



THE ROLE OF LEAF NITROGEN CONTENT IN PROTECTING PHOTO-INHIBITORY DAMAGE IN *HEVEA* SEEDLINGS UNDER LOW TEMPERATURE STRESS

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Received on 24 June, 2008, Revised on 19 March, 2009

SUMMARY

An experiment was conducted on *Hevea brasiliensis* seedlings with different levels of leaf chlorophyll content index (CCI) to study the role of leaf nitrogen in protecting young leaves from photoinhibitory damage during winter period. Seedlings were categorized as low nitrogen (LN), medium nitrogen (MN) and high nitrogen (HN) with corresponding 6.14, 16.41 and 34.84 CCI values and 0.51%, 1.18% and 1.95% leaf nitrogen content. During peak winter period, the photosynthetic rate was significantly lower ($1.48 \mu\text{mol CO}_2 \text{ m}^{-2}\text{s}^{-1}$) in LN in comparison to MN ($5.9 \mu\text{mol CO}_2 \text{ m}^{-2}\text{s}^{-1}$) and HN ($7.2 \mu\text{mol CO}_2 \text{ m}^{-2}\text{s}^{-1}$) seedlings. The mesophyll efficiency (C_i/g_s) was higher in HN plants than in LN plants. The potential quantum efficiency of PSII (dark adapted F_v/F_m) was reduced by 67%, 22% and 17% in LN, MN and HN leaves respectively at peak winter period (January) in relation to an optimum period (September). Similarly, the efficiency of PSII photochemistry (Φ_{PSII}) was reduced by 65%, 30% and 25% in these three categories of seedlings respectively. The results of this experiment suggest that higher leaf nitrogen content can protect the plants from photosynthetic down regulation caused by low temperature stress during winter season.

Key words: Chlorophyll content index, chlorophyll fluorescence, *Hevea*, leaf nitrogen, photoinhibition.

INTRODUCTION

Plant nutrients are one of the major factors that is responsible for proper growth and development of plants under any environmental condition. The role of the nutrients becomes more crucial under any stressful environment such as temperature extremes (Caporn *et al.* 1994, Saebo *et al.* 2001), high light intensities (Müller *et al.* 2001, Fernandes *et al.* 2002) and drought (Damatta *et al.* 2002). The impact of these environmental adversities becomes more severe when the plants are nutritionally deficient. Among all the nutrients, nitrogen usually remains deficient in plants grown in denuded marginal land. This is the most important nutrient that acts as prime constituent of

chlorophyll, protein molecules, vitamins and cellular membranes and many other macro molecules. Thereby, it becomes the integral part of light harvesting process of photosynthesis and other components of various metabolic pathways of plants. Hence, leaf nitrogen content becomes very crucial to optimize various metabolic processes.

In general, a positive correlation between light saturated photosynthesis of a leaf and its nitrogen concentration is well recognized (Field and Mooney 1986, Evan 1989). This relationship is mainly due to the large proportion of leaf nitrogen allocated only in chloroplast, most of which is present in photosynthetic enzymes and other intermediates (Evans and Seemann 1989, Makino

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and Osmond 1991). Out of chloroplast protein, a major portion is allocated to the primary carboxylating enzyme, Rubisco (Ribulose 1-5 bisphosphate carboxylase/oxygenase) (Evans 1989, Makino *et al.* 1992). Therefore, a close interaction between photosynthesis and nitrogen nutrition is likely to exist depending on the severity of stress. Photosynthetic response is shown to be dependent on leaf nitrogen content in N-deficient and N-sufficient plants (Sugiharto *et al.* 1990). However, another report says that maximum photochemical efficiency of PSII reaction centers (dark adapted Fv/Fm) was not influenced by increased N supply (Pankovic *et al.* 2000). On the contrary, recovery from high light stress in *Coffea arabica* was N dependent (Ramalho *et al.* 2000). Osmond and co-workers have made it evident that high nitrogen plants grown under high light are less susceptible to photoinhibition (Balachandran and Osmond 1994, Skillman and Osmond 1998). Moreover, some extrinsic proteins of PSII such as water splitting enzyme complex degrade easily at low temperature (Terashima *et al.* 1989, Aro *et al.* 1993). In general, plants have numerous anti-oxidant enzyme systems to prevent calamitous damage to component proteins and lipids. It is increasingly being evident that regeneration of antioxidant system such as ascorbate (Vitamin C) can be a variable electron sink (Asada 1998). Therefore, greater nitrogen assimilation in the form of proteins in photosynthetic tissue represents a substantial electron sink which might protect PSII against photo damage eventually.

Protection of PSII reaction centers of photosynthetic machinery or reduction of photoinhibitory damage becomes very crucial for survival and growth of the seedlings of *Hevea brasiliensis* when young plants grow through a period of cold stress, especially when they are grown in north eastern region of India. In this region, *Hevea* trees that are originated from tropical humid climate become vulnerable to sub-optimal temperature during winter season (Jacob *et al.* 1999, Alam and Jacob 2002, Ray *et al.* 2004). The susceptibility to low temperature is mainly due to reduction in photochemical efficiency and thereby photon energy absorbed by the plants becomes excess than required for CO₂ reduction (Fryer *et al.* 1998, Alam *et al.* 2005). Excess energy in

photosynthetic apparatus leads to photo oxidative damage (Fryer *et al.* 1998).

Hevea brasiliensis, being a highly cross pollinated tree species, produce largely heterozygous seeds and hence polyclonal seedling population. As phenotypic characteristics, seedlings show variable leaf chlorophyll content and other growth parameters etc. (Ray *et al.* 2003). The variability in leaf chlorophyll content usually reflects the different leaf nitrogen status of the seedlings. We have used these seedlings with variable leaf nitrogen content as model plants to study the role of nitrogen nutrition in protecting plants from oxidative stress such as low temperature coupled with high light intensities during winter period. Our hypothesis is that leaves with higher nitrogen content can have substantial electron sink for photochemistry than the leaves with low nitrogen content. Hence, those plants can minimize the diversion of excitation energy towards production of reactive oxygen species which otherwise can substantially damage the photosynthetic machinery. Thereby, leaves with high nitrogen can protect the plants from photoinhibitory damage during cold stress. In the present study, *Hevea* seedlings with different level of leaf nitrogen were subjected to gas exchange studies to determine their photosynthetic capability under stress condition. Further, same plants were also used to study the chlorophyll fluorescence characteristics to assess the extent of photoinhibition caused by prevailing stress.

MATERIALS AND METHODS

Experimental site and plant materials: The experiment was conducted on 5 months old *Hevea* seedlings grown in a seedlings nursery at Taranagar research farm, regional research station of the Rubber Research Institute of India (RRII), Agartala, Tripura (91°15' E, 23°25' N; 30 m above MSL) during January 2007. The soil (0-30 cm) of the experimental site contains 21% clay, 25% silt and 54% sand with pH of 4.6. The mean monthly maximum temperature ranged from 25.8°C to 29.0°C and the minimum temperature varied from 7.8°C to 12.9°C during December 2006 to February 2007 period. A subsequent experiment was conducted during the first week of September 2007 when the average

maximum and minimum temperatures were recorded as 31.5°C and 25 °C respectively. During that week, the total rainfall of 83.4 mm was well distributed leaving two days moderately sunny. The average relative humidity during the week was ranging from 97-87 % from morning to evening hours.

Selection of seedlings based on CCI, an estimate of Chlorophyll content: Polyclonal seedlings of *Hevea brasiliensis* are usually raised in nursery beds at a spacing of 23cm x 23cm for multiplication through bud grafting. From these seedlings, three categories, 10 plants in each group, were selected based on leaf chlorophyll content index (CCI) measured by a Chlorophyll meter (CCM 200, Opti-Sciences, MA, USA). Seedlings were categorized based on the assumption that dark green leaves are of high nitrogen types (HN), light green types as low nitrogen (LN) and intermediate green types were grouped as medium nitrogen (MN). The average CCI for LN seedlings was 6.14 ranging from 4.3 to 8.17. The MN seedlings recorded average CCI of 16.41 with the minimum of 13.77 and maximum CCI of 20.90. Similarly, higher average CCI of 34.84 was recorded by HN seedlings. The range of higher values of CCI is 26.87 to 41.37 (Table 1). Leaf N was also determined by analytical method and expressed in percentage. A correlation was drawn between the CCI and leaf N (%) to determine the reliability of CCI values to predict leaf nitrogen status. In each group, representative plants were taken for measuring growth parameters and other physiological traits. Nursery management practices were carried out as per the recommended procedures. Mulching between rows was done using dried forest undergrowths and grass cuttings. Irrigation was undertaken twice a week during the period of winter experiment. Plant height and leaf

number were recorded as an indication of growth of five months old seedlings.

Leaf nitrogen content: Three leaves from each plant were sampled randomly for N estimation. The samples without the midribs were oven-dried at 70°C till a constant weight was obtained. The dried leaf samples were ground to fine powder and a sub sample of 50 mg was taken for nitrogen analysis by Kjeldhal method (Wolf 1982). Nitrogen was determined after distillation of the digested leaf sample in an alkaline medium and NH₃ released was trapped into Boric acid, which is then back titrated with 0.02N H₂SO₄ using a automated Nitrogen analyzer (Kjeltec™2100, Foss, Sweden)

Gas exchange parameters: Photosynthetic rate (P_N), stomatal conductance (g_s), and CO₂ concentration in sub stomatal spaces (C_i), were measured with an open-flow portable infrared gas analyzer (CIRAS II, PP systems, UK). Measurements were recorded on five physiologically matured leaves taken from each plant. During the month of January, the gas exchange measurements were taken between 0900-1000 hours at 1500 mmolm⁻²s⁻¹ PPF (Photosynthetic photon flux) using automatic light (LED) source of the CIRAS II photosynthetic system which was comparable to ambient irradiance. Measurements were taken on a 2.5cm² area in the central part of the leaf blade leaving the mid rib away. The flow rate of air through the chamber was set to 250 ml min⁻¹. The CO₂ concentration of the intake air was maintained at 350 ppm at air vapour pressure deficit (VPD) was approximately 1.5 Kpa during measurements. Plants were irrigated on the previous day of gas exchange measurements.

Fluorescence studies: Chlorophyll fluorescence was measured with a portable fluorimeter (FMS2, Hansatech, UK) on the same leaves and the positions that was used to measure photosynthesis. The initial florescence (F_o) was recorded on leaves adapted to darkness for more than 20 minutes by using leaf clips supplied by Hansatech Instruments Ltd, UK. A holder that can fix the end of fiber-optic probe of FMS2 chlorophyll fluorimeter is at 60 ° angle at the surface of the leaf. It was taken care that the fiber optics of the fluorimeter does not shade the leaf surface. A single saturating pulse of actinic light was applied to obtain the maximum

Table 1. Classification of *Hevea* seedlings based on the leaf nitrogen content expressed both in CCI and analytical values

Category of seedlings	CCI	%N
Low leaf nitrogen (LN)	6.14(±0.90)	0.51(±0.09)
Medium leaf nitrogen (MN)	16.41(±1.65)	1.18(±0.11)
High leaf nitrogen (HN)	34.84(±2.67)	1.95(±0.1)

fluorescence (Fm) when all PSII reaction centers are in reduced form. The maximum efficiency of PSII photochemistry in dark adapted state (Fv/Fm) was calculated following Schreiber *et al.* (1998).

The maximum photochemical efficiency of PSII was calculated by the ratio Fv/Fm

$$Fv/Fm = (Fm-Fo)/Fm$$

Fo is the back ground fluorescence measured after 20 minutes of dark adaptation, Fv is the variable fluorescence which is the difference between Fo and Fm. The quantum yield of PSII photochemistry (Φ_{PSII}) was calculated as

$$\Phi_{PSII} = (Fm'-Fs)/Fm'$$

This parameter measures the proportion of light absorbed by the antennae pigments of PSII that is utilized for photochemistry. As such it reveals that rate of electron flow through the PSII for photochemistry and an indication of extent of probable photo oxidative damage occurring in photosynthesizing machinery (Genty *et al.* 1989).

Another widely used fluorescence parameter, photochemical quenching, q_p was calculated using the formula

$$q_p = (Fm'-Fs)/(Fm'-Fo')$$

Though it is superficially very similar to Φ_{PSII} , q_p gives an indication of proportion of PSII that are open. An alternative expression of this is $1 - q_p$ that is the proportion of closed reaction centers. Sometimes, it is termed as 'excitation pressure' on PSII (Maxwell and Johnson 2000). The leaf clips supplied by Hansatech, UK is specially designed to keep a small portion of leaf under dark while far red light is switched on. Under that condition, Fo' is measured. Non-photochemical quenching (q_N) was calculated using the following formula:

$$q_N = 1 - (Fm'-Fo')/(Fm-Fo)$$

The study on mid day depression of certain fluorescence parameters were conducted in two different seasons such as in peak winter (first week of January) period as well as pre-winter (first week of September) season when all environmental conditions were favorable. During the month of September, there was intermittent rainfall resulting in optimum soil moisture. The minimum and maximum temperature was recorded as 23.9°C and 29°C respectively. The average PAR during the experimental period was around 1000 $\mu\text{mol m}^{-2}\text{s}^{-1}$. Where as, at the peak winter period, i.e. last week of January 2007, the seedlings were grown under well irrigated condition. The minimum and maximum temperature was recorded as 11.9°C and 23°C respectively. The average PAR during the experimental period was around 1600 $\mu\text{mol m}^{-2}\text{s}^{-1}$. Photoinhibition was assessed as percent reduction in dark adapted leaf Fv/Fm of seedlings with different leaf nitrogen content grown in peak winter (January) in compared to that of non-winter condition (September). The quenching component of excitation energy (q_p and q_N) were also measured at the same time.

RESULTS AND DISCUSSION

Leaf nitrogen content: Leaf nitrogen content revealed that there is a positive relation between CCI values and leaf nitrogen (Fig.1). Similar trend of relationship was also observed in rice (Takebe and Yoneyama 1989), maize (Wood *et al.* 1992) wheat (Follet *et al.* 1992),

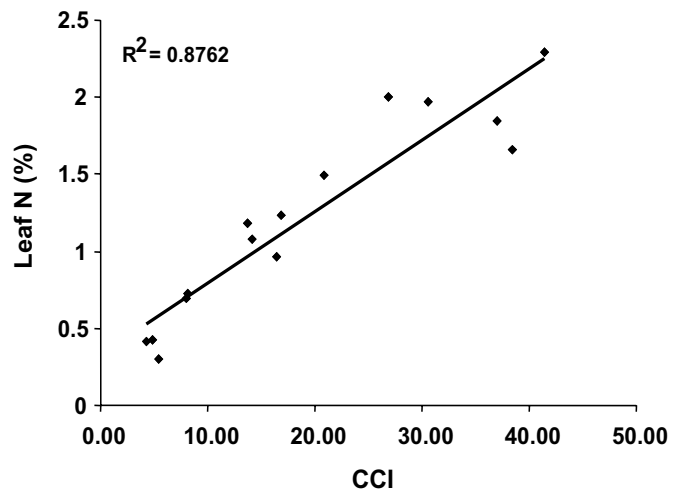


Fig. 1. Relationship between CCI values and leaf nitrogen content (%) in selected polyclonal seedlings

conifers (Linder 1980), tree species (Felix *et al.* 2002). In the present study, LN, MN and HN type seedlings recorded the average leaf nitrogen content of 0.51%, 1.18% and 1.95% respectively.

Growth of seedlings: Growth of the seedlings differed significantly among the groups. Seedlings of LN, MN and HN groups had an average height of 56.88cm, 76.8cm and 85.3cm respectively. Number of leaves per plant also varied similarly among the groups. However, there was no statistically significant difference in leaf number among the different types of seedlings. Nevertheless, the average leaf number of LN type seedlings were only 15 per plant and 28 leaves were borne by each HN type seedling per plant basis. On an average, 24 leaves per plant were found in MN types seedlings (Fig. 2).

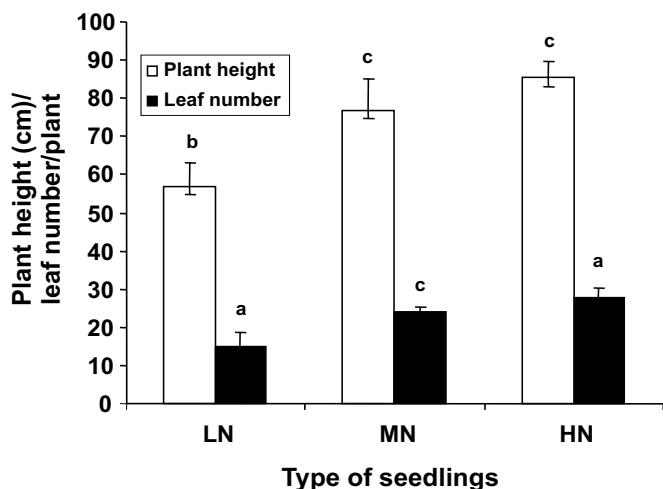


Fig. 2. Plant height (cm) and number of leaves per plant in three groups of seedlings

Gas exchange parameters: Seedlings of variable leaf nitrogen content showed significant difference in some of the major photosynthetic parameters. Light saturated net photosynthetic rate (P_N) was less in LN type seedlings ($1.48 \mu\text{mol CO}_2 \text{ m}^{-2}\text{s}^{-1}$) and high in HN types ($7.2 \mu\text{mol CO}_2 \text{ m}^{-2}\text{s}^{-1}$). However, MN seedlings recorded $5.9 \mu\text{mol CO}_2 \text{ m}^{-2}\text{s}^{-1}$. N-deficient seedlings (LN types) showed the stomatal conductance (g_s) of $47.25 \text{ mmol m}^{-2} \text{ s}^{-1}$ where as other two types of seedlings (MN and HN types) recorded g_s of 79.37 and $81.4 \text{ mmol m}^{-2} \text{ s}^{-1}$, indicating the stomatal regulation of photosynthesis under extreme N-deficient situations. A calculated parameter, C_i/g_s which is an indirect estimate of mesophyll efficiency in photosynthesizing tissue, was compared among the seedlings of different groups. A negative correlation between C_i/g_s and mesophyll efficiency does exists in several field crops (Krishnaprasad *et al.* 1996). C_i/g_s was found to be 7.18 in LN seedlings, 3.29 in MN and 2.8 in HN type of seedlings indicating that *in vivo* mesophyll activity was maintained higher in N- sufficient plants (HN seedlings) in compared to LN plants (Table 2). It appears that LN seedlings accumulated higher intercellular CO_2 of 318 ppm in spite of lower stomatal conductance because of less consumption of CO_2 in the process of carboxylation catalyzed by Rubisco. This enzyme content is the most susceptible component to get depleted under N-limited condition (Sugiharto *et al.* 1990). In many studies, it has been observed that additional application of N can enhance the Rubisco content which can improve net carbon fixation through better photosynthetic rate at a given level of activation state of the enzyme. This additional storage of leaf N in the form of Rubisco or other stress proteins may be useful under stressed condition.

Table 2. Photosynthetic characteristics of *Hevea* seedlings differing in leaf nitrogen content in peak winter season.

Seedling groups	Photosynthetic rate (P_N) ($\mu\text{mol CO}_2 \text{ m}^{-2}\text{s}^{-1}$)	Stomatal conductance (g_s) ($\mu\text{mol m}^{-2}\text{s}^{-1}$)	Internal CO_2 concentration (Ci) (ppm)	Mesophyll efficiency (C_i/g_s)
LN	1.48(\pm 0.09)	47.2(\pm 6.54)	318(\pm 8.98)	7.18(\pm 1.06)
MN	5.9(\pm 0.81)	79.3(\pm 2.82)	261(\pm 10.46)	3.29(\pm 0.32)
HN	7.2(\pm 0.42)	81.4(\pm 4.96)	224(\pm 6.39)	2.8(\pm 0.16)
CD _{0.05}	0.9	NS	NS	-

Chlorophyll fluorescence: The potential quantum efficiency (dark adapted Fv/Fm) was recorded as 0.813, 0.812, and 0.82 in LN, MN and HN type of seedlings when measured at favorable environmental conditions during the first week of September 2007. The efficiency of PSII photochemistry (Φ_{PSII}) was 0.626, 0.570, and 0.681 (Fig. 3A). It indicated that major proportion of energy absorbed was utilized in photochemistry of PSII. In other words it is a measure of better photosynthesis (Maxwell and Johnson 2000). The potential quantum efficiency of PSII (dark adapted Fv/Fm), when measured during the peak winter period (January) showed that plants with adequate leaf nitrogen (HN) had consistently higher Fv/Fm in compared to nitrogen deficient plants

(LN). The Fv/Fm was recorded 0.680 in HN, 0.631 in MN and 0.263 in LN type of seedlings. In terms of percent reduction of Fv/Fm during January, it is 67%, 22% and 17% in the leaves of LN, MN and HN types of seedlings in compared to September. Similarly, the efficiency of PSII photochemistry (Φ_{PSII}) was found to be reduced by 65%, 30% and 25% in these three kinds of seedlings respectively (Fig.3B). The photochemical inhibition was reflected more in LN plants by greater reduction of Fv/Fm, Φ_{PSII} and q_p in compared to MN and HN plants. These fluorescence parameters were also used to indicate oxidative stress in *Hevea* plants under low temperature condition.

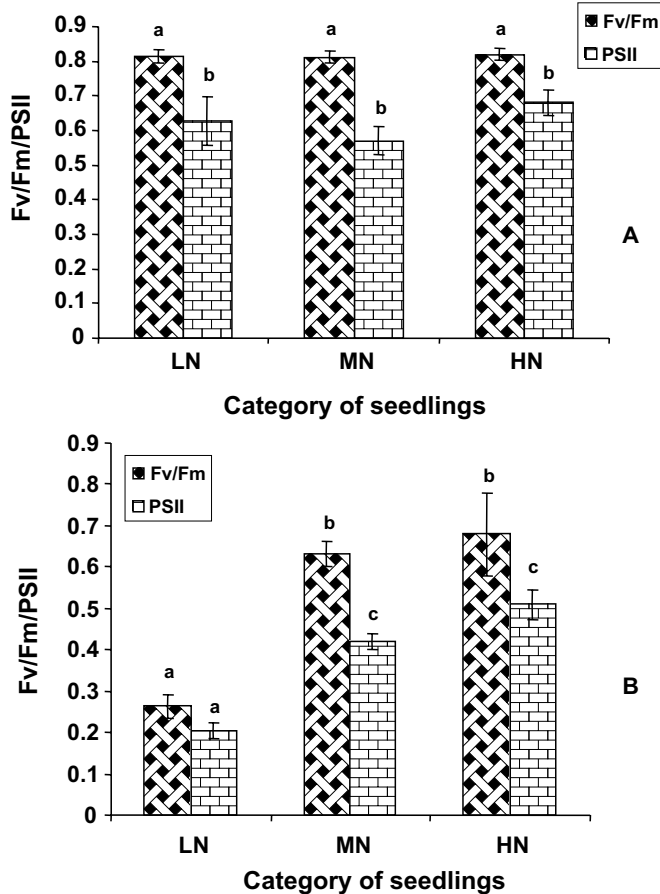


Fig. 3. Effect of leaf nitrogen content on Chlorophyll fluorescence in *Hevea brasiliensis* seedlings during two seasons: (B) Peak winter period (January 2007) and (A) subsequent pre-winter season (September 2007). (Plants were dark-adapted for 20 minutes prior to taking the measurements. Mean \pm S.E., n = 3. * p<0.05)

Under nitrogen limiting condition (LN) a significant decline in Fv/Fm to 0.263 during the mid day of cold period indicates severe oxidative stress acting on those plants. Nitrogen limitation may lead to limitation in enzymes content involved in photosynthetic carbon assimilation (Sugiharto *et al.* 1990). In particular, low nitrogen status was often found to limit Rubisco content of leaves in several plants (Sage *et al.* 1987). Differences in Rubisco content are accompanied by concomitant changes in other chloroplast protein and chlorophyll as a result of inadequate supply of nitrogen to leaf.

Photosynthetic energy utilization or dissipation was assessed through chlorophyll fluorescence measurements in leaves differing in its nitrogen content during two contrasting environmental conditions (September and January). During the month of September, when all environmental conditions were more favorable, there was no significant difference in photochemical (q_p) and non-photochemical (q_N) quenching among the LN, MN and HN leaves. The photochemical quenching was recorded as 0.78, 0.75 and 0.85 in LN, MN, and HN types of seedlings respectively during the month of September (Fig. 4A) where as the q_p was recorded as 0.48, 0.67 and 0.86 in LN, MN and HN type seedlings respectively under low temperature condition (January) (Fig. 4B). The non-photochemical quenching (q_N) was found to be 0.27, 0.36 and 0.31 respectively under September condition and it was 0.44, 0.23 and 0.19 in LN, MN and HN seedlings respectively under January situation. Therefore, the photochemical quenching was decreased by 38% and 10% in leaves of LN and MN types of

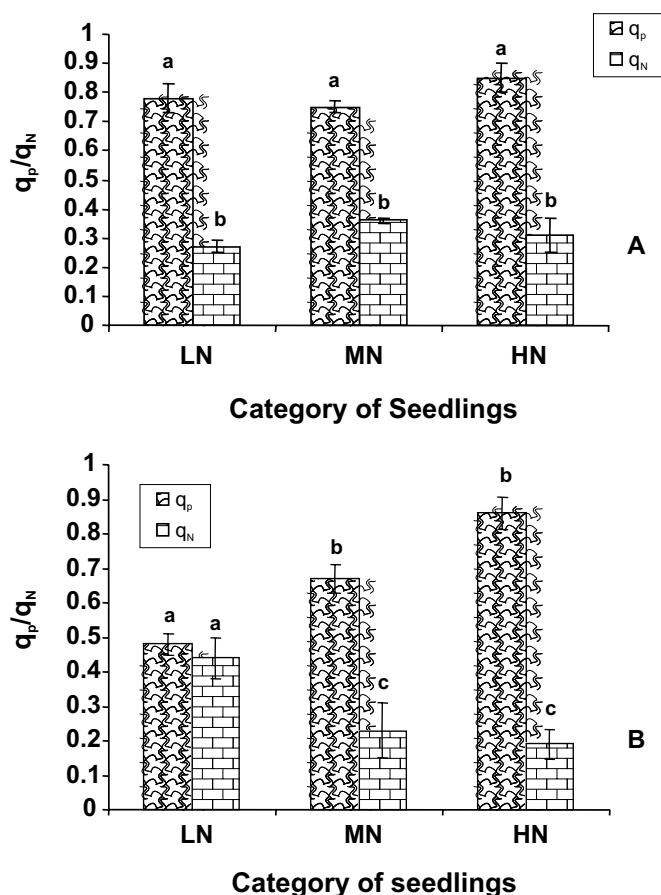


Fig. 4. Photosynthetic energy utilization /dissipation through photochemical (q_p) and non-photochemical (q_n) quenching in *Hevea* leaves having different nitrogen status during two seasons: (A) September 2007 and (B) January 2007. Mean \pm S.E., $n = 4-5$. * $p < 0.05$

seedlings respectively and there was no significant reduction in HN types seedlings under low temperature condition (Fig. 4A). This reduction in dark adapted F_v/F_m , Φ_{PSII} and q_p can be considered as percent photoinhibition in photosynthesizing leaves of three groups of seedlings with variable leaf nitrogen content (Fig. 5).

Our findings in this study show that plants with sufficient leaf nitrogen did not exhibit drastic decline in F_v/F_m values under low temperature and high irradiance stress. In other words, HN plants could overcome better the low temperature stress than LN plants. The proper leaf nitrogen content may lead to appropriate protein or enzyme levels that can optimize photochemical electron sink resulting better photosynthetic capacity. Earlier it

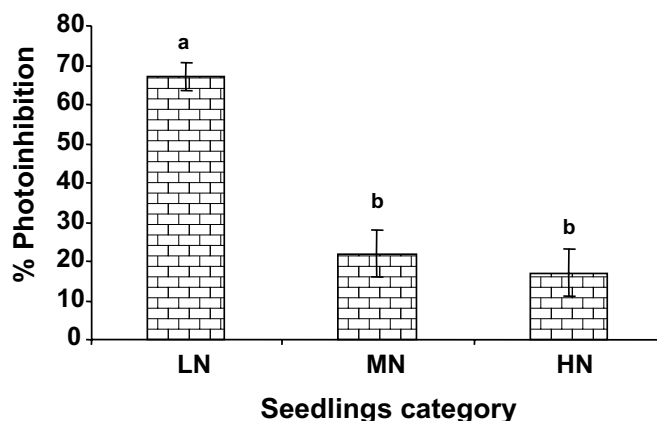


Fig. 5. Photoinhibition during peak winter season in three different categories of *Hevea* seedlings having difference in leaf nitrogen content. Mean \pm S.E., $n = 4-5$. * $p < 0.05$

was proposed that plant can use oxidized nitrogen anion as electron sink to unload excess reducing power (Aparicio *et al.* 1985). In our present study also, higher leaf nitrogen status in *Hevea* seedlings has resulted in better stress tolerance for photoinhibition as also observed in other plant species such as *Neoregelia cruenta* (Fernandes *et al.* 2002).

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