**Research article**

**Intra-specific variation in response of Neem** (*Azadirachta indica* A. Juss) **to elevated CO$_2$ levels and biochemical characterization of differently responding plants**


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**Abstract:** Global climate change the looming environmental threat is mainly due to the increase in atmospheric CO$_2$ levels, which was increasing earlier by about 1.55 ppm per year and currently by 2.76 ppm per year. Thus, CO$_2$ concentration has reached 400.16 ppm in 2015. To understand the response of various tropical tree species to such an elevated CO$_2$, experiments were conducted in Automated Open Top Chambers (AOTC) facility at Institute of Forest Genetics and Tree Breeding (IFGTB), Coimbatore (India). The results of initial studies indicated the scope for exploring intra-specific variation in response of tropical trees to elevated CO$_2$. Subsequently, experiments were carried out to assess intra-specific variation in Neem (*Azadirachta indica*). The selected phenotypes of Neem (varieties or clones) were exposed to four treatments viz., i) CO$_2$ of 600 ppm, ii) CO$_2$ of 900 ppm, iii) chamber control- without any CO$_2$ enrichment and iv) ambient conditions. The parameters studied were shoot length, root length, dry matter accumulation in shoot and root. The results of the study showed that there existed significant variation among different treatments of CO$_2$ as well as among various phenotypes or clones in terms of growth characteristics. This intra-specific variation in biomass accumulation under elevated CO$_2$ levels could be exploited for future breeding programmes in developing climate ready genotypes having greater potential to sequester more carbon and produce greater biomass under forecasted elevated levels of atmospheric CO$_2$. Another objective in this study was to analyze intra-specific variation in selected biometrical and biochemical characteristics of leaf samples of neem trees in relation to their differential response to elevated CO$_2$. Among parameters of leaf, Fumaric acid, Malic acid and Oxalic acid, leaf Nitrogen and Specific Leaf Weight may be considered as a biochemical and biometrical marker to categorize the plants adapted to elevated CO$_2$ environments.

**Keywords:** Response to elevated CO$_2$ - Neem (*Azadirachta indica*) - Open Top Chambers - Within species variation in growth.


**INTRODUCTION**

Even the most environment friendly emission scenarios lead to an increase in atmospheric CO$_2$ concentration over the next 100 years, to about double the pre-industrial levels up to 550 ppm (IPCC 2007). Currently, CO$_2$ concentration has reached 400.16 ppm in 2015 Mike McGee (2015). This increasing concentration of carbon dioxide (CO$_2$) in the atmosphere may have a direct effect on the physiology of plants: higher CO$_2$ tends to suppress plant transpiration through reduced stomatal conductance (Field *et al.* 1995). In tree species, short term experiments with *Pinus ponderosa*, *Quercus coccinea*, *Pinus radiata* and *Populus deltoids* have shown a definite increase in photosynthesis rate up to 40–80% under 600 ppm levels of CO$_2$ (Couteaux *et al.* 1992). Studies conducted at Institute of Forest Genetics and Tree Breeding (IFGTB) showed the existence of greater inter- and intra-specific variation in tropical tree species in response to elevated CO$_2$ and temperature (Buvaneswaran *et al.* 2010). With reference to intra-specific clonal variation in tree species, Johanna *et al.* (2003) reported that two clones of silver birch (*Betula pendula*) responded differently to elevated CO$_2$ levels. In one clone, total biomass increased by 40% under elevated CO$_2$, but in another clone no response was found.
Similar such intra-specific clonal variations have been reported in Sitka spruce (Murray et al. 1994), Poplar (Ceulemans et al. 1995), Hevea brasiliensis (Devakumar et al. 1998), Populus tremuloides (Isebrands et al. 2003). However, not much study has been conducted on intra-specific variation in response of tree species to elevated CO₂ levels, particularly the plantation forestry species of India. Hence, the present study was conducted to generate knowledge on intra-specific variation in important tropical plantation species besides helping to screen assembled productive clones of Neem (Azadirachta indica A. Juss) for higher carbon sequestration potential under elevated CO₂. This selection of clones adapted to elevated CO₂ levels may also be used for developing ‘climate ready genotypes’ in future through biotechnological interventions. The information generated could also help in identification of biometrical and biochemical markers, if any, for higher carbon sequestration potential of the selected species under elevated CO₂.

MATERIALS AND METHODS

The present research experiment was conducted using Automated Open Top Chambers (AOTC) facility at Institute of Forest Genetics and Tree Breeding (IFGTB), Coimbatore, India. This study site is located on 11°59’0.69” N, 76°57’2.32” E and 437 m MSL. The facility of AOTCs has chambers which are cubical type structures of 3 × 3 × 3 m dimension, fabricated with galvanized iron (GI) pipe frames and covered with polyvinyl chloride (PVC) sheet of 120 micron gauge to have more than 90% transmittance of light. The upper portion of the chamber is kept open to maintain near-natural conditions. A software facility called Supervisory Control and Data Acquisition (SCADA) was used to continuously control record and display the actual and desired CO₂ level, relative humidity and temperature in each OTC by feedback control loop passing through Programmable Logical Controllers (PLC).

The species selected for the present study was Neem (Azadirachta indica A. Juss) in which 12 clones (varieties) were selected and subjected to various treatments. The initial shoot length was recorded for all the plants. Then, these plants were exposed to four treatments with the different levels of CO₂ viz., i) chamber with 600 ppm of CO₂, ii) chamber with 900 ppm concentration of CO₂, iii) chamber without any elevation of CO₂ and iv) ambient control- in open area. CO₂ enrichment was done by using CO₂ cylinders. The design of the experiment adopted is Completely Randomized Block Design (CRBD).

At end of exposure period under various treatments, plants were sampled and growth parameters were measured like shoot length, root length, and fresh weight of leaf, shoot and root. Then, the plant samples were kept in hot air oven at 60°C till the constant dry weight is obtained. Then dry weight was recorded for in root, shoot and leaves. The data on growth and dry matter production were subjected to analysis of variance for completely randomized design with replications.

To analyze intra-specific variation in few biometrical and biochemical characteristics of Neem trees in relation to their differential response to elevated CO₂, Four different clones (varieties) of Neem viz. IFGTB-AI-9, IFGTB-AI-11, IFGTB-AI-3 and IFGTB-AI-5 were used to assess intra-specific variation in biochemical characteristics in neem trees in relation to their differential response to elevated CO₂. Among these four clones selected for the present study, two clones IFGTB-AI-9 and IFGTB-AI-11 were responding positively under elevated CO₂ environments in the earlier experiments conducted under Open Top Chambers at Institute of Forest Genetics and Tree Breeding, Coimbatore. On the other hand, clones IFGTB-AI-3 and IFGTB-AI-5 were responding poorly to the elevated CO₂ conditions by recording lesser dry matter accumulation in biomass of various plant parts.

The mother plants of these four selected clones of neem, belonging to two categories with respect to their response to elevated CO₂ environments - were studied for variation in chlorophyll content (chlorophyll - a, chlorophyll - b and total chlorophyll), Organic acids (Fumaric acid, Malic acid, Citric acid and Oxalic acid), Nutrient elements (Nitrogen, Phosphorus, Potassium, Calcium and Magnesium) and also for leaf weight, leaf area and Specific Leaf Weight. All these parameters were assessed using leaf samples collected from the respective clones available in the germplasm assemblage of neem in IFGTB campus, Coimbatore.

RESULTS AND DISCUSSIONS

A) Response of Azadirachta indica to elevated CO₂ in Open Top Chambers

A set of 12 phenotypes (clones) of Neem (Azadirachta indica A. Juss) were used in the experiments conducted in Automated Open Top Chambers (AOTC) facility at IFGTB, Coimbatore. The results of the study showed that there existed significant variation among different treatments of CO₂ as well as among various
clones in terms of growth characteristics. Overall mean shoot length was 59.73 cm for plants exposed to 600 ppm of CO₂ and it was only 28.25 cm under ambient conditions, irrespective of clones (Table 1). Root length was greater (39.33 cm) under 900 ppm CO₂ level than that recorded in ambient conditions (30.07 cm). Dry matter accumulation in shoots was 78% more under 600 ppm CO₂ level than that of in ambient conditions. Similarly, 58% more dry matter accumulation was recorded in roots of plants grown under 600 ppm of CO₂ levels when compared to the plants grown in ambient conditions (Table 1).

Among 12 clones studied, clone IFGTB-AI-12 recorded 86% more total dry matter accumulation in biomass of plants grown under 600 ppm of CO₂ as compared to that observed for the same clone under ambient conditions. Clone IFGTB-AI-9 accumulated greater amount of total dry matter in biomass under 900 ppm CO₂ levels that is 89% over and above the total dry matter accumulated in the same clone under ambient environments (Fig 1). In Neem, only one clone that is IFGTB-AI-5 showed negative response in term of total dry matter accumulation under 900 ppm CO₂ level by registering 3% less dry matter accumulation in biomass when compared to that of ambient grown plants of the same clone. Followed by clone IFGTB-AI-3 which recorded lesser accumulation of total dry weight when compared to all other positively responding clones.

| Table 1. Shoot and root characteristics of Azadirachta indica A. Juss plants exposed to various levels of CO₂ in open top chamber experiments conducted in IFGTB, Coimbatore, India. |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | 600 ppm         | 900 ppm         | Chamber control | Ambient         |
| Mean shoot length (cm) | 59.73 ± 3.48 | 54.69 ± 2.12 | 62.05 ± 2.56 | 28.25 ± 1.59 |
| Mean root length (cm) | 36.10 ± 3.11 | 39.33 ± 2.15 | 33.20 ± 2.79 | 30.07 ± 1.01 |
| Leaf dry weight (g per plant) | 6.22 ± 0.26 | 5.52 ± 0.48 | 6.85 ± 0.40 | 4.53 ± 0.29 |
| Shoot dry weight (g per plant) | 12.24 ± 0.80 | 10.72 ± 0.61 | 11.50 ± 0.55 | 6.86 ± 0.54 |
| Root dry weight (g per plant) | 9.12 ± 0.90 | 8.29 ± 0.61 | 7.54 ± 0.57 | 5.78 ± 0.49 |

**Note:** Values shown are Mean ± Standard Error

In the present study, shoot length was increased under elevated CO₂ conditions when compared to ambient conditions. As observed in the present study, Pokorny et al. (2012) observed that the shoot length was increased frequently under elevated CO₂ conditions. With regard to increased root length under elevated CO₂ environment, it is reported that the responses of roots to CO₂ are dependent on experimental conditions (Ceulemans & Mousseau 1994). When the plants exposed to increased CO₂, root have observed to become more numerous, longer, thicker and faster growing in crops (Chandhuri et al. 1990) with increased root length in many plant species (Norby 1994, Pritchard & Rogers 2000, Bernecchi et al. 2000). Lengths and volumes of tap root and fine roots were higher for CO₂ enhanced cotton plants (Rogers et al. 1993). 110% increase in root length of soybean was observed as CO₂ concentration increased (Rogers et al. 1992). Increased root length and number under elevated CO₂ environments has also been reported in sweet potato (Bhattacharya et al. 1990) and Phaseolus acutifolius and P. vulgaris (Salsman et al. 1999).

In the present study, dry matter accumulation both in shoot and root biomass was greater in plants grown under elevated CO₂ environments as compared to that in ambient grown plants. Similar findings were reported.
by Cao et al. (2008) in Gossypium hirsutum and the above ground biomass was increased under 600 ppm of elevated CO₂ level. Lukac et al. (2003) revealed that the CO₂ enrichment increases the belowground biomass allocation in three *populus* species were investigated and the standing root biomass enhanced by 47–76%. Moreover the growth of Minjiang fir showed significantly positive responses of elevated CO₂ with greater increases of total biomass than the control conditions (Hou et al. 2011). Ghasemzadeh & Jaafar (2011) reported in two varieties of *Zingiber officinale* were exposed to different CO₂ concentration (400 and 800 ppm) resulted in increasing total plant biomass over the ambient conditions. Mohamed (2013) reported that there is no significant intra specific difference in dry matter production in the tropical dry land forest species of Neem. But when the plants were exposed on increased atmospheric CO₂ the final plant biomass, above ground biomass and below ground biomass was significantly increased in tree species (Madhu & Hatfield 2013). Similarly, the shoot biomass was approximately 35% greater for creeping bentgrass plants grown under elevated CO₂ compared to plants maintained under ambient CO₂, while the root biomass increased by 37% due to elevated CO₂ (Burgess & Huang 2014). Wang et al. (2015) studied the responses of rice production under elevated CO₂, there was significant stimulation in above ground biomass 28 per cent and below ground biomass of rice was 42 per cent increases. Similar findings of root biomass was higher under elevated CO₂ levels has been reported by several authors and it was increased of 55 per cent in *Pinus sylvestris* (Jach et al., 2000), 32 per cent in *Pinus taeda* by Jackson et al. (2009).

With reference to intra-specific clonal variation in tree species, it is observed in the present study that huge variation existed among the clones in terms of total dry matter accumulation in Neem. Similarly, Johanna et al. (2003) also reported that two clones of silver birch (*Betula pendula*) responded differently to elevated CO₂ levels. In one clone, total biomass increased by 40% under elevated CO₂, but in another clone no response was found. Similar such intra-specific clonal variations have been reported in Sitka spruce (Murray et al.1994), Poplar (*Cleulmans et al. 1995*), *Hevea brasiliensis* (Devakumar et al. 1998), Populus tremuloides (Isebrands et al. 2003).

**B) Foliar analysis of differently responding clones for biometrical and biochemical traits**

In the present study, the existing variation in chlorophyll contents among the clones studied were not corresponding to the growth response observed for the respective clones under the elevated CO₂ environments (Table 2). Similarly, Nicolas et al. (2007) also reported that there is no link of chlorophyll with tree growth irrespectively of site and family in hybrids of poplar. However, Kumar & Paramathama (2005) reported that all the traits studied, via., plant height, collar diameter. Number of branches, survival percent, chlorophyll content and suitability index were strongly associated with volume index in 44 clones of Casuarina equisetifolia assembled from Tamilnadu, Andhra Pradesh, Orissa and Pondicherry. Reddy et al. (2003) also reported that chlorophyll a, b and total chlorophyll showed significant positive correlation with leaf area and yield in different genotype of Mulberry.

### Table 2. Intra-specific Variation in Chlorophyll contents (mg chlorophyll per g leaf tissue) in selected Neem clones.

<table>
<thead>
<tr>
<th>Clones</th>
<th>Chlorophyll-a</th>
<th>Chlorophyll-b</th>
<th>Total Chlorophyll</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFGTB-AI-9</td>
<td>0.0135 ± 0.0016</td>
<td>0.0115 ± 0.0037</td>
<td>0.0359 ± 0.0072</td>
</tr>
<tr>
<td>IFGTB-AI-11</td>
<td>0.0074 ± 0.0025</td>
<td>0.0155 ± 0.0046</td>
<td>0.0298 ± 0.0049</td>
</tr>
<tr>
<td>IFGTB-AI-3</td>
<td>0.0049 ± 0.0023</td>
<td>0.0048 ± 0.0025</td>
<td>0.0245 ± 0.0079</td>
</tr>
<tr>
<td>IFGTB-AI-5</td>
<td>0.0140 ± 0.0014</td>
<td>0.0196 ± 0.0028</td>
<td>0.0490 ± 0.002</td>
</tr>
</tbody>
</table>

**Note:** Values shown are Mean ± Standard Error.

Among various organic acids, Fumaric and Malic acid was in greater quantity in clones positive to elevated CO₂ when compared to other two clones which are responding poorly to elevated CO₂. On the contrary, Oxalic acid content was observed to be lesser in quantity in clones adaptive to elevated CO₂ than in clones less adaptive to elevated CO₂. However, there is not much difference among clones in respect of Citric acid content (Table 3). Similar variation in organic acid contents under exposure to stress environments has been reported by Silva et al. (2004) who reported that Gas chromatography/mass spectrometry and ion chromatography analyses indicated that root exposure to AI led to a greater than 200% increase in Malic acid concentration in the root tips of all eucalypt species. The increase in Malate concentration in response to AI treatment is correlated with the tree species. A small increase in citric acid concentration was also observed in all species, but there were no consistent changes in the concentration of other organic acids in response to AI treatment. In all eucalypt
species, AI treatment induced the secretion of citric acid and Malic acid in root exudates. But no trend with respect to AI tolerance was observed. Thus, although malate and citrate exudation by roots may partially account for the overall high AI tolerance of these eucalypt species, it appears that tolerance is mainly derived from the internal detoxification of AI by complexation with malic acid.

Table 3. Intra-specific Variation in organic acids (mg per g leaf tissue) in selected Neem clones.

<table>
<thead>
<tr>
<th>Clones</th>
<th>Fumaric Acid</th>
<th>Malic acid</th>
<th>Citric acid</th>
<th>Oxalic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFGTB-AI-9</td>
<td>11.56 ± 0.022</td>
<td>11.70 ± 0.322</td>
<td>1.80 ± 0.027</td>
<td>0.049 ± 0.001</td>
</tr>
<tr>
<td>IFGTB-AI-11</td>
<td>10.36 ± 0.093</td>
<td>11.34 ± 0.305</td>
<td>2.04 ± 0.002</td>
<td>0.2697 ± 0.012</td>
</tr>
<tr>
<td>IFGTB-AI-3</td>
<td>9.40 ± 0.066</td>
<td>10.40 ± 0.907</td>
<td>1.79 ± 0.032</td>
<td>0.4767 ± 0.065</td>
</tr>
<tr>
<td>IFGTB-AI-5</td>
<td>8.32 ± 0.057</td>
<td>10.54 ± 0.636</td>
<td>2.42 ± 0.099</td>
<td>0.3967 ± 0.085</td>
</tr>
</tbody>
</table>

*Note: Values shown are Mean ± Standard Error*

Significant observation in the present study was that among various nutrient elements studied, Nitrogen content was greater in amount in clones which are categorized as adaptive to elevated CO₂ (0.112 to 0.14%) when compared to that in clones which are not adaptive to elevated CO₂ environments (0.028 to 0.056%) (Table 4). It is reported that the largest single pool of nitrogen in leaves is RuBisCO and hence it can be inferred that the greater amount of Nitrogen in the leaves of adaptive clones may indicate greater amount of RuBisCO availability. This higher RuBisCO availability is related to higher biomass production, as photosynthesis is catalysed by this RuBisCO, which fixes carbon to form carbohydrates. Moore et al. (1998) also reported that short term exposure of elevated CO₂ for plants generally leads to increased rates of leaf-level photosynthesis due to enhanced activity of RuBisCO. On the other hand, photosynthesis down regulation is characterized at the biochemical and leaf levels by reduced chlorophyll content, reduced RuBisCO content and activity and decreased leaf nitrogen concentration on a leaf mass basis (Sage 1994, Tissue et al. 1995). It warrants further research to understand the reason for higher leaf N in some species and lower N levels in leaf in other species when plants are exposed to elevated CO₂.

Table 4. Intra-specific Variation in nutrient elements (% in leaf tissue) in selected Neem clones.

<table>
<thead>
<tr>
<th>Clones</th>
<th>N %</th>
<th>P %</th>
<th>K %</th>
<th>Ca</th>
<th>Mg %</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFGTB-AI-9</td>
<td>0.140</td>
<td>0.196</td>
<td>2.00</td>
<td>0.56</td>
<td>0.08</td>
</tr>
<tr>
<td>IFGTB-AI-11</td>
<td>0.112</td>
<td>0.136</td>
<td>2.87</td>
<td>0.28</td>
<td>0.21</td>
</tr>
<tr>
<td>IFGTB-AI-3</td>
<td>0.056</td>
<td>0.143</td>
<td>1.97</td>
<td>1.24</td>
<td>1.87</td>
</tr>
<tr>
<td>IFGTB-AI-5</td>
<td>0.028</td>
<td>0.135</td>
<td>2.22</td>
<td>1.16</td>
<td>1.74</td>
</tr>
</tbody>
</table>

Another important observation made in the present study was that the actual dry weight and leaf area of clones were not differentiated in terms of clonal variation in respect of their response to elevated CO₂. However, when Specific Leaf Weight (SLW) was worked out, this SLW parameter clearly got differentiated in clones. Greater SLW was recorded in clones which were responding positively to the elevated CO₂ enforcing. Hence, clones IFGTB-AI-9 and IFGTB-AI-11 recorded greater SLW values of 13.06 and 10.75 mg per cm² respectively (Table 5). On the other hand, clones IFGTB-AI-3 and IFGTB-AI-5 recorded lesser SLW, which were the clones responded poorly to the elevated CO₂ environments in the earlier experiments. Sicho et al. (1994) who studied the photosynthetic acclimation to elevated CO₂ occurs in transformed Tobacco and reported that the dry weight gain was due to increased specific leaf weight. Specific leaf weight was shown to be a valuable index for comparing photosynthesis various parts of a tree canopy over a season or throughout an entire year. Mean annual photosynthetic rate in five separate portions of a spruce canopy was directly proportional to observed differences in specific leaf weight (Ram 1984).

In brief, among various parameters of leaf, organic acids particularly Fumaric acid, Malic acid and Oxalic acid, leaf Nitrogen content and Specific Leaf Weight may be considered to act as a biochemical and biometrical marker to categorize the plants adapted to elevated CO₂ environments, specifically to neem trees.

Table 5. Intra-specific variation in leaf dry weight, leaf area and Specific Leaf Weight in clones of Neem.

<table>
<thead>
<tr>
<th>Clones</th>
<th>Dry weight</th>
<th>Leaf area (cm²)</th>
<th>Specific Leaf Weight (mg per cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFGTB-AI-9</td>
<td>389.6 ± 0.129</td>
<td>31.67 ± 5.89</td>
<td>13.06 ± 2.16</td>
</tr>
<tr>
<td>IFGTB-AI-11</td>
<td>451.7 ± 0.075</td>
<td>43.00 ± 7.00</td>
<td>10.75 ± 2.31</td>
</tr>
<tr>
<td>IFGTB-AI-3</td>
<td>519.7 ± 0.087</td>
<td>56.67 ± 6.64</td>
<td>9.36 ± 0.84</td>
</tr>
<tr>
<td>IFGTB-AI-5</td>
<td>221.2 ± 0.037</td>
<td>28.00 ± 3.05</td>
<td>7.99 ± 0.68</td>
</tr>
</tbody>
</table>

*Note: Values shown are Mean ± Standard Error*
CONCLUSION
The present study evidently proves that there exists significant intra-specific variation in _Azadirachta indica_ in response to elevated CO₂. Greater variation existed in dry matter accumulation in biomass among different clones under elevated CO₂ conditions. This variation could be explored in all other commercially important tropical tree species and superior tree varieties can be identified for higher productivity and carbon sequestration potential under forecasted elevated CO₂ levels of the future environment.

When four different clones (varieties) of Neem (_Azadirachta indica_ var. IFGTB-AI-9, IFGTB-AI-11, IFGTB-AI-3 and IFGTB-AI-5) were used to assess intra-specific variation in biochemical characteristics in neem trees in relation to their differential response to elevated CO₂, it is observed that among various parameters of leaf, organic acids particularly Fumaric acid, Malic acid and Oxalic acid, leaf Nitrogen content and Specific Leaf Weight may be considered to act as a biochemical and biometrical marker to categorize the plants adapted to elevated CO₂ environments, specifically to neem trees. Further research is suggested to confirm these observations in other tropical tree species which will aid in large scale screening of various tree species for their adaptation potential to ever increasing atmospheric CO₂.

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