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Nitrogen Metabolising Enzymes and Photosynthetic Pigments as Influenced by Nitrogen Application in Oilseed Rape (*Brassica napus* L.)

Nimrat Kaur Gill[1], Pushp Sharma[2] and Virender Sardana[2]

1. Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana, Punjab 141004, India

2. Department of Botany, Punjab Agricultural University, Ludhiana, Punjab 141004, India

Abstract: Nitrogen (N) assimilation is a wide pathway in plants because of its fundamental importance for growth and development. The transport, assimilation and recycling of nitrogen is a highly complex and regulated process, as it is the mineral nutrient that is required in great abundance by the plants. Basic approach to enhance agriculture sustainability is dependent on exploration of the elite germplasm where new cultivars could perform better even under low N. To test the effect of nitrogen levels at 100 and 125 kg ha^{-1} on photosynthetic pigments and N assimilation in oilseed rape (*B. napus* L.) canola variety GSC-7 and hybrid Hyola PAC 401 were selected for comparison with new hybrid PGSH-52 in pipeline. N assimilating enzymes were assayed at vegetative, flowering and siliquing stages of crop growth to visualise the impact of N on the productivity. Nitrogen assimilating enzymes were highest at flowering stage and enhanced with increased N level. Nitrate reductase (NR) activity improved by 11.5%, nitrite reductase (NiR) by 24.2%, glutamine synthetase (GS) by 12.2% and glutamate synthase (GOGAT) by 35.2% over recommended N dose (N_{100}) at flowering stage. Hyola PAC 401 registered maximum enzymatic activities trailed by GSC-7 at all the three stages of crop growth. Differences existed within the genotypes for photosynthetic pigments which varied with N levels. Chlorophyll *a*, chlorophyll *b*, total chlorophyll and carotenoids increased with nitrogen at 125 kg N ha^{-1} whereas chlorophyll *a*/*b* declined at three stages of crop growth. At flowering total chlorophyll and carotenoids were maximum and enhanced by 12.8% and 5% respectively with higher nitrogen level.

Key words: *B. napus*, nitrogen, biochemical estimations, photosynthetic pigments.

1. Introduction

Nitrogen (N) is an essential macronutrient having a strong impact on growth, development and productivity of a plant and is related with many of its metabolic processes associated with lush green colour due to rise in chlorophyll. N is sharply linked to control the vegetative growth of plant and also determines the developmental events of the reproductive cycle [1]. An important role of nitrogen is noticed in plant growth and synthesis of proteins, protoplasm, cell size and its photosynthetic activity [2]. Nitrogen acts as a limiting factor in agro-ecosystems [2], and improves soil fertility to increase crop production [3]. Some studies have found that *B. napus* has a high N demand and by improving soil N availability, its biomass and seed production can be increased [4]. Crop plants use nitrogen in the form of nitrate (NO_3^-) and ammonium (NH_4^+) which is available in the soil. After the internal demand for nitrogen has been met, there is accumulation of nitrogen in plant. If there is excessive intake of nitrate-N than the physiological demand, it gets accumulated in vacuoles. There is reduction of nitrate (NO_3^-) to nitrite (NO_2^-) and then to ammonium (NH_4^+) during the process of nitrate uptake and assimilation. Through the glutamine synthetase (GS) pathway there is primary assimilation of ammonia in the plants [5]. The glutamate dehydrogenase (GDH) pathway works

Corresponding author: Pushp Sharma, Ph.D., professor, research fields: abiotic stresses & its mitigation, input use efficiencies and source-sink alteration with PGRs.

for the assimilation of ammonia when it is present in excess [6]. This process of nitrate uptake and assimilation depends on the major nitrogen assimilating enzymes. The first two enzymes involved in nitrate assimilation are nitrate reductase (NR) and nitrite reductase (NiR). NiR requires ferredoxin to reduce nitrite to ammonia, which is subsequently assimilated via glutamine synthetase (GS) and glutamate synthase (GOGAT) [7]. NiR, GS and GOGAT are present in enzyme complexes within the chloroplasts [8].

With the help of amino transferases, glutamate amino group can be transferred to different amino acids [9]. The information on germplasm variability for enzymes of N metabolism, N uptake and assimilation at different growth stages in *Brassicas* are limited [5]. In fruits of green bean (*Phaseolus vulgaris*), with increase in N doses the activity of NR also increased. The expected increase with the increase in N level was related to NR acting as a substrate inducible enzyme [10]. In rice leaves higher NR activity was with either with NO_3^- or NH_4^+ nitrogen form. The leaves of the NO_3^- grown plants exhibit higher NR activity as compared to those of NH_4^+ grown plants [11]. Maximum enhancement in the activity of ammonium assimilating enzymes was reported during flowering at 100 kg ha^{-1} of nitrogen in Indian mustard (*B. juncea*) [12]. Enhanced enzymatic activities (NR, NiR, GS and GOGAT) along with protein content were reported in both *B. juncea* and *B. napus* germplasm with increased N doses [5]. During the pre-anthesis stages the increase was much higher in *B. juncea*, as the NR, NiR, GS and GOGAT were found to be maximum at 100 kg N ha^{-1} [13].

In the cytosol, nitrate reduction into nitrite is catalysed by the enzyme NR [14]. With the help of NiR, nitrite is trans-located to the chloroplast where it is reduced to ammonia. By the GS/GOGAT cycle, ammonia which originates from nitrate reduction and photorespiration or amino acid recycling mainly assimilates in the plastid/chloroplast [15, 16]. N is also exported from the leaves, during the post-anthesis stages. More than 48% of the N entering the plant gets accumulated in the pods and was derived from the remobilization of N from other tissues on an average [17]. There was no increased expression of NR and *Os*NiR found in nitrate deficient side roots, while GS and GOGAT were induced in both nitrate deficient and nitrate supplied side roots. From this it was concluded that most of the absorbed NO_3^- could be quickly reduced in nitrate supplied roots and leaves, and the assimilated N metabolites will be transported from shoot to the whole root system through downward phloem within a short time after absorption of NO_3^-. For nitrogen re-distribution, GS and GOGAT are involved in plant tissues during later growth stages, and nitrogen use efficiency (NUE) can be regulated via physiological mechanisms [18].

Rapeseed is one of the most important oilseed crops produced worldwide, having annual production of 26 million tonnes of oil, which accounts for approximately 15% of the world's vegetable oil production [19] and 7.91 million tonnes of the total seven oilseeds produced in the country with an area of 6.32 million hectare [20]. The major rapeseed-mustard producing states in India are Rajasthan, Uttar Pradesh, Haryana, Madhya Pradesh and Gujarat while Punjab contributes 44.8 thousand tonnes to the total production and has an area of 31.7 thousand hectare [21].

After soybean and palm oil, canola has the world's highest production of oilseeds in recent decades [22]. *B. napus* is commonly known as canola or oilseed rape and is grown in more than 120 countries in the world for edible vegetable oil because of low content of saturated fatty acids with about 7-10% alpha linolenic and 17-21% linoleic acid [1]. Canola has low fiber and high protein content in leaves and stem and also describes the double low oil and meal quality i.e. low erucic acid (less than 2%) in oil and low glucosinolate in meal (less than 30 μg g^{-1} of defatted meal). Canola oil is a magnificent feedstock for

bio-fuel production [23]. The intend of the present investigation was to identify the effect of N on nitrogen assimilating enzymes along with the photosynthetic pigments at different growth stages and to test the performance of PGSH-52, a newly developed canola hybrid.

2. Material and Method

2.1 Plant Material and Treatment

The field experiments were conducted at the research farm and biochemical estimations were carried out in laboratories of Oilseeds Section at Punjab Agricultural University, Ludhiana. The experimental site is located at 30°56′ N latitude and 75°48′ E longitude and at an altitude of 247 m above the mean sea level. The soil is loamy textured with low organic carbon, high plant-available phosphorus and moderate available nitrogen. Released canola variety GSC-7 and hybrid Hyola PAC 401 were selected to test the performance of new hybrid PGSH-52 in pipeline. The sowing was done on 20 October 2015. The experiment was conducted in split plot design with three replications and two nitrogen levels i.e. 100 kg N ha^{-1} (N_{100}) and 125 kg N ha^{-1} (N_{125}). Nitrogen in the form of urea was added in two split doses to the soil half at time of sowing and the remaining half at the time of first irrigation. Photosynthetic pigments, enzymatic activity and protein content were estimated from third or fourth fully opened leaves on main raceme at three stages: vegetative (60 DAS), flowering (80 DAS) and siliquing (100 DAS) stages. The leaves were stored in liquid nitrogen and kept at -80 °C.

2.2 Determination of Photosynthetic Pigments

Chlorophyll content was estimated by Hiscox and Israelstam [24] method. Leaf samples of 0.1 g were placed in vial containing 10 mL of dimethyl sulphoxide (DMSO). Vials were then kept into the boiling water bath at 65 °C for 30 minutes. Absorbance was recorded at 645 nm and 663 nm to measure the concentration of chlorophyll *a*, *b* and total chlorophyll which were calculated by using Arnon's equations and for carotenoid at 480 nm using UV 2600 spectrophotometer (Techcomp). The concentrations of chlorophyll *a*, chlorophyll *b* and total chlorophyll were calculated by using Eqs. (1)-(3):

$$\text{Chlorophyll } a \text{ (mg g}^{-1}\text{ FW)} = 12.7 \times A_{663} - 2.69 \times A_{645} \times \frac{\text{Volume}}{1000 \times \text{Weight}} \quad (1)$$

$$\text{Chlorophyll } b \text{ (mg g}^{-1}\text{ FW)} = 22.9 \times A_{645} - 4.68 \times A_{663} \times \frac{\text{Volume}}{1000 \times \text{Weight}} \quad (2)$$

$$\text{Total Chlorophyll (mg g}^{-1}\text{ FW)} = 20.2 \times A_{645} + 8.02 \times A_{663} \times \frac{\text{Volume}}{1000 \times \text{Weight}} \quad (3)$$

Absorbance was again measured at 480 nm in UV-spectrophotometer to estimate the carotenoid content by using Eq. (4):

$$\text{Carotenoids (mg g}^{-1}\text{ FW)} = \frac{1000 \times A_{480} - 1.29 \times \text{Chl a} - 53.78 \times \text{Chl b}}{220} \times \frac{\text{Volume}}{1000 \times \text{Weight}} \quad (4)$$

(A = Absorbance at respective wave length)

2.3 Estimation of N Assimilating Enzymes

NR activity (EC-1.6.6.1) was estimated by Jaworski [25] method. Leaves weighing 200 mg were suspended in a covered screw cap vial containing 5 mL of 0.1 M phosphate buffer (pH 7.5), 0.05 mL of *n*-propanol and 0.25 g KNO_3. The vials were sealed, shaken and incubated in dark for 90 min. After this, the release of NO_2^- (nitrite) into the medium was determined by treating 1 mL of aliquot with 1 mL of 1% sulphanilamide in 1 N HCl and 0.02% N-napthylethylenediamine dihydrochloride. The total volume was made to 10 mL with distilled water. After 20 minutes, the absorbance was recorded at 540 nm using UV 2600 spectrometer (Techcomp). The standard curve was prepared using 0-10 μg of KNO_2. NR activity was expressed as μmol NO_2^- formed $h^{-1}g^{-1}$ tissue FW.

$$\text{NR activity} = \frac{\text{Concentration of std}}{\text{O.D. of std}} \times \frac{\text{O.D. of sample}}{\text{Fresh weight}} \times \frac{\text{Total volume}}{\text{Aliquot taken}} \times \text{Dilution factor} \times \text{Incubation time}$$

NiR activity (EC-1.7.7.1) was estimated by Verner & Ferari [26]. Leaves weighing 200 mg were suspended in a covered screw cap vials containing 2 mL of 0.1 M phosphate buffer (pH 4.5), chloramphenicol (20 µL) and $NaNO_2$ (50 µL). Nitrite ($NaNO_2$) was added to start the assay. And 0.1 mL aliquots in two separate tubes were immediately removed to provide an accurate measure of the initial nitrite concentration of the medium with tissue present. After 40 minutes, 0.1 mL aliquots of the medium in two test tubes were removed for nitrite determination. One milliliter (1 mL) dimethyl sulfoxide (50% *v*/*v* DMSO) was then added to the medium and the vials were placed in water bath until the medium came to boil (about 10-12 minutes). Under these conditions, the nitrite of tissue rapidly leaked back into the medium. After cooling, in two separate test tubes 0.2 mL aliquots were removed for nitrite determination. Nitrite was determined colorimetrically by adding to aliquots 0.3 mL of 1% sulphanilamide prepared in 3 N HCl and 0.02% N-1-napthylethylene diaminedihydrochloride. Absorbance was read at 540 nm after centrifugation at 200 g for 10 minutes. NiR activity was expressed as µmol NO_2^- released $h^{-1}g^{-1}$ tissue FW. The amount of nitrite taken up by the tissue was determined from the difference between the final and the initial nitrite concentration of the medium which measures the amount of nitrite reduced.

$$\text{NiR activity} = \frac{\text{Concentration of std}}{\text{O.D. of std}} \times \frac{\text{O.D. of sample}}{\text{Fresh weight}} \times \frac{\text{Total volume}}{\text{Aliquot taken}} \times \text{Dilution factor} \times \text{Incubation time}$$

GS (EC 6.3.1.2) was estimated by the method of Kanamori and Matsumto [27]. Leaves weighing 0.5 g were macerated in 5 mL of 0.1 M phosphate buffer (pH 7.5) containing 5 mM cysteine and 300 mg polyvinyl pyrollidone (PVP). After centrifugation at 10,000× *g* for 10 minutes, the proteins in the supernatant were precipitated using ammonium sulphate and aged overnight. After this step, centrifugation was done at 15,000× *g* for 20 minutes. The supernatant so obtained was dissolved in 0.1 M phosphate buffer (pH 7.5) and dialysed for 24 hour using dialysing bags. This extract was then used for assay of both GS and GOGAT enzymes. The assay protocol consisted of 0.5 mL of 0.2 M tris-HCl buffer (pH = 7.5), 0.2 mL of 0.05 M ATP (pH = 7.0), 0.5 mL of 0.5 M sodium glutamate, 0.1 mL of 1 M NH_2OH (pH = 7.0) (freshly prepared), 0.1 mL of 0.1 M cystiene and appropriate enzyme extract. The final volume was made 3 mL with distilled water. The reaction was started by addition of sodium glutamate (1 mL) which was omitted in blank. After incubation at 30 °C for 15 minutes, γ-glutamylhydroxymate formed was determined by adding 1 mL of ferric chloride reagent (0.37 M $FeCl_3 \cdot 6H_2O$, 0.067 N HCl and 0.20 M TCA). The reaction mixture was centrifuged and absorbance was read at 540 nm, using γ-glutamylhydroxymate as standard. The GS activity was expressed as moles γ-glutamylhydroxymate formed per gram tissue FW.

GOGAT activity (EC-1.4.7.1) was estimated by Bulen [28] method. The assay system consisted of 1.7 mL of 0.2 M TrisHCl buffer (pH 8.3), 0.5 mL of 0.2 M α-ketoglutaric acid, 0.5 mL of 1.5 M glutamine, 0.5 mL of 4.0 mM NADH and appropriate (0.1 mL) enzyme extract. The decrease in absorbance at 340 nm was recorded every 2 seconds for a period of 120 seconds. The enzyme activity was expressed as µmol NADH oxidized $min^{-1}g^{-1}$ tissue FW.

$$\text{GOGAT activity} = \frac{\Delta A_{340}/\text{minute}}{6.22} \times \frac{\text{Volume of reaction mixture}}{\text{Volume of sample taken for reaction}} \times \text{Dilution factor} \times \frac{\text{Total volume of extract}}{\text{Tissue weight taken}}$$

2.4 Estimation of Total Soluble Proteins

Total soluble proteins were estimated by the method of Lowry *et al.* [29]. Five hundred milligrams (500 mg) leaf samples were weighed, macerated in pestle and mortar in 2 mL of phosphate buffer (pH 7.4) and the material was transferred to centrifuge tubes. The homogenate was centrifuged at 10,000 rpm for 20 minutes and the supernatant was collected. Ten microliters (10 µL) of this supernatant was taken in duplicate and diluted to 1.0 mL with distilled water. Five milliliters (5 mL) of reagent C {freshly prepared by mixing reagent A [4% sodium carbonate (4 g/100 mL) in 0.2 N NaOH (0.8 g/100 mL)] and reagent B [1% $CuSO_4 \cdot 5H_2O$ (1 g/100 mL) in 2% sodium potassium tartrate (2 g/100 mL)] in ratio of 25:1 (*v*/*v*)} was added in each tube, the contents were shaken and after 10 minutes, 0.5 mL of Folin-Ciocalteau's reagent was added. Contents were shaken and after 30 minutes, absorbance was read at 750 nm, using UV 2600 spectrophotometer (Techcomp). The concentration of protein samples was calculated from the standard curve of Bovine Serum Albumin (10-70 µg).

$$\text{Proteins} = \frac{\text{Concentration of std}}{\text{O.D. of std}} \times \frac{\text{O.D. of sample}}{\text{Fresh weight}} \times \frac{\text{Total volume}}{\text{Aliquot taken}}$$

2.5 Statistical Analysis

The statistical significance of the differences in the measured parameters between the treatments was tested by the analysis of variance (ANOVA) test using SPSS (Version 16.0 for Windows). The values shown in the figures are the mean values ± SE. Mean values are significantly different at $p < 0.05$.

3. Results

3.1 Photosynthetic Pigments

Photosynthetic pigments varied significantly within the genotypes at the three stages of crop growth. N did not vary significantly for chlorophyll *a* at vegetative and siliquing stages but had marked impact on chlorophyll *b*, total chlorophyll, chlorophyll *a*/*b* and carotenoids at all the studied three stages of crop growth (Table 1).

At vegetative stage chlorophyll *a* increased marginally with increased N at 125 kg/ha in GSC-7, Hyola PAC 401 and PGSH-52. Chlorophyll *b* was 0.39, 0.44 and 0.52 mg/g FW with N_{100} and increased to 0.47, 0.49 and 0.52 mg/g FW in GSC-7, Hyola 401 and PGSH-52 respectively. Total chlorophyll improved significantly in GSC-7 and PGSH-52 with N_{125}. Total chlorophyll improved marginally with N_{125} in Hyola PAC 401. Decline in chl *a*/ chl *b* was recorded in all the cultivars with N_{125}. Carotenoids increased in GSC-7, Hyola PAC 401 and PGSH-52 with increase in nitrogen (N_{125}). Mean increase in chlorophyll *a* content was 5.0%, chlorophyll *b* 9.3%, total chlorophyll 6.3% and carotenoids 12.3% over N_{100}. However, chl *a*/chl *b* registered a drop of 6.1% with N_{125} over N_{100} (Table 1 and Fig. 1).

At flowering stage there was a significant increase in chlorophyll *a* and chlorophyll *b* with N_{125} in GSC-7, Hyola PAC 401 and PGSH-52. Total chlorophyll increased in GSC-7 and Hyola PAC 401. Total chlorophyll improved significantly from 2.60 to 3.05 mg/g FW with increased N in PGSH-52. Decline in chl *a*/chl *b* was recorded in all the cultivars with N_{125}. Carotenoids were recorded maximum in Hyola PAC 401 and increased in GSC-7 and PGSH-52 with increased N. Mean upsurge in chlorophyll *a* was 10.0%, chlorophyll *b* 11.3%, total chlorophyll 12.8% and carotenoids 5.0% over N_{100}. However, chl *a*/chl *b* registered a decrease of 6.7% with N_{125} over N_{100} (Table 1 and Fig. 1).

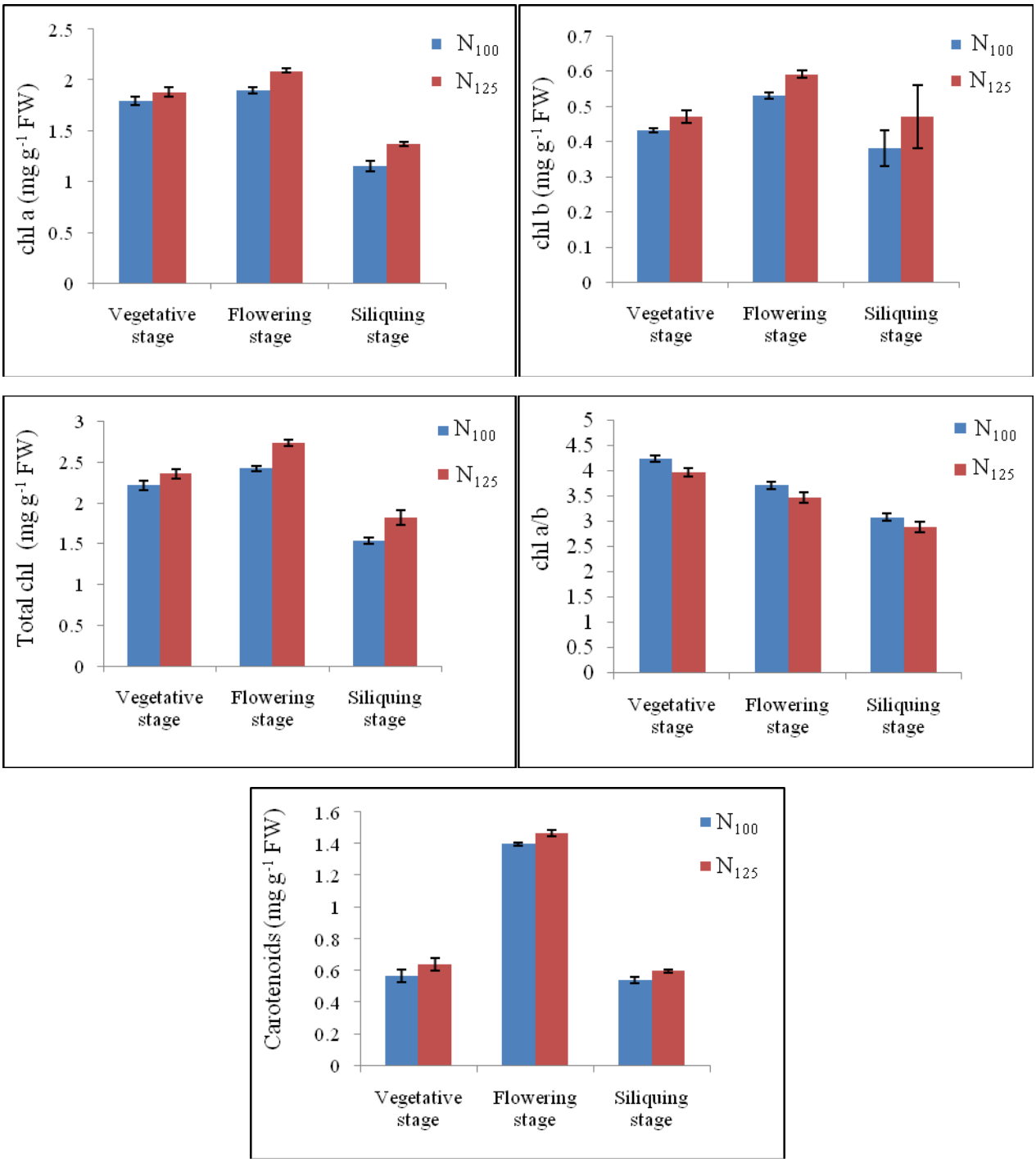

Fig. 1 Effect of nitrogen on photosynthetic pigments (mean values) in *Brassica napus* genotypes.

At siliquing stage chlorophyll *a* content improved with increased N at 125 kg ha^{-1} in GSC-7, Hyola PAC 401 and PGSH-52. Chlorophyll *b* increased marginally with N_{125}. Total chlorophyll was 1.48 mg/g FW in GSC-7 and increased to 1.77 mg g $^{-1}$ FW with N_{125}. Total chlorophyll of 1.81 was recorded at recommended N dose and improved marginally to 1.96 mg g $^{-1}$ FW with N_{125} in Hyola PAC 401. Total chlorophyll improved

Table 1 Effect of nitrogen on chlorophyll content and carotenoids (mg g $^{-1}$ FW) at three stages in *Brassica napus*.

Genotypes	Chl *a*			Chl *b*			Total chl			Chl *a*/*b*			Carotenoids		
	N_{100}	N_{125}	Average	N_{100}	N_{125}	Average	N_{100}	N_{125}	Average	N_{100}	N_{125}	Average	N_{100}	N_{125}	Average
							Vegetative stage								
GSC-7	1.72 ± 0.003	1.89 ± 0.005	1.81 ± 0.03	0.39 ± 0.004	0.47 ± 0.003	0.43 ± 0.013	2.11 ± 0.002	2.36 ± 0.003	2.24 ± 0.04	4.41 ± 0.02	4.02 ± 0.17	4.22 ± 0.04	0.51 ± 0.004	0.59 ± 0.004	0.55 ± 0.005
HyolaPAC401	1.78 ± 0.002	1.79 ± 0.005	1.79 ± 0.02	0.48 ± 0.005	0.49 ± 0.003	0.49 ± 0.009	2.26 ± 0.003	2.28 ± 0.003	2.27 ± 0.03	3.71 ± 0.02	3.65 ± 0.08	3.68 ± 0.04	0.60 ± 0.004	0.70 ± 0.007	0.65 ± 0.015
PGSH-52	1.95 ± 0.004	2.00 ± 0.005	1.99 ± 0.01	0.43 ± 0.003	0.52 ± 0.004	0.48 ± 0.012	2.37 ± 0.003	2.52 ± 0.003	2.45 ± 0.01	4.53 ± 0.11	3.85 ± 0.02	4.19 ± 0.08	0.63 ± 0.004	0.77 ± 0.005	0.70 ± 0.020
Mean	1.81 ± 0.003	1.89 ± 0.005		0.43 ± 0.004	0.49 ± 0.003		2.23 ± 0.003	2.37 ± 0.003		4.16 ± 0.05	3.95 ± 0.09		0.58 ± 0.004	0.67 ± 0.005	
CD (p = 0.05)	G = 0.007, N = NS G × N = 0.009			G = 0.01, N = 0.01 G × N = 0.02			G = 0.005, N = 0.005 G × N = 0.008			G = 0.01, N = 0.009 G × N = 0.02			G = 0.02, N = 0.01 G × N = 0.02		
							Flowering stage								
GSC-7	1.82 ± 0.006	2.09 ± 0.003	1.96 ± 0.02	0.42 ± 0.005	0.52 ± 0.004	0.47 ± 0.02	2.24 ± 0.007	2.61 ± 0.006	2.43 ± 0.03	4.33 ± 0.03	4.02 ± 0.03	4.18 ± 0.02	1.33 ± 0.003	1.47 ± 0.002	1.40 ± 0.019
HyolaPAC401	2.01 ± 0.003	2.16 ± 0.003	2.01 ± 0.02	0.69 ± 0.004	0.71 ± 0.003	0.70 ± 0.02	2.70 ± 0.003	2.95 ± 0.004	2.83 ± 0.04	2.91 ± 0.04	2.73 ± 0.03	2.82 ± 0.04	1.60 ± 0.004	1.62 ± 0.004	1.61 ± 0.004
PGSH-52	1.99 ± 0.004	2.20 ± 0.005	2.01 ± 0.01	0.61 ± 0.005	0.68 ± 0.005	0.65 ± 0.02	2.60 ± 0.005	3.05 ± 0.003	2.83 ± 0.03	3.26 ± 0.05	3.23 ± 0.05	3.25 ± 0.10	1.54 ± 0.003	1.58 ± 0.004	1.56 ± 0.006
Mean	1.94 ± 0.004	2.15 ± 0.004		0.57 ± 0.005	0.64 ± 0.004		2.51 ± 0.005	2.87 ± 0.004		3.50 ± 0.004	3.33 ± 0.004		1.49 ± 0.003	1.56 ± 0.003	
CD (p = 0.05)	G = 0.006, N = 0.009 G × N = 0.02			G = NS, N = 0.005 G × N = 0.009			G = 0.01, N = 0.007 G × N = 0.01			G = 0.009, N = 0.007 G × N = 0.01			G = 0.0005, N = 0.0003 G × N = 0.005		
							Siliquing stage								
GSC-7	1.16 ± 0.004	1.37 ± 0.004	1.27 ± 0.05	0.32 ± 0.004	0.40 ± 0.005	0.36 ± 0.01	1.48 ± 0.007	1.77 ± 0.005	1.63 ± 0.06	3.63 ± 0.04	3.43 ± 0.03	3.53 ± 0.07	0.53 ± 0.003	0.62 ± 0.005	0.58 ± 0.013
HyolaPAC401	1.31 ± 0.005	1.40 ± 0.006	1.36 ± 0.05	0.50 ± 0.004	0.56 ± 0.005	0.53 ± 0.01	1.81 ± 0.007	1.96 ± 0.006	1.89 ± 0.05	2.62 ± 0.02	2.50 ± 0.01	2.56 ± 0.07	0.55 ± 0.003	0.60 ± 0.005	0.58 ± 0.010
PGSH-52	1.29 ± 0.003	1.53 ± 0.005	1.41 ± 0.01	0.42 ± 0.003	0.56 ± 0.005	0.49 ± 0.01	1.71 ± 0.010	2.09 ± 0.008	1.90 ± 0.01	3.07 ± 0.03	2.73 ± 0.02	2.90 ± 0.03	0.57 ± 0.010	0.63 ± 0.030	0.60 ± 0.016
Mean	1.16 ± 0.004	1.43 ± 0.005		0.41 ± 0.004	0.51 ± 0.005		1.67 ± 0.008	1.94 ± 0.007		3.11 ± 0.03	2.89 ± 0.02		0.55 ± 0.005	0.62 ± 0.013	
CD (p = 0.05)	G = 0.007, N = NS G × N = 0.009			G = 0.01, N = 0.01 G × N = 0.02			G = 0.005, N = 0.005 G × N = 0.008			G = 0.01, N = 0.009 G × N = 0.02			G = 0.02, N = 0.01 G × N = 0.02		

significantly from 1.71 to 2.09 mg g^{-1} FW with increased N in PGSH-52. Decline in chl *a*/chl *b* was recorded in all the cultivars with N_{125}. Carotenoids increased marginally in all the cultivars with improved N content. Mean increase in chlorophyll *a* was 19.1%, chlorophyll *b* 23.7%, total chlorophyll 19.6% and carotenoids 11.1% over N_{100}. However, chlorophyll *a*/*b* registered a decline of 6.5% with N_{125} over N_{100} (Table 1 and Fig. 1).

3.2 Activities of the N Assimilating Enzymes

N assimilating enzymes varied significantly with the genotypes at the three stages of crop growth. N improved the activities of all the four studied N-assimilating enzymes at the three stages of crop growth and development in the cultivars. Activities of all the enzymes were maximum at flowering followed by a decline. Hyola PAC 401 had higher enzymatic activities followed by GSC-7 at the studied stages of crop growth.

NR activity at vegetative stage increased in GSC-7, Hyola PAC 401 and PGSH-52 with the increased N level to 125 kg N ha^{-1}. At flowering, with N_{100} NR activity maximum in Hyola PAC 401 followed by PGSH-52. Upsurge in NR activity was recorded with N_{125} in GSC-7. Hyola PAC 401 and PGSH-52 registered improved NR activity. At siliquing stage, with increase in N level to 125 kg N ha^{-1}, NR activity was maximum in Hyola PAC 40. Treatment mean revealed 15.4% increased NR activity at vegetative stage, 11.5% at flowering and 15.4% at siliquing stage with N_{125} (Table 2 and Fig. 2).

Hyola PAC 401 had maximum NiR activity with N_{100} at vegetative stage. N level at 125 kg N ha^{-1} enhanced NiR activity in GSC-7 being highest in Hyola PAC 401. With recommended N-dose, NiR activity was maximum in Hyola PAC 401 trailed by GSC-7 and with lesser increase in PGSH-52. With increased N level the NiR activity also increased in GSC-7, Hyola PAC 401 and PGSH-52 at the flowering stage. At siliquing stage GSC-7 and Hyola PAC 401 had comparable NiR activity whereas PGSH-52 had lower NiR activity with N_{100}. With higher N level, NiR activity improved in GSC-7, maximum activity assayed was in Hyola PAC 401 and improved in PGSH-52. Treatment mean revealed 41.1% increase in NiR activity at vegetative stage, 24.2% at flowering and 41.3% at siliquing stage with N_{125} (Table 3 and Fig. 2).

At vegetative stage, Hyola PAC 401 had maximum GS activity with N_{100}. The GS activity increased in *B. napus* variety and both the canola hybrids with increased dose of nitrogen. At flowering, with N_{100} maximum activity was in Hyola PAC 401. With increased N level GSC-7, Hyola PAC 401 and PGSH-52 witnessed an increase in the activity. At siliquing stage with increase in N level to 125 kg N ha^{-1}, Hyola PAC 401 had maximum activity followed by GSC-7. Treatment mean revealed 11.4% increased GS activity at vegetative stage, 12.2% at flowering and 15.7% at siliquing stage with N_{125} (Table 4 and Fig. 2).

Table 2 Effect of nitrogen on NR activity (µmoles NO_2^- formed g^{-1} h^{-1}) in *Brassica napus* genotypes.

Genotypes	Vegetative stage			Flowering stage			Siliquing stage		
	N_{100}	N_{125}	Average	N_{100}	N_{125}	Average	N_{100}	N_{125}	Average
GSC-7	0.420 ± 0.001	0.489 ± 0.004	0.455	0.485 ± 0.003	0.535 ± 0.003	0.510	0.320 ± 0.0	0.393 ± 0.003	0.357
Hyola PAC 401	0.488 ± 0.003	0.505 ± 0.003	0.497	0.555 ± 0.003	0.603 ± 0.003	0.579	0.380 ± 0.004	0.410 ± 0.004	0.395
PGSH-52	0.375 ± 0.003	0.488 ± 0.005	0.432	0.423 ± 0.003	0.493 ± 0.003	0.458	0.295 ± 0.003	0.345 ± 0.003	0.320
Mean	0.428 ± 0.002	0.494 ± 0.004		0.488 ± 0.003	0.544 ± 0.003		0.332 ± 0.002	0.383 ± 0.003	
CD (p = 0.05)	G = 0.009, N = 0.006, G × N = 0.01			G = 0.008, N = 0.004, G × N = 0.006			G = 0.01, N = 0.004, G × N = 0.006		

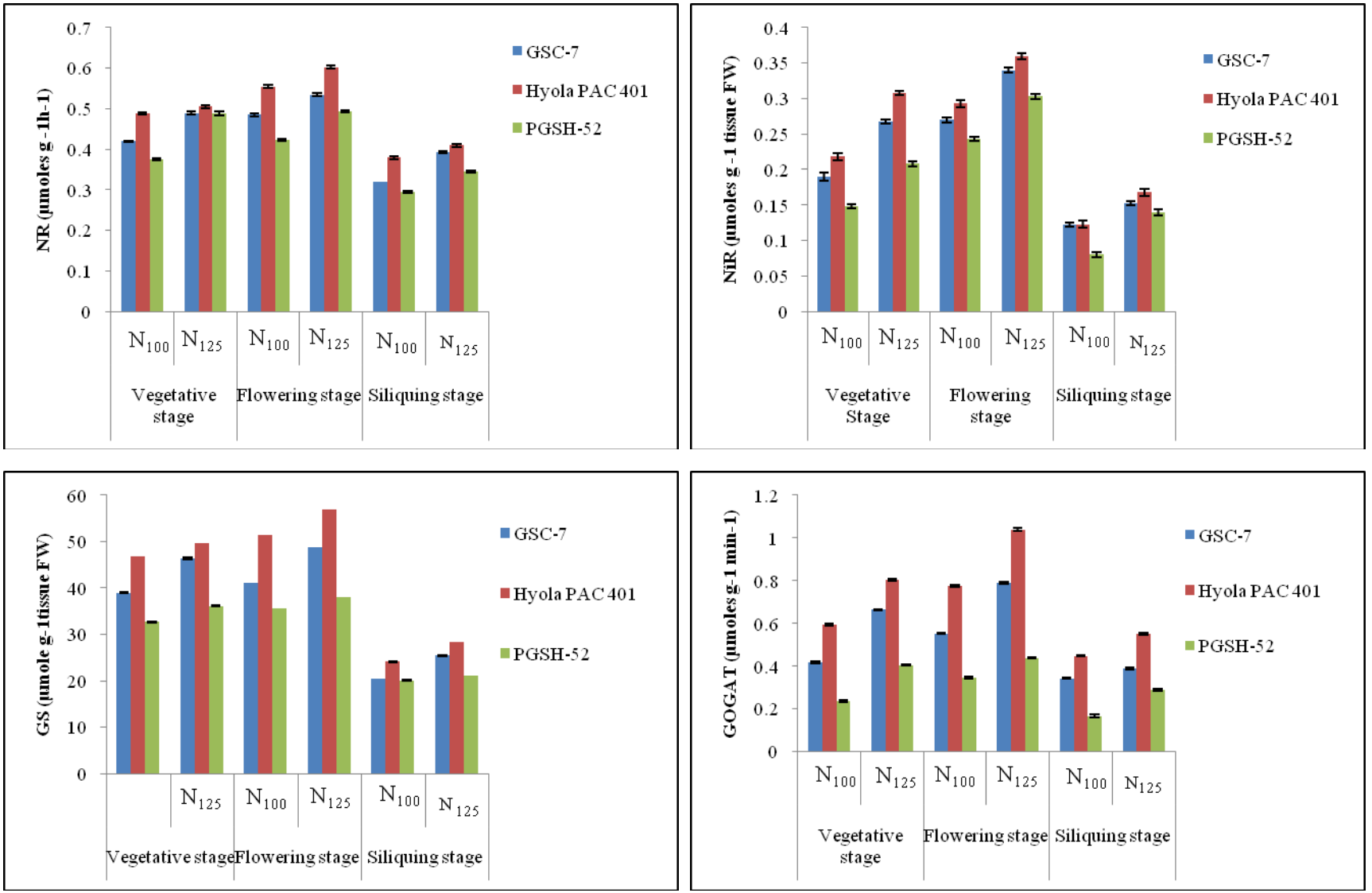

Fig. 2 Effect of nitrogen on N assimilating enzymes in *Brassica napus* genotypes.

Table 3 Effect of nitrogen on NiR activity (µmoles NO_2^- released g^{-1} FW) in *Brassica napus* genotypes.

Genotypes	Vegetative stage			Flowering stage			Siliquing stage		
	N_{100}	N_{125}	Average	N_{100}	N_{125}	Average	N_{100}	N_{125}	Average
GSC-7	0.190 ± 0.006	0.268 ± 0.003	0.229	0.270 ± 0.004	0.340 ± 0.004	0.305	0.123 ± 0.003	0.153 ± 0.003	0.138
Hyola PAC 401	0.218 ± 0.005	0.308 ± 0.003	0.263	0.293 ± 0.005	0.360 ± 0.004	0.327	0.123 ± 0.005	0.168 ± 0.005	0.146
PGSH-52	0.148 ± 0.003	0.208 ± 0.003	0.178	0.243 ± 0.003	0.303 ± 0.003	0.273	0.080 ± 0.004	0.140 ± 0.004	0.110
Mean	0.185 ± 0.005	0.261 ± 0.003		0.269 ± 0.004	0.334 ± 0.004		0.109 ± 0.004	0.154 ± 0.004	
CD (p = 0.05)	G = 0.01, N = 0.005, G × N = 0.008			G = 0.007, N = 0.008, G × N = NS			G = 0.01, N = 0.006, G × N = 0.01		

Table 4 Effect of nitrogen on GS (µmol ã-glutamylhydroxomate formed g^{-1} tissue FW) in *Brassica napus* genotypes.

Genotypes	Vegetative stage			Flowering stage			Siliquing stage		
	N_{100}	N_{125}	Average	N_{100}	N_{125}	Average	N_{100}	N_{125}	Average
GSC-7	39.0 ± 0.12	46.4 ± 0.14	42.7	41.1 ± 0.06	48.8 ± 0.08	45.0	20.5 ± 0.01	25.4 ± 0.06	23.0
Hyola PAC 401	46.8 ± 0.08	49.6 ± 0.04	48.2	51.4 ± 0.08	56.9 ± 0.02	54.2	24.1 ± 0.06	28.4 ± 0.07	26.3
PGSH-52	32.7 ± 0.09	36.1 ± 0.13	34.4	35.6 ± 0.03	38.1 ± 0.02	36.9	20.1 ± 0.05	21.2 ± 0.02	20.1
Mean	39.5 ± 0.1	44.0 ± 0.1		42.7 ± 0.06	47.9 ± 0.04		21.6 ± 0.04	25.0 ± 0.05	
CD (p = 0.05)	G = 0.41, N = 0.10, G × N = 0.18			G = 0.07, N = 0.13, G × N = 0.22			G = 0.08, N = 0.12, G × N = 0.21		

Table 5 Effect of nitrogen on GOGAT (µmoles NADH oxidized g^{-1} min^{-1}) in *Brassica napus* genotypes.

Genotypes	Vegetative stage			Flowering stage			Siliquing stage		
	N_{100}	N_{125}	Average	N_{100}	N_{125}	Average	N_{100}	N_{125}	Average
GSC-7	0.418 ± 0.005	0.665 ± 0.003	0.542	0.553 ± 0.003	0.789 ± 0.005	0.671	0.345 ± 0.003	0.390 ± 0.004	0.368
Hyola PAC 401	0.595 ± 0.003	0.803 ± 0.005	0.699	0.775 ± 0.003	1.040 ± 0.008	0.908	0.448 ± 0.003	0.550 ± 0.004	0.499
PGSH-52	0.238 ± 0.005	0.405 ± 0.003	0.322	0.348 ± 0.005	0.438 ± 0.003	0.393	0.168 ± 0.005	0.290 ± 0.004	0.229
Mean	0.417 ± 0.004	0.624 ± 0.004		0.559 ± 0.004	0.756 ± 0.005		0.320 ± 0.004	0.410 ± 0.004	
CD (p = 0.05)	G = 0.007, N = 0.008, G × N = 0.01			G = 0.01, N = 0.009, G × N = 0.02			G = 0.01, N = 0.007, G × N = 0.01		

Table 6 Effect of nitrogen on protein content (mg g^{-1} FW) in leaves of *Brassica napus* genotypes.

Genotypes	Vegetative stage			Flowering stage			Siliquing stage		
	N_{100}	N_{125}	Average	N_{100}	N_{125}	Average	N_{100}	N_{125}	Average
GSC-7	43.3 ± 0.5	51.6 ± 0.4	47.46	67.9 ± 0.3	73.8 ± 0.3	70.8	42.0 ± 0.4	42.8 ± 0.3	42.4
Hyola PAC 401	46.7 ± 1.1	48.2 ± 0.7	47.42	65.9 ± 0.3	71.8 ± 0.2	68.9	44.5 ± 0.5	47.0 ± 0.7	45.7
PGSH-52	44.2 ± 0.2	44.7 ± 0.4	44.43	59.5 ± 0.3	68.6 ± 0.6	64.1	40.9 ± 0.6	48.9 ± 0.1	44.9
Mean	44.7 ± 0.6	48.1 ± 0.5		64.42 ± 0.3	71.4 ± 0.4		42.5 ± 0.5	46.2 ± 0.4	
CD (p = 0.05)	G = 1.12, N = 1.43, G × N = 2.47			G = 1.07, N = 0.67, G × N =1.16			G = 1.01, N = 0.69, G × N = 1.19		

GSC-7 possessed increased GOGAT activity at vegetative stage along with Hyola PAC 401 and

PGSH-52 with increasing N level to 125 kg N ha-1. At flowering, with N_{100} the trend in GOGAT activity remained the same maximum in Hyola PAC 401 trailed by GSC-7 and PGSH-52. With higher N-dose GOGAT activity improved marginally in GSC-7 whereas significant increase was registered in Hyola PAC 401 and PGSH-52. At siliquing stage, with increase in N level to 125 kg N ha^{-1}, GOGAT activity improved as in the previous two stages of crop growth to maximum in Hyola PAC 401 followed by GSC-7 and lesser increase was in PGSH-52. Treatment mean revealed 49.6% higher GOGAT activity at vegetative stage, 35.2% at flowering and 28.1% at siliquing stage with N_{125} (Table 5 and Fig. 2).

3.3 Total Soluble Protein

Total soluble proteins varied significantly with the genotypes at the three stages of crop growth. Total soluble protein content was maximum at flowering stage. Hyola PAC 401 had maximum protein content of 46.7, 65.9 and 44.5 mg g^{-1} FW at vegetative, flowering and siliquing stages respectively with N_{100}. Increased N level of 125 kg N ha^{-1} enhanced protein content at the three studied crop growth stages. Maximum protein content was 51.6 mg g^{-1} FW in GSC-7 at vegetative stage, 71.8 mg g^{-1} FW in Hyola PAC-401 at flowering and 48.9 mg g^{-1} FW in PGSH-52 at siliquing stage. Treatment mean revealed 7.6% higher protein content at vegetative stage, 10.9% at flowering and 11.1% at siliquing stage with N_{125} (Table 6).

4. Discussion

Nitrogen is an important component of several important compounds in plant cells. Plant N status is highly dependent on N-fertilization, which is also a major component of chlorophyll and amino acids, the building blocks of proteins. Increase in N supply can stimulate plant growth and productivity as well as photosynthetic activity through increased amounts of stromal and thylakoid proteins in leaves. Several studies have shown the availability of N has influenced the growth and photosynthetic functions to a great extent in oilseed brassicas [30]. Photosynthetic pigments along with other accessory pigments play an important role in photosynthesis as they capture and transfer light energy. The present investigation revealed increase in chlorophyll *a*, chlorophyll *b* and total chlorophyll with increase in N level while a decrease was observed in chl *a*/ chl *b*. Further, chlorophyll *a*, chlorophyll *b*, total chlorophyll and carotenoids were higher in plants which received 125 kg N ha^{-1} as compared to 100 kg N ha^{-1} as depicted in Fig. 1. Our findings are in accordance with those of Ogunlela *et al.* [31] where contents of leaf chlorophyll *a*, chlorophyll *b* and total chlorophyll enhanced with N supply but resulted in very little influence on chlorophyll *a*/*b* ratios except that increasing N supply tended to reduce these ratios. These authors concluded that the variations in the leaf chlorophyll content of rape plants in response to N nutrition may be function of leaf age and position which may have great significance on physiological implications. The highest levels of Chl *a*, Chl *b*, and Chl *a* + *b* were found in the plants that received both 150 and 200 kg N ha^{-1}, followed by the plants that received 100 kg N ha^{-1} and their lowest levels were noted in the control plots [32]. The maximum leaf chlorophyll content was seen in accessions ACN 44 and ACN 81 of *B. napus* and accession MLM 41 of *B. juncea* (except N_0 at pre-anthesis stage), regardless of growth stage and nitrogen status [5]. Chlorophyll content of leaves during vegetative and generative phases increased significantly with N supply up to 100 ppm. N supply of 30 ppm was found to create N stress while 170 ppm was observed as an excessive supply. However, N supply of 100 ppm was found to enhance the leaf expansion [33].

N has a strong impact on growth, development and productivity of a plant and is associated with many of its metabolic processes. Nitrogen assimilation is not a process in isolation, as it needs to be tightly linked to

carbon metabolism and photosynthesis. NR and NiR are reliant on photosynthetic activity and in particular NiR due to its localization in the chloroplast underpins the strong relationship between C and N metabolism. Crop plants use nitrogen in the form of nitrate (NO_3^-) and ammonium (NH_4^+) which is available in the soil. There is reduction of nitrate (NO_3^-) to nitrite (NO_2^-) and then to ammonium (NH_4^+) during the process of nitrate uptake and assimilation. Through the glutamine synthase pathway there is primary assimilation of ammonia in the plants [5]. The GDH pathway works for the assimilation of ammonia when it is present in excess [6]. This process of nitrate uptake and assimilation depends on the major enzymatic activities namely, NR, NiR, GOGAT, GS and GDH. In present study NR, NiR, GS and GOGAT activity was up regulated during flowering stage trailed by siliquing stage and was least at vegetative stage. However, enzymatic activities increased linearly with the increase in N dose. Our studies are concomitant with the findings of Gupta *et al.* [5] in *B. napus*, Saberi and Hassan [34] in *B. juncea* and Kaur [35] in *B. carinata*.

With the help of amino transferases, glutamate amino group can be transferred to different amino acids [9]. In fruits of green bean (*Phaseolus vulgaris*), with increase in N doses the activity of NR also increased. The expected increase with the increase in N level was related to NR acting as a substrate inducible enzyme [10]. Higher NR activity in rice leaves was either with NO_3^- or NH_4^+ nitrogen form. The leaves of the NO_3^- grown plants exhibit higher NR activity as compared to those of NH_4^+ grown plants [11]. The nitrate is translocated to the chloroplast where it is reduced to ammonium by NiR. Glutamine is formed as the glutamine synthase which fixes ammonium on a glutamate molecule and this glutamine reacts subsequently with 2-oxaloglutarate, being catalyzed by GOGAT. NiR activity is increased by 8.5% with N_{125} as compared to N_{100} in *B. carinata* [35]. Earlier, Nathawat *et al*. [12] reported maximum enhancement in the activity of ammonium assimilating enzymes during flowering with 100 kg ha $^{-1}$ of N. GOGAT activity increased by 13.6% and protein content by 11.1% with N_{125} over N_{100} [35]. The enhancement in protein content with increase in N-level has been reported previously in *B. napus* [35], in *B. juncea* [36] and later in both *B. napus* and *B. juncea* by Gupta *et al.* [5] and recently by Brennan [37] in *B. napus*. Protein content was higher at flowering stage in the present study and improved by 8.6% in GSC 7, 8.9% in Hyola PAC 401 and maximum of 15.2 % in PGSH52 out of two hybrids in *B. napus* with mean increase of 10.9% with 125 kg N ha^{-1} as compared to 100 kg N ha^{-1}, the recommended dose.

5. Conclusions

Photosynthetic pigments increased with increase in nitrogen to 125 kg ha^{-1} except for chlorophyll *a*/*b*. At flowering stage, total chlorophyll, accessory pigments/carotenoids, all the four nitrogen assimilating enzymes and protein content were maximum and enhanced with higher N-dose. Hyola PAC 401 possessed maximum enzymatic activities and protein content followed by GSC7 at the studied 3 stages of crop growth except for NiR activity which was at par in GSC7, a canola variety and Hyola PAC 401, a hybrid at recommended N dose (N_{100}) only at siliquing stage. N at 125 kg ha^{-1} was beneficial in enhancing photosynthetic pigments along with the nitrogen assimilation particularly GS/GOGAT.

Author Contribution

P.S. contributed to the design, methodology of the experiments and V.S. field trails. N.K.G. performed biochemical analysis and wrote the paper. P.S. reviewed and edited the manuscript.

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