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Influence of Inorganic Nutrients on the activity of Enzyme, Nitrate reductase in the leaves of Mulberry, Morus alba (L) (M-5 variety)

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Abstract

The protein content of mulberry leaves is directly related with the potential of nitrate reductase enzyme. Effect of kinetic parameters and inorganic mineral nutrients (Mg; Zn and Mo) on the velocity of nitrate reductase catalyzed biochemical reaction was studied using the leaves of mulberry, Morus alba (L) (M-5 variety). Maximum velocity (Vmax) was found registered for PH=7.4; temperature=32^{0}C; incubation period= 30 minutes with vaccum infiltration manually at 5 minutes interval. For the purpose to determine Michaelis Menten constant (Km), the substrate concentration at which, the velocity of enzyme catalyzed biochemical reaction attain half of its maximum, attempt has been made towards the transformation of data on [S] and v. The key quotient: [(2v Vmax +S) ÷ v] – [S(1+Vmax) ÷ Vmax] was calculated. Plotting the key quotient verses the substrate concentration [S] has illuminated into a straight line intersecting both, X and Y axes at a point which correspond to : [(2Vmax^2 + Km)÷Vmax]. The equation of the plot correspond to be derived as: Y = - [S] + [(2Vmax^2+Km)÷ Vmax]. This plot is to be recognized as Punyamayee plot of enzyme kinetics. Accordingly the Michaelis Menten constant (Km) of nitrate reductase catalyzed biochemical reaction in assay sample of mulberry leaves was found elevated in assay sample of leaves of mulberry plant recipient of foliar spray of magnesium sulphate, Zinc sulphate and ammonium molybdate. The optimum dose for magnesium sulphate and Zinc sulphate was 2.5mM, while with the ammonium molybdate, it was 0.01mM. The enzyme nitrate reductase was found significantly influenced with the optimum dosage of inorganic nutrients like $MgSO_4$; Zn and $(NH_4)_2$ Mo O_4. The nitrate reductase activity may be considered as predictive test for protein rich yield of leaves in mulberry. Efficient use of inorganic nutrients for qualitative protein levels in mulberry leaves serve to orchestrate the moriculture practices and thereby the qualitative improvement in cocoon yield of silkworm, Bombyx mori(L).

Keywords: Michaelis Menten constant, reductase enzyme, Mulberry.

Introduction

Nitrogen is having Key position in the protein and therefore a constituent of every living cell. It is a basic nutrient in the synthesis of proteins, amino acids, chlorophyll and alkaloids. In nitrogen deficient mulberry plant, the vegetative growth is stunted, the leaves dry up or shed prematurely. Nitrogen deficiency reduces the protein and water content of the leaves, thereby reducing the nutritive value of the leaves. When nitrogen supplied in optimum quantity and the plants put forth vigorous vegetative growth, the leaves enlarge in the size, become deep dark green, indicating the increase in chlorophyll content. Nitrogen, thus, increase the vegetative growth, number, size and weight of leaves. Ultimately, the nitrogen help to increase the yield. Also, the leaves become succulent, improving their feed value to the silkworms[1].

Silk protein produced by silkworm, Bombyx mori (L) is directly derived from broad spectrum of proteins and amino acids available in the leaves of mulberry, Morus alba (L)[2]. Growth and development of silkworm larvae and economic characters of cocoon are greatly influenced by the nutritional status of mulberry leaves[3]. Biochemical constituents of mulberry leaves play important role for successful cocooning in silkworm, Bombyx mori (L)[4]. The quality of nutrition in the larval stage in silkworm is having significant influence on the life of pupa, adult and silk production[5]. Improvement of quality foliage seems to be essential part of sericulture. In this respect most of the work seems to pertain application of fertilizers[6-10]. Efficient utilization of exotic genetic resources resulted in promising hybrid with very good quality foliage[11]. Most of these studies confined to growth, yield parameters, biochemical components of mulberry leaves and their influence on the economic parameters of silkworm, Bombyx mori (L).

The nitrate reductase enzyme serves to interplay the orchestration of fixation nitrogen through loss of oxygen. The converse of the concept of oxidation is true for reaction. The enzyme nitrate reductase is the key enzyme for the metabolism of proteins and carbohydrates. It has been demonstrated that, the nitrate reductase is inducible enzyme, found to be stimulated in rice plant by monovalent cations, such as Na^+ and K^+ and in vigna mungo (L) by divalent cations, such as Ca^{++}. For the purpose to study the influence of various parameters on the

velocity of nitrate reductase in mulberry leaves, the present study was carried out[12,13].

Material and Methods

The saplings of mulberry, Morus alba (L) (M-5 variety) were procured from mulberry garden at Malegaon farm of Agriculture Development Trust. They were raised in the nursery using earthen pots having soil mixed with farm yard manure in the proportion of 3:1 and sterilized with five percent formaldehyde. The plants of M-5 Variety of mulberry were raised in natural light through the recommended cultural practices[14].

The compounds selected for foliar treatment include Magnesium sulphate (source of Mg), zinc sulphate (source of Zn) and ammonium molybdate (source of Mo). According to recommendations[15], four concentratons of each compound were prepared by dissolving appropriate quantity in distilled water. The concentrations for magnesium sulphate and zinc sulphate were 0.00, 1.0, 2.5, 5.0 and 10.0mM each. For ammonium molybdate, the concentrations were 0.00, 0.005; 0.01, 0.05, 0.01, 0.05, and 0.1mM. Two months old mulberry plants were selected for the experimentation. They were divided into one control group and twelve experimental groups, each group consisted of 25 plants. The foliar spray of distilled water (control group), magnesium sulphate, zinc sulphate and ammonium molybdate (experimental groups) was used twice a day (8.0am and 4.0pm.) up to drain out point (5-7 ml Per plant). The foliar spray was carried out for 20 days using hand sprayer. The plants were allowed to grow in natural conditions with daily watering (7 a.m.).The activity of nitrate reductase was carried out daily on: 0th, 5th, 10th, 15th and 20th. For homogeneous sampling, three leaves were plucked off each from the base, middle and from the region below the shoot apex. The leaves were cut into narrow strips (2-3mm) and homogenized in n-propanol using morter and pestle (250mg/ml). The homogenate was filtered in muslin cloth and the filtrate was used as assay sample. Half the volume of assay sample was used for determination of proteins (S.P. and T.P.)[16]. The nitrate reductase activity was determined through the method of Hageman and Hucklesby[17] with modifications suggested by Srivastava[18]. The assay mixture consisted of one ml Of substrate potassium nitrate (100mM); one ml Assay sample; two ml Phosphate buffer solution (pH=7.4). The assay mixture was taken in a 15ml Capacity dark brown serum vial with air tight rubber cap. The vials were incubated for 30 minutes at 30^0C in water bath. During incubation period, vacuum infiltration was done manually at five minutes of interval with the help of hypodermic syringe with No.20 needle. After incubation, vials were cooled. The NO_2 released in assay mixture was determined calorimetrically by using one ml Of sulphanilamide (one percent in 1.5 N HCl, W/V) and 1ml of N- (1 – napthyl) ethylene diamine dichloride (0.02% in distilled water). This addition made the assay mixture purple color complex of azo dye, which was measured at 540nm, using Bausch and Lomb's spectronic-20. Sodium nitrate ($NaNO_2$) was used as standard to determine NO_2 content. In order to characterize the nitrate reductase enzyme in the assay sample of mulberry leaves, the factors influencing the velocity were introduced in the general set-up, which include : pH, temperature, incubation period and substrate concentration.

Punyamayee plot for determination of Michaelis Menten constant (Km): For the purpose to determine the Michaelis Menten constant (Km), attempt has been made towards calculation of key Quotient and plotting it verses the substrate concentration [S]. For this purpose, the very first step is to point out/mark the initial velocities and corresponding substrate concentration, that deserve Michaelis Menten stream. The (Vmax – v) was obtained and multiplied with respective substrate concentration [S]. This product [S(Vmax –v)] exhibit increasing tendency as biochemical reaction proceed along substrate concentration [S]. Practically the product [S (Vmax – v)] that correspond to the velocities less than half of its maximum (Vmax÷2) (up to some extent) (3Vmax÷4) seems to deserve the increasing tendancy. Therefore the initial velocities belong to increasing tendency in their, product: [S (Vmax – v)] were marked as the 'velocities of Michaelis Menten, stream' and considered for the plot. The ratio: [S (1+Vmax)÷Vmax] for each substrate concentration was calculated. Another ratio [(2vVmax + S)÷v] was also calculated. The figure obtained by subtraction of [S(1+Vmax)÷Vmax]from [(2vVmax+S)÷v] was designated as Key Quotient. Substrate concentration [S] were arranged on x-axis and key Quotient on y – axis. The line, slope of which correspond to one was obtained. Calculation of Key Quotients and plotting them verses the substrate concentrations help to transform the data on substrate concentration [S] and velocities [v] into linear form. The values of points intersecting x and y axes are one and same. This point correspond to: $[(2Vmax^2+Km)÷Vmax]$. This figure help for calculation of Michaelis Menten constant (Km), the substrate concentration at which velocity of enzyme catalyzed biochemical reaction attain half of its maximum. The graphical representation of substrate concentration and key Quotient for determination of Km value, is to be recognized as "Punyamayee plot of enzyme kinetics."

All the experiments were conducted in triplicate. The data was subjected for statistical analysis[19].

Results and Discussion

The data pertaining to the effect of inorganic mineral nutrients on the nitrate reductase activity in the leaves of mulberry Morus alba (L) (M-5 variety) is summarized into table 1-6. Use of inorganic mineral nutrient – foliar spray has found resulted into significant increase in the soluble and total protein content in the leaves of mulberry, Morus alba (L) (M-5 variety). Maximum increase in soluble protein was recorded on 10th day in 10mM $MgSO_4$ treated group.

Table – 1
Influence of inorganic nutrients on Nitrate Reductase activity in the leaves mulberry, Morus alba (L) (M-5 variety)

Conc. Of inorganic foliar spray (mM)	Nitrate Reductase activity (Micromol. NO_2 released/mg protein/min.) Age of mulberry (Days after spraying micronutrients)				
	0	**5**	**10**	**15**	**20**
0.00	4.374 (+0.93)	4.461 (+0.482)	4.962 (+0.327)	4.716 (+0.995)	4.739 (+0.931)
Magnesium Sulphate					
1.0	5.667 (+0.0243) 29.561	5.581 (+0.364) 25.106	5.672 (+0.358) 14.308	5.169 (+0.635) 9.605	5.094 (+0.763) 7.491
2.5	5.712 (+0.189) 30.589	5.923 (+0.276) 32.772	5.765 (+0.178) 16.182	5.813 (+0.579) 23.261	5.771 (+0.864) 21.776
5.0	5.419 (+0.674) 23.891	5.728 (+0.681) 28.401	5.683 (+0.717) 14.304	4.978 (+0.684) 5.555	4.381 (+0.652) 2.996
10.0	5.354 (+0.663) 22.405	4.694 (+0.519) 27.639	5.012 (+0.339) 1.007	4.958 (+0.573) 5.131	4.845 (+0.748) 2.236
Zinc. Sulphate					
1.0	5.148 (+0.576) 17.695	6.185 (+0.432) 38.646	7.056 (+0.359) 42.200	6.547 (+0.491) 38.825	6.176 (+0.712) 30.322
2.5	6.769 (+0.882) 54.755	6.743 (+0.916) 51.154	7.861 (+0.638) 58.424	7.123 (+0.889) 51.039	6.486 (+0.756) 36.864
5.0	5.591 (+0.627) 27.823	6.921 (+0.579) 55.144	7.542 (+0.768) 51.995	5.653 (+0.671) 19.868	5.492 (+0.843) 15.889
10.0	5.378 (+0.653) 22.953	6.638 (+0.874) 18.939	6.715 (+0.562) 35.328	5.582 (+0.913) 18.363	5.457 (+0.629) 15.150
Ammonium Molybdate					
1.0	6.347 (+0.712) 45.107	7.063 (+0.754) 58.327	7.887 (+0.641) 58.949	5.892 (+0.679) 24.936	5.669 (+0.654) 19.624
2.5	8.893 (+0.712) 103.315	9.887 (+0.814) 121.631	9.896 (+0.726) 99.435	7.485 (+0.862) 58.715	7.213 (+0.756) 52.205
5.0	9.158 (+0.553) 109.373	9.979 (+0.847) 123.694	9.376 (+0.813) 99.435	9.392 (+0.541) 99.151	8.789 (+0.627) 85.461
10.0	8.396 (+0.681) 91.952	9.251 (+0.526) 107.35	9.859 (+0.612) 98.691	10.082 (+0.576) 113.78	9.849 (+0.587) 107.828

Total proteins of $MgSO_4$ treated group were found increasing with increase in the concentration of treatment and number of days of treatment. Zinc sulphate and ammonium molybdate registered increase in both, soluble and total proteins for all the four concentrations.

The nitrate reductase activity in untreated group was found measured 4.374; 4.461; 4.962; 4.716 and 4.739 units respectively on 0th, 5th, 10th, 15th and 20th days of foliar spraying. With all the inorganic nutrients, the nitrate reductase activity was found enhanced with increase in the concentration to certain level (table – 1). However, in the case of 10.0mM magnesium Sulphate and 0.1mM ammonium molybdate, the nitrate reductase activity was noticed nonsignificantly increased. The Zinc sulphate was found influencing the increase in enzyme activity significantly at all the concentrations. All the concentrations of three inorganic nutrients were registered no inhibition in the nitrate reductase activity. The optimum concentration of the three inorganic nutrients through direct method and excised shoot dipping treatment was recorded 0.0075; 0.10 and 0.50mM for Ammonium molybdate; Zinc sulphate and Magnesium sulphate respectively. With optimum dose of magnesium sulphate 18-60 percent (Direct treatment) and 12-45 percent (shoot dipping), increase in enzyme activity was registered. Zinc sulphate and ammonium molybdate were found enrolling: 4.46-26 (Direct treatment); 13 (Shoot dipping) and 3-47(Direct treatment); 11-25 (shoot dipping) percent increase in the nitrate reductase activity. The excise shoot dipping method of inorganic nutrient seems to be significant over the direct method.

Enhancement of nitrate reductase activity in the leaves of mulberry plants sprayed with different doses of various inorganic mineral nutrients is of physiological significance as well as utilization value. The importance of minerals like Mg, Zn, MO for general physiology of cell, especially in enzyme activation is well recognized[20-27] Therefore, increased level of activity of nitrate reductase in mulberry leaves in the study would have been caused through general stimulatory effect of inorganic mineral nutrients. This enzyme is inducible found to be stimulated in rice plants by monovalent cations such as Na^+, K^+[12] and in Vigna mungo by divalent cations such as ca^{++}[13]. Increase in nitrate reductase by magnesium, Zinc and molybdate seems to be in the same way as it induced by monovalent cations. It has been well established that, nitrate reductase is to considered as predictive index of crop yield through proteins of foliage[28 and 29].

With the reference to kinetic parameters, the nitrate reductase activity in the leaves of mulberry, the enzyme activity was found maximum for pH=7.4; 32^0C and 30 minutes of incubation period in control group and inorganic nutrients treated groups. The treated groups exhibited significant increase in the enzyme activity. The optimum conditions (pH, temperature and incubation period) allow intramolecular reorientation of enzyme molecule leading probable exposure of more active sites, contributing improved catalytic potential of system. Activation energy for any enzyme is concerned with temperature and incubation period of reaction mixture. Most of the enzymes exhibit deflecting tendency in their optimum conditions for maximum velocity[30]. Nitrate reductase in the leaves of mulberry Morus alba (L) (M-5 variety) seems to be unaffected by inorganic nutrients in its optimum conditions. Whether the reaction mixture is with inorganic nutrients or without, the optimum pH, temperature and incubation period were found unaltered. The only difference was in the percent change in the velocity of nitrate reductase catalyzed bio chemical reaction.

Substrate concentration [S] is the key factor influencing the intensity of velocity of enzyme catalyzed biochemical reaction. Hydrolysis of varying concentrations of substrate [S] (mM KNO_3) by the leaf homogenate (assay sample) from Morus alba (L) (M-5 variety), in both groups (control and inorganic mineral nutrient treated) exhibited gradual increase in velocity of biochemical reaction. The incubation mixture of control group was found elevated upto 40mM of substrate with 4.786 units of velocity. The inorganic mineral nutrients treated group of incubation mixtures was found achieving the increased level of maximum velocity (which correspond 4.913 units for $MgSO_4$; 7.062 units for $ZnSO_4$ and 6.983 units for ammonium molybdate).

The substrate concentration [S] at which reaction mixture attains its maximum was found same for control and Magnesium sulphate treated group. The reaction mixtures belong to Zinc sulphate and ammonium molybdate treated groups were found stabilizing earlier. Increase in velocity of enzyme catalyzed biochemical reaction corresponds to increase in the substrate concentration in a definite pattern. Practically, this pattern of increase in velocity corresponds to certain specific substrate concentration. This is because; the substrate binds to active sites in the enzyme-protein. When only a small amount of substrate is present in a system (reaction mixture), some active sites centers in enzyme-protein are left free and enzyme use to work at a rate less than its maximal. The maximal rate (Vmax) is achieved when all the active sites/centers of enzyme protein get saturated. Theoretically, a typical hyperbolic curve explains the velocity of enzyme catalyzed biochemical reaction for varying concentration of substrate. Inorganic mineral nutrient treatment may serve to orchestrate earlier saturation (especially for Zinc sulphate and ammonium molybdate)of substrate for nitrate reductase.

Maximum velocity of enzyme catalyzed biochemical reaction and Michaelis menten constant (Km) are the pace makers for enzyme kinetics. The Km represent quality of reaction, especially, the kinetic status of enzyme catalyzed turnover. Several methods have been proposed for the purpose of plotting the data pertaining substrate concentration and velocity. The hyperbolic curve obtained by plotting velocity of reaction [v] verses the substrate concentration [S] gives the quantitative idea of reaction, but not the qualitative. Therefore, the data is to be

more conveniently plotted after transforming it into a linear form. The attempts in this regard include: Line weavers- Burk plot; Hans-Woolf plot; Woolf- Augustinsson- Hoffstee plot; Eadie – Scatcherd plot …….. etc.[30]. One more attempt has been made to determine the kinetic constant, Km from the data obtained in the experimentation, under the heading of "Punyamayee plot of enzyme kinetics." mathematical equation of the plot was found derived as :

$$Y=-S+[(2Vmax^2+Km)\div Vmax].$$

Plotting the key Quotient $[(2vVmax+S)\div v] - [S(1+Vmax)\div Vmax]$ verses the substrate concentration [S] has illuminated into a linear transformation exhibiting a line having slope=1 and intersecting both the axes at a point corresponding to $[(2Vmax^2+Km)\div Vmax$. Accordingly, this point of intersection (the constant from equation (Y=mx+c) for control, Magnesium sulphate, Zinc sulphate and ammonium molybdate treated groups in the study found calculated 13.572; 13.618; 16.270 and 15.45 respectively. This figure help to calculate the value of Km. The km for control, magnesium sulphate, Zinc sulphate and ammonium molybdate treated groups in the study found calculated as : 19.144; 18.632, 15.162 and 10.356 mM KNO_3 respectively. Experimental tissue enzyme has higher Vmax and lesser Km over the control tissue.

Significance of the plot is summarized as: i. Marking the substrate concentrations of Michaelis Menten stream for consideration for the plot deserve applicable influence for linear transformation of the data on [S] and v. ii. Calculation of Key Quotient $[(2Vmax+S)\div v] - [S(1+Vmax)\div Vmax]$ and plotting it verses substrate concentration [S] are simple mathematical operations. iii. The lower substrate concentrations [of Michaelis Menten stream] yield precise and significant key quotients in the plot. iv. The points of intersection of the line in the plot on both the axes are one and same which, correspond to $[(2Vmax^2+Km)\div Vmax]$. This will give value of Km. v. The plot seems to be more reliable, excellent and therefore deserve wider use.

Inorganic mineral nutrient foliar treatment in mulberry affects the leaf biomass and protein content. Efficient use of such foliar spray serves to interplay for orchestration of foliar improvement through nitrate reductase.

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Table – 2
Effect of substrate concentration [S] on the nitrate reductase activity[v] in the leaves of mulberry, Morus alba(L) (M-5 variety)

Group → Concentration of KNO_3 (mM)[S] ↓	Control	$MgSO_4$	$ZnSO_4$	$(NH_4)_6MoO_4$
5	0.991 (+0.047)	1.039 (+0.021)	1.751 (+0.132)	2.273 (+0.119)
10	1.642 (+0.327)	1.715 (+0.168)	2.806 (+0.159)	3.431 (+0.138)
20	2.445 (+0.361)	2.543 (+0.276)	4.117 (+0.188)	4.603 (+0.169)
30	3.784 (+0.439)	3.889 (+0.354)	6.912 (+0.243)	6.698 (+0.264)
40	4.396 (+0.621)	4.517 (+0.409)	7.062 (+0.342)	6.983 (+0.264)
50	4.786 (+0.538)	4.913 (+0.476)	7.062 (+0.379)	6.983 (+0.213)
60	4.786 (+0.661)	4.913 (+0.324)	7.062 (+0.296)	6.983 (+0.237)

Each figure is the mean of three replications, Figure in parenthesis with + sign indicate the standard deviation, Figure below the standard deviation is the percent change over the control.

Table – 3
Data Pertaining Key Quotient of Nitrate reductase activity in the leaves of mulberry, Morus alba (L) (M-5 variety) (Group :- Control)

Substrate conc.[S] (mM KNO_3)	v	S(Vmax-v)	[S(1+Vmax)]÷Vmax	[(2vVmax+S)÷v]	Key Quotient
5	0.991	*18.975	6.0447	14.6174	8.5727
10	1.642	*31.440	12.0894	15.6621	3.5727
20	2.445	*46.820	24.1788	10.1924	13.8864
30	3.784	30.060	36.2682	17.5001	18.7681
40	4.396	15.600	48.3577	18.6711	29.6866
50	4.786	0.000	60.4471	20.0191	40.428
60	4.786	0.000	72.5365	22.1085	50.428

Table – 4
Data pertaining Key Quotient of Nitrate reductase activity in the leaves of mulberry, Morus alba(L) (M-5 variety) (Group : $MgSO_4$ treated)

Substrate conc.[S] (mM KNO_3)	v	S(Vmax-v)	[S(1+Vmax)÷Vmax]	[(2vVmax+S)÷v]	Key Quotient
5	1.038	*15.775	6.0177	14.6429	8.6252
10	1.718	*31.950	12.0354	15.6467	3.6113
20	2.543	*47.400	24.0708	17.6907	-6.3801
30	3.889	30.720	36.1062	17.5400	-18.2662
40	4.517	15.840	48.1416	18.6814	-29.4602
50	4.913	0.000	60.1771	20.0030	-40.1741
60	4.913	0.000	72.1249	22.0384	-50.0865

Table – 5
Data pertaining Key Quotient of Nitrate reductase activity in the leaves of mulberry, Morus alba (L) (M-5 variety) (Group : $ZnSO_4$ treated)

Substrate conc.[S] (mM KNO_3)	v	S(Vmax-v)	[S(1+Vmax)÷Vmax]	[(2vVmax+S)÷v]	Key Quotient
5	1.753	*26.545	5.7080	16.9762	11.2682
10	2.809	*42.531	11.4160	17.6839	6.2679
20	4.118	*58.880	22.8321	18.9807	-3.8514
30	6.912	4.500	34.2481	18.4642	-15.7839
40	7.062	0.000	45.6641	19.7861	-28.878
50	7.062	0.000	57.0731	21.2041	-35.869
60	7.062	0.000	68.4961	22.6201	-45.876

Table – 6
Data pertaining Key Quotient of Nitrate reductase activity in the leaves of mulberry, Morus alba (L) (M-5 variety) (Group : Ammonium molybdate treated)

Substrate conc.[S] (mM KNO_3)	v	S(Vmax-v)	[S(1+Vmax)÷Vmax]	[(2vVmax+S)÷v]	Key Quotient
5	2.276	*22.11	5.7465	15.5928	9.8463
10	3.432	*32.66	11.4930	16.3097	4.8167
20	4.603	10.26	22.9856	17.7409	-5.2447
30	6.356	0.000	34.4790	18.116	-16.363
40	6.698	0.000	45.9720	19.3679	-26.6042
50	6.698	0.000	57.4650	20.8609	-36.6041
60	6.698	0.000	68.9579	22.3538	-46.6041

* indicate that, the corresponding [S] and v belong to the “stream of Michaelis Menten”