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Full Length Research Paper

Effect of nitrogen fertilizer on the in-vivo nitrate reductase activity in Dalbergia sissoo plants under nursery conditions

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Dalbergia sissoo is multipurpose Nitrogen (N) fixing tree species cultivated extensively in the Indian plains. It accumulates a considerable amount of N-rich litter which decomposes quickly and improves the organic matter as well as the nutrient status of the soil. It is generally recommended for afforestation of degraded lands in arid and semiarid regions, where soil N status is poor. N is one of the most important elements for the growth and development of plants. In the present communication, an attempt was made to study the N assimilation behavior of individual plant parts in D. sissoo under nursery conditions. Urea was used as the N source and applied at 0, 50,100 and 200 Kg/ha in two split doses in pot grown plants. The objective was to observe the effect of N application on the in-vivo nitrate assimilation pattern in different plant parts of D.sissoo at monthly interval.

The results reveal that two peaks, i.e. one large in April and another small in August were observed in all four N treatments for leaf, stem, and root nitrate reductase (NR) activity (g^{-1} fresh wt h^{-1}). However, in nodule two peaks one large in April and another small in July was observed. In total leaf (NR) activity ($pl^{-1}h^{-1}$), a large peak was observed in August and a small in June. In stem, large and small peak were observed in April and August respectively in all the treatments. Whereas in the root, a large peak in April and small in August were observed in both g^{-1} fresh wt h^{-1} and $pl^{-1}h^{-1}$ NR activity in all treatments. In leaf, stem and root higher NR activity was observed in 200 kg N/ha as compared to other treatments. However, in the nodule, maximum activity was observed in 50 kg N/ha as compared to others. On the basis of seasonal effect, higher NR activity (g^{-1} fresh wt h^{-1}) was recorded in summer followed by rainy and minimum in the winter season in different plant parts. The total in-vivo NR activity ($pl^{-1}h^{-1}$) was observed maximum in rainy followed by summer and winter in leaf and nodule. Whereas, in the stem and root, maximum activity was observed in summer followed by rainy and lowest in winter. Total NR pl^{-1} activity was recorded higher in rainy as compared to summer and winter.

Key words: shisham, seasonal effect, nitrogen, In-vivo nitrate reductase (NR) activity

INTRODUCTION

Low fertility is a common problem in establishing vegetation on eroded and barren degraded lands. Nitrogen (N) is generally limited in soils of these areas, hence the most conventional way by which soil fertility can be maintained for optimization of productivity of our plantations are by using chemical fertilizers, which are scarce and expensive for developing countries like India. The suitable alternate is to plant such tree species in a large scale which can utilize soil N as well as atmospheric N more efficiently (Chaukiyal et. al, 2000). Plants assimilate N in combined form usually as NO_3- or NH_4+ via their root system. N is readily lost from the soil due to the processes of nitrification, denitrification, and

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leaching. The predominant form of N available to the plant is NO_3-. Nitrate is highly mobile in soil and its availability can be affected by water transport in roots (Clarkson et. al., 2000; Gloser et. al., 2007). The utilization of soil N by higher plants involves several steps of reduction by the participating enzymes. One of the important key enzymes involved is nitrate reductase (NR) which determines the rate of reduction as a whole (Beevers and Hagemans, 1969).

Dalbergia sissoo Roxb. commonly known as shisham, is a multipurpose, drought resistant, frost hardy and widely distributed indigenous N fixing tree species, valued for fuel, fodder, and timber. It provides important raw material for a variety of wood based industries, especially furniture and construction as well. It is an excellent species for agro-social forestry and wasteland afforestation programs in the native areas. Initially, the emerging seedling has small demand for N but the requirement increases rapidly as the plant grows. In many situations, soil N availability does not match the plant requirements and therefore, fertilizer may be required to maintain maximum growth rate (Williams and Haynes, 1995). Due to multifarious uses of this species, an attempt was made to study the N assimilating behavior with respect to fertilizer application on the growth in relation with seasonal changes so that N utilization behavior can be worked out in detail for the various plantation programs in the country.

MATERIAL AND METHODS

Fresh Dalbergia sissoo seeds were collected and sown in the germination boxes. Eight months old seedlings were transplanted in earthen pots filled with well-sieved soil, sand and farm yard manure mixture in 2:1:1 ratio and kept in the glass house premises of Plant Physiology Discipline, Forest Research Institute, Dehradun. Urea as a source of N was applied at 0, 50,100 and 200 kg/ha. Two equal split doses of N in aqueous solution were applied in March and April respectively. A randomized block design was used and three replications were maintained for each treatment. In-vivo NR activity in leaf, stem, root and nodule in individual seedlings were recorded at monthly intervals. The observations were pooled and divided into three seasons, viz., winter (November, December, January and February), summer (March, April, May and June) and rainy (July, August, September and October).

In-vivo NR activity in different plant parts was assayed periodically and the procedure as described by Klepper et al. (1971) and method adopted by Pokhriyal and Raturi (1985) was followed. Three uniform size plants were selected from each treatment, uprooted carefully and brought to the laboratory. Different plant parts were separated, washed with distilled water and kept in moist blotting papers. Plant parts (except nodules) were chopped into small pieces of about 2 to 3 mm^2. Approximately 500 mg of the chopped plant tissue was taken in flat bottom culture tube (30 ml capacity), containing 3 ml of 0.20 M phosphate ($KH_2 PO_4$) buffer mixed with 3 ml substrate of 0.20 M KNO_3 and embedded in ice trays. These tubes were evacuated with the help of vacuum pump for about 2 minutes. The process was repeated until the plant tissues were fully submerged into the incubation medium. These tubes were transferred into a shaking water bath at 30°C in dark for incubation. The tubes were removed after one hour and then immersed into a boiling water bath for about 4 minutes to stop the reaction and effective removal of the nitrite accumulated in the plant tissue.

A known amount of aliquot was taken for the estimation of nitrate reduced into nitrite during enzyme activity by the method described by Evans and Nason (1953). A required amount of aliquot from each tube was pipetted into clean test tube and 1 ml sulphanilamide (1%, sulphanilamide in conc. hydrochloric acid) was added, followed by 1 ml of NEDD (0.01%, 1-Nepthyethylene diamine dihydrochloride) and mixed thoroughly .The color was allowed to develop for 25 minutes and final volume was made up to 6 ml with distilled water. A change in color intensity was estimated at 540 nm in Bausch & Lamb Spectornic-20 colorimeter. The recorded data was pooled and analyzed on monthly, seasonally and yearly basis. The statistical analysis was done using two-way ANOVA technique and for two main factors i.e., N treatment and the months or seasons.

RESULTS

Leaf NR (g^{-1} fresh wt. h^{-1}) activity showed two peaks i.e., a high in April and a low in August in all four N treatments. In the case of total NR (pl^{-1} h^{-1}) activity, a high peak was observed in August and a low in June. Maximum (3670.22 nmol) and minimum (82.98 nmol) NR activity was observed in 200 kg N/ha and control in April and December respectively. Highest (127355.46 nmol) and lowest (62527.02 nmol) total NR activity (pl^{-1} h^{-1}) was observed in 100 kg N/ha and control respectively in August as shown in figure 1.

Figure 1. Leaf and stem nitrate reductase (nmol NO_3^- reduced per gram and per plant) activity as influenced by different nitrogen treatments.

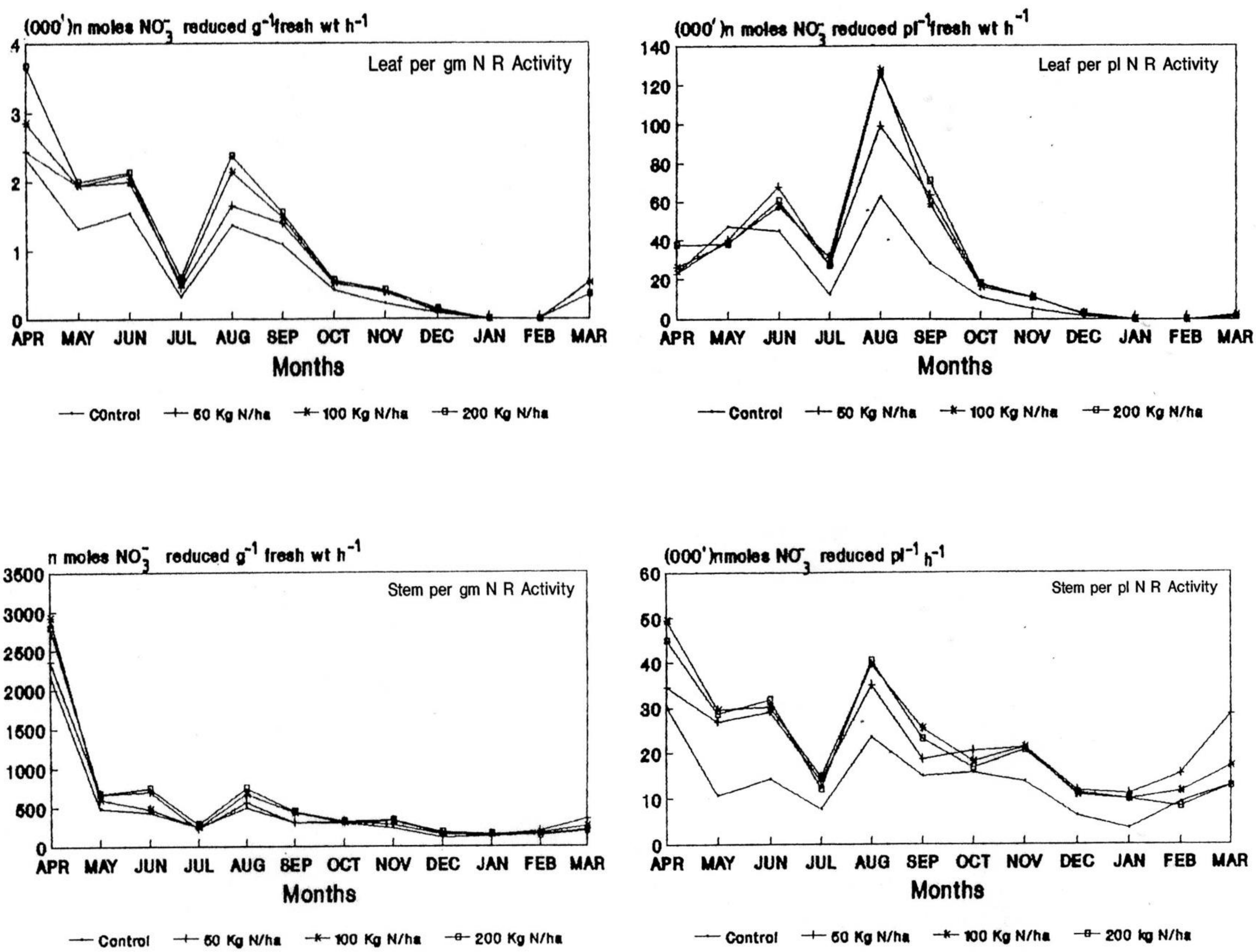


Table 1: Monthly analysis of nitrate reductase activity as influenced by different nitrogen treatments in the seedlings of *Dalbergia sissoo*.

NR activity	Months and treatments													CD	SIG.
(A). NRA g^{-1} leaf	Months	Apr 2991.9	Jun 1936.2	Aug 1875.0	May 1795.1	Sep 1396.7	Oct 510.6	Jul 481.9	Mar 455.7	Nov 363.8	Dec 113.3			452.86	***
	Treatments	N200 1381.7	N100 1247.7	N50 1219.1	N0 908.9									189.25	***
(B). NRA pl^{-1} leaf	Months	Aug 103451	Jun 57612	Sep 55225	Apr 37087	May 33324	Jul 24503	Oct 23971	Nov 9750	Dec 2314	Mar 1707			29232	***
	Treatments	N200 139196	N50 39173	N100 37170	N0 24019									12216	*
(c). NRA g^{-1} stem	Months	Apr 2566.2	Aug 1205.2	May 607.5	June 589.5	Sep 373.4	Oct 311.9	Nov 291.7	Mar 251.6	July 249.4	Dec 146.9	Jan 141.9	Feb 134	806.6	***
	Treatments	N200 779.3	N100 559.3	N50 520.7	N0 438.7									NS	….
(D). NRA pl^{-1} Stem	Months	Apr 39833	Aug 34595	June 25629	May 24879	Sep 20491	Nov 19239	July 18955	Mar 17844	Oct 17791	Dec 14475	Jan 9443	Feb 3634	16112	***
	Treatments	N200 23476	N100 22902	N50 21395	N0 16247									5695	*
(E). NRA g^{-1} Root	Months	Apr 3050.4	May 1883	Jun 1686.2	Aug 1571.8	Sep 1348.4	Jul 697.8	Mar 543.6	Oct 519.2	Nov 373.4	Feb 209.6	Dec 172.1	Jan 163.3	557.1	***
	Treatments	N200 1196.9	N100 1069.3	N50 1018.6	N0 782.1									196.9	…
(F). NRA pl^{-1} Root	Months	Apr 69449	Aug 67882	May 65652	Sep 60631	Jun 57184	Mar 33225	Oct 26238	Nov 24823	Jul 21867	Feb 10839	Dec 9331	Jan 8875	19844	***
	Treatments	N200 44744	N100 41405	N50 40158	N0 25691									7225.4	***
(G). NRA g^{-1} nodule	Months	Apr 17439	Jul 8126	May 2831	Jun 2010	Nov 1059	Aug 706	Dec 648	Oct 646	Sep 592	Mar 328			7494.4	***
	Treatments	N50 4835	N100 3755	N200 2899	N0 2245									NS	-----
(H). NRA pl^{-1} nodule	Months	Jul 15337	Apr 2536	May 1879	Jun 1573	Aug 1242	Sep 509	Oct 254	Dec 189	Jul 15337	Apr 2536			2520.2	***
	Treatment s	N50 2848.8	N200 2433`1	N0 2362.5	N100 1838.7									NS	----
(I). Total NRA	Months	Aug 207170	Jun 144103	Sep 136856	Apr 133478	May 133234	Jul 73772	Oct 59920	Nov 53951	Mar 49441	Feb 22042	Dec 21879	Jan 17473	51533	***
	Treatments	N200 100476	N100 97041	N50 92257	N0 61334									18262	***

Note: *****, **, *** indicates significant at 0.1%, 1% and 5% level of probability respectively; **NS** indicates not significant even at 5% level of probability.
N0 = Control (without N); **N50**= 50kg N/ha; **N100**= 100 Kg N/ha; **N200**= 200 Kg N/ha.

A significant difference in the leaf NR (g-1 h-1 and pl^{-1} h^{-1}) activity due to different N doses was observed (Table 1, A & B); whereas on the seasonal average basis, the differences between the treatments were non-significant. Significantly, higher NR (g^{-1} h^{-1}) activity was recorded in summer as compared to rainy and winter season, whereas, total NR (pl^{-1} h^{-1}) activity was significantly higher in rainy and summer season as compared to winter (Table 2, A & B).

Stem NR activity have also followed an almost similar pattern as in the case of the leaf. Highest and lowest NR activity was observed in April and February respectively. The maximum value was observed in 100 Kg N/ha (2925.53 nmol) in April. In the case of total NR (pl^{-1} h^{-1}), activity, a high and a low peak was observed in April and August respectively in all the treatments. However, maximum values were observed in 100 Kg N/ ha and minimum in control (Fig 1). The differences among treatments were observed non-significant in the case of g^{-1} h^{-1} NR activity and significant in total (pl^{-1} h^{-1}) NR activity (Table 1, C & D).

The season wise analysis of NR (g^{-1} h^{-1} and pl^{-1} h^{-1}) activity showed that the variations among different N treatments were non-significant. A significantly higher (g^{-1} h^{-1}) NR activity was observed in summer as compared to rainy and winter season. Whereas, in total NR (pl^{-1} h^{-1}) activity, summer and rainy seasons showed significantly higher values as compared to winter (Table 2, C & D).

In root also, two peaks for NR (g^{-1} h^{-1}) activity were observed in April as well as in August. However, maximum NR activity was observed in 200 Kg N/ha (3734.05 nmol) and minimum in control (2180.85 nmol) in April. Almost similar pattern was observed in total root NR activity. NR activity remained low during the winter season (Fig. 2). The pooled seasonal data analysis showed that the variation among different treatments was significant. NR activity also showed an increased with increase in N treatments. However in both the cases (g^{-1} h^{-1} and $pl^{-1}h^{-1}$) the treatments effects were synonymous (Table 1, E & F).

Significant variations among different N treatments were observed as compared to control. The differences between the treatments under different seasons were observed non-significant. Significantly higher NR activity was observed during summer as compared to rainy and winter seasons. However, total NR activity was significantly higher in summer and rainy season as compared to winter (Table 2, E & F).

In root nodule, two peaks *i.e,* one high in April and another low in July was observed for nodule NR (g^{-1} h^{-1}) activity. A fluctuation in the nodules NR activity was also recorded as in other plant parts like leaf, stem, and root. NR activity remained low during the winter season. Overall the lowest NR activity was observed in December. Highest total nodular NR activity was observed in 50 kg N/ha (18903.83 nmol) and minimum in 200 kg N/ha (102.38 nmol) in April and December respectively. No nodular NR activity was observed in January and February (Fig. 2). In the case of monthly pooled values no significant variations among different N treatments for both the parameters i.e. g^{-1} h^{-1} and $pl^{-1}h^{-1}$ in nodules was observed (Table 1, G & H).). In case of seasonally pooled data for g^{-1} h^{-1} NR activity, significantly higher value was observed in summer season as compared to rainy and winter, whereas, in the case of total nodule NR activity ($pl^{-1}h^{-1}$) significantly higher values were observed in rainy season as compared to summer and winter (Table 2, G & H).

Figure 2. Root and nodule nitrate reductase (nmol NO_3^- reduced per gram and per plant) activity as influenced by different nitrogen treatments.

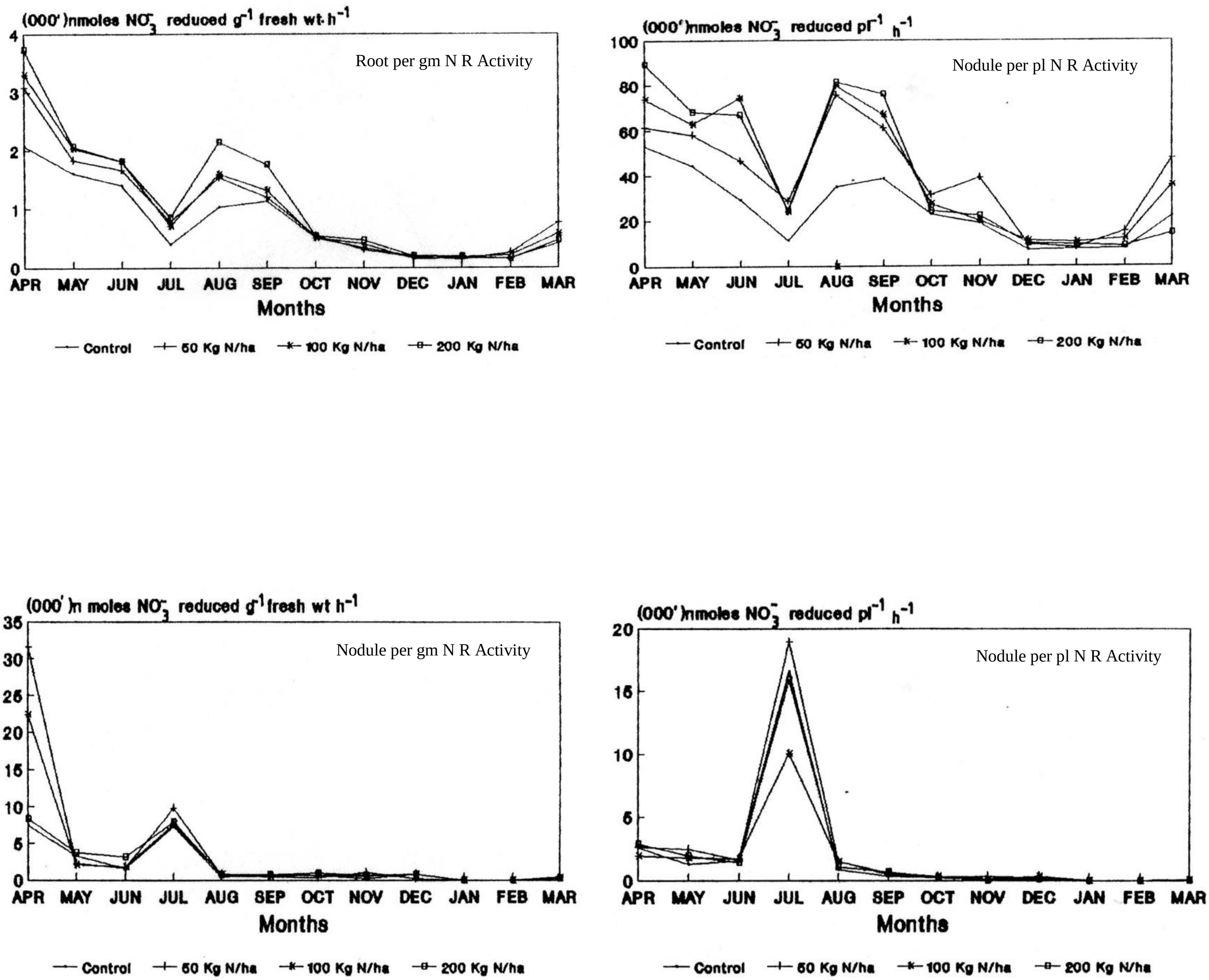


Table 2: Seasonal analysis of nitrate reductase activity as influenced by different nitrogen treatments in the seedlings of *Dalbergia sissoo*.

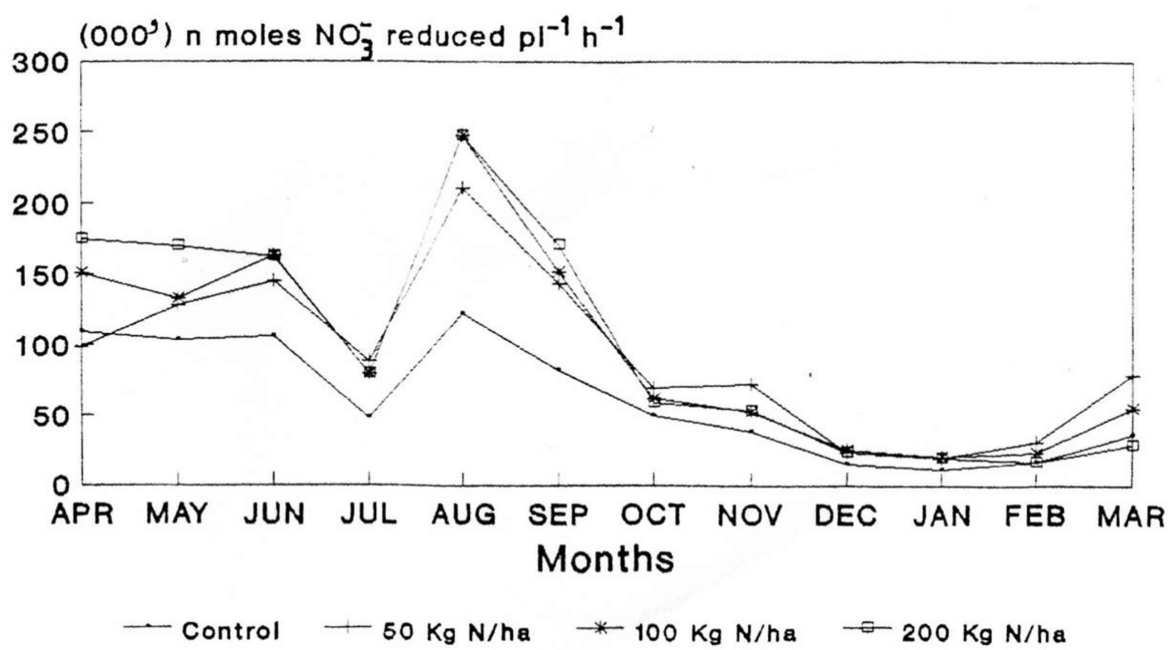
Plant part NR activity	Seasons and treatments								CD	Sig.
(A). NRA g^{-1} leaf	Seasons	S:1797.8 >	R: 1059.3 >	W: 119.3					440.8	***
	Treatments	N200: 1151.4	N100: 1039.7	N50: 1015.9	N0: 757.4				NS	-----
(B). NRA pl^{-1} leaf	Seasons	R: 51787.4	S: 32432.4	W: 3016					16480	***
	Treatments	N200: 32663.1	N50: 2661.0	N100: 30974.9	N0: 20015.5				NS	-----
(C). NRA g^{-1} stem	Seasons	S: 1003.6 >	R: 388.6 >	W: 185.3					368.5	…..
	Treatments	N200: 583.9	N100: 559.7	N50: 520.9	N0: 438.7				NS	…..
(D). NRA pl^{-1} stem	Seasons	S: 27046.2	R: 23021.4	W: 13572.5					5757.3	…..
	Treatments	N200: 23476.2	N100: 22902.2	N50: 22228.4	N0: 16246.6				NS	-----
(E). NRA g^{-1} root	Seasons	S: 1790.6 >	R: 1029.8 >	W: 229.6					411.79	***
	Treatments	N200: 1196.9	N100: 1069.3	N50: 1018.4	N0: 782.1				NS	----
(F). NRA pl^{-1} root	Seasons	S: 56380.4	R: 44154.4	W: 13467.7					11635.2	***
	Treatments	N200: 44743.9	N100: 41405.4	N50: 40158.2	N0: 25724.5				NS	-----
(G). NRA g^{-1} nodule	Seasons	S: 5570.1	R: 2504.9	W: 414.1					3569.7	*
	Treatments	N50: 3993.6	N100: 3101.2	N200: 2378.7	N0: 1845.3				NS	-----
(H). NRA pl^{-1} nodule	Seasons	R: 4335.5 >	S: 1509.3 >	W: 92.5					2596.6	*
	Treatments	N50: 2374	N200: 2041.4	N0: 1968.8	N100: 1532.3				NS	-----
(I). Total plant NRA	Seasons	R: 119432.4	S: 115064.3	W: 28836.6					30448	**
	Treatments	N200: 100479	N100: 97040.6	N50: 92257.1	N0: 61334.4				NS	-----

Note: ***, **, * indicate significant at 0.1%, 1% and 5% level of probability respectively;
NS indicates not significant even at 5% level of probability.
N0 = Control (without N);
N50= 50kg N/ha;
N100= 100 Kg N/ha;
N200= 200 Kg N/ha.
W = winter season,
S = summer season,
R = rainy season.

The total NR (leaf + stem + root + nodule) activity showed a distinct peak in the rainy season (August) in all the treatments. The effect of N treatments on the total NR activity showed low activity with the lower doses (Fig. 3). Statistical analysis showed that treatments effect was significantly different and N doses were equally effective as compared to control (Table 1, I). The results obtained from seasonal analysis revealed that both rainy and summer seasons were effective and significantly higher than winter. However, differences among various treatments were observed non- significant (Table 2, H). Among different treatments, overall maximum NR activity was observed in 200 kg N/ha followed by 100 kg N/ha, 50 kg N/ha and minimum in control in all three seasons.

seasons. Conversely in nodules, maximum activity was observed in 50 kg N/ha during rainy seasons.
Among different plant parts, minimum activity was reported in the nodules and leaf in winter followed by stem and roots in summer and rainy seasons (Fig. 1 & 2). Among different seasons, overall maximum NR activity was observed in rainy followed by summer and winter season (Table 2, I). Total leaf NR activity was markedly influenced by the higher N doses (Fig. 3). Almost similar results were reported by Pokhriyal et al. (1988) in Leucaena leucocephala; Uniyal (1997) and Mir (2012) in Dalbergia sissoo Chaukiyal and Pokhriyal (2005) in Pongamia pinnata; Kumar (2005) in Acacia catechu and recently Chaukiyal et al. (2014) in Myrica esculenta and Kandpal and Chaukiyal (2016) in Albizia

Figure 3. Total nitrate reductase (nmol NO^-_3 reduced pl^{-1} h^{-1}) activity as influenced by different nitrogen treatments.



DISCUSSION

Nitrate (NO_3-) is one of the major sources of N, taken up by roots of higher plants, which is translocated to the shoot, as well as stored in the vacuole and assimilate into reduced N products. The process of NO_3- uptake, translocation and assimilation are interdependent and closely regulated in higher plants (Huber, et al., 1996; Sivasankar and Oaks, 1996). Overall maximum NR activity was observed in 200 kg N/ha followed by 100 kg N/ha, 50 kg N/ha and minimum in control in different plant parts *i.e.,* leaf, stem and root in all the three

The capacity of root to reduce NO_3- appear to be particularly intense during the early stage of plant developments (Oaks et al., 1972: Wallace, 1975 and Oaks, 1978) as the plant tend to reduce an increasing proportion of absorbed NO_3-. To some extent, the contribution of root and shoot tissue to the overall NR assimilation process may depend on the external NO_3-concentrations. An increase in the concentration of NO_3- in the external medium can greatly increase NR activity in a shoot with a concomitant decrease in activity in the root (Wallace and Pate, 1967). NR activity is influenced by site factor which affect the regulation of nitrate assimilation (Lillo, 1994; Crawford, 1995; Ruis et al., 1998). It is well known that NR represents a classical example of a substrate induced enzyme (Zabeen and Ahmad, 2011) its activity is decisively determined by NO_3- supply (Black, et al., 2002). Similar types of observations were reported by Gibson and Pagan (1977); Bazzaz, (1990) and Gifford (1992). In a survey of 555 woody species, only 41% showed NR activity in leaves (Smirnoff et al., 1984) and among legume, high leaf NR activity was reported in those of tropical origin (Sprent, 1980).

Differences in nitrate-induced NR activity between leaves and roots have been reported for other species also. Both red oak (Quercus rubra) and red ash (Fraxinus pennsylvanica) have slightly higher leaf NR activity than root at low N availability (Traux et al., 1994). Leaves are the major site of NR activity in Acer rubrum (Downs et al., 1993): F. excelsior (Gebaur and Stadler, 1990; Stadler and Gebaur, 1992) F. pennsylvanica and Q. rubra (Traux et al., 1994). However, in another study roots are predominant site of NR activity in Malus (Lee and Titus, 1992); Pinus resinosa, P. rigida, P. strobus (Downs et al., 1993); Pinus sylvestris (Sarjala et al., 1987) and Prunus persica (Gojon et al., 1991). Sellstedt (1986), reported that the partitioning of NR activity among different plant parts varies with species, for instances, Alnus incana has high NR activity in both root and stem tip but not in leaves. Whereas, Beevers and Hageman (1969); Harper and Hageman (1972); Brunetti and Hageman (1976); Johnsen et al., (1991) and Pokhriyal et al. (1993) reported that the leaf is the major site of nitrate reduction. Thus the location of nitrate assimilation varies from species to species, habitat of species (Black et al., 2002); age (Semwal *et al*., 2012 a & b; Ratrey et al., 2013; Chaukiyal et al., 2014; Vandana et al., 2016)), environmental factors (Chaukiyal and Shams, 2014) and in most herbaceous plants the main site of nitrate reduction are the leaves (Andrews, 1986; Gojon et al., 1994; Black et al., 2002). The partitioning of nitrate reduction between root and shoot is not necessarily constant for every species and may vary with plant growth conditions and plant development (Andrews, 1986).

It is evident from the results derived from a present study that the higher N doses were not found effective as far as the N assimilation is concerned in Dalbergia sissoo seedlings. Therefore, an excessive N fertilizer application in the leguminous tree species like D. sissoo should either be avoided or given in more splits after assessing the requirement to maintain a consistent availability of N to the plants. Initially, low N starter dose will be helpful in boosting up the seedling growth whereas, excessive N fertilizer application will inhibit the process of N fixation on one hand and depletion through volatilization and leaching and ultimately polluting atmosphere and soil respectively on the other.

CONCLUSION

Dalbergia sissoo (shisham) is an economically valued N-fixing tree and hence it becomes important to study its various aspects of N metabolism. Study of variation in NR activity with varying N doses and changing season has been assessed. Results obtained leads to a conclusion that on one side NR activity increases with increasing the N dose in leaf, stem, and root but on the other hand, it is inversely related in nodules and therefore, shows maximum activity in lowest (50 kg N/ha) dose of N. In addition, rainy and summer seasons seem to be favorable for NR activity in D. sissoo as compared to winter when activity was found minimum. Higher doses of N and low temperature were found to have a negative effect on NR activity in nodules which is the site of N fixation. Therefore, it is recommended that D. sissoo should be planted at the beginning of rainy season and N treatment at 50 kg N/ha should be applied for better establishment of the seedlings.

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