CHAPTER-5

ENHANCEMENT OF ROOT GROWTH AND NITROGEN FIXATION IN TRIGONELLA BY UV-EXCLUSION

Approximately 78% of the atmosphere is nitrogen gas (N\(_2\)). All organisms use the ammonia (NH\(_3\)) form of nitrogen to manufacture amino acids, proteins, nucleic acids and other nitrogen-containing components necessary for life. Most plants live in close relationship with microbial partners or symbionts, which provide nutrients and thereby assist their host to grow on poor soils. The roots of many legumes are colonised by N\(_2\)-fixing bacteria, which are beneficial for plant growth. The most common endosymbionts found in the roots of legume crops are bacteria of the genera *Rhizobium* and *Bradyrhizobium*. The symbiotic association between the roots of leguminous plants and soil rhizobia results in the development of specific organs, called nodules, whose primary function is N\(_2\) fixation. The products of symbiotic N-fixation (amides in temperate legumes and ureides in tropical legumes) are exported from the nodules to the rest of the plant, where they are incorporated into essential macromolecules such as amino acids and proteins that drive plant growth, development and, in the case of agriculture, crop yields. Root nodules not only make a crucial contribution to the N-economy of leguminous crops but also enhance the N-content of the soil, and thus they have a key role in environment-friendly agricultural practices. Leghemoglobin plays an essential role in the nitrogen fixation process, by providing oxygen to the bacteroids at a low, but constant, concentration, which allows for simultaneous bacteroid respiration and nitrogenase activity. Leghemoglobin facilitates the diffusive flux of O\(_2\) to the vigorously respiring, phosphorylating and N\(_2\)-fixing *Rhizobium* bacteroids at very low concentrations of free, dissolved O\(_2\) (Appleby, 1984). In this process, provided the leghemoglobin is partially oxygenated, the very small diffusive flux of free, dissolved O\(_2\) is greatly augmented by the diffusion flux of oxyleghaemoglobin in the cytoplasm of the infected cells. In fenugreek, root nodules are formed both in the main tap root and in the branches. Nitrogenase requires a continued and abundant supply of suitable reductant and ATP for conversion of N\(_2\) to NH\(_3\) (Halbleib et al., 2000). The process of nitrogen fixation is depressed during midday when visible and UV irradiance is higher in aquatic algae that were directly exposed to UV (Peterson et al., 1977). UV-B supplementation induced
reduction in nitrogen fixation in tropical crops like *Phaseolus mungo* and *Vigna radiata* (Pinto et al., 2002).

The other most important enzyme in the assimilation of exogenous nitrate is the Nitrate reductase. Nitrate is the predominant form of nitrogen available to green plants growing in soil. Activity of this enzyme in plants gives a good estimate of the nitrogen status of the plant and is very often correlated with growth and yield. Little is known about the effects of UV radiation on the nitrogen economy of fenugreek, particularly on the nitrate reductase (NR) activity in tropical country like India where nitrogen is a growth limiting factor. Nitrate reductase has a key role in nitrogen fixation and assimilation in most terrestrial plants as it is the enzyme responsible for the reduction of nitrate (NO$_3^-$) to nitrite (NO$_2^-$). This step includes the entire metabolic pathway leading to incorporate inorganic forms of nitrogen (nitrate) into amino acids. Although the availability of nitrate from soil is believed to be the most important factor controlling NR activity; one of the most important stress ie. UV radiation may influence enzyme activity (Sinha et al., 1998). Measuring NR activity gives important information about the intensity of nitrate assimilation and when combined with other parameters, it provides a view on the general physiological status of the plants being investigated (Norby et al., 1989; Krywult et al., 1996; Krywult & Bytnerowicz, 1997). In Trigonella, both the roots and foliage participate in NO$_3^-$ reduction. The use of NADH or NADPH as reducing agents makes leaf NO$_3^-$ assimilation dependent on the photosynthesis process, while in roots the NR activity depends on the carbohydrate made available to the plant from the shoots.

In the present chapter, data is presented to on the impact of UV exclusion on nitrogenase activity in the nodules of *Trigonella foenum-graecum* along with root growth, nodulation and leghemoglobin content. The direct impact of exclusion of UV from sunlight on the activity of nitrogenase enzyme was not analysed in the previous studies. In addition data is also presented on the impact of UV exclusion on the activity of nitrate reductase in the leaves.
5.1 Results

5.1.1. Root growth

Exclusion of solar UV-B and UV-A+B enhanced root growth (Figure. 5.1[A]). Enhancement in root length (length of longest root) and fresh weight were prominent at later stages of growth (45 DAE and 60 DAE; Figure. 5.1[A] and 5.1[B]). Maximum promotion in root length was observed at 45 DAE (***P<0.001) (40% in -UV-B and 35% in -UV-A+B; Fig. 5.1[A]) and at 60 DAE it was promoted by 26% (Figure. 5.1[A]). A similar difference was recorded in fresh weight of roots (Figure. 5.1 [B]). Enhancement in fresh weight was higher at 45 DAE (228% in -UV-A+B and 263% in -UV-B; Fig. 5.1[B]) compared to 60 DAE 57% in -UV-A+B and 84% in -UV-B (***P<0.001). Enhancement in dry weight of roots by exclusion of UV-B was higher (87%) compared to exclusion of UV-A+B (59%) at 45 DAE (Figure. 5.2[A]).

5.1.2 Nitrogenase activity, leghemoglobin content, hemechrome content and protein

The activity of nitrogenase measured as n mol ethylene produced from acetylene per gram fresh weight of the nodules was significantly enhanced by the exclusion of solar UV (Figure. 5.2 [B]). The activity was enhanced by 80% (UV-A+B) and 120% (UV-B) after exclusion (Figure. 5.2 [B]) (***P<0.001). There was also an enhancement in the total protein content, leghemoglobin, and heme chrome in the nodules after exclusion of UV (Table 3). An enhancement of 26% (-UV-A+B) and 30% (-UV-B) was recorded in total protein (**P<0.01). Leghemoglobin per gram fresh weight of the nodules was enhanced by 36% (-UV-A+B) and 63% (-UV-B) (**P<0.001). Enhancement in heme chrome content per mg protein was by 57% (-UV-A+B) and 74% (-UV-B). All these parameters were slightly enhanced in the roots of the plants grown under polythene permissible to solar UV compared to the plants grown in the open field. (Table 3). The spectra of leghemoglobin from various treatments is presented in Figure.5.3.
**Fig.5.1:** Effect of solar UV-B+A and UV-B exclusion on (A) Root length and (B) Root fresh weight of *Trigonella* (Pusa early bunching) at different days after emergence of the seedlings (15 to 60 DAE). The vertical bars indicate ± SE for mean. UV-B+A and UV-B excluded plants show significant difference at (**P<0.01 ***P<0.001) compared to open control. (Newman–Keuls multiple comparison test).
Fig. 5.2. Effect of solar UV-B+A and UV-B exclusion on (A) Root Dry weight and (B) Nitrogenase activity of *Trigonella* (Pusa early bunching) at 45 DAE. The vertical bars indicate ± SE for mean. UV-B+A and UV-B excluded plants show significant difference at (***P<0.001) compared to open control. (Newman-Keuls Multiple Comparison Test).
<table>
<thead>
<tr>
<th>S.No.</th>
<th>Parameter</th>
<th>Open control</th>
<th>Filter control</th>
<th>-UV-A+B</th>
<th>-UV-B</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Protein (µg/gm FW)</td>
<td>8.692 ± 0.0320</td>
<td>8.941 ± 0.287</td>
<td>10.849 ± 0.106**</td>
<td>11.228 ± 0.010**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(3.9%)</td>
<td>(26.15%)</td>
<td>(30.56%)</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Leghemosglobin content/gm FW</td>
<td>0.011 ± 0.0001</td>
<td>0.0125 ± 0.0004</td>
<td>0.015704 ± 0.0002***</td>
<td>0.0182 ± 0.0003***</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(7.7%)</td>
<td>(35%)</td>
<td>(56%)</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>Heme chrome content/ mg protein</td>
<td>0.1756 ± 0.0021</td>
<td>0.1834 ± 0.0049</td>
<td>0.2756 ± 0.0047***</td>
<td>0.3047 ± 0.009***</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(4%)</td>
<td>(57%)</td>
<td>(74%)</td>
<td></td>
</tr>
</tbody>
</table>

*Table 3.* Total protein, leghemosglobin and hemechrome contents in the root nodules of *Trigonella* on 45th DAE (Day after emergence) of seedlings by the exclusion of solar UV-B. and UV-A+B. The values are mean ±SE. [Values in parenthesis show percent increase]. ***, ** indicate significance at P < 0.001 and 0.01, respectively, compared to control.
**Fig.5.3.** Spectral analysis of leghemoglobin isolated from root nodules of Trigonella plants at 45\textsuperscript{th} DAE.

### 5.1.3 Nodulation

Exclusion of UV-B and UV-A+B enhanced both the number and the size of the nodules recorded at 45 DAE. Number of nodules were higher by 75\% after exclusion of UV-A+B and 102\% after UV-B exclusion (Figure.5.4 [A]) (**P<0.001). The fresh weight of the nodules was also enhanced by 142\% (UV-A+B) and 178\% (UV- B) after exclusion (Figure.5.4 [B]).

### 5.1.4 Nitrate reductase

Although legumes can fix nitrogen directly through the root nodules, the activity of nitrate reductase assumes importance particularly at the stage of pod filling at maturity, when the nodular activity declines. UV exclusion enhanced the activity of nitrate reductase in fenugreek at 45 DAE. An enhancement of 96\% (-UV-B) and 75\% (-UV-B+A) has been recorded in our experiments (Figure.5.5) (**P<0.001). This enhancement may contribute to the yield in particular.

(A)
Fig. 5.4. Effect of solar UV-B+A and UV-B exclusion on (A) Number of nodules and (B) Fresh weight of nodule of *Trigonella* (Pusa early bunching) at 45 DAE. The vertical bars indicate ± SE for mean. UV-B+A and UV-B excluded plants show significant difference at (***P<0.001) compared to open control. (Newman-Keuls Multiple Comparison Test).
Fig. 5.5. Effect of solar UV-B+A and UV-B exclusion on Nitrate Reductase activity in the leaves of *Trigonella* (Pusa early bunching) at different days after emergence of the seedlings (30 to 60 DAE). The vertical bars indicate ± SE for mean. UV-B+A and UV-B excluded plants show significant difference at (**P<0.001) compared to open control. (Newman-Keuls Multiple Comparison Test).
5.2. Discussion

Exclusion of UV-B and UV-B+A enhances the activity of nitrogenase and nitrogen fixation in *Trigonella foenum graecum* nodules as evident in the results. Earlier studies on UV exclusion had only obtained indirect evidence for increased nitrogen fixation in legumes like soybean (Singh, 1997; Chouhan et al., 2008). In soybean plants grown under UV-excluded sunlight, nodule number and weight of nodules, leghemoglobin and heme-chrome were higher compared to plants grown under normal sunlight (Chouhan et al., 2008). Reduction in nitrogen concentration on exposure to supplemental UV-B was observed in the roots of *Glycine max* and *Phaseolus vulgaris* indicating suppressive action of UV-B on nitrogen fixation (Singh, 1995). Besides enhancing nitrogenase activity, exclusion of UV also enhanced the leghemoglobin and heme-chrome along with the accessory protein required for efficient fixation of nitrogen in the present study. Since there was no significant difference in PAR intensity among treatments so, enhancement is due to the exclusion of UV components and not because of difference in PAR intensity. Enhancement in root biomass as observed here in *Trigonella* (Figure 5.6) has also been reported after UV exclusion on leguminous plants like soybean (Varalakshmi et al., 2003; Chouhan et al., 2008). In the results presented here enhancement in root growth (root length, root fresh weight with nodules and root dry weight with nodules), nodulation (number of nodules and fresh weight of nodules) and nitrogenase activity in *Trigonella* are more prominent by the elimination of UV-B from sunlight whereas, elimination of UV-A along with UV-B reduces the extent of enhancement. This indicates that UV-B is the suppressor of nitrogen fixation, since presence of UV-A enhances the observed parameters. It has earlier been reported in soybean that UV-A promotes nodulation and nitrogenase activity (Tezuka et al., 1998), similarly near UV promoted nodulation and nitrogen fixation in pea plants (Shiozaki et al., 1999). UV exclusion promotes the growth of aerial parts of the plants, especially leaf area (Pal et al., 2006; Dehariya et al., 2012; Kataria and Guruprasad, 2012) and enhances photosynthesis.
Fig. 5.6. Photographs showing effect of UV-A+B and UV-B exclusion on Root length with number of nodules of *Trigonella*. 
In leguminous plants like *Trigonella* the enhanced rate of photosynthesis increase the amount of substrates and ATP as a reducing power source to support the activity of nitrogenase. The requirement of UV-A for the maximum enhancement in the activity of nitrogenase may be due to enhanced level of flavonoids in the nodules induced by UV-A (Shiozaki et al., 1999). Flavonoids may activate the expression of nodulation genes and symbiotic nitrogen fixation in alfalfa (Redmond et al., 1986). Since flavonoids in leguminous plants are associated with nodulation on the roots (Redmond, 1986; Kapulnik et al., 1987). Several flavonoids that are exuded from plant roots act as signals, which induce the transcription of bacterial genes, where protein products are required for the infection process (Hungria and Stacey 1997; Broughton et al., 2003; Mathesius, 2003; Cooper, 2004; Kobayashi et al., 2004). In trigonella UV-A is also required for the maximum enhancement of photosynthesis under exclusion of UV-B as observed in chapter-4. Enhanced rate of nitrogen fixation due to UV-A may also be related to this enhanced rate of photosynthesis.

Nitrate Reductase catalytic flux is controlled by substrate availability, by the level of functional enzyme and by the activity level of functional NR and the overall nitrate reduction capacity is regulated in relation to overall plant metabolic level by metabolic sensors and signal transduction pathways (Campbell et. al., 1999). Nitrate and light are thought to be important environmental signals for NR regulation (Hageman and Flesher, 1960) and nitrate reductase that is located at the junction of two energy-consuming pathways, nitrate assimilation and carbon fixation, results in a controlled response to the environmental changes that affect photosynthesis (Cheng et. al., 1992). Exposure to UV-B resulted in a decrease in the activity of nitrate reductase in maize (Quagggiotti et al., 2004) and barley (Ghisi et al., 2002). Recent study has shown that UV-B radiation led to the inhibition in the activities of the key enzymes (nitrate reductase) in the nitrogen assimilation, the decrease in the contents of nitrate and soluble proteins, in soybean seedlings (Huang et al., 2013). However there was an enhancement in the activity of nitrate reductase in sun hemp *Croatalaria juncea* as reported by (Saralabai et al., 1989; and Rail, 1998). Ghisi et al., (2002) observed significant reductions in the activities of nitrate reductase and glutamine synthetase in the root and leaves exposed to UV-B radiation. Enhanced UV-B has also been found
to inhibit growth and decrease NR activity in dragon spruce (*Picea asperata Mast.*) needles (Yao and Liu, 2007) and in young crop seedlings (Balakumar et al., 1999; Ghisi et al., 2002; Quaggiotti et al., 2004; Rajendiran and Ramanujam, 2006). NR activity in the leaves of trigonella was enhanced by the exclusion of UV. This is particularly significant at the pod filling stage when the nodules activity declines. In conclusion solar UV-B and UV-A regulate nodulation, nitrate reductase and nitrogen fixation in *Trigonella foenum graecum*. Enhanced rate of photosynthesis may be one of the possibilities for the enhancement in nitrogen fixation by the exclusion of UV-B although there may be other factors contributing to this. UV-A is required for this enhancement as it seems to regulate nitrogen fixation in the nodules through increased rate of photosynthesis and flavonoids. Developing technologies to eliminate UV-B from solar radiation may increase productivity in *Trigonella foenum graecum* besides reducing the use of fertilizers. Exclusion of solar UV is beneficial to agriculture in terms of nitrogen fixation as exclusion of UV-B and UV-B+A enhanced root biomass, and number of nodules in the roots.