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IMPACT OF ELEVATED CO_2 ON *in vitro* PROPAGATION OF IMPORTANT MEDICINAL PLANT *Nyctanthes arbor-tristis L.*

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ABSTRACT
Nyctanthes arbor-tristis L. is an important ornamental as well as medicinal plant known for its flowers. The axillary nodes of this plant are cultured on Murashige and Skoog medium, which results in the direct organogenesis. The M.S. medium is supplemented with 3 mg/L Indole-3-acetic acid. The in-vitro regenerated shoots are sub-cultured on the MS medium supplemented with IAA and BAP and subjected to elevated level of CO_2 viz 0.5% and 2% in airtight CO_2 chamber. The comparative analysis is performed on various physical and biochemical parameters after an 8-week treatment at elevated CO_2. The results of the present study show that the enhanced CO_2. level proves beneficial for invitro growth of Nyctanthes arbor-tristis L.

KEYWORDS:- *Nyctanthes arbor-tristis L., Elevated CO_2, M.S. Medium, Direct Organogenesis*

INTRODUCTION
***Nyctanthes arbor-tristis* L.**, commonly referred to as Night-flowering Jasmine or Parijat, is a plant renowned for its significant medicinal, ornamental, and cultural value. This versatile plant has been utilized extensively in traditional medicine, with every part of the plant, including its leaves, roots, stems, bark, and seeds, demonstrating therapeutic properties.(Himanshi Rawat *et. al*., 2021 ; Jaspal Singh *et.al*., 2021.). The leaf juice is commonly used to aid digestion, act as an antidote to reptile venom, and serve as a tonic, laxative, diaphoretic (promoting sweating), and diuretic (increasing urine output). Additionally, the leaves are employed in the treatment of an enlarged spleen, highlighting their role in addressing specific organ-related conditions.

Traditionally, powdered dried leaves are known to alleviate malaria symptoms and rheumatic joint pain, reflecting their importance in treating both infectious diseases and inflammatory conditions.(Ashokkumar, K., Dharshini, M., Janani, T. *et al. ; 2024)* Beyond these uses, the plant exhibits a wide array of pharmacological activities, including antihistaminic effects (to manage allergic reactions) and central nervous system (CNS) actions such as hypnotic (sleep-inducing), tranquilizing (calming), and anesthetic properties. Its pharmacological spectrum further includes analgesic (pain-relieving), anti-inflammatory, antipyretic (fever-reducing), antiulcer, anti-amoebic, anti-helminthic (against parasitic worms), anti-trypanosomal (against trypanosome parasites), antidepressant, antiviral, and immunomodulatory (immune-regulating) activities.

Phytochemical studies of *Nyctanthes arbor-tristis L.* have revealed the presence of a wide range of bioactive compounds, including phytosterols, phenolics, tannins, flavonoids, glycosides, and saponins. (Mansi , Rajhans, Sanjukta *et.al*., 2021; Samir Panda *et al*.; 2024) Glycosides and alkaloids are particularly abundant in the plant, with iridoid glycosides and phenylpropanoid glycosides being the most frequently identified glycosides. Among the alkaloids, nyctanthine is found to be the most prominent, playing a significant role in the plant's therapeutic efficacy.

The seeds of the plant are rich in iridoid compounds and arbortristosides (A, B, D, and E), which have demonstrated immunomodulatory properties and antileishmanial activity (effective against *Leishmania* parasites). The leaves contain a diverse array of bioactive compounds, such as 6,7-di-O-benzoylnystantoside, 6-O-trans-cinnamoyl-6β-hydroxy loganin, 7-O-trans-cinnamoyl-6β-hydroxy loganin, and desrhamnosylverbascoside, which are known for their potent anti-inflammatory and antipyretic effects. The stem is characterized by the presence of naringenin-4-O-α-glucopyranoside and 1-β-xylopyranoside, which contribute to the plant's overall medicinal value.(Champa Rani, Sunaina Chawla *et.al*.; 2012)

Environmental changes, particularly the rising levels of carbon dioxide (CO_2) due to anthropogenic activities, have forced plants like *Nyctanthes arbor-tristis L.* to adapt by altering their secondary metabolism. Elevated CO_2 levels are known to stimulate the production of secondary metabolites, which are often associated with enhanced pharmacological activities. Studies involving ***In vitro*** cultivation of *Nyctanthes arbor-tristis L.* Under elevated CO_2 conditions have provided insights into these adaptive changes, offering valuable knowledge about how such environmental factors influence the plant's phytochemistry and potential medicinal applications.

MATERIAL AND METHODS
Young axillary buds of *Nyctanthes arbor-tristis L .which was collected from the campus* of IITE, Gandhinagar, were used as an explants. The explants were surface sterilized with 0.1% $HgCl_2$ for 2 minutes, followed by washing with sterilized distilled water, and then sterilized with 1% sodium hypochlorite for 2 minutes. After sterilization, the explants were washed with distilled water 5-6 times and then inoculated onto MS (Murashige and Skoog) media, supplemented with 3 mg/L Indole-3-acetic acid (IAA).

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Direct Organogenesis

Axillary buds of approximately 1 cm^2 in length were inoculated onto MS media containing 3% sucrose and 0.8% Agar-agar. The medium was supplemented with 3 mg/L IAA.

Once the shoots were regenerated and the axillary buds reached sufficient length, they were sub-cultured onto MS media with 2% sucrose, and supplemented with 3 mg/L IAA and 1 mg/L BAP (6-benzylaminopurine). The inoculated culture bottles were placed in an airtight CO_2 chamber with CO_2 levels maintained at 0.5% to 2%.

The pH of the medium was adjusted to 5.8 and autoclaved at 121°C (15 psi) for 20 minutes. The cultures were incubated at 25°C under a 16-hour photoperiod.

RESULT AND DISCUSSION

The current study investigated the *in vitro* propagation of ***Nyctanthes arbor-tristis* L** through direct organogenesis, focusing on the role of varying carbon dioxide (CO_2) concentrations in influencing growth, physiological responses, and biochemical composition. Axillary nodes of approximately 1 cm^2 length were inoculated on MS medium supplemented with 3 mg/L indole-3-acetic acid (IAA). Within 10 days, initial shoot formation was observed, indicating that the explants were responsive to the culture medium and hormonal treatment. By the 20^{th} day, multiple shoots were observed, each producing an average of 3 ± 0.8 leaves. This robust growth suggests that the chosen medium and hormonal conditions effectively supported organogenesis. However, unhealthy explants showed reduced growth and organogenesis, underscoring the importance of explant health in achieving optimal results.

After 25 days of culture, the shoots were sub cultured and transferred to a fresh MS medium containing 3 mg/L IAA and 20 g/L sucrose and placed in airtight chambers with controlled CO_2 concentrations of 0%, 0.5%, and 2.5%. Organogenesis was observed in all treatments by the 12th day, but significant differences in growth were noted based on the CO_2 levels. Plants cultured under 2.5% CO_2 demonstrated the most vigorous growth, producing an average of 6 ± 0.1 leaves per shoot, which was significantly higher compared to the 0.5% CO_2 treatment, where shoots produced only 5 ± 0.3 leaves on average. Notably, no shoot or leaf formation was observed in the absence of CO_2 (0% treatment), highlighting the essential role of CO_2 in plant development and organogenesis.

Micropropagation can also be conducted on sugar-free media under photoautotrophic conditions, utilizing elevated CO_2 as a carbon source. This method has been demonstrated to lower the risk of microbial contamination (Kozai, 1991; Pospisilova *et al*., 1999; Deng and Donnelly, 1993; Yoon *et al*., 2009). However, in the current study, when CO_2 was used as the sole carbon source (without sucrose supplementation), leaf explants survived only for 6–8 days before deteriorating. Similar outcomes were reported in photoautotrophic cultures of *tobacco* (Solarova et al., 1989; Ticha, 1996), *Wrightia tomentosa* (Vyas and Purohit, 2003), and *Chlorophytum borivilianum* (Joshi *et al*., 2009), where plantlets failed to sustain growth in sucrose-free media (Solarova and Pospisilova, 1997). In contrast, potato plantlets were able to grow in sugar-free media, although media containing sucrose resulted in superior growth (Rahman and Alsadon, 2007). Additionally, shoot cultures of *Achras zapota* cultivated on sucrose-containing media under CO_2 free conditions exhibited a significant decline in all growth parameters.

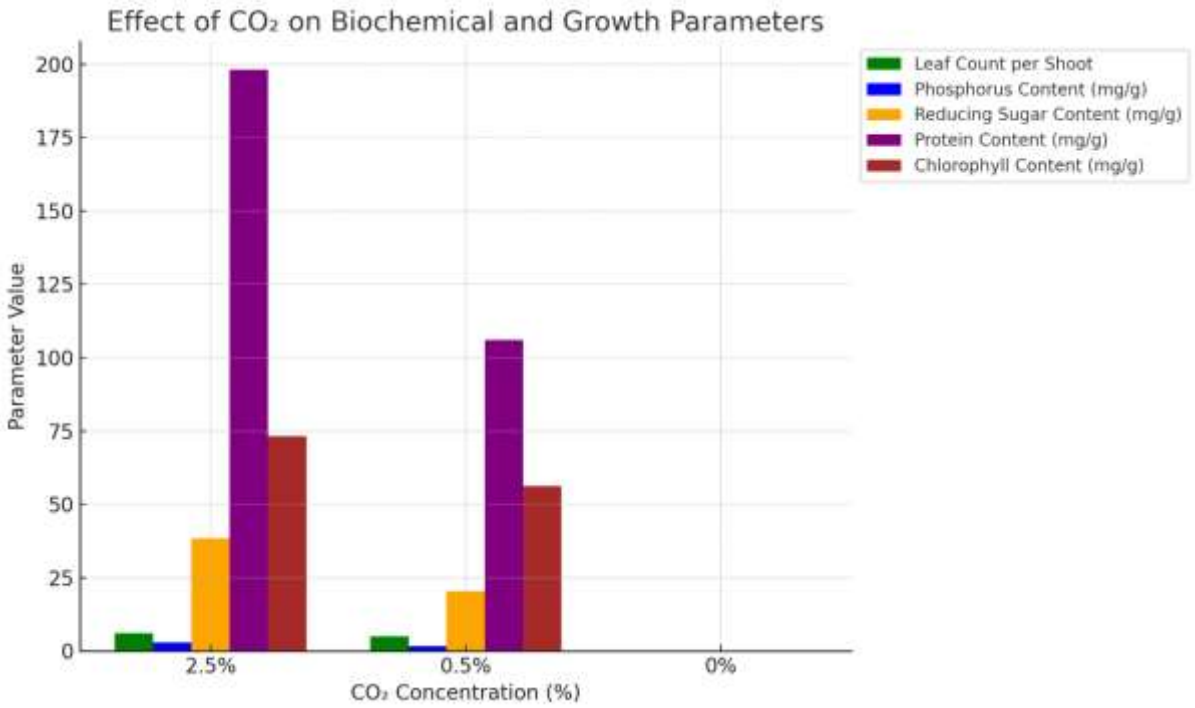
Biochemical analyses revealed clear trends in nutrient and metabolite accumulation. Plants grown under 2.5% CO_2 exhibited the highest phosphorus content (*2.85*$\pm$1.04*mg/g of leaf*)***, followed by the control plants, while those grown under 0.5% CO_2 showed the lowest phosphorus levels*** *(1.65mg/g of leaf*). Similar trends were observed for reducing sugar and protein content, with plants at 2.5% CO_2 recording the highest levels (38.5 ± 2.04 mg/g and 198 ± 4.02mg/g of leaf *respectively*)***, followed by the control plants, and plants at 0.5% CO_2 having the lowest values (*** $20.34\pm1.09 mg/g$ and $106\pm2.08 mg/g\ of\ leaf$ respectively). These results suggest that elevated CO_2 levels enhance nutrient assimilation and promote the synthesis of critical biomolecules required for plant growth.

Physiological parameters such as stomatal frequency and chlorophyll content also varied significantly with CO_2 levels. Plants grown under 2.5% CO_2 exhibited the highest stomatal frequency (22.34 ± 2.12)***, facilitating efficient gas exchange, while those under 0.5% CO_2 recorded the lowest frequency (***19.8 ± 1.2). Chlorophyll content was likewise highest in plants cultured at 2.5% CO_2 (73.208mg/g)***, correlating with increased photosynthetic efficiency, and lowest in plants at 0.5% CO_2 (***56.178mg/g of leaf). The absence of CO_2 in the 0% treatment resulted in no detectable growth, indicating a complete lack of physiological activity.

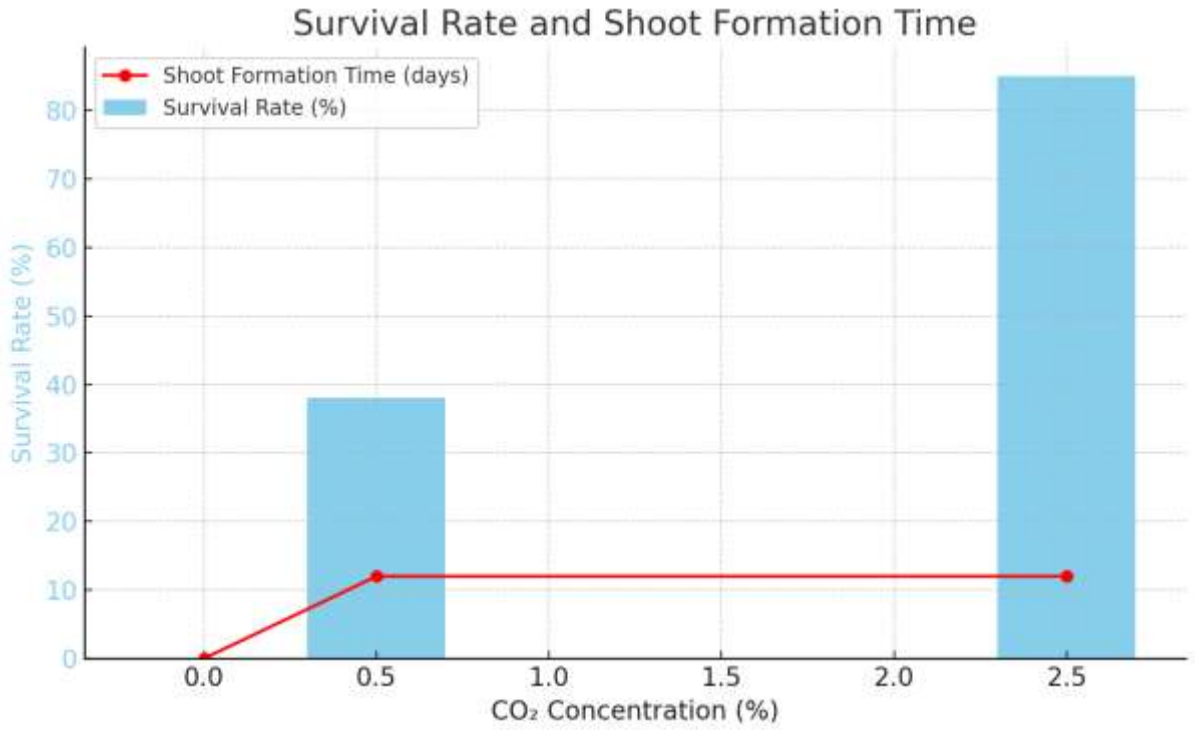
Following the culture phase, plantlets were transferred to thermocol cups containing a 1:1:1 mixture of soil, sand, and compost for the hardening process. Plants grown at 2.5% CO_2 showed exceptional adaptation to the *Ex-Vitro* environment, achieving a survival rate of 85% and demonstrating vigorous growth. Conversely, plantlets from the 0.5% CO_2 treatment exhibited limited growth and a lower survival rate of 38%, reflecting the adverse effects of inadequate CO_2 on plantlet Vigor and acclimatization.

The findings of this study emphasize the critical role of CO_2 in driving in vitro organogenesis, enhancing biochemical composition, and supporting robust plant growth. Among the tested concentrations, 2.5% CO_2 emerged as the most favourable, promoting superior shoot formation, metabolic activity, and adaptability to *Ex Vitro* conditions. In contrast, lower CO_2 levels (0.5% and 0%) negatively affected all parameters, including growth, nutrient assimilation, and survival rates. These results highlight the need to optimize CO_2 levels during *In Vitro* culture to maximize propagation efficiency and plantlet quality.

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Graph 1



Graph 2

CONCLUSION

The research on the in vitro cultivation of **Nyctanthes arbor-tristis L.** emphasizes the essential influence of carbon dioxide (CO_2) levels on plant development, physiological traits, and biochemical properties during direct organogenesis.. The findings demonstrate that elevated CO_2 concentrations significantly enhance shoot formation, nutrient assimilation, and overall plant vigor, with 2.5% CO_2 proving to be the most effective concentration. Plants grown under this condition exhibited superior growth parameters, including a higher number of leaves, enhanced phosphorus, protein, and reducing sugar content, as well as increased stomatal frequency and chlorophyll levels, which collectively indicate improved metabolic and photosynthetic efficiency.

In contrast, suboptimal CO_2 conditions (0.5%) and the absence of CO_2 (0%) resulted in reduced growth and organogenesis, with the latter condition leading to complete failure of shoot and leaf formation. These results underscore the critical necessity of CO_2 for successful in vitro plant culture, especially under conditions designed to simulate photoautotrophic growth.

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Plantlets propagated under 2.5% CO_2 also showed the highest adaptation and survival rates during the *Ex-Vitro* acclimatization phase, indicating their superior robustness and readiness for transplantation. The study confirms that optimizing CO_2 levels during in vitro culture not only enhances the efficiency of propagation protocols but also improves the quality and resilience of the plantlets.

Overall, this research provides valuable insights into the role of environmental factors in plant tissue culture and suggests further exploration of the interaction between CO_2 and other variables, such as light and humidity, to refine propagation techniques for diverse plant species.

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