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ENHANCEMENT IN THE YIELD OF DIOSGENIN BY NITROGEN AND SULPHUR NUTRIENTS IN *TRIBULUS TERRESTRIS* L.

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ABSTRACT

Field experiments were conducted to study the effect of nitrogen and sulphur nutrients on growth performance of a medicinal plant, *Tribulus terrestris* and yield of diosgenin. Application of urea at the rate of 60, 100 and 150 kg N ha^{-1} significantly enhanced the growth of plants and also the yield of secondary metabolite, diosgenin. Combined effect of Sulphur x Nitrogen (SxN) along with neem cake proved better than nitrogen alone. Our results indicate that specific and balanced application of inorganic, and/or organic nutrients can be effective in increasing the productivity of medicinal plants.

KEY WORDS: **Biomass, diosgenin content, neem cake, nitrogen and sulphur nutrients, *Tribulus terrestris.***

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INTRODUCTION

Over exploitation of medicinal plants from the wild is resulting in their rapid depletion and, consequent loss in biodiversity. This has necessitated the need for developing technologies which can integrate traditional practices with environmental and economic perspectives. In addition to crop plants, productivity of medicinal plants is an area that also requires sustained efforts. Unfortunately, medicinal plants have not received the attention they deserve. To boost the yield of plants, nitrogen is considered most important among all the major elements required. However, a major part of fertilizer N is lost through leaching or denitrification after its conversion to nitrate.[1, 2] This loss of ammonium-N can be minimized by delaying the conversion of ammonium to nitrate through the use of nitrification inhibitors.[3] Nitrification inhibitors have shown potential for reducing fertilizer N losses and improving its uptake as well as N-use efficiency.[4, 5] An attempt was made in the present study to optimize and enhance the yield of drug component of *Tribulus terrestris* through agrotechnology. It could be the first step to ensure higher yield and encourage farmers to adopt medicinal plants for better economic gains. *Tribulus terrestris* L. is an indigenous drug plant commonly used in Ayurvedic and Unani system of medicine. Presence of steroidal sapogenins in glycolysated forms has been documented in various morphological parts of the plant.[6,7,8,9] The flour from fruits is rich in calcium and is credited with diuretic and tonic properties. It is also prescribed in Bright's disease.[10] In traditional Chinese medicine the fruits are used for the treatment of eyes, edema and the abdominal distention, emission and morbid leucorrhea as well as vitiligo. The leaf paste is used for the treatment of stones in the bladder and applied for treatment of corns.

MATERIALS AND METHODS

2.1 Experimentation

The experiments were laid out in randomized block design plots (3x3 m) with three replicates of each treatment at experimental field at Hamdard University, New Delhi, India, located at $28^0$38' N, $77^0$11' E and 228m on asl. The weather conditions of the location are semi arid with sandy loam soil having N and S content of 700 and 100 μg g^{-1}, respectively.[11] *Tribulus* seeds soaked in water for 24 hours were sown in plots at equal distance of 15-20cm. Gypsum and urea blended with neem cake, were used to evaluate the interaction of sulphur and nitrogen on accumulation of biomass and its impact on the yield of diosgenin. The fertilizer treatments included three levels of nitrogen in the form of urea, viz. 60, 100 and 150 kg N ha^{-1} and sulphur, 30 kg ha^{-1} along with neem cake. 100 kg of commercial grade urea (46%) was coated with 20 or 40 kg of neem cake in a fertilizer mixing drum. The neem cake (Nc) contained 4.7% N and thus the amount of N added as Nc was only 2% of total N applied per treatment; this was, however, an organic N, which mineralizes slowly. Benzene hexachloride (BHC) was applied during preparation of beds to protect the plants from termites. Hand weeding was done regularly during the entire crop season. One uniform irrigation was provided after sowing and then subsequently, when required. The sampling was performed after 30-, 45- and 60-days of fertilizer treatment. Ten plants taken out randomly from each plot were washed properly to evaluate the growth parameters such as number and dry weight (DW) of shoots and fruits, chlorophyll a, b, protein and sulphur content, and nitrate reductase activity (NRA) in leaves. Total soluble protein in leaves was estimated by Bradford method.[12] 100 mg fresh tissue was homogenized in 1.5 ml (0.2M) phosphate buffer (pH-7.2) and centrifuged at 11,000 rpm for 10 min, supernatant was mixed with 10% Tri acetic acid (TCA) and recentrifuged at 3,300 rpm for 15 min. The pellet was dissolved in 1 ml (0.1N) NaOH. To 0.1 ml of aliquot, 5.0 ml Bradford's reagent was added and mixed vigorously. A blue colour developed within 2 min. The absorbance was taken at 595 nm with Lambda-20 spectrophotometer. The calibration curve was drawn using different concentrations of bovine serum albumin, treated similarly as that of aliquots to calculate protein content (mg g^{-1}fw). Chlorophyll a and b were estimated by the method of Hiscox and Israelstam by using Dimethyl sulphoxide.[13] Freshly harvested leaves (100 mg) were chopped and collected in test tubes each containing 7.0 ml DMSO. The tubes were covered with black paper and incubated at 65^0c for 60 min. The reaction mixture was transferred to a graduated tube and the final volume made up to 10 ml by adding DMSO. The chlorophyll content was then measured immediately. The activity of NR in the fresh leaves was assayed by the method of Srivastava.[14] Small pieces of leaves were collected in the test tube covered with black paper. In each tube 8.0 ml of 1.0M sodium phosphate buffer (pH 7.4), 1.0 ml of 2.0M KNO_3 and 1.0 ml of 25% n-propanol were added, sealed and incubated at 33^0C for 30 min. In 2.0 ml aliquot, 2.0 ml of 1% sulphanilamide and 0.02% N-naphthylethylenediaminedihydrochloride (NEDD) were added. The OD of the developed pink colour was measured at 540 nm using spectrophotometer Lamda-20. The amount of nitrate was calculated in μmoles $N0_2$ g^{-1} fw h^{-1} with the help of a standard curve prepared from sodium nitrate.

2.2 Screening for diosgenin

The active compound diosgenin was analyzed in treated and control plants.

2.3 High performance liquid chromatography (HPLC) of Diosgenin

Reverse phase HPLC (Perkin-Elmer) was performed with Petroleum ether (bp 60-80^0 C) and Isopropanol (12:1) as mobile phase, at a flow rate of 1 ml min^{-1}. A pressure of 500 psi was maintained by Binary LC pump 250. Separation was achieved by Waters Spherisorb cum silica analytical column (4.6x250 mm). A Perkin Elmer LC-30 RI detector was used with standard energy at 0.5, response time 2 and range (x 10^{-3} ΔRI) 0.005. Attentuation was set at 16 with the help of integrator. The retention time (RT) of diosgenin was 5.4-5.7. Calculations were made with the help of a Perkin Elmer LC1-100 Laboratory Computing Integrator.

2.4 Extraction Procedure for the Estimation of Diosgenin

Plant samples (500 g) were dried in oven at 90 ^{0}C and pulverized with a grinder. Each of the powdered sample was hydrolysed in conical flasks with 2.5 N HCl for 4 h in

a water bath adjusted at 100 ^{0}C. The content of the flask was cooled, filtered through Whatman No. 41 filter paper and washed until the residue was free from acid ascertained by measureing pH. The residue was dried at 100^{0}C and then extracted exhaustively with Petroleum ether (bp 60-80^{0}C) and Isopropanol (12:1) in a soxhlet for 4 h. The solvent was evaporated and the residue was used for HPLC analysis.

2.5 Sample Preparation for Diosgenin Estimation

The residue was dissolved as 1 mg ml^{-1} of HPLC grade Petroleum ether (12) :Isopropanol (1) and filtered through sartorius filters (0.2 μm). 20 μl of the sample solution was injected with an Exmire Microsyringe (MS-R25)[26] .

2.6 Standard Curve of Diosgenin

Stock solution (2.0 mg ml^{-1}) of diosgenin (Sigma, USA, ~98% purity) was prepared in HPLC grade Petroleum ether:Isopropanol (12:1). From the stock solution various dilutions were made in 1.0 ml solvent (Petroleum ether:Isopropanol). Three replicates of each concentration were analyzed through HPLC and the curve plotted between concentration and peak height showed good linearity.

2.7 Calculations

The relevant peaks of each sample were interpolated with the standard curve to determine the quantity of diosgenin (mg g^{-1} DM).

RESULTS

Interactive effect of organic and inorganic fertilizers.All the growth parameters measured showed maximum response after 45 days of treatment.

3.1 Shoot length

Maximum increase (235%) in shoot length was recorded after treatment with 100 kg N ha^{-1}. When S 30 kg ha^{-1} was also applied, 248% improvement in shoot length was observed. N (Urea) 100 kg ha^{-1} with 20% of Nc resulted in further increase in length by
298%, addition of S 30 kg ha^{-1} induced 300% increase in shoot length (Table 1).

3.2 Fruit number

216% increase in the number of fruits was noticed after treatment with N 100 kg ha^{-1}, alongwith 20% neem cake it resulted in 264% increase, with 40% Nc it was 238%. Combined treatment with S proved best (Table 2).

3.3 Dry matter of shoots

Maximum increase (178%) in dry matter of shoots was recorded treatment with 100 kg N ha^{-1}, alongwith 30 kg S ha^{-1} 191% improvement in dry matter was observed. 20% Nc ha^{-1} induced further increase in dry matter by 245%. The three, nitrogen 100 kg N ha^{-1}, 20% neem cake and 30 kg S ha^{-1} applied together induced 252% increase (Fig. 1).

3.4 Dry matter of roots

Maximum increase (200%) in dry matter of roots was after treatment with 100 kg N ha^{-1}. The increase was 209% with S 30 kg and N 100 kg ha^{-1} and with 20% Nc the dry matter increased to 254% (Fig. 1).

3.5 Dry matter of fruit

Application of nitrogen (100 kg N ha^{-1}) resulted in 200% enhancement in dry matter. Nitrogen along with S or with neem cake blended urea (20 or 40%) proved better. Urea blended with 20% Nc promoted 240% increase. 100 kg N ha^{-1} along with 30 kg S ha^{-1} induced increase by 200%. Of all the treatments, a combination of N (100 kg ha^{-1}) with Nc (20%) blended urea along with S (30 kg ha^{-1}) resulted in maximum increase (280%) in dry matter (Fig. 1).

3.6 Chlorophyll content

The percent increase in Chlorophyll a as influenced by 100 kg N ha^{-1}was 225. Corresponding figure for chlorophyll b was 184%.Same dose of urea when blended with neem cake (20%) proved better; chlorophyll a rose to 240% and b to 231%. All the three when applied together resulted in maximum yield of chlorophyll a and b (Fig. 2).

3.7 Soluble protein content

The percent increase in protein content over the control was 225 with 100 kg N ha^{-1}, N (urea) blended with 20% Nc elicited 278% increase. Maximum improvement (317%) in soluble protein was observed with 100 kg N ha^{-1}, 30 kg S ha^{-1} and neem coated urea (20%) (Table 3).

3.8 Nitrate reductase activity (NRA)

Nitrogen 100 kg ha^{-1} improved NRA by 218% over control. Sulphur 30 kg ha^{-1} along with 100 kg N ha^{-1} although proved better than N alone, the response was similar to those observed with blended urea (20 or 40%). When sulphur was applied with 20% Nc blended urea, it resulted in 350% increase in NRA (Fig. 3).

3.9 Diosgenin content

Urea (N) at the rate of 100 kg ha^{-1} enhanced the diosgenin content by 146%. When 30 kg S ha^{-1} was used along with Nc blended urea (20%) and 100 kg N ha^{-1}, 292% increase in diosgenin content was recorded. 100 kg urea (N) ha^{-1} blended with 40% Nc applied along with sulphur 30 kg ha^{-1} resulted in 267% increase (Fig. 4)

DISCUSSION

Nitrogen in soil occurs as organic nitrogen (N) and mineral N. The supply of soil inorganic nitrogen may be limiting for plant growth in many environments. Efficient use of N in plant production is essential. For improving N efficiency, inhibitors which retard nitrification for sufficiently longer time leading to reduction in the leaching loss of nitrate-N and improving N uptake have been used.[15] The effect of nitrification inhibitors, neem cake and dicynamide (DCD) on the efficiency of applied prilled urea nitrogen in a maize-wheat cropping system has been demonstrated.[16] Walters and Malzer have observed that nitrogen inhibitors increase the efficiency of N use even if potential for nitrate leaching was high.[17] Nitrification inhibiting properties of neem seed and its cake have earlier been reported by Reddy and Prasad.[18] In rice, neem cake coated urea has proved superior to prilled urea.[19] Our results also proved that urea applied

with neem cake significantly enhanced the growth performance measured as shoot growth, chlorophyll, total soluble protein content, NR activity and diosgenin content. Combined effect of SxN alongwith neem cake (20%) proved better. A number of studies have demonstrated the synergistic effect of combined application of S and N.[20, 21] Randall et al. observed that S application increased the yield of wheat grains.[22] Sulphur is reported to favour dry matter accumulation/plant and yield of secondary metabolites due to proper partitioning of photosynthates from source to sink.[23, 21] The present investigation also confirms that SxN interaction induces better response in comparison to nitrogen alone. A direct positive correlation between nitrate reductase activity and total organic nitrogen and protein content has been reported by Pandey and Singh.[24] Any variation in this enzyme affects nitrogen assimilation pathways and hence plant growth. The improvement in crop yield by enhancement of efficiency of the nitrate reduction process is amply demonstrated in a variety of studies on nitrate assimilation.[25] Our results demonstrate that application of urea alongwith neem cake can significantly increase the NR activity and growth performance coupled with increase in secondary metabolite of a medicinal plant, diosgenin content in this case.

Table 1

Tribulus terrestris– Length of shoot (cm) after 45 days of treatment with urea or neem cake (Nc) blended urea in the presence of sulphur.

	Neem cake 0	Neem cake 20%	Neem cake 40%
Treatments Kg ha^{-1}	**X ± S.E**	**X ± S.E**	**X ± S.E**
CONTROL	20.06±1.5	20.06±1.5	20.06±1.5
N 60	40.12±0.5	53.56±1.2	42.12±1.8
N 100	47.14±2.2	59.77±2.1	56.56±2.0
N 150	40.52±1.5	49.94±2.1	45.13±1.0
	S30 + Nc 0	S30 + 20%	S30 + 40%
N 60	41.12±1.4	59.17±1.0	46.13±1.6
N 100	49.74±1.9	60.18±1.0	48.14±1.5
N 150	44.13±1.0	41.12±1.1	59.57±0.6

N=Nitrogen, X=Mean, S.E=Standard error, 10 samples for each experiment.

Table 2

Tribulus terrestris– Number of fruits after 45 days of treatment with urea or neem cake (Nc) blended urea in the presence of sulphur.

	Neem cake 0	Neem cake 20%	Neem cake 40%
Treatments Kg ha^{-1}	**X ± S.E**	**X ± S.E**	**X ± S.E**
CONTROL	45±1.5	45±1.5	45±1.5
N 60	80±2.1	112±1.8	105±1.8
N 100	97±1.0	119±1.2	107±1.0
N 150	83±1.0	111±1.1	109±2.5
	S_{30} + Nc 0	S_{30} + 20%	S_{30} + 40%
N 60	94±1.2	124±1.2	111±1.5
N 100	108±1.3	134±1.1	112±1.2
N 150	94±1.6	119±1.5	118±1.5

N=Nitrogen, X=Mean, S.E=Standard error, 10 samples for each experiment.

Table 3

Tribulus terrestris– Protein content (mg g^{-1} fw) after 45 days of treatment with urea or neem cake (Nc) blended urea in the presence of sulphur.

	Neem cake 0	Neem cake 20%	Neem cake 40%
Treatments Kg ha^{-1}	**X ± S.E**	**X ± S.E**	**X ± S.E**
CONTROL	254.74±1.0	254.74±1.2	254.74±1.2
N 60	509.48±0.8	659.77±2.1	632.46±1.4
N 100	573.16±1.3	708.17±1.2	647.70±1.4
N 150	573.16±1.5	659.77±1.3	716.28±1.6
	S30 + Nc 0	S30 + 20%	S30 + 40%
N 60	607.06±1.9	736.19±0.5	662.32±1.3
N 100	635.00±1.9	807.60±1.5	776.95±1.4
N 150	533.44±1.6	700.53±0.6	807.60±1.1

N=Nitrogen, X=Mean, S.E=Standard error, 10 samples for each experiment.

Figure 1

Tribulus terrestris- Dry matter of shoot, root and fruit after treatments

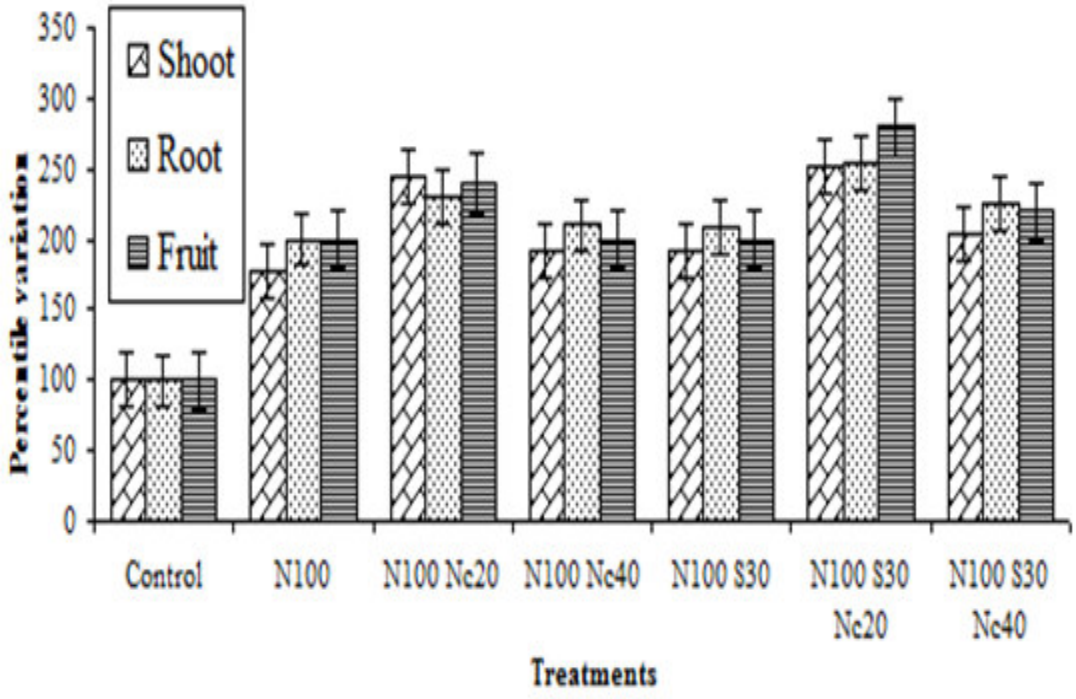

Fig. 1.

Figure 2
Tribulus terrestris- Chlorophyll a and b content after treatments

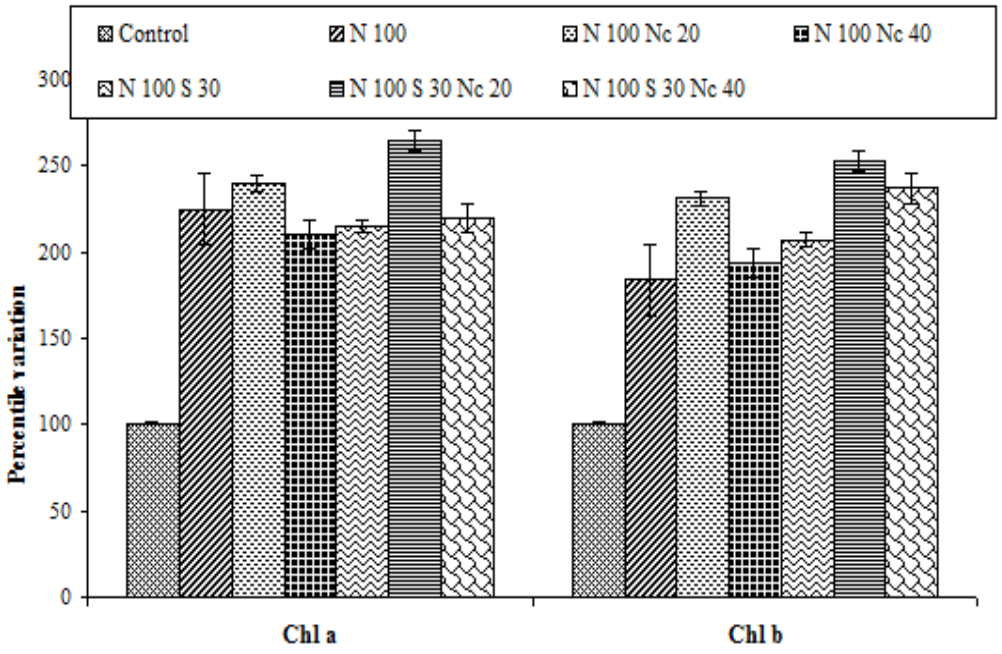

Fig. 2.

Figure 3
Tribulus terrestris- Nitrate reductase activity after treatments

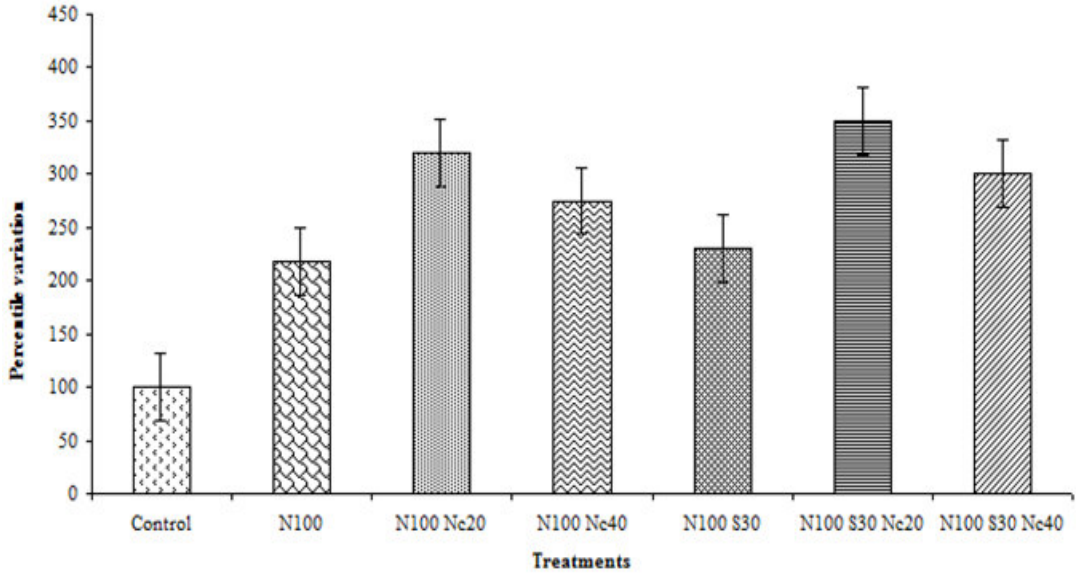

Fig. 3.

Figure 4
Tribulus terrestris- Diosgenin content after treatments

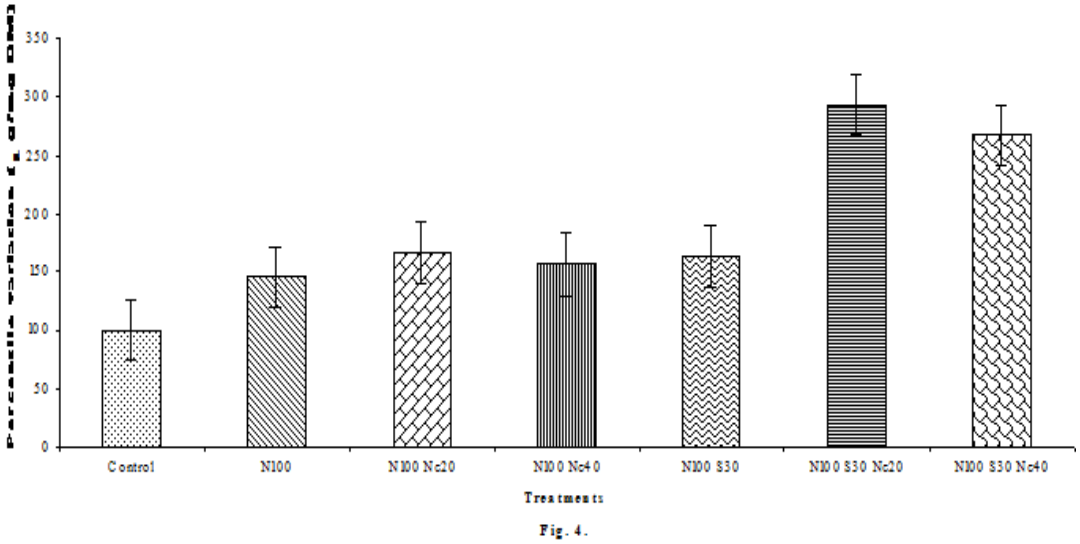

Fig. 4.

CONCLUSIONS

It is thus apparent that balanced application of nutrients significantly improves growth performance, yield and most importantly secondary metabolite content in medicinal plants. With resurgence of herbal system of medicine and to counter the enhanced related imbalance in supply and demand, it is imperative that sustained efforts are made to enhance the yield of drug component. Hitherto, little attempt was made on systematic study of medicinal plants, their growth requirement and correlative yield of secondary metabolite. Our report is one of the few attempts in this direction.

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