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Interactive Effect of Sulfur and Nitrogen on Nitrate Reductase and ATP-Sulfurylase Activities in Relation to Seed Yield from *Psoralea corylifolia* L.

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Field experiments were conducted to determine the interactive effect of sulfur (S) and nitrogen (N) applications on seed yield from *Psoralea corylifolia* L. Six treatments were tested: T_1 = control (without manure and fertilizers), T_2 = manure @ 9 kg plot^{-1} (10 t ha^{-1}), $T_3 = S_0 N_{20} K_{40} P_{40}$, $T_4 = S_{20} N_{20} K_{40} P_{40}$, $T_5 = S_{20+20} N_{20} K_{40} P_{40}$, and $T_6 = S_{20+20} N_{20+20} K_{40} P_{40}$. Activities of nitrate reductase (NR) and ATP-sulfurylase in the leaves were measured at various phenological stages. These two enzymes catalyze the rate-limiting steps in the respective assimilatory pathways for nitrate and sulfate. Enzyme activity was strongly correlated with seed yield, with the greatest performance being achieved with treatment T_5. This might be attributed to the optimization of leaf soluble protein and photosynthetic rate, both of which are influenced by S and N assimilation.

Keywords: ATP-sulfurylase, nitrate reductase, nitrogen, *Psoralea corylifolia* L., seed yield, sulfur

Psoralea corylifolia L. (Fabaceae), commonly known as Babchi in India, has been used worldwide for centuries as a pharmaceutical. Its seeds are extensively utilized in Ayurvedic and Unani medicine as a stomachic, deobstruent, antihelmintic, and diuretic, as well as for protection against such skin diseases as leucoderma and leprosy (Ahmad et al., 2006a). It also possesses cytotoxic, anti-cancer, and immunomodulator properties (Latha and Panikkar, 1998). The seeds contain an essential oil, a non-volatile terpenoid oil, a dark brown resin, a monoterpenoid phenol (i.e., bakuchiol), a brown fixed oil, reffinose, and coumarin compounds, viz. psoralen, isopsoralen, psoralidin, isopsoralidin, and corylifolin (Ahmad et al., 2006a). Psoralen, the most important component, is the fundamental linear furocoumarin (Rangari and Agrawal, 1992), supplies of which are currently limited. Because its chemical synthesis is still very expensive, the molecules are commercially extracted from *Psoralea* seeds, although this means of production is insufficient to meet growing demand by pharmaceutical industries. Therefore, better agrotechnological methods are necessary if we are to increase metabolite yields. So far, no efforts have been made to breed or select strains of certain genotypes with higher harvest indices and levels of active ingredients, and little information is available for optimizing its cultivation and the harvesting of larger seed crops.

Sulfur (S) is gaining greater recognition as the fourth major plant nutrient, after nitrogen, phosphorus, and potassium. Its uptake and assimilation in higher plants is a crucial factor in determining seed yield and quality, as well as resistance to pests and environmental stresses. Combined applications of sulfur and nitrogen (N) have an interactive effect on the performance of agricultural crops (Ahmad et al., 2001; Fazli et al., 2005a, b; Jamal et al., 2005, 2006). To our knowledge, no investigation other than that described here has been conducted on the impact of this interaction on growth and seed development in medicinal plants, particularly *P. corylifolia*. Here, we report our analysis of the physiochemical basis for yield responses to these nutrients.

MATERIALS AND METHODS

Plant Materials

Seeds of *P. corylifolia* L. were collected from the herbal garden at Hamdard University, New Delhi, India (28° 38'N, 77° 11'E; elevation of 228 m). These were sown during 2004 and 2005 at the University experimental field. The sandy-loam soil (pH 7.3) was deficient in S (0.001%).

Treatments

Six treatments were tested: T_1 = control (without any manure or fertilizers), T_2 = manure, mixture of animal's feces and bedding straw applied at 9 kg per plot (10 t ha^{-1}), $T_3 = S_0 N_{20} K_{40} P_{40}$, $T_4 = S_{20} N_{20} K_{40} P_{40}$, $T_5 = S_{20+20} N_{20} K_{40} P_{40}$, and $T_6 = S_{20+20} N_{20+20} K_{40} P_{40}$. For T_4, T_5, and T_6, N and S were applied at the rate of 20 and 40 kg ha^{-1} as urea and gypsum ($CaSO_4$), respectively. In treatment T_3, the same rate of N was applied, but without gypsum. Sulfur as gypsum was applied in two equal splits in treatments T_5 and T_6 while N as urea was applied in equal splits in treatment T_6. The first dose of S was given at the time of sowing (basal) and the second at 45 d after sowing (before flowering). All plots

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received 40 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 9 m^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.

Sampling

Samples were collected at 45-d intervals, and the seeds were packed in polythene bags for transport to the laboratory.

Plant Dry Weights

Leaves, stems, roots, and seeds were sampled at several phenological stages (vegetative, pre-, and post-flowering). At each collection point, three plants were randomly selected, then cut at the root-shoot junction, and oven-dried to constant weight at 65 ± 2°C.

Estimation of Soluble Protein

Soluble protein content in the developing leaves was estimated by the method of Bradford (1976) after precipitation with trichloroacetic acid. Bovine serum albumin was used as the standard.

Measurement of Photosynthetic Rate

The photosynthetic rate of intact leaves in the field was measured at different phenological stages with a portable photosynthesis system (Li 6200; Li-COR, USA).

Assay of Nitrate Reductase Activity

In vivo nitrate reductase activity in leaves was monitored at various stages according to the procedure of Hageman and Hucklesby (1971), with slight modification (Ahmad et al., 1998). Fresh leaf tissue was cut into 2-mm slices and placed in an ice-cold incubation medium containing 3.0 mL of potassium phosphate buffer (0.15 M; pH 7.2) and 3.0 mL of 0.4 M KNO_3 solution. The tubes were evacuated with a vacuum pump and then incubated in a water bath at 33°C for 90 min under dark conditions. Afterward, they were placed in a boiling water bath for 5 min to stop enzyme activity and complete the leaching of nitrite in the medium. Nitrite content was estimated by the method of Evans and Nason (1953). From the reaction mixture, 0.2 mL of the aliquot was removed and 1.0 mL each of 1.0% sulfanilamide in 1N-HCl and 0.02% N-(1-Napthyl)-ethylene diammonium dichloride (NEDD) in distilled water were added. Pink coloration due to diazotization was allowed to develop for 30 min, after which the volume was made up to 6.0 mL with distilled water. Absorbance was read at 540 nm on a DU 640B uv-vis spectrophotometer (Beckman, USA). A calibration curve was prepared using sodium nitrite solution, and enzyme activity was expressed as μmole NO_2 g^{-1} fw h^{-1}.

Assay of ATP-Sulfurylase Activity

In vitro ATP-sulfurylase activity in fresh leaves was determined by the method of Wilson and Bandurski (1958). To do so, 0.5 g of fresh tissue was homogenized on ice with mortar and pestle in 5 mL of extraction buffer [0.1 M Tris (pH 8) that contained 2 mM magnesium chloride, 100 mM potassium chloride, and 10 mM Dithioerythritol]. The homogenate was centrifuged at 5000 rpm for 15 min at 4°C. Afterward, a 0.1 mL aliquot was placed in each test tube, to which was added 0.4 mL of reaction mixture [8 mL DD H_2O, 4 mL $MgCl_2 \cdot 6H_2O$ (40 mM), 3 mL of 0.4 M Tris buffer (pH 8.0), 40 mg Na_2MoO_4, 45 mg Na_2ATP, and 20 μL of inorganic pyrophosphatase]. This was then incubated in a water bath at 33°C for 30 min. After the reaction was terminated in hot water, 1 mL of 2.5% ammonium molybdate solution and 0.1 mL of reducing agent (1 g of 1-amino-naphthol sulfonic acid, 3 g of Na_2SO_3. $7H_2O$, and 6 g of $Na_2S_2O_5$) were added. The volume was made up to 10 mL with DD H_2O. After 20 min, absorbance was read at 660 nm. A calibration curve was prepared using different concentrations of KH_2PO_4 solution, and enzyme activity was expressed as μmol Pi mg^{-1} protein min^{-1}.

Seed Yield

To determine crop yield, we removed and cleaned all the seed that had been 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

Statistical analyses were performed according to the methods of Cochran and Cox (1957).

RESULTS AND DISCUSSION

Dry Matter, Soluble Protein, and Photosynthetic Rate

Biomass accumulation in *P. corylifolia* L. was 2.73 g per plant at the vegetative stage, but values then peaked to 73.88 g per plant at the post-flowering stage. At harvest, a slight decrease was observed in those amounts. Among the various treatments, T_5 (S_{20+20} N_{20} K_{40} P_{40}) resulted in the greatest increment in biomass accumulation, i.e., 29.81 and 43.30% at the vegetative stage and at harvest, respectively (Fig. 1). When sulfur was applied in two equal splits (T_5), the plants accumulated more biomass, maintained a higher leaf area index, and demonstrated greater leaf area duration (data not shown) at all phonological stages compared with the control (T_1). Previous researchers have also reported that application of these plant nutrients causes significant increases in the growth and yield of medicinal plants (Randhawa et al., 1985; Gill and Samra, 1986; Lakshmipathaiah et al., 1999; Ahmad et al., 2006b). Furthermore, Ramesh et al. (1989) has observed improvements in plant height, number of branches, and leaf area per plant in Isabgol when a balanced dose of macronutrients, such as N, P, and K, is used.

Photosynthesis was maximal at the pre-flowering stage (25.33 μmole CO_2 m^{-2} s^{-1}) compared with the periods of vegetative growth (24.13 μmole CO_2 m^{-2} s^{-1}) or post-flowering (20.68 μmole CO_2 m^{-2} s^{-1}). Thereafter, it declined steadily. Treatments with different combinations of S and N

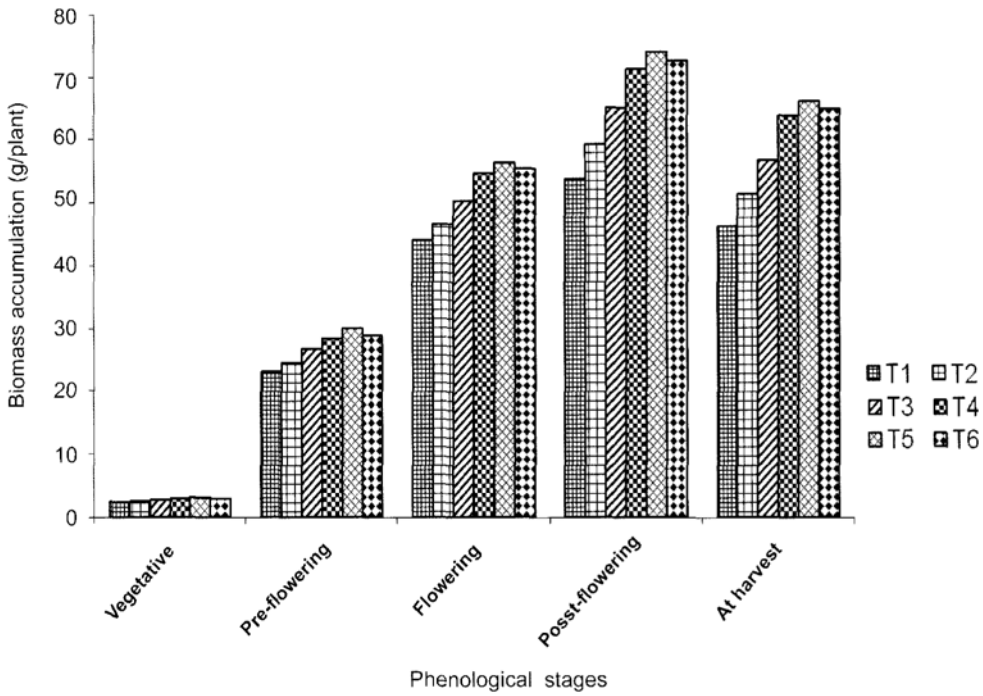

Figure 1. Effect of S and N on biomass accumulation (g plant $^{-1}$) in *P. corylifolia* L. at different phenological stages.

(T_1 through T_6) significantly enhanced this rate. Among all treatments, T_5 presented the best results at each stage. For example, compared with the control (T_1), the percent increment in photosynthesis due to Treatment T_5 was 16.16% and 13.28% at the vegetative and post-flowering stage, respectively. An examination of the average mean values over treatments revealed that the content of leaf soluble protein attained peak values at pre-flowering, before declining. Values varied from 12.65 mg g^{-1} fw at the vegetative stage to 11.18 mg g^{-1} fw post-flowering. Compared with the control (T_1), the percent increment in leaf soluble protein due to Treatment T_5 was 24.60 and 26.43% at the vegetative and post-flowering stages, respectively. Again, data for leaf soluble protein (Fig. 2) and photosynthesis (Table 1) demonstrated that the split application of S and N (T_5) resulted in significant enhancement over the control values for both parameters. The interrelationship between these two has also been reported with other species (Evans, 1983; Makino et al., 1984; Lawlor et al., 1987a, b, 1989; Sinclair and Horie, 1989). Findings have been similar in oilseed crops (Ahmad and Abdin, 2000; Fazli et al., 2005a, b; Jamal et al., 2005). The better response by plants of *P. corylifolia* to Treatment T_5 may have been due to the availability of these nutrients in appropriate amounts throughout the growth

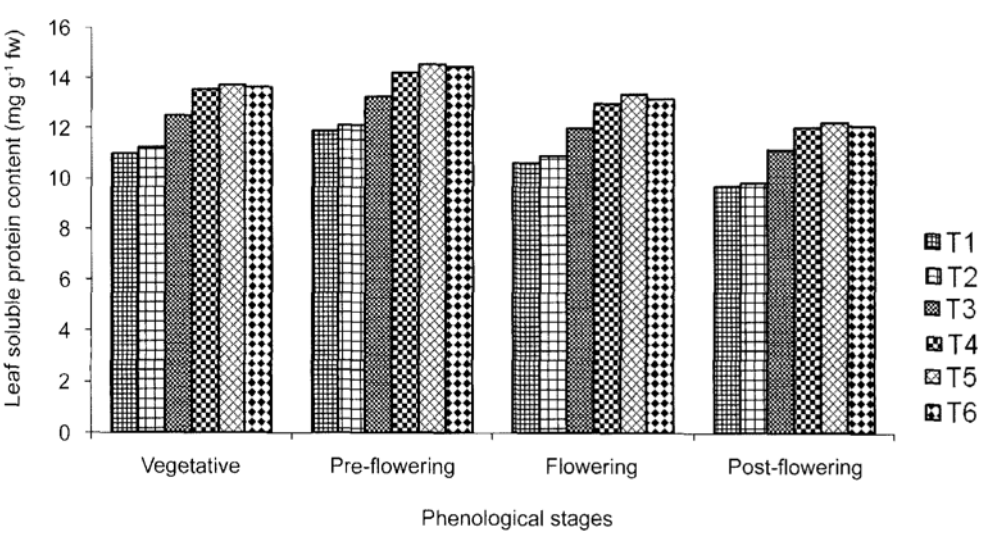

Figure 2. Effect of S and N on soluble protein content (mg g^{-1} fw) in leaves of *P. corylifolia* L. at different phenological stages.

Table 1. Effect of sulfur and nitrogen treatments on photosynthesis in the leaves of *P. corylifolia* L. at different phenological stages.

Treatment	Photosynthetic rate (μmole CO_2 m^{-2} s^{-1})				Mean
	Vegetative stage	Pre-flowering stage	Flowering stage	Post-flowering stage	
T_1 control, no manure or fertilizers	24.13	25.33	23.65	20.68	**23.45**
T_2 manure at 10 t ha^{-1}	24.76	25.92	24.01	21.01	**23.92**
T_3 S_0 N_{20} K_{40} P_{40}	26.22	27.44	25.38	22.17	**25.30**
T_4 S_{20} N_{20} K_{40} P_{40}	27.36	28.25	26.22	23.13	**26.24**
T_5 S_{20+20} N_{20} K_{40} P_{40}	28.03	28.92	26.62	23.43	**26.75**
T_6 S_{20+20} N_{20+20} K_{40} P_{40}	27.53	28.31	26.34	23.32	**26.38**
GRAND MEAN	26.33	27.36	25.37	22.29	

L.S.D. ($p > 0.05$)
S 0.4926
T 0.6033
S x T NS (T, treatment; S, phenological stage; NS, non significant)

period, especially because they are all involved in the biosynthesis of amino acids, the building blocks of proteins. This type of balanced and combined applications has also been critical to the production of more dry matter and higher seed yields in other crops (Dev and Saggar, 1974; Lakkineni and Abrol, 1992; Zhao et al., 1993). A strong metabolic coupling between these nutrients also has been demonstrated (Ahmad et al., 1998; Abdin et al., 2003). Adequate supplies of sulfur and nitrogen lead to better utilization of carbohydrates in forming more protoplasm. Under such conditions, plant cells tend to be large and have thin walls (Black, 1967), which may cause an increase in leaf area. Higher leaf area indices indicate better leaf expansion, further assisting in the subsequent interception and efficient utilization of solar radiation to assimilate carbon. This leads to better accumulation and distribution of dry matter in various plant organs, including the seeds. Leaf area duration, which is the product of leaf area index X time, is also affected by changes in leaf area index over the growing season and in response to S and N applications.

In Vivo Nitrate Reductase and *in Vitro* ATP-Sulfurylase Activities

NR activity in the leaves was maximal at the pre-flowering stage (3.30 μmol NO_2 g^{-1} fw h^{-1}), when compared with the vegetative (3.14 μmol NO_2 g^{-1} fw h^{-1}) and post-flowering stages (1.73 μmol NO_2 g^{-1} fw h^{-1}). Among our different treatments, T_5 showed the greatest increase over time (Fig. 3), with the percent increments calculated as 14.12% (vegetative) and 46.44% (post-flowering) higher than the control (T_1). ATP-sulfurylase activity also peaked at the pre-flowering stage (24.28 μmol Pi mg^{-1} protein min^{-1}); other values included 23.63 μmol Pi mg^{-1} protein min^{-1} (vegetative) and 19.15 μmol Pi mg^{-1} protein min^{-1} (post-flowering). Treatment T_5 was associated with the greatest rise in ATP-sulfurylase activity at all phenological stages (Fig. 4), with values of 16.14% (vegetative) and 19.46% (post-flowering) over the control (T_1).

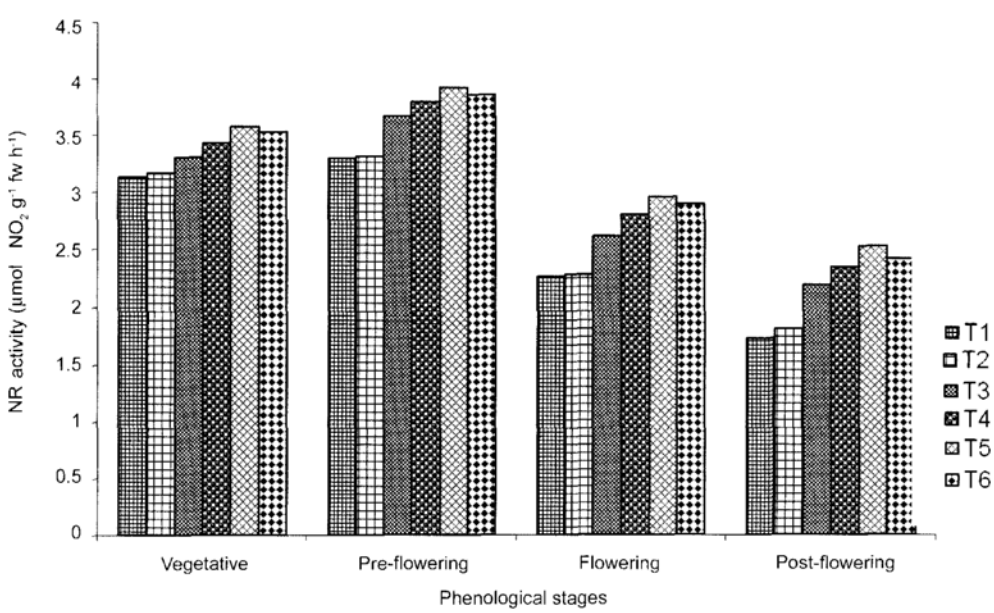

Figure 3. Effect of S and N on NR Activity (μmol NO_2 g^{-1} fw h^{-1}) in leaves of *P. corylifolia* L. at different phenological stages.

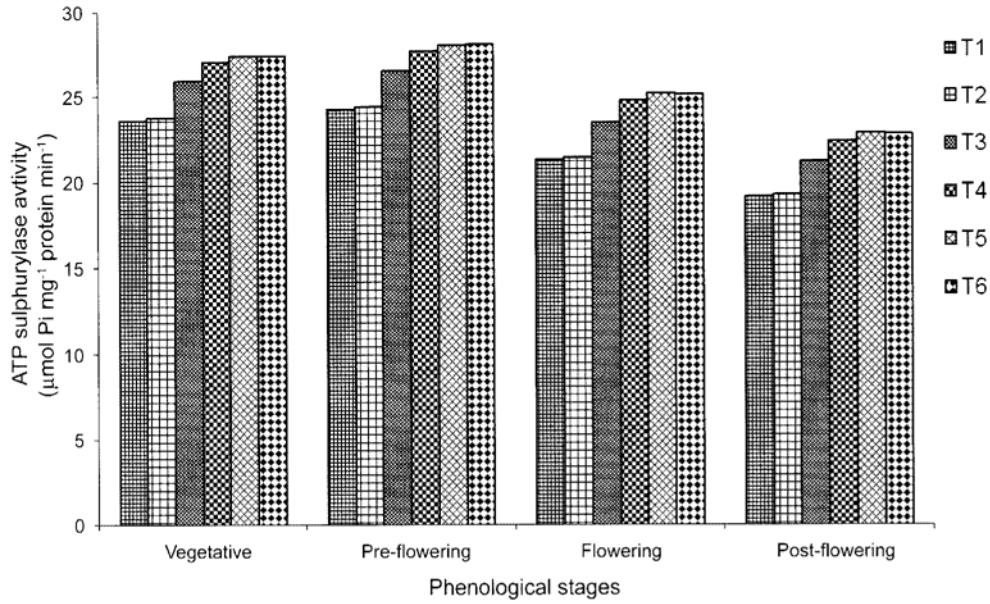

Figure 4. Effect of S and N on *in vitro* ATP sulphurylase activity (µmol Pi mg^{-1} protein min^{-1}) in leaves of *P. corylifolia* L. at different phenological stages.

When one considers the key role played by nitrate reductase in nitrate assimilation and of ATP-sulfurylase in sulfate assimilation, it is reasonable to conclude that these enzyme activities are related to seed yield. The combine application of S plus N enhanced their activities compared with the results from applying N alone (T_3). In contrast to the control (T_1), the highest NR and ATP-sulfurylase activities were observed with the treatment that consisted of 40 kg S ha^{-1} applied in two equal splits along with 20 kg N ha^{-1} applied as a single basal dose (T_5). This indicated that metabolic activity related to N and S assimilation was increased in response to the balanced supply of nitrogen and sulfur to our crop. These results are consistent with those reported for other species (Smith, 1975; Reuveny et al., 1980; Barney and Bush, 1985; Clarkson et al., 1989; Ahmad et al., 1999; Ahmad and Abdin, 2000; Fazli et al., 2005a). For example, Barney and Bush (1985) showed that +N-S-treated tobacco had very low NR activity because of the lack of sulfur. Moreover, when those plants were transferred from +N-S to -N+S, NR activity still remained very low because nitrogen was absent. Similarly, -N+S-treated plants exhibited very little ATP-sulfurylase activity, perhaps because the li3mited N supply prevented the translocation of SO_4^{2-} from roots to shoots. 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 appears to be the meeting point between N and S assimilation. Our observation of enhanced NR activity, greater ATP-sulfurylase activity, and improved seed yields in response to Treatment T_5 suggests a strong correlation between yield and enzyme activities at different phenological stages. Similar findings have been noted with other crops, e.g., maize (Deckard et al., 1973; Balasubramanian et al., 1977), wheat (Croy and Hageman, 1970; Abrol et al., 1976), and rapeseed-mustard (Ahmad et al., 1999).

Seed Yield

Among all treatments, T_5 resulted in optimum seed yield (21.14 q ha^{-1}), followed by T_6 (20.64 q ha^{-1}). Compared with the control (T_1), this means that production was 67.94% greater when Treatment T_5 was applied (Table 2).

Growth attributes, the primary components that determine the success of a particular crop, are positively correlated with seed yield (Salimath and Bahl, 1986; Ahmad et al., 1998). Our data revealed that the combined application of S and N in Treatment T_5 resulted in significant enhancement of both soluble protein content (Fig. 2) and photosynthetic rate (Table 1) over the control (T_1). In contrast, the low protein levels observed in leaves of *Psoralea* treated with another combination of S and N (T_2) could have been due to an imbalanced nutrient supply. When plants are grown with insufficient sulfur, non-protein nitrogen is stored in the vegetative tissue at the expense of protein N, and growth is retarded (Eppendorfer, 1971). This increase in non-protein N in S-deficient plants is characterized by an accumulation of amides, usually asparagines (Stewart and Porter, 1969). Therefore, any decline in soluble protein due to either low S or an imbalanced supply of

Table 2. Effect of sulfur and nitrogen treatments on seed yield from *P. corylifolia* L.

Treatment	Seed yield (q ha^{-1})
T_1	12.588
T_2	15.064
T_3	17.112
T_4	20.156
T_5	21.140
T_6	20.640
GRAND MEAN	17.783

L.S.D. (0.05)
T (Treatment) 2.262
q ha^{-1}, – quintals per hectare (100 kg = 1 quintal).

nitrogen and sulfur is mainly a consequence of the linkage between N and S metabolism at the level of protein synthesis. Because RUBISCO constitutes 50 to 70% of total soluble protein, any change in that content affects the photosynthetic rate. Maintenance of photosynthesis by leaves throughout the growing season, but especially at the seed-filling stage, is a major requirement for ensuring that adequate carbohydrate is produced to form large seeds and high yields (People et al., 1980). Hence, the improved yield obtained here when Treatment T_5 was used could have occurred because sufficient carbohydrates were produced during seed development. This was evidenced by the maximum levels of both soluble protein and photosynthesis associated with this treatment.

From these observations, we can conclude that the optimization of both NR and ATP-sulfurylase activities via balanced doses of sulfur and nitrogen fertilizers may lead to higher seed yield in *P. corylifolia*.

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