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ASSESSMENT OF NITRATE REDUCTASE ACTIVITY IN LEAF OF *Albizia chinensis* (OSB.) MERR

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Abstract: The combination of different concentration of substrate (0.10M, 0.15M, 0.20M and 0.25M, KNO_3) with different pH of buffer (0.10 M and 0.20 M, KH_2PO_4 of the pH 6.5, 7.0, 7.5, 7.6, 7.7, and 7.8) solutions were tried for the nitrate reductase (NR) activity in *Albizia chinensis* leaf. Maximum nitrate reductase activity was observed in the combination of buffer solution (KH_2PO_4) 0.10M having pH 7.6 and substrate solution of the concentration 0.15M. Among different individual leaf, NR activity increased up to 6th leaf from top to bottom and then decreased afterwards. Biomass was observed increasing from 1st to 6th and then decreases after seventh leaf. Moisture content was observed between 60 to 70% in 1st to 9th leaf and further decreased from 10th to 12th leaf. It was also found that the nitrate reductase activity increases from 9.00 am till 1.00 pm and thereafter, it starts decreasing till 5.00 pm. In countries like India where soil is nitrogen (N) deficient, restoration of soil fertility is a critical problem. N is the most abundant element on the earth and acquired by plants though fertilizers, mineralization of organic matter and biological nitrogen fixation (BNF). Overuse of fertilizers has lead to surface and ground water pollution. Therefore, BNF is a sustainable method for plants to acquire nitrogen. N fixing trees are widely used in agroforestry and soil improvement in degraded lands. *A. chinensis* is a multipurpose nitrogen-fixing tree that can be used in various forestry programs for increasing the fertility of soil.
Keywords: *Albizia chinensis;* Nitrate reductase (NR) activity; Substrate and buffer solutions.
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INTRODUCTION

Nitrogen is the key element for the normal growth and development of plants. It is taken up from the soil mainly in the form of nitrate (NO_3^-) which is reduced to nitrite (NO_2^-) and then to ammonium with the help of enzymes, nitrate reductase (NR) and nitrite reductase (NiR) respectively. Nitrate is one of the major sources of nitrogen, taken up by roots of higher plants, translocate to the shoot, store in vacuole and assimilate into reduced nitrogen products. The process of nitrate uptake, translocation and assimilation are interdependent and closely regulated in higher plants (Huber *et al*., 1996; Sivasankar and Oaks, 1996). Nitrate reductase catalyses the first committed step of nitrate assimilation (Crawford *et al*., 2000). Because of the importance of nitrogen to plant productivity, nitrate assimilation pattern have been studied in many annuals, perennials and tropical plants (Beevers and Hageman 1980; Pate, 1980; Chaukiyal, 2008 a,b,c,d, 2009 and Chaukiyal *et al.,* 2014). Nitrate enters into the cell where it is reduced to NO_2^- by the enzyme NR in both root and shoots tissues (Li and Oaks, 1995). Therefore, this enzyme is the key factor for assessing the nitrogen assimilation and hence nitrogen fixing ability of a species. Though the work has been done on this enzyme in the field of agriculture (Brunetti and Hageman, 1976) but very few attempts have been made in forestry species and need to be extended. *Albizia chinensis* is one of the important fast growing multipurpose leguminous tree species.

It is used for establishment of high shade tree in tea orchads and can be planted in mid and low elevation range of 1370-1500m (TRI Srilanka, 2003). Several flavinoids has been extracted from its leaves which show antimicrobial activity (Ghaly *et al.,* 2010). Besides this, its bark contains terpenoids known as albizosides has antitumor property (Lui *et al*., 2009). The species is also planted for agroforestry slope stabilization, reforestation and soil improvement in degraded land (Khaleque and Gold, 1993). Considering the importance of this species as a potential source for medicine, timber, manure and other purposes, its nitrogen uptake pattern becomes important to be studied to screen out the most nitrogen assimilatory traits among the populations. For this purpose, suitable buffer and substrate solution must be determined so that the nitrogen assimilation behavior of this species can be studied.

EXPERIMENTAL

Fresh leaves of *A.chinensis* were collected randomly from pot grown plants and washed thoroughly in tap water followed by distilled water and chopped into small pieces of about 2-3 mm length. Five hundred mg of the chopped plant leaf were taken in a flat bottom (30 ml capacity) culture tubes containing 3 mL phosphate buffer and 3ml potassium nitrate of different concentrations 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. Tubes were transferred in shaking water bath at 30^0C in dark for incubation. After incubation for one hour, tubes were removed and immersed into boiling water bath for 5 minutes to stop the reaction and effective removal of the nitrite accumulated in plant tissues after completion of the enzyme reaction. The same method was adopted for NR (E.C. 1.6.6.1) activity as earlier described by Klepper *et al.* (1971) with some modification by Nair and Abrol (1977).

The amount of nitrite (end product) produced by reduction of nitrate during enzyme activity was determined by the method described by Evans and Nason (1953). A required amount of sample aliquot was pipetted in a test tube; 1 mL sulphanilamide was added to it followed by 1mL NEDD (1-Naphthylethylene Diamine Dihydrochloride) and mixed thoroughly. Colour was allowed to develop for 25 minutes and final volume was made upto 6 ml with distilled water. A change in colour intensity was estimated at 540 nm in Systronics, Visiscan 167 spectrophotometer. Different pH solutions of phosphate buffer (0.1M) along with different substrate (potassium nitrate) concentrations were tested to find out a suitable incubation medium for the estimation of optimum NR activity in the leaves of *A. chinensis*. *In- vivo* NR activity was assayed as described by Klepper *et al*. (1971) and method adopted by Pokhriyal and Raturi (1985). The standardization of buffers and substrate combinations were carried out in three steps after initial preliminary testing as follows:

The initial buffer and substrate concentrations were considered on the basis of literature surveyed (Chaukiyal, 2008a, b, c, d; 2009; Chaukiyal and Mir, 2010; Kandpal and Chaukiyal, 2013; Ratrey *et al*., 2013, Chaukiyal *et al.,* 2014 and Sharma *et al.,* 2015). Thus 0.10 M and 0.20 M concentration of phosphate buffer having different pH ranging from 6.5, 7.0, 7.5, 7.6, 7.7 and 7.8 along with the combinations of different substrate concentrations *i.e.* 0.10 M, 0.15 M, 0.20 M and 0.25 M were taken for the of enzyme assay (Table 1).

Nitrate Reductase activity in individual leaf: A separate experiment was performed in *A. chinensis* leaf with an objective to determine that, at which leaf number, maximum NR activity occurs. The topmost fully expanded young leaf was marked as leaf no.1 proceeding in descending order up to 12^{th} the lowermost mature leaf and the same leaf samples were used for NR assay. Fresh weight, dry weight and moisture content were also measured in all individual leaf.

Variation in Nitrate reductase activity during day length: Fresh leaves were harvested in quadruplicate for the estimation of NR activity after every two hours interval starting from 9:00

a.m. to 5:00 p.m. The experiment was replicated three times (three consecutive days). Atmospheric temperature was also recorded each time the leaves were harvested.

RESULTS AND DISCUSSION

In the first phase of the experiment the buffer solution strength 0.10M with six different pH ranging from 6.5, 7.0, 7.5, 7.6, 7.7 and 7.8 were taken in combination with substrate solutions of the strength 0.10 M, 0.15 M, 0.20 M, and 0.25 M. In these combinations as the pH of the buffer solutions increased, the NR activity also increased up to the pH 7.6 after that the activity decreased in all the substrate combinations. Again when the substrate concentration increased from 0.10M to 0.25M, NR activity also increased up to 0.15 M and after this concentration, the activity decreased. Among all the combinations higher NR activity (934.47$\pm$ 77.84 n moles nitrate reduced per gram fr wt per hour) was recorded in the combination of 0.15M substrate with buffer pH 7.6 (Table 1). The experiment was repeated with same substrate solutions i.e., 0.10M, 0.15, 0.20M, 0.25M and buffer solutions (0.20M) ranging from 6.5 to 7.8 pH as it was done in phase I. In the second phase, it was again observed that as the substrate concentration increased the NR activity also increased up to the substrate concentration of 0.15M, after that it decreased. Secondly, among different pH combination of 0.20M buffer, the activity increased up to the pH 7.6 and after that it decreased in all the combinations. However, maximum (204.68 $\pm$ 18.66 n moles NO_3^- reduced per gram fr wt per hour) activity was recorded in the combination of 0.15M, KNO_3 substrate with buffer pH 7.6 (Table 2).

Result of Table 1 and Table 2 shows that NR activity was lowest in combination of 0.10M substrate and 6.5 pH buffers. Therefore, this combination of substrate concentration and buffer pH was discarded and rest all was considered in third phase. It was again observed that NR activity was highest in the combination of 0.15M substrate and 7.6 pH of both strength of the buffer solution (Table 3). However, on comparing the two buffer concentrations (0.1M and 0.20M) higher NR activity (225.11+ 10.35 n moles NO_3^- reduced per gram fr. wt. per hour) was recorded in the combination of 0.10M buffer (pH 7.6) and substrate solution 0.15M. Therefore, the above mentioned solutions strengths and pH of the incubation media should be considered for the NR activity in *A. chinensis* leaves.

The rate of per gram fr. wt. per hour and per leaf fr. wt. per hour NR activity from newly emerged to mature leaves increased up to 6th and further decreased after 7th with increase in leaf age (Figure 1a&b). Similar observations were recorded by Pokhriyal and Raturi, 1985 in *Populus deltoids* where maximum activity was observed in 6th leaf blade. *Eucalyptus* hybrid shows maximum NR activity in 4th, 5th & 6th leaf blades (Pokhriyal and Raturi, 1984) and in *Myrica esculenta*, NR activity increased up to 8th leaf afterwards it decreased (Chaukiyal *et. al.,* 2014). Therefore, different leaves of the same plant vary in NR activity. Statistically, significant differences (P≤0.05) were observed from newly emerging to mature leaves of the *A. chinensis* plants for per gram and per leaf NR activity (Table 4).

Fresh and dry weight of individual leaf increased up to 6th leaf, remained constant up to 7th and afterwards it started decreasing gradually from 8th to 12th leaf (Figure 1 c). Mean differences for fresh and dry weight were found significant (P≤ 0.05) (Table 4). Similar type of results were reported by Pokhriyal and Raturi (1984 and 1985); Chaukiyal and Pokhriyal (1996) and Chaukiyal *et al*. (2014). Moisture content in first to ninth leaf was recorded between 60 to 70% and further decreased from tenth to twelfth leaf (Figure 1d). Similarly, significant differences (P≤0.05) were also found from newly formed to 12th (mature) leaf for moisture content (Table 4).

In the further study on variation in NR activity with increasing the time during a day, it was observed that the activity increased from 9:00 a.m. to 1:00 p.m. and afterwards it started to decrease from 3:00 p.m. to 5:00 p.m. (Figure 2 a). Difference between at least two means for per gram NR activity was found significant (P ≤ 0.05) (Table 5). Temperature and light has long been recognized to have stimulated effects on

NR activity. In green plants, both intensity and duration of light affects level of the enzyme (Lillo, 1994; Nicholas *et al.,* 1976 and Kenjebaeva and Natasha, 1995). The report shows that dark inactivation was reversed by illumination of the seedlings. Beevers and Hageman (1969) have shown that there is a decrease in the amount of extractable NR enzyme when plants are placed in the dark. They suggested that light may affect the uptake and utilization of nitrate by changing cell membrane permeability. Hatam (1980) reported that NR activity shows diurnal variation and observed that maximum NR activity in soybean occurred in the early afternoon and declined to minimum at night.

In agriculture, comprehensive work has been done on nitrogen assimilation as compared to forestry. In a few economically important forestry species like *Eucalyptus, Populus deltoides, Albizia lebbeck, Acacia nilotica* and *Dalbergia sissoo* standardization of buffer and substrate has already been done by Pokhriyal and Raturi (1984 and 1985) and Pokhriyal *et al.* 1988. In some dry zone species like *Clitoria ternatea, Mucuna pruriens, Rhynchoaia minima Crotalaria burhia,* and *Mimosa hamata,* buffer and substrate solutions were also standardized by Chaukiyal (2008 a, b, c, d; 2009). Recently, Chaukiyal and Mir (2010) in *Terminalia chebula*, Semwal *et al.* (2012) in *Grewia optiva*, Rautela *et al.* (2013) in *Castanospermum australe*, Ratrey *et al*. (2013) in *Erythrina blakei* and Chaukiyal *et al.* (2014) in *Myrica esculanta* also assessed the buffer and substrate solutions for nitrogen assimilation study. Therefore in *A. chinensis*, a buffer solution of 0.10M (pH 7.6) and substrate solution of 0.15M was observed optimum for maximum nitrate reductase activity in the leaves. There is a need to understand the nitrogen assimilatory processes in relation to growth and development in the fast growing nitrogen fixing tree species, so that their nitrogen assimilation potential can be screened and used to exploit for increasing the productivity in per unit area.

Table 1. *In-vivo* assay of NR (n moles NO_3^- reduced per gram fr wt per hour) activity in different incubation medium containing different buffer and substrate concentrations in *A. chinensis* leaves

0.10M KH_2PO_4 with different pH	Substrate concentrations (KNO_3)			
	0.10M	0.15M	0.20M	0.25M
6.5	311.50±20.39	471.06±6.10	174.89±17.04	61.27±2.23
7.0	420.85±126.09	562.97±23.02	278.30±35.06	91.91±13.66
7.5	439.57±90.57	618.72±58.72	304.68±33.77	139.14±19.44
7.6	492.76±31.19	934.47±77.84	483.83±42.20	375.32±47.27
7.7	354.89±28.55	788.93±95.007	406.38±32.93	349.36±9.90
7.8	347.66±27.44	782.55±25.07	354.04±13.32	157.45±31.46

Table 2. *In-vivo* assay of NR (n moles NO_3^- reduced per gram fr. wt. per hour) activity in different incubation medium containing different buffer and substrate concentrations in *A. chinensis* leaves

0.20MKH_2PO_4 with Different pH	Substrate concentrations (KNO_3)			
-	0.1M	0.15M	0.20M	0.25M
6.5	95.32±1.63	127.23±3.18	118.29±4.73	81.98±15.85
7.0	105.96±6.60	140.43±6.12	128.72±8.33	127.87±6.4
7.5	130.64±6.77	200.43±8.46	167.66±16.66	161.70±38.50
7.6	143.62±17.86	204.68±18.66	190.43±15.69	178.51±8.28
7.7	85.32±4.13	177.44±6.84	124.04±9.64	114.04±8.23
7.8	85.10±2.92	147.23±16.60	104.04±7.1	94.68±5.08

Table 3. *In-vivo* assay of NR (n moles NO_3^- reduced per gram fr. wt . per hour) activity in different incubation medium containing different buffer and substrate concentrations in *A. chinensis* leaves

0.10 M KH_2PO_4 with different pH	Substrate concentrations (KNO_3)			0.20 M KH_2PO_4 with different pH	Substrate concentrations (KNO_3)		
	0.15M	0.20M	0.25M		0.15M	0.20M	0.25M
7.0	169.79 ±10.64	160.63 ±3.55	138.94 ±6.79	7.0	93.61 ±4.50	68.93 ±6.15	51.06 ±2.87
7.5	178.72 ±6.55	170.47 ±10.79	145.53 ±4.38	7.5	108.93 ±13.89	93.19 ±9.47	82.98 ±9.92
7.6	225.11 ±10.35	189.57 ±39.56	154.04 ±9.45	7.6	163.40 ±9.59	106.80 ±9.14	94.04 ±2.41
7.7	160.00 ±12.44	154.25 ±9.09	130.42 ±8.54	7.7	91.00 ±4.69	80.85 ±2.34	41.27 ±1.38
7.8	136.81 ±3.28	129.57 ±4.01	117.87 ±9.63	7.8	79.57 ±1.79	53.19 ±3.75	34.89 ±1.70

Table 4. Variations in NR activity, moisture content, fresh and dry weight among different leaf blades of *A. chinensis*

	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12	F	P	Sig.	CD
NRA per gram fr. wt. per hour	86.00 ±16.62	129.25 ±15.80	172.34 ± 28.22	275.27 ± 24.24	337.50 ± 18.58	530.58 ± 23.82	251.33 ± 13.26	250.13 ± 17.41	186.30 ± 35.49	163.56 ± 15.36	99.33 ±12.88	51.86 ±15.94	17.90	≤ 0.001	S	89.06
NRA per leaf fr. wt. per hour	244.9 ±66.97	428.6 ±85.88	603.79 ±137.02	1003.01 ±80.19	1380.77 ±109.31	2450.31 ±167.31	1129.66 ±107.23	932.58 ±90.78	652.38 ±186.74	467.8 ±45.92	215.16 ±36.45	79.00 ±35.16	37.68	≤ 0.001	S	305.70
Fresh wt. (gm)	2.80 ±0.24	3.27 ±0.14	3.56 ±0.27	3.74 ±0.29	4.09 ±0.04	4.63 ±0.17	4.49 ±0.22	3.77 ±0.25	3.51 ±0.09	2.92 ±0.19	2.22 ±0.13	1.66 ±0.15	19.96	≤ 0.001	S	0.5634
Dry wt. (gm)	0.85 ±0.03	1.12 ±0.09	1.22 ±0.1	1.22 ±0.12	1.31 ±0.14	1.54 ±0.11	1.59 ±0.12	1.36 ±0.11	1.22 ±0.06	1.2 ±0.06	1.05 ±0.06	0.9 ±0.04	5.69	≤ 0.001	S	0.2667
Moisture %	69.15 ±1.81	65.42 ±3.42	65.56 ±2.92	67.36 ±2.28	67.99 ±3.34	66.48 ±2.98	64.14 ±3.98	63.87 ±2.22	65.2 ±1.31	58.87 ±2.13	52.94 ±1.09	45.25 ±2.16	7.29	≤ 0.001	S	7.478

Table 5. Daytime fluctuation of NR activity in *A. chinensis* leaves

	9:00 a.m.	11:00 a.m.	1:00 p.m.	3:00 p.m.	5:00 p.m.	F	P	S/NS	CD
NRA g^{-1} fr. wt. h^{-1}	136.1 ± 8.33	272.5 ± 18.32	404.9 ± 10.83	323.1 ± 16.48	220.5 ± 14.36	51.68	≤ 0.001	S	40.63

S= Significant; NS= Non-significant; CD= Critical difference

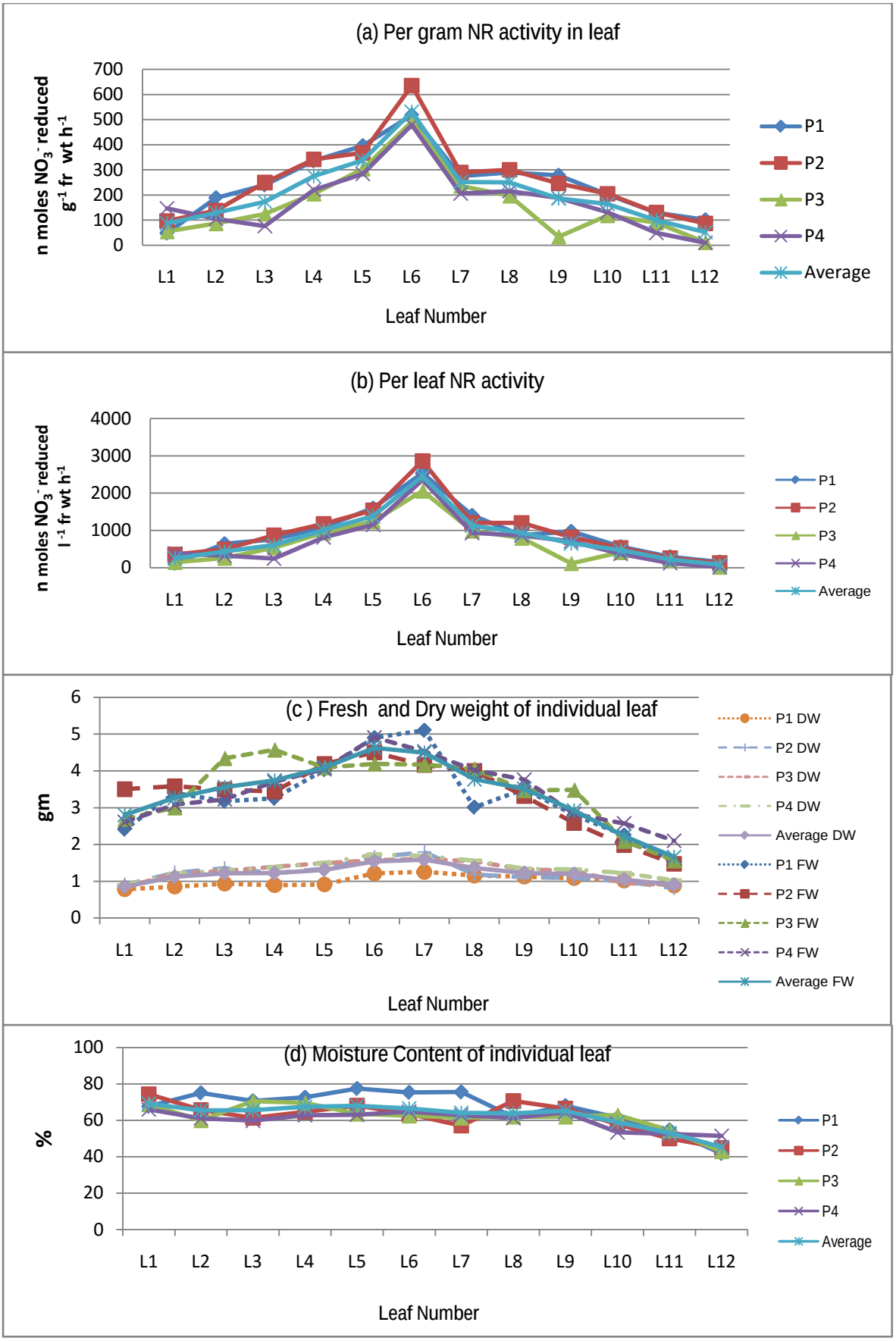

Figure 1(a, b, c and d): Nitrate reductase activity (a,b), fresh and dry weight (c) and moisture content (d) in individual leaf of *A. chinensis:* P1, P2, P3, P4 represents plant numbers; FW= Fresh weight; DW= Dry weight.

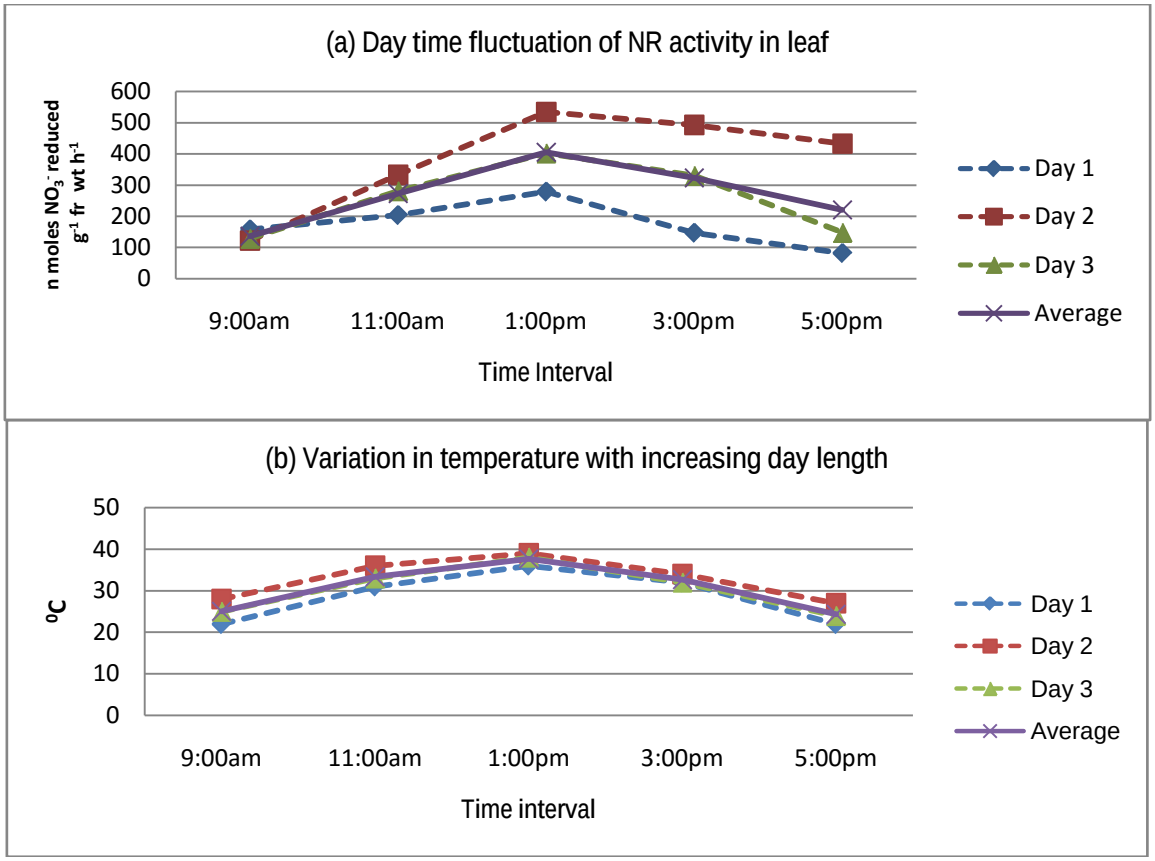

Figure 2 (a, b): Variations in day time temperature and NR activity in *A. chinensis* leaf

CONCLUSION

In the present study, maximum NR activity in the leaves of *Albizia chinensis* was observed in 0.15M substrate (KNO_3) in combination with 0.10M buffer (KH2PO4) of pH 7.6 solution. Using this combination, incubation media was prepared to further study the variation in NR activity with increasing leaf age (1st to 12th leaf) and also with increasing day length. It was observed that NR activity increased from 1st to 6th leaf and further decreases from 7th onwards. Similar pattern was observed for biomass also. Moisture content was recorded between 60 to 70% from 1st to 9th and further decreased up to 12th leaf. In a day time, NR activity increased from 9.00 am to 1.00 pm and after that it started to decrease.

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