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Exploring *Chrysopogon nodulibarbis* (Hochst. ex Steud.) Henrard – A Prospective species for grassland restoration through *In vitro* Regeneration

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Abstract:

The genus *Chrysopogon* is renowned for its culinary, medicinal, and aromatic uses as well as practical applications. However, research on the regeneration methods for *Chrysopogon nodulibarbis* (podhigar grass) remains absent to date. This study attempted to achieve a protocol for plantlet regeneration in *C. nodulibarbis*, initiating successful *in vitro* regeneration from basal shoot explants through direct organogenesis. The explants were cultured in Murashige & Skoog's (MS) media with growth regulators such as 2,4-D, NAA, BAP, and IAA individually and also in various combinations with bovine serum albumin, charcoal, ascorbic acid and citric acid supplements. 17 distinct combinations of growth hormones were evaluated for shoot proliferation. The maximum induction frequency (90.7% and 80.4%) was noticed in BAP (3 mg/l) + NAA (3 mg/l) + Citric acid (25 mg/l) and BAP (3 mg/l) + NAA (3 mg/l) respectively. Lower induction frequencies were marked in BAP (3 mg/l), and BAP (4 mg/l) individually yielding 60.2% and 50.1%. 2,4-D (1 to 7 mg/l) and IAA (1 to 5 mg/l) of different doses did not favour callus induction. Although these are preliminary findings, further investigation is employed for fine-tuning the concentrations with the extension of sufficient growth enhancers to optimize callus induction and somatic embryogenesis.

Keywords: *C. nodulibarbis*, *in vitro* regeneration, growth hormones, shoot, organogenesis.

Introduction

The genus *Chrysopogon* encompasses, 49 species with diverse medicinal applications most of which are employed in the food and pharmaceutical fields. It has been widely cultivated for the production of essential oils used as active ingredients and fragrances in foods, cosmetics and aromatic products. Moreover, some species of this genus are medicinally used as a carminative, diaphoretic, diuretic, emmenagogue, refrigerant, stomachic, tonic, antispasmodic,

anthelmintic and sudorific. It helps to treat fever and gastric disorders and the fresh rhizome is used as a stimulant drink (Satapathy *et al.,* 2019). Certain species exhibit potent antioxidant, anti-inflammatory, antimicrobial, and antifungal properties. With the rise of the adverse effects of synthetic drugs, man has rebounded to nature and employed medicinal plants as a remedy for a range of ailments. Medicinal herbs, the cornerstone of traditional medicine for thousands of years, are practised by over 3.3 billion people in less developed countries (Ajlan, 2016; Tsabang *et al.,* 2016). Besides playing a significant role in the fodder as nutritive and appetising (Shukla, 2019), the genus also plays a crucial role in ecological conservation, agriculture, and various industries, demonstrating its versatility and economic significance across distinct domains. (Chou *et al.,* 2016).

Plant tissue culture methods enable the regulated aseptic raising of complete plants, organs, tissues, or cells in the lab. Basal media, controlled incubation conditions, better temperature & light conditions and the addition of plant growth regulators (natural or synthetic phytohormones) contribute to regulated plant development (Phillips and Garda, 2019). This technique offers a substitute method of vegetative propagation of several species. For instance, the potential to introduce, develop, transfer, and protect the fascinating plant science gene pool through the use of tissue culture procedures creates new chances for academics and business people (Bijalwan, 2021).

The genus *Chrysopogon* is mostly distributed in the Old-World tropics. In India, the genus is known to comprise 16 species. *C. nodulibarbis* commonly known as podhigar grass, is an endemic species found in the high-altitude grasslands surrounding Udhagamandalam, Nilgiris, Tamilnadu, India and it belongs to the Poaceae or Gramineae family which is the fourth largest flowering plant. It is employed for restoration activities to maintain ecological harmony (Jones, 2017).

2. Materials and Methods

The plant *C. nodulibarbis* was collected from Udhagamandalam and identified at the Department of Botany, PSG College of Arts & Science, Coimbatore, Tamil Nadu, India and authenticated.

Surface Sterilization of Explants

The basal shoot explant of *C.nodulibarbis* was taken as 2-3cm and washed with running tap water for 7 minutes followed by distilled water three times, each for 5 minutes. It was then

treated with teepol for 10 minutes to eliminate any dirt or surface impurities, followed by rinsing with distilled water three times, each for 5 minutes. The explants were induced into a 0.1% bavistin solution (a fungicide) for 20 minutes and then washed with distilled water three times, each for 5 minutes. The explant was washed with sterile water inside a laminar airflow cabinet (Model: BBS-V1300) to maintain sterility followed by exposure to sodium hypochlorite for 10 minutes, followed by washing with sterile water three times, each for 5 minutes. The explants were rinsed with 70% ethanol for 1-2 minutes, followed by a sterile water wash three times, each for 5 minutes. Finally, the explants were treated with 0.1% streptomycin solution (an antibiotic) for 15 minutes and rinsed with sterile water three times, each for 5 minutes to remove traces of sterilizing agents.

Growth media and conditions

MS basal medium (Murashige and Skoog, 1962) was added with different doses of PGRs (Plant growth regulators) including BAP (6-Benzylaminopurine), 2,4-D (2,4-Dichlorophenoxyacetic acid), NAA (1-Naphthaleneacetic acid), IAA (Indole-3-acetic acid) with addition of BSA (Bovine Serum Albumin), charcoal, ascorbic acid and citric acid. Sucrose (30 g/l) was incorporated into the medium as a carbon source and agar (8 g/l) was chosen as a solidifying agent. The pH of the medium was varied to 5.7-5.8 with 1 N HCl or 1 N NaOH. The autoclaving of the culture media was done in an autoclave using steam sterilization on 1.5 kg/cm^2 pressure and 121℃ for 15 min.

Inoculation and Incubation of Explants

The sterilised explants (plant parts to be cultured) were inoculated onto the prepared MS medium. Depending on the tissue type and desired outcome, the explants were placed directly onto the medium or slightly inserted under aseptic conditions, such as within a laminar flow hood. Cool white fluorescent tubes were used in the plant growth chamber to maintain (photoperiod - 16/8 hours; light intensity - 3000 lux and relative humidity (RH) - 70 to 80%) (Santos *et al.*, 2012). The cultured explants in the glass jars were labelled and randomly arranged on the shelves of the growth room under sterile conditions. After 6 weeks, the average number of shoots (%) and shoot height (cm) of explants was calculated. The shoot height was measured with a calliper to evaluate each shoot per explant across replication (Eliwa *et al.*, 2024).

Statistical analysis of data

All experiments were conducted with triplicates. Statistical calculations were done by Microsoft Excel plug-in software. Values are derived as Mean ± Standard Error of various growth hormone concentrations.

3. Results and discussion

Establishing a micropropagation procedure for *C. nodulibarbis*

Auxin and cytokinin, the very important plant hormones that regulate several aspects of growth and development are usually employed for the differentiation of explants during plant tissue cultures. Auxins typically play a significant role in plant growth, but responses can vary among species (Woodward and Bartel, 2005). The explants failed to respond on growth regulator-free MS medium (control), a similar observation was recorded in the shoot organogenesis of *Ficus religiosa* (Hesami *et al.,* 2018). The responses of *C. nodulibarbis* in various growth regulator concentrations are tabulated (Table 1-2).

BAP is a widely used and effective cytokinin (Sakakibara, 2006). In our study, MS media comprising BAP (3 and 4 mg/l) individually exhibited lower induction frequencies with average growth percentage and shoot length of 60.2% (5.5 ± 0.53 cm) and 50.1% (4.7 ± 0.52 cm) respectively. Similarly, shoot regeneration of *Lathyrus sativus* (Grass Pea) from nodal explants was achieved on MS medium supplemented with BAP, with the highest shoot percentage and shoot length (56% and 4.6 ± 0.2 cm) at 3 mg/l (Saha *et al.,* 2015). Likewise, organogenesis of *Sansevieria trifasciata* cultured on MS medium with BAP 3mg/l reported maximum yield (García-Hernández *et al.,* 2022). Additionally, the highest frequency (71% and 5.1 ± 1.2 cm) of axillary shoots of *Pennisetum glaucum* regenerated in a media containing BAP 4 mg/l (Jha *et al.,* 2009). Comparable findings were reported by Eldessoky *et al.,* 2011; de Almeida *et al.,* 2012; Rathinapriya *et al.,* 2019 and Balamurugan *et al.,* 2021. These minor deviations perchance due to genetic variability between species, differences in tissue sensitivity to BAP, variations in cultural conditions, and the presence of other interacting compounds in the media.

Combining BAP and NAA in the medium generated superior shoot induction from the explants (Hassanein *et al.,* 2015). In our study, MS media containing BAP (3 mg/l) + NAA (3 mg/l) + Citric acid (25 mg/l) and BAP (3 mg/l) + NAA (3 mg/l) resulted in maximum shoot

induction with the percentage and average number of 90.7% (12.4 ± 0.51 cm) and 80.4% (8.4 ± 1.05 cm) respectively. In concordance with our work, nodal explants of *Vitex agnus-castus*, subjected to MS medium in combination with BAP and NAA resulted in 90.3% (6.7 ± 0.4 cm) induction of shoots (Balaraju *et al.*, 2008). According to Goswami *et al.*, 2022, the mergence of BAP and NAA along with MS media was effective in the 100% (10.2 ± 1.5 cm) shoot regeneration of *Oryza sativa*. In comparison to our studies, Behera & Sahoo (2009) reported multiple shoot induction in *Saccharum officinarum* on MS medium employing BAP and NAA. Similarly, Carra *et al.,* (2016) established an efficient protocol for plant multiplication via direct organogenesis from endemic *Calendula maritima* exhibiting 60% (2.5 ± 0.6 cm) of shoots with the sequence of BAP and NAA. In a similar vein, employing BAP alone or in combination with NAA on MS media, 80% (7.4 ± 0.5 cm) of shoot buds appeared on the cut surfaces of *Limonium wrightii* explants (Huang *et.al.,* 2000). Most of the investigations indicated BAP and NAA had potent multiple-shoot induction citing synergistic effects in stimulating cell division and a balanced hormone environment associated with shoot proliferation.

In the present study, the inclusion of citric acid (25 mg/l) in the media noted resistance to browning in the appropriate media and explant. In comparison, the embodiment of citric acid with BAP alone or a combination of NAA in a culture medium of *Geranium wallichianum* explant resulted in decreased browning severity (Masood *et al.,* 2023). Similar results were inscribed by Lakshmi *et al.,* (2010) and Naz *et al.,* (2015).

2,4-D (1 to 7 mg/l) and IAA (1 to 5 mg/l) concentrations did not favour callus induction in our initial results, the lack of response from 2,4-D and IAA suggests *C. nodulibarbis* has unique physiological characteristics and hormone sensitivity. Further examinations are in progress to explore the potential of these growth hormones for favourable results.

4. Conclusion

The results of our study suggest valuable insights for enhancing the regeneration process of this endemic species. This micropropagation methodology offers a viable strategy for the conservation efforts of *C. nodulibarbis*, aiding the preservation of biodiversity in high-altitude grasslands. Future investigations endeavour to trigger indirect organogenesis to induce callus, which is presently ongoing.

Tables:

Table 1. Average shoot number and length of *C. nodulibarbis* in various growth regulator concentrations

S. No	Growth regulators	Concentration	Average No. of shoots (%)	Average shoot length (cm)
1.	BAP	3 mg/l	60.2	5.5 ± 0.53
2.	BAP + NAA	3 mg/l + 3 mg/l	80.4	8.4 ± 1.05
3.	BAP + NAA + Citric acid	3 mg/l + 3 mg/l + 25 mg/l	90.7	12.4 ± 0.51
4.	BAP	4 mg/l	50.1	4.7 ± 0.52

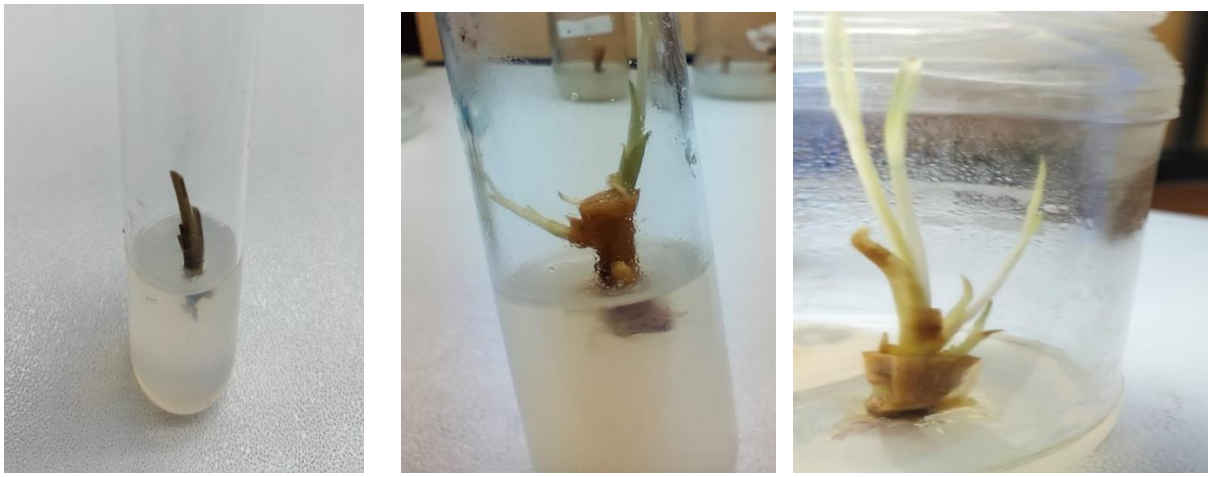
Values are expressed as Mean ± S.E.

Table 2. Negative Response of *C. nodulibarbis* to various growth regulators concentration

S.No	Growth Regulator	Concentration
1.	2,4 - D	1 to 5 mg/l
2.	2,4 - D + Ascorbic Acid	1 to 5 mg/l + 25 mg/l
3.	2,4 –D + BAP	1 to 5 mg/l + 0.5 to 2 mg/l
4.	2,4 – D + BAP + Ascorbic Acid	1 to 5 mg/l + 0.5 to 2 mg/l + 25 mg/l
5.	2,4 – D + BAP + Ascorbic Acid + BSA	1 to 5 mg/l + 0.5 to 2 mg/l + 25 mg/l + 5 mg/l
6.	2,4 –D + NAA	1 to 5 mg/l + 0.5 to 2 mg/l
7.	2,4 –D + IAA	1 to 5 mg/l + 0.5 to 2 mg/l
8.	2,4 –D + BAP	1 to 5 mg/l + 3 mg/l
9.	2,4 –D + BAP + NAA	1 to 5 mg/l + 3 mg/l + 3mg/l
10.	2,4 –D + BAP + Citric acid	1 to 5 mg/l + 3 mg/l + 25 mg/l
11.	2,4 –D + Citric acid	1 to 5 mg/l + 25 mg/l
12.	BAP	0.5, 1, 1.5, 2, 2.5, 3.5, 4.5, 5, 5.5, 6, 6.5, 7 mg/l
13.	BAP + NAA	1, 2 ,4, 5 mg/l + 1, 2, 4, 5 mg/l

Figures:

Fig. 1 (a-c): Initiation and Multiplication of Shoots in *C. nodulibarbis* - MS + BAP 3 mg/l



(a) (b) (c)

Figure 1a. Inoculation of shoot basal explants in full-strength MS Media supplemented with BAP 3 mg/l concentration individually.

Figure 1b. Initiation of shoot basal explants in full-strength MS Media supplemented with BAP 3 mg/l concentration individually.

Figure 1c. Multiplication of shoot basal explants in full-strength MS Media supplemented with BAP 3 mg/l concentration individually.

Fig. 2 (a-c): Initiation and Multiplication of Shoots in *C. nodulibarbis*

- **MS + BAP 3 mg/l + NAA 3 mg/l**

(a) (b) (c)

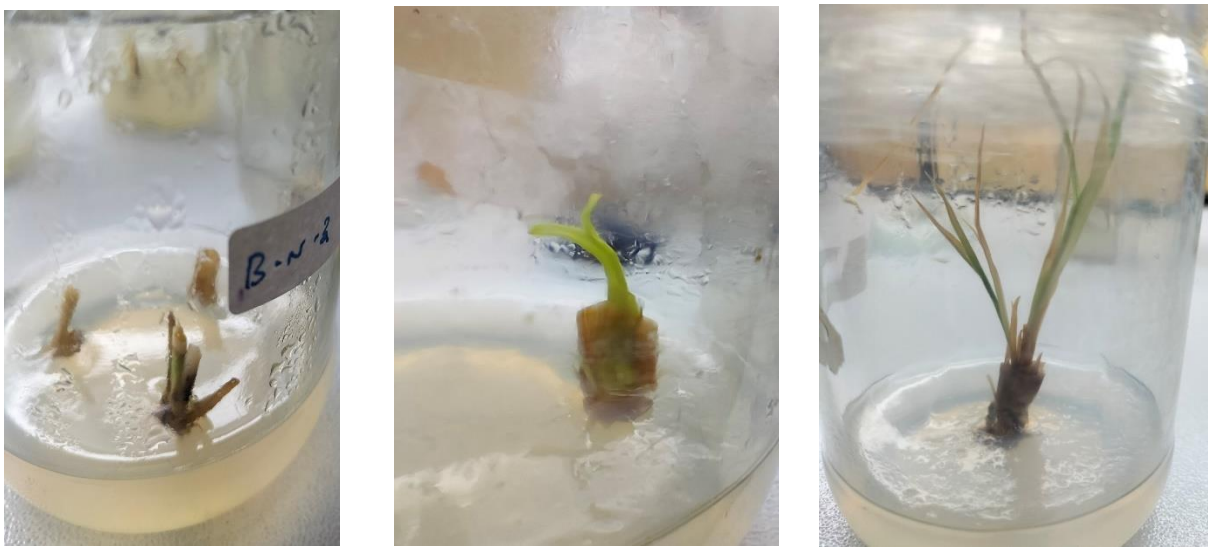
Figure 2 a. Inoculation of shoot basal explants in full-strength MS Media supplemented with a combination of BAP 3 mg/l + NAA 3 mg/l concentrations.

Figure 2 b. Initiation of shoot basal explants in full-strength MS Media supplemented with a combination of BAP 3 mg/l + NAA 3 mg/l concentrations.

Figure 2 c. Multiplication of shoot basal explants in full-strength MS Media supplemented with a combination of BAP 3 mg/l + NAA 3 mg/l concentrations.

Fig. 3 (a-c): Initiation and Multiplication of Shoots in *C. nodulibarbis*

- **MS + BAP 3 mg/l + NAA 3 mg/l + Citric acid 25 mg/l**



(a) (b) (c)

Figure 3 a. Inoculation of shoot basal explants in full-strength MS Media supplemented with a combination of BAP 3 mg/l + NAA 3 mg/l + Citric acid 25 mg/l concentrations.

Figure 3 b. Initiation of shoot basal explants in full-strength MS Media supplemented with a combination of BAP 3 mg/l + NAA 3 mg/l + Citric acid 25 mg/l concentrations.

Figure 3 c. Multiplication of shoot basal explants in full-strength MS Media supplemented with a combination of BAP 3 mg/l + NAA 3 mg/l + Citric acid 25 mg/l concentrations.

Fig. 4 (a-c): Initiation and Multiplication of Shoots in *C. nodulibarbis* - MS + BAP 4 mg/l

(a) (b) (c)

Figure 4 a. Inoculation of shoot basal explants in full-strength MS Media supplemented with BAP 4 mg/l concentration individually.

Figure 4 b. Initiation of shoot basal explants in full-strength MS Media supplemented with BAP 4 mg/l concentration individually.

Figure 4c. Multiplication of shoot basal explants in full-strength MS Media supplemented with BAP 4 mg/l concentration individually.

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Conflicts of interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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