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Standardization and estimation of nitrate reductase activity in the leaves of *Ammi majus* L. (Bishops weed) in relation to sulphur deficiency and seed yield

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Abstract

Field experiments were conducted to determine the interactive effect of sulphur (S) and nitrogen (N) in relation to nitrate reductase (NR) activity as the enzyme catalyze rate-limiting step of the assimilatory pathway of nitrate, and seed yield of *Ammi majus* L. (commonly called as atrilal). To carry out this study, we cultivated atrilal in experimental field and used two combinations of sulphur (S) and nitrogen (N) nutrient (-S+N & +S+N in kg ha^{-1}) along with control group (without S & N) in soil during sowing. Sulphur & nitrogen nutrients were applied into field as single basal dose. To the best of our knowledge, no report has been published so far to get the reliable protocol for NR activity in terms of medicinal plant productivity and quality; hence, we standardized the NR activity in leaves prior to its estimation in the experimental materials. Results show that sulphur application significantly enhanced NR activity and physiological changes which resulted in higher seed yield ($P<0.05$) when compared with the sulphur deficient (-S+N) and control group. Internal CO_2 concentration and stomatal conductance were also high in the leaves of Atrilal at different growth stages and this increase may be correlated with increased photosynthesis rate and higher NR activity in the leaves of plants grown with combined application of S and N when compared with N alone. The results obtained in this study suggested that sulphur is involved along with nitrogen in regulation of enhanced NR activity and physiological changes which results higher seed yield.

Keywords: *Nitrate reductase activity*, *Ammi majus* L., *Sulphur, nitrogen, seed yield*
Abbreviations: S-sulphur, N-nitrogen, NR-nitrate reductase, q ha^{-1}–100 kg/hectare, ATP- adenosine tri phosphate, T-treatment, Acetyl-CoA-acetyl coenzyme A

Introduction

Ammi majus L., commonly called as Atrilal in India is widely used in the treatment of certain skin diseases like vitiligo since ancient times (Ahmad *et al*., 2004, 2007b). It synthesizes furocoumarins such as xanthotoxin and bergaptons, which are used to prepare cream and lotions to cure the skin diseases (Ahmad *et al*., 2006, 2007a). Seeds are the main source of furocoumarins and that is the reason for high demand of this plant in pharmaceutical companies. There are not many reports on its cultivation and enhanced production. Hence, considerable attention is now being paid to develop good agro-technology to enhance the seed as well as secondary metabolites yield of *Ammi majus* to fulfill the demand of pharma sector. It is well known that sulphur (S) and nitrogen (N) are essential plant nutrients and play an important role in plant growth and development (Fazili *et al*., 2010; Chandana *et al*., 2010; Kazemeini *et al*., 2010; Zhao *et al*., 1999), but much of the work has been done on crop productivity in relation to sulphur and nitrogen application and their interaction. Fazili *et al*. (2005, 2006) reported that increased sulphur and nitrogen nutrition can affect the lipid accumulation, acetyl-CoA concentration and acetyl-CoA carboxylase activity in developing seeds of oilseed crops that leads to enhanced crop productivity. It was reported by several investigators that initially S was met by its incidental presence in NPK fertilizers like ammonium sulfate, ammonium sulfate nitrate, single super phosphate, sulfate of potash, etc. Thus, S deficiency was not reported. At present, the use of high analysis fertilizers and pesticides lacking sulphur, however, has resulted in increased incidence of S deficiency in aerable crops (Németh, 2006; Jamal *et al*., 2005, 2006, 2010).

Sulphur (S) is an essential nutrient for all plants and animals, as it is a constituent of cysteine, methionine, several coenzymes (e.g. biotin, coenzyme A, thiamine pyrophosphate and lipoic acid), thioredoxins and sulpholipids. S is increasingly being recognized as the fourth major plant nutrient after nitrogen, phosphorus and potassium. The level of S in the soil is one of the critical factors determining the growth and yield of the

plants (Lakkineni & Abrol, 1994). The S deficiency is, however, an important nutrient disorder in agricultural production in all continents (Scherer, 2001). Besides a decrease in crop productivity and negative influence on crop quality (Abdin *et al*., 2003; Ahmad *et al*., 2001), a higher susceptibility of crops to certain diseases was observed in S deficient soils (Schnug *et al*., 1993). Sulphur is required along with nitrogen in the synthesis of proteins and enzymes (Schnug and Hanneklaus, 1998). Previous studies show that combined and balanced application of S and N resulted in increased oil accumulation and reduced ratio of erucic to oleic acid in rapeseed-mustard (Ahmad and Abdin, 2000). In our earlier study, we have shown that the sufficient amount of S along with N increases the productivity of *Psoralea corylifolia* L. (Ahmad *et al*., 2007a).

There is ample evidence that the plant nutrients, S and N are involved in enhanced nitrate reductase activity in plants (Jamal *et al*., 2007; Ahmad *et al*., 2007). Nitrate reductase (NR) is the enzyme, which is involved in nitrogen metabolism, play important role in amino acid biosynthesis, and regulates the protein synthesis (Nair and Abrol, 1977; Harris *et al*., 2000). Nitrate is assimilated through a pathway involving nitrate uptake steps and by two reductive steps catalyzed by the enzymes NR and nitrite reductase (NiR). Of these two enzymes, NR is considered to catalyze the rate-limiting step in NO_3^- assimilation because it initiates the reaction when NO_3^- is available (Fonseka *et al*., 1997; Osaki *et al*., 1997). Studies suggest that there are regulatory coupling between nitrate and sulphate assimilation pathways, and it shows the strong relationship between S and N (Wilson and Bandursky, 1958; Stewart and Porter, 1969; Smith, 1975; Reuveny *et al*., 1980). To the best of our knowledge, much study has not been done in relation to *Ammi majus* L. productivity in terms of S and N interaction. In this study therefore, we focused on the effect of – S and + S along with N on the nitrate reductase activity and seed yield of *Ammi majus*.

Material and methods

Experimental material

Ammi majus L. was used as experimental material. Seed material for cultivation was collected from the herbal garden at Hamdard University, New Delhi, India (28° 38'N, 77° 11'E; elevation of 228 m). Seeds were sown during fall at the University experimental field. The sandy-loam soil (pH 7.3) was deficient in S (0.001%).

General chemicals

All the chemicals used were of AR or GR quality. Most of the chemicals were the products of BDH, IDPL, E.Merck, Aldrich-Sigma, Qualigen, S.D. fines and Loba.

Treatments and Sowing

The fertilizer treatments included two levels of sulphur viz., 0 and 40Kg ha^{-1} in the following combinations (T_1 = control-without manure and fertilizers, $T_2 = S_0 N_{50} K_{25} P_{25}$, $T_3 = S_{40} N_{50} K_{25} P_{25}$). In treatment T_3, sulphur was applied in the form of single basal dose. N and S were applied at the rate of 50 and 40 kg ha^{-1} as urea and gypsum ($CaSO_4$), respectively. S and N were given at the time of sowing. All plots received 25 kg ha^{-1} each of potassium and phosphorus at the time of sowing. The experiments were conducted using a randomized block design, with three replicates per treatment. Plots were $9m^2$ (3 x 3 m), comprising nine rows spaced 45 cm apart. Sixteen periods of irrigations were utilized, at different intervals, over the entire growing season. Plots were weeded frequently to reduce site competition (Fig A).

In vivo nitrate reductase (NR) assay

The *in vivo* assay of NR activity in leaf was done according to the procedure of Hageman and Hucklesby (1971) with slight modifications. Fresh tissue of *Ammi majus* was cut into 2 mm slices and placed in ice-cold incubation medium containing 3.0ml of 0.05M potassium phosphate buffer (pH-7.8) and 3.0ml of 0.4M KNO_3 solution. The tubes were evacuated with a vacuum pump and then incubated in water bath at 35ºC for 75 min under dark conditions. At the end of incubation period, tubes were kept in boiling water bath for 5min to stop the enzyme activity and complete leaching of the nitrite in the medium. Nitrite was estimated by the method of Evans and Nason (1953). 0.2 ml of the aliquot from reaction mixture was taken and 1.0ml each of 1.0% sulphanilamide in 1N-HCl and 0.025% N-(1-Napthyl)-ethylene diammonium dichloride (NEDD) in double distilled water were added. The pink colour due to diazotisation was allowed to develop for 30 min after which the volume was made upto 6.0ml with double distilled water. The absorbance was read at 540 nm, using uv-vis spectrophotometer (Model DU 640B, Beckman, USA). The calibration curve was prepared using sodium nitrite solution. The enzyme activity was expressed as µmole NO_2 g^{-1}fwhr $^{-1}$.

Measurement of Internal CO_2 concentration and stomatal conductance

The measurement of internal CO_2 concentration and stomatal conductance in intact leaves of Atrilal in the field was recorded during winter season (November-March) in sunny days at different growth stages with a portable photosynthesis system (Li 6200; Li-COR, USA). The measurements were repeated three times at all the development stages and mean values were taken for the calculation.

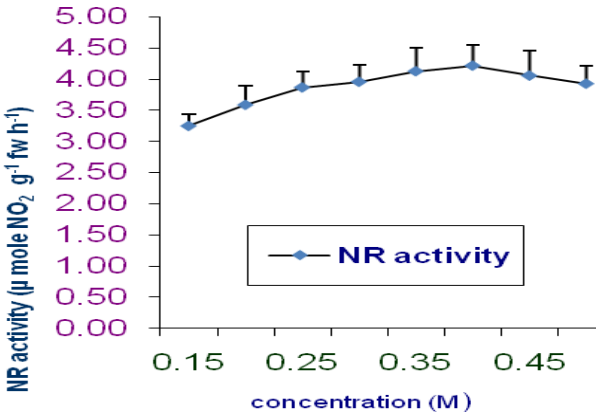

Fig 1. Standardizatin of *in vivo* NR activity (µ mole NO_2 g^{-1} fw h^{-1}) in leaves of *Ammi majus* L. at different concentration (M) of substrate (KNO3). Each point is the mean of three replications and the bars indicate ±S.E.

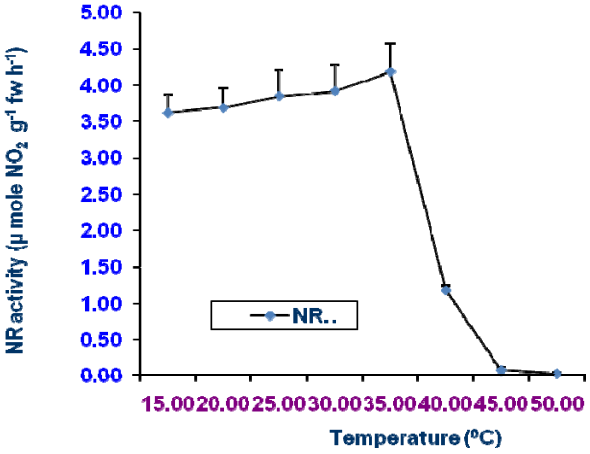

Fig 2. Standardization of *in vivo* NR activity (µ mole NO_2 g^{-1} fw h^{-1}) in leaves of *Ammi majus* L. at different tmperature. Each point is the mean of three replications and the bars indicate ±S.E.

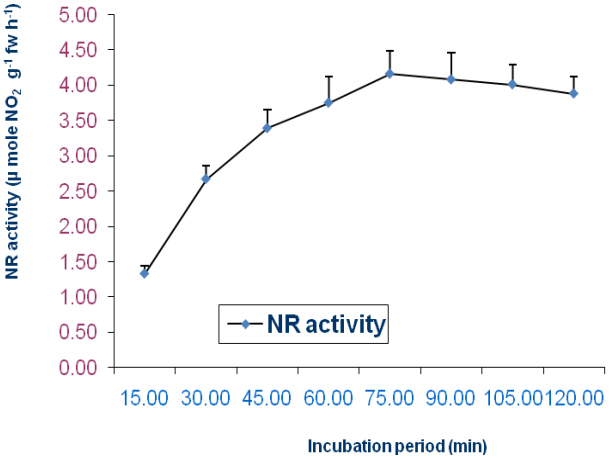

Fig 3. Standardization of *in vivo* NR activity (µ mole NO_2 g^{-1} fw h^{-1}) in leaves of *Ammi majus* L. at different Incubation period (min).Each point is the mean of three replications and the bars indicate ±S.E.

Seed Yield

To determine yield, we removed and cleaned all the seeds produced within a 1-m^2 area in the field. Yield was defined in terms of grams per square meter and quintals per hectare.

Statistical Analysis

Data collected from the experiments was analyzed statistically by using Analysis of Variance as described by Nageswar RG (1983).

Results

Stadardization of in vivo assay of NR in Ammi majus L.

In vivo assay of NR at different molarity and pH of phosphate buffer

The fully expanded leaves were separated and cut into small pieces of about 8-10 mm devoid of midrib. Assay of NR was done as described in Materials and Methods, but the incubation was done using phosphate buffer of varying molarity (0.025, 0.05, 0.075, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40) and pH (6.8, 7.0, 7.2, 7.5, 7.8, 8.0, 8.2, 8.5). The experiment was repeated three times and the results representing the mean values are depicted in figs (4, 5). Significant differences in NR activity was observed at different molarity and pH of phosphate buffer. Maximum NR activity was noted at 0.05 molarity and pH 7.8, when compared with the pH 7.5 used in Hageman and Hucklesby's method.

In vivo assay of NR at different substrate concentrations, temperatures and incubation periods

In this experiment, assay of NR was done at different temperatures (20, 25, 30, 35 and 40) and at different periods of incubation, varying from 15 min to 120 min. The pH of the incubation mixture was 7.8 and the concentration of KNO_3 was varied (0.2, 0.25, 0.30, 0.35, 0.40, 0.45, 0.50M). The optimum NR activity was noted at the substrate (KNO_3) concentration of 0.40 M, temperature of 35^0 C and incubation period of 75 min (fig 1, 2 & 3). Thus, in the subsequent field experiments, NR activity was assayed by modification of the methods of Hageman and Hucklesby (1971) as follows:

Molarity of the phosphate buffer = 0.05M
pH of the phosphate buffer = 7.8
Concentration of the substrate (KNO_3) = 0.4M
Incubation temperature = 35ºC
Incubation period = 75min

In vivo nitrate reductase (NR) activity in leaves at different phenological stages

The effect of S and N interaction on *in vivo* Nitrate reductase (NR) activity of fully expanded leaves of *Ammi majus* L. at different phenological stages was determined and the result obtained is shown in Fig 6. The variability in terms of NR activity was existed at different phenological stages. The NR activity increased continuously till pre-flowering stage and was recorded maximum at this stage when compared with vegetative and flowering stages, respectively, but declined thereafter at subsequent phenological stages. The NR activity was not determined at post-flowering stage because almost all the leaves had withered. Among the treatments, T_3 showed best results at different phenological stages when compared with others. The percent increment in NR activity with T_3 at pre flowering stage was 17.5 % and 5.7 %, respectively when compared with control (T_1) and T_2.

Stomatal conductance

Data on stomatal conductance (mmole m^{-2} sec^{-1}) as influenced by S and N interaction at different phenological stages is shown in Table 1. Stomatal conductance was recorded optimum at pre-flowering stage (0.54mmole m^{-2} sec^{-1}) when compared with vegetative (0.43mmole m^{-2} sec^{-1}) and flowering stage (0.48mmole m^{-2} sec^{-1}), respectively, which was followed by a steady decline. The stomatal conductance was not determined at post-flowering stage because almost all the leaves had withered.

Table 1. Effect of Sulphur and nitrogen on Stomatal conductance (µ mole CO_2m^{-2} sec^{-1}) in leaves of *Ammi majus* L. at different phenological stages

Genotype /(G) Treatment (T)	Phenological stages (S)				Mean
Ammi majus L. (G)	Vegetative stage	Pre-flowering stage	Flowering stage	Post-flowering stage	
T_1	0.43	0.54	0.48	ND	0.48
T_2	0.53	0.65	0.59	ND	0.59
T_3	0.62	0.75	0.69	ND	0.69
GRAND MEAN	0.52	0.64	0.58		
LSD at 5 % P	0.0199				
S	0.0282				
T	NS				
S x T					

Among the treatments, T_3 showed best result at different phenological stages. The percent increment at vegetative and flowering stage was 19 and 21%, respectively when compared with control (T_1).

Internal CO_2 concentration

Data on internal CO_2 concentration (ppm) as influenced by S and N application at different phenological stages is presented in Table 2. Internal CO_2 concentration increased continuously until pre-flowering stage (255.70ppm) when compared with vegetative (232.17 ppm) and flowering stage (206.20ppm), respectively, but declined thereafter at subsequent phenological stages. The internal CO_2 concentration was not determined at post-flowering stage because almost maximum leaves had withered. Among the treatments, T_3 showed best result at different phenological stages. The percent increment at vegetative and flowering stage was 3.9 and 4.16%, respectively, when compared with control (T_1).

Seed yield (q ha^{-1})

Data pertaining to seed yield as influenced by S and N interaction is depicted in fig 7. Combination of S and N (T_3) enhanced the seed yield significantly, when compared with control (T_1) and T_2. Among the treatments, T_3 resulted in optimum seed yield (7.8 q ha^{-1}). The percent enhancement in seed yield at T_3 treatment due to the combined application of S and N was 33 % and 15 % when compared with control (T_1) and T_2 respectively.

Discussion

In this study, we found that sulphur deficiency in soils may lead to the low productivity of *Ammi majus* plant and the application of sulphur along with nitrogen may help to overcome this problem. First, we standardized and slightly modified the NR activity in fresh leaves of *Ammi majus* (Fig 1, 2, 3, 4, 5). Our data shows that S and N interaction resulted in higher NR activity in leaves (fig 6). In earlier reports, it has been demonstrated that the enhanced sulphur application along with

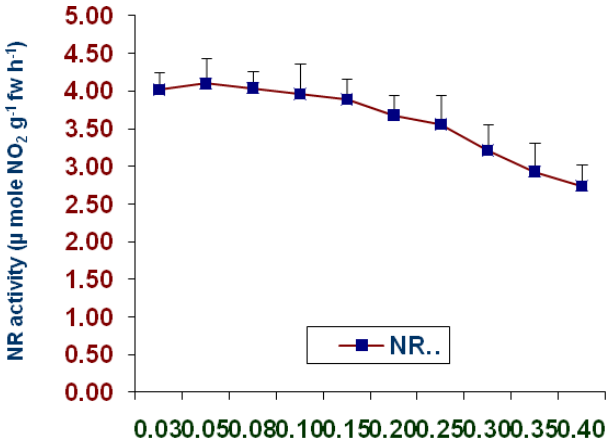

Fig 4. Standardization of *in vivo* NR activity (µ mole NO_2 g^{-1} fw h^{-1}) in leaves of *Ammi majus* L. at different concentration (M) of phosphate buffer. Each point is the mean of three replications and the bars indicate ±S.E.

nitrogen can increase the yield of oilseeds crop (Ahmad *et al*., 2001; Ahmad and Abdin, 1998, 2000, Fazili *et al*., 2008, 2010b). Many attempts have been made to relate nitrate reductase activity (NRA) to agricultural productivity as nitrate reductase is a rate-limiting enzyme of nitrate-assimilation pathway and is also involved in protein biosynthesis (Ahmad *et al*., 1999). Plants obtain their nitrogen requirements mainly from the soil as nitrates. Most of this nitrate is utilized in the synthesis of proteins and nucleic acids. This assimilatory nitrate reduction occurs via two enzyme-catalyzed reactions, which uses eight electrons and does not require ATP. Nitrate is first reduced to nitrite, which is subsequently reduced to ammonia and then incorporated into the amino acids glutamine/glutamate using the C-skeletons produced via other metabolic pathways such as respiration and photosynthesis. Sulphur is a structural component of some amino acids and vitamins, and is essential in the development and functioning of chloroplasts. It has been

Table 2. Effect of Sulphur and nitrogen on Internal CO_2 concentration (ppm) in leaves of *Ammi majus* L. at different phenological stages

Genotype /(G) Treatment (T)	Phenological stages (S)				Mean
Ammi majus L. (G)	Vegetative stage	Pre-flowering stage	Flowering stage	Post-flowering stage	
T_1	232.17	255.70	206.20	ND	231.36
T_2	257.65	277.63	225.40	ND	253.56
T_3	272.13	297.03	247.83	ND	272.33
GRAND MEAN	253.98	276.78	226.47		
LSD at 5 % P					
S	8.2340				
T	11.6447				
S x T	NS				

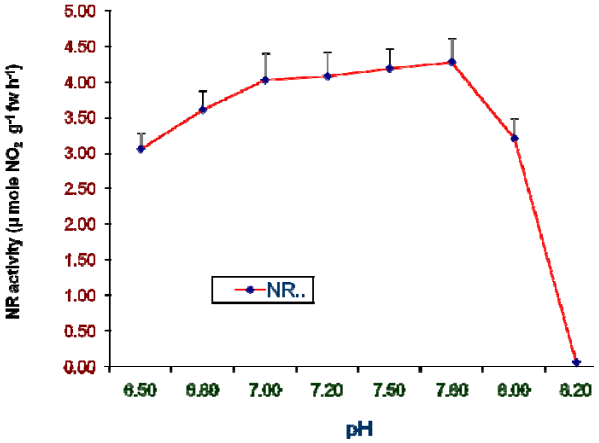

Fig 5. Standardization of the *in vivo* NR activity (µ mole NO_2 g^{-1} fw h^{-1}) in leaves of the *Ammi majus* L. at different pH of phosphate buffer. Each point is the mean of three replications and the bars indicate ±S.E.

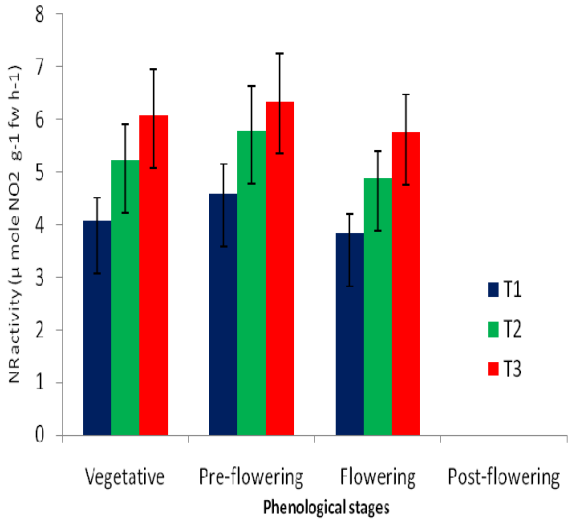

Fig 6. Effect of Sulphur and Nitrogen on NR activity (µ mole NO2 g-1 fw h-1) in leaves of *Ammi majus* L.

reported that the presence of S enhanced the seed and oil yields (Ahmad *et al.,* 2007a; Fazli *et al.,* 2008; Ahmad and Abdin, 2000). Hue *et al*. (1991) has reported earlier that under S-deficient conditions, an excess of unassimilated NO_3^--N and free amino acids accumulate in leaves. Our data suggested that the combined application of S and N in treatment T_3 resulted in significant enhancement of seed yield (Fig. 7). In contrast, the low NR activity in leaves of *Ammi majus* treated with another combination of S and N (T_2) could have been due to an imbalanced nutrient supply. Eppendorfer (1971) reported that non-protein nitrogen is stored in the vegetative tissue at the expense of protein N and growth is retarded in plants grown with insufficient sulphur. This increase in non-protein N in S-deficient plants is characterized by an accumulation of amides, usually asparagines (Mathot *et al*., 2009; Stewart and Porter, 1969). The other researchers also reported low protein levels and decreased NR as well as ATP sulphurylase activities in crop plants grown with imbalanced supply of S and N (Jamal et al., 2010; Reuveny et al., 1980; Migge et al., 2000; Prosser et al., 2001) which supports our results. On the other hand in our earlier report, we have shown that the balanced nutrient supply

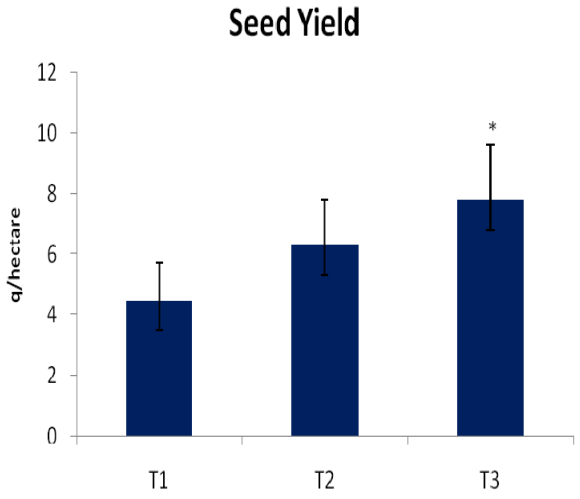

Fig 7. Sulphur and Nitrogen interaction effect on seed yield of *Ammi majus* L. Data Mean ± S.E., *P<0.05

8a	8b	8c	8d	8e	8f	8g
Vegetative stage	Preflowering stage	Flowering stage	At harvest stage	Control group (T_1)	T_2 group ($S_0N_{50}P_{25}K_{25}$)	T_3 group ($S_{40}N_{50}P_{25}K_{25}$)

Fig 8. Effect of S and N interaction on growth and development of *Ammi majus* L., at different growth stages (picture a, b ,c, d show plant growth stages and e, f and g show plants development. Fig 8a- Vegetative stage, Fig 8b- Preflowering stage, Fig 8c- Flowering stage, Fig 8d- At harvest stage, Fig 8e- Control group, Fig 8f- T2 group ($S_0N_{50}P_{25}K_{25}$), Fig 8g- T3 group ($S_{40}N_{50}P_{25}K_{25}$)

have resulted in enhanced photosynthetic rate and chlorophyll content in the leaves of *Psoralea corylifolia*, which lead to higher biomass accumulation and biological yield (Ahmad *et al*., 2007a). In the present study, our data shows slight increased in stomatal conductance and internal CO_2 concentration rate with treatment T_3 when compared with T_1 and T_2 (Table 1 and 2). It can be correlated with increased photosynthesis rate (data not shown). Maintenance of photosynthesis by leaves throughout the growing season, especially at the seed-filling stage, is a major requirement for ensuring the adequate carbohydrate supply to form large seeds and high yields (People *et al*., 1980). Hence, the improved yield obtained in this study with treatment T_3 (Fig 6) could be because of supply of sufficient carbohydrates to developing seeds. We have also shown earlier that sulphur application together with nitrogen and other nutrients enhanced the leaf area index and leaf area duration in *Psoralea corylifolia* over the –S group and control (Ahmad *et al*., 2007a). In the present study, the higher biomass accumulation in plants during various development stages with treatment T_3 could be due to the balanced supply of S and N (Fig 8). We also studied the reduced nitrogen content and total sulphur in different organs of *Ammi majus* plant at different growth stages and found increased trend with T_3 treatment over T_2 and T_1. Apparently, this data was not statistically significant (data not shown). Similar findings have been reported with other crops, e.g., maize (Deckard *et al*., 1973; Balasubramanian *et al*., 1977), wheat (Croy and Hageman, 1970; Abrol *et al*.,

1976; Rahimizadeh *et al*., 2010), and rapeseed-mustard (Fismes *et al*., 2000; Ahmad *et al*., 1999).

Barney and Bush (1985) has demonstrated that tobacco plants treated with –S and +N had very low NR activity. However, when these plants were transferred from +N-S to -N+S, NR activity remained very low because nitrogen was absent. Similarly, -N+S-treated plants exhibited very little ATP sulfurylase activity, perhaps because the limited N supply prevented the translocation of SO_4^{2-} from roots to shoots (Fazili *et al*., 2010). Increased nitrate reductase activity due to S fertilization has been demonstrated in tobacco (Pal *et al*., 1976). The synthesis of cysteine that results from the incorporation of a sulfide moiety into O-acetyl serine (OAS) appears to be the meeting point between N and S assimilation. Recently Kaur *et al*. (2010) reported that sulphur is involved in nitrate uptake in rapeseed, which is in agreement to our study. Our observation of enhanced NR activity, internal CO_2 concentration and stomatal conductance and improved seed yield in response to Treatment T_3 thus suggests a strong correlation between yield and enzyme activities at different phenological stages. Further studies are needed to understand the mechanism of N and S interaction at molecular level in *Ammi majus* L.

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