW-1994 during the pre-anthesis stage and post-anthesis stage, signifying better N assimilation efficiency. Under limiting nitrogen conditions, the GS2 gene was upregulated in N-efficient genotypes HD-2781 and NIAW-1994 compared to N-inefficient genotypes C-306 and HD-2932. Fd-GOGAT expression was higher at the post-anthesis stage as compared to the pre-anthesis stage, which is more important for the evaluation of NUE.

Nitrate content and NR activity increased in both N-efficient and N-inefficient wheat genotypes after omeprazole treatment. However, the C-306 N-inefficient wheat genotype with omeprazole supplement had similar nitrate content and *in vivo* NR and *in vitro* NR activity to the HD-2781 N-efficient wheat genotype. OP increases nitrate assimilation by changing the activation state and substrate affinity of enzyme nitrate reductase.

The root of nitrogen-efficient and inefficient genotypes had higher G6PDH activity when treated with omeprazole than the shoot. G6PDH activity remained unchanged in the shoot, indicating that OPPP and chloroplast nitrogen assimilation are unrelated. The increase in G6PDH rate can be linked to a rise in cytosolic enzyme activity and the appearance of the plastidic G6PDH isoform after omeprazole treatment in the roots.

Chlorophyll content, leaf nitrate content, *in vivo* NR, *in vitro* NR, and *in vitro* NiR were significantly higher in the pre-anthesis stages than the post-anthesis stages. The significantly higher *in vitro* NR activity compared to *in vivo* NR activity at saturating nitrate concentration (+KNO<sub>3</sub>) signifies that, beside nitrate, NADH appears to be a limiting factor. Glutamine synthetase, Fd-GOGAT, and NADH-GOGAT activities were higher at the post-anthesis stage, which signifies their importance in N assimilation and remobilization under low-nitrogen conditions. The HD-2781 N-efficient wheat genotype had a considerably lower NADH/NAD<sup>+</sup> ratio than the C-306 N-inefficient wheat genotype at all three nitrogen levels. As compared to the C-306 (N-inefficient) wheat genotype, the HD-2781 (N-efficient) wheat genotype had a lower NADH/NAD<sup>+</sup> ratio, which means a higher NADH level.

Four wheat genotypes: two nitrogen-use efficient HD-2781 and NIAW-1994 and two nitrogen-uses inefficient C-306 and HD-2932 were screened for carbon monoxide sensitivity of cytochrome oxidase to understand the coordination of redox state in integration of C and N metabolism. Carbon monoxide (CO) is an inhibitor of cytochrome oxidase, as it forms a binary complex with cytochrome a<sub>3</sub> only when it is in the reduced state. Thus, carbon monoxide sensitivity gives an idea regarding the redox state of cytochrome oxidase during steady state respiration. In tightly coupled mitochondria, the cytochrome a<sub>3</sub> is most likely in the oxidized form by donating electrons to O<sub>2</sub> and under the situation carbon monoxide cannot form a complex with cytochrome a<sub>3</sub>. When CO forms adduct with cytochrome a<sub>3</sub> (reduced), the NADH is exported to the cytosol for the reduction of nitrate to nitrite. Thus accumulation of nitrite under CO-aerobic condition is indication of redox state and thus tightness of coupling.

In HD-2781 and NIAW-1994, the cytochrome a<sub>3</sub> was found to be more in a reduced state, thereby indicating a lower extent of coupled mitochondria; however, in wheat genotype C-306 and HD-2932, the cytochrome a<sub>3</sub> is more in an oxidised form, thereby indicating that mitochondria are tightly coupled.

Infiltration with 2 mM DNP an uncoupler of the oxidative phosphorylation before CO exposure enhanced the CO aerobic response significantly in C-306 and HD-2932, as evident from the amount of nitrite formed, thereby confirming that mitochondrial electron transport

and the oxidative phosphor relation are tightly coupled in the nitrogen use inefficient wheat genotype.

### **Conclusion**

The optimum dose of nitrogen has various advantages, including saving energy for fertilizer manufacturing (mainly using the Haber–Bosch technique), lowering farmer expenses, and lessening the negative environmental consequences of leached and gaseous nitrogen used by plants. Wheat genotypes with different levels of nitrogen metabolizing enzyme activity differ in the amount of nitrogen assimilated throughout the course of their lives. The N metabolising enzymes were more active in the flag leaves of the high nitrogen use efficient genotype until later stages of growth. Seedlings of the high nitrogen use efficient genotype uptake more nitrate from the medium than seedlings of the low nitrogen use efficient genotype. The increased activity of all nitrogen-metabolizing enzymes resulted in higher grain nitrogen and grain yield. A large part of the nitrate is not retranslocated to the harvested tissues in the N-inefficient genotype, as GS / GOGAT activity in the flag leaves during senescence decreases significantly compared to the N-efficient genotype leaves. The changes in mitochondrial redox systems can affect plant responses to N as oxidized nitrate ( $\text{NO}_3^-$ ) or reduced ammonia ( $\text{NH}_4^+$ ). Thus, mitochondrial-driven effects on NAD status exert a major influence on the rate of nitrate assimilation. As a result, the HNE genotypes ability to harvest more nitrogen and reassimilate it to grains is due to a well coordinated nitrate uptake, assimilation, and remobilization mechanism.

### **Further lines of work**

Beside the expression and activity profile of the nitrogen assimilation enzyme, the availability of reductant (NADH) is a critical factor in governing the improvement of nitrate assimilation and hence a better response to low nitrogen status. The plant mitochondria have non-energy linked mitochondrial electron transport, and under the situation of high energy status, these are operative.

The abundance and activity of non-energy linked mitochondrial electron transport enzymes can be directly modified in transgenic plants either by T-DNA insertion, RNAi, or CRISPR-Cas9 or indirectly by inactivating other components, which need to be investigated. The activity of enzymes is regulated by substrate limitation, and end product inhibition also needs due consideration.

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## 7. VITAE

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**ABUJ BHAGYASHREE BHASKARRAO**  
**DOCTOR OF PHILOSOPHY (AGRICULTURE)**  
**IN**  
**BIOCHEMISTRY**  
**2023**

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