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EFFECTIVENESS OF PGR FOR *IN VITRO* CLONAL PROPAGATION OF *LINDERNIA DUBIA* (L.) PENNELL

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ABSTRACT

The study focused on standardizing the *in vitro* organogenesis process for *Lindernia dubia* (L.) Pennell, a less-concerned emergent plant of the Linderniaceae family, utilizing a micropropagation technique in hormonal MS medium. Two concentrations of MS medium were tested: half-strength and full-strength, with the half-strength medium showing superior results in regeneration. Various concentrations of BAP, KIN, and GA3 were employed for shoot induction, while NAA and IAA were used for root induction. After a month post-inoculation, developed shootlets were evaluated, and statistical analyses, including ANOVA and Tukey's Multiple Comparative Analysis, were performed. Regeneration rates fluctuated from 70% to 100%, with half-strength medium outperforming full-strength in all parameters. The peak performance was noted with 8 µM BAP, yielding 11.9 cm shoot lengths and 16.2 roots averaging 10.5 cm in length in the NAA rooting medium. The full-strength medium averaged 46 shoots and 5.7 roots. In combinations of auxins and cytokinins, the 4+2 µM BAP + KIN combination produced 64.9 shoots, while KIN + GA3 yielded 63.9 shoots at 8+4 µM. Auxin-rich media (NAA + IAA) facilitated root regeneration across all combinations, recording a maximum of 2.4 roots with an average length of 1.2 cm in the BAP + KIN combination. The conclusion drawn emphasizes BAP and NAA as optimal hormones for regenerating *L. dubia* in half-strength medium, with BAP + KIN and KIN + GA3 being effective combinations. This established micropropagation procedure aims to enhance the population of this plant to support sustainable conservation and provide essential raw materials for future medicinal research benefiting society.

INTRODUCTION

A healthy environment is mandatory for developing rich biodiversity that is essential for mortals to live a harmonious life. The possible number of unknown species adorns the least understanding of biodiversity. Human communities depend on plant diversity, recent science, technology and anthropogenic urges have equally contributed to the current pace and intensity of biodiversity loss. The biodiversity loss has weakened of ecological food webs, agricultural decline, and economic losses (Cunsolo and Ellis, 2018). Thus, loss of diversity is considered as a trouble with no linear solutions and "as a common denominator to biological and societal challenges" (Bradshaw et al., 2021). Research indicates that successful plant conservation strategies take into account both biological and social aspects. The special ability of plants to reproduce asexually, create a lot of plantlets using propagation techniques. These techniques repopulate significant Rare, Endangered and Threatened (RET) species, endemics, therapeutic plants, ornamentals, seeds to achieve conservation objectives (Kulak et al., 2022).

The majority of seed-cultivated plants are highly heterozygous, resulting in significant variations in growth and production, which may lead to discarding inferior products. Additionally, many plants cannot be propagated vegetatively through methods like grafting and cutting, limiting the cultivation of preferred cultivars. Moreover, systemic pathogens such as bacteria, fungi, and viruses in vegetatively propagated plants can adversely affect product quality and appearance (Sharma et al., 2010).

Currently, technoscience is confined to aseptic, controlled and automated environments either naturally (fields) or constructed (laboratories) to foresee, keep record on and manipulate living organisms. The term *in vitro*, is widely used in the life sciences (Corlett, 2017) aims in differentiation and dedifferentiation of tissues and cell that morphogenesis to a genetically cloned whole plant. This technology induces explants from wild to grow by aseptic conditions (Corlett 2017; Stephens et al., 2019) creating a stress condition to stimulate vegetative, reproductive and physiological growth for various purposes. *In vitro* technology of plant propagation is a combination of micropropagation (Multiple clones of wild plants and seeds), somatic embryogenesis (plantlet from an embryo), cryopreservation, and slow-growth storage. Micropropagation and somatic embryogenesis follow two different pathways; still contribute in tremendous clone regeneration of traits like uniformity, pathogen free plants, resistant species (Kulak et al., 2022) secondary metabolites rich plants for medications, and economically valuables.

Recent years have seen an increase in *in vitro* culture techniques, which offer a useful tool for secondary metabolite production, quick regeneration, germplasm conservation of uncommon, endangered, threatened, and noteworthy plants, and mass multiplication (Baskaran & Jayabalan, 2005; Lozovaya et al., 2006; Maruyama et al., 2007). From Haberlandt's 1902 concept of plant totipotency, Schleiden and Schwann's (1838 and 1839) cell theories, and the first successful reports of tissue culture from mesophyll tissue, the long history of plant tissue culture has continuously changed to establish its influence in the fields of genetic biodiversity conservation, breeding, and biopharmaceutical production (Tang et al., 2020; Sussex, 2008). Using this characteristic, micropropagation of phytomedicinal species allows us to produce identical offspring that express the same or higher quantities of their advantageous metabolites by carefully regulating plant growth hormones and nutrients. Phytohormones are given to the culture medium to redirect somatic cell proliferation and differentiation (Skoog & Miller, 1957).

One of the key components of developing tissue culture procedure is the alteration of hormones in the growing media. Plant growth regulators (PGRs) must be present in the culture conditions in specific combinations and quantities for callogenesis and organogenesis from explants (Ehsanpour & Jones, 2000). Auxin and cytokinin are inevitable among other natural and artificial growth hormones for plants (Gasper et al., 1996). Auxin triggers cell division, leaf initiation prior to root initiation (Marhavý et al., 2016), upon proper concentration assist in initiating roots (Ludwig-Müller, 2011). Cytokinin stimulates cell division, initiate the growth and proliferation of buds, shoots and slow down root formation (Saad et al., 2012). Adventitious shoots are enhanced by BAP, zeatin or kinetin for shoot regeneration (Ghaffoor *et al.*, 2003). Wendt *et al.*, 2001 reported the pre-treatment of internode with zeatin shows shoot regeneration with elevated shoot numbers than the ones treated with BAP. TDZ an important regulator for morphogenic responses (Ricci *et al.*, 2001) and shoot organogenesis (Yucesan *et al.*, 2007) while combination of BAP and TDZ used to induce shoot (Yadav & Sticklen, 1995). Phytohormones not only promote plant growth, they are applied in oxidation prevention, pharma, cosmetics, signals for plant-animal-microbe interactions (Mukherjee et al., 2022).

The annual wetland species *Lindernia dubia*, often known as yellow-seeded false pimpernel, is found in rice fields throughout Eurasia. The family linderniaceae accommodates 17 genera and 303 species (Roskov et al., 2021, 2025). *Lindernia dubia* is American native, introduced and widespread weed in Europe and Asia, but in India the species is least distributed with low frequency (Uniyal et al., 2007; Krishnasamy and Arumugam, 2015; Kottaimuthu 2017; Prajapati et al., 2021). The genus include medicinal *(L. crustacea, L. ciliata, and L. ruellioides*), ornamental (the whole *Lindernia* genus), desiccation-tolerant (*L. brevidens*), sensitive (*L. subracemosa*), and weed-like (*L. dubia & L. procumbens*) species. As the sole published work, the taxonomical and morphological articles (Vishnyakov, 2025; Curt et al., 2024; Prajapati et al., 2021; Prasad and Sunojkuma, 2014) help to clarify the necessity of exploring this species for its therapeutic abilities.

The genus accounts for medications like emmenagogue, root with astringent used for diarrhea, decoction of root and leaf is anthelmintic, used for vertigo, cough, and jaundice from *L. antipoda* (Si, 2016), Neuritogenic in *Lindernia crustacea* (Cheng *et al.*, 2017), Anti-tumor from *L. procumbens* (Jie *et al.*, 2010), Influenza by *L. ruelloides* (Aththorick & Berutu, 2018), antioxidant *L. antipoda* (Ho *et al*, 2012), Whole plant of *Lindernia crustae* is applicable for dysentery and ringworm; *Lindernia Cordifolia* leaves for gonorrhea (Singh *et al.*, 2013), cytotoxic (Chiranjeevi *et al.*, 2017) and anthelmintic activity of from *L. madayiparense* (Umakrithika *et al.*, 2018), hepatoprotective in *L. ciliata* (Parella *et al.*, 2018). Though species from the genus *lindernia* account for pharmaceutical properties to various ailments it is still least explored. From the available literatures, it is confirmed that the plant least explored, neglected and requires conservation for its therapeutic exploration, the current study act as the bridge for the above mentioned research gaps. The present investigation focuses on establishing a standard protocol to increase the population of *L. dubia* under controlled environment to accomplish solutions to afore mentioned research gaps and it serves as the first report on this wetland species *Lindernia dubia*.

MATERIALS AND METHODS

Elite plant and explant selection: Explants from a healthy environment must be pathogen free in order to be used in microbial-free cultures. Using sterile scissors and a container, newly grown, mature *Lindernia dubia* plant pieces were chosen and gathered from the rice fields in sithalavai, Krishnarayapuram, Karur district, Tamil Nadu, India. The plants were removed for aseptic culturing and digitally captured in their natural environment. Nodal areas were excised from the elite plant and used as explants for additional cultivation.

Sterilization, inoculation, and incubation: The explants were properly cleaned with running tap water for 30 minutes to remove any dirt or microorganisms. A drop of liquid soap was then added and sterilized for an additional 10 minutes before being thoroughly cleaned with double-distilled water. The nodal explants were inoculated in the Laminar airflow chamber. To prepare the explants for regeneration (the production of disease-free plantlets), they were disinfected using ethanol and mercuric chloride. Micronutrients, macronutrients, vitamins, carbon sources, and solidifiers were added to the MS basal medium, along with PGRs cytokinin (BAP, KIN & GA3) for shoot regeneration and auxin (NAA & IAA) for root regeneration. The

hormones were treated individually (2 µM to 10 µM) and also in combinations with concentrations ranging from 2+1 µM to 10+5 µM vice versa. Similarly, the strength of MS salts was changed to half-strength (half the amount of MS salt is mixed in 1L of distilled water) and full-strength (1L stalk). After adjusting the pH to 5.8, the autoclave was run at standard pressure and protocol. The autoclaved medium was used to inoculate the nodal explants, and they were then incubated under ideal conditions for regeneration. The well-established cultures were hardened using garden soil: farmyard Manure: soil in the ratio of 2:1:1 and the survivality percentage were calculated for field transfer.

Experimental Analysis

After seven days of inoculation, the initiation percentage of the incubated nodal explants was measured. The proportion of initiation was determined using

$$\textbf{Initiation percentage} = \frac{\text{Total number of nodes initiated}}{\text{Total number of inoculated nodal explants}} \times 100$$

Daily morphological observations were made, and after 15 days, the regenerated nodes were sub-cultured. At the 50th day after inoculation, data on characteristics such as shoot and root length and number were recorded. In order to conduct a thorough data analysis, the experiment was conducted five times. Using Graphpad Prism, the collected data were organized in randomized blocks and subjected to a two-way ANOVA with Tukey's multiple comparison tests to achieve significance at $p \leq 0.05$ and $p \leq 0.0001$.

RESULT AND DISCUSSION

Efficacy of Individual Hormones

To standardize a micropropagation procedure for *L. dubia* the nodal explants were excised and inoculated in different strengths of MS media supplemented with BAP, KIN at 2 to 10 µM for shoot regeneration and the same concentration for root concentration for NAA and IAA plant hormones. Morphological parameters like shoot, root number and length were tabulated (Table No 1; Fig 1) Half-strength BAP, KIN recorded better significance than full strength media. A maximum of 68 shoots in BAP and 57.1 in KIN were recorded in 8 µM HS media while at the same concentration only 46 and 32 shoots were regenerated respectively. Highest shoot length of 10.4 cm is observed in 10 micromolar BAP HS media followed by 9.6 in KIN, 4.2 cm in 2 micromolar concentration marked the higher length in full strength media of KIN. Regarding the root number and length NAA served better shoots than IAA. A maximum of 16.2 roots and of length 7.6 cm was recorded in 8 and 4 µM of HS and FS respectively in NAA. 8 µM NAA showed better root length than other hormonal concentrations.

Table No 1: Hormonal efficiency on nodal explant regeneration of *Lindernia dubia* (L.) Pennell

Strength	Concentration (µM)	Shoot Number		Shoot length (cm)		Root number		Root length (cm)	
		BAP	KIN	BAP	KIN	NAA	IAA	NAA	IAA
Half - Strength	2	62.3	53.2	9.1	8.2	13.4	9.3	8.9	6.5
	4	58****	60****	7.3****	7.6****	11**	7.8	9.6	5.9**
	6	60****	56.2****	8.6	8.4****	12.4**	6.7	9.3	5.3**
	8	68****	57.1****	11.9****	9.2	16.2	8.5	10.5	6.9**
	10	54	57.5	10.4	9.6	9.6	7.3**	7.5	5.1**
Full - Strength	2	29	26	3.2	2.8	7.4	6.9	3.2	2.9
	4	38**	29**	4.1*	4.2*	7.6	7.1	2.9*	3.1*
	6	32**	34**	2.8*	2.6*	5.2*	4.*3	3.3	2.2*
	8	46**	32**	2.7*	1.9*	5.9	5.7	3.4*	2.1
	10	42**	38**	1.8*	1.7*	6.5	4.9	2.1*	1.9

Mean of five replicates; **- significance at p<0.0001; ** - p<0.001; * - p<0.05**

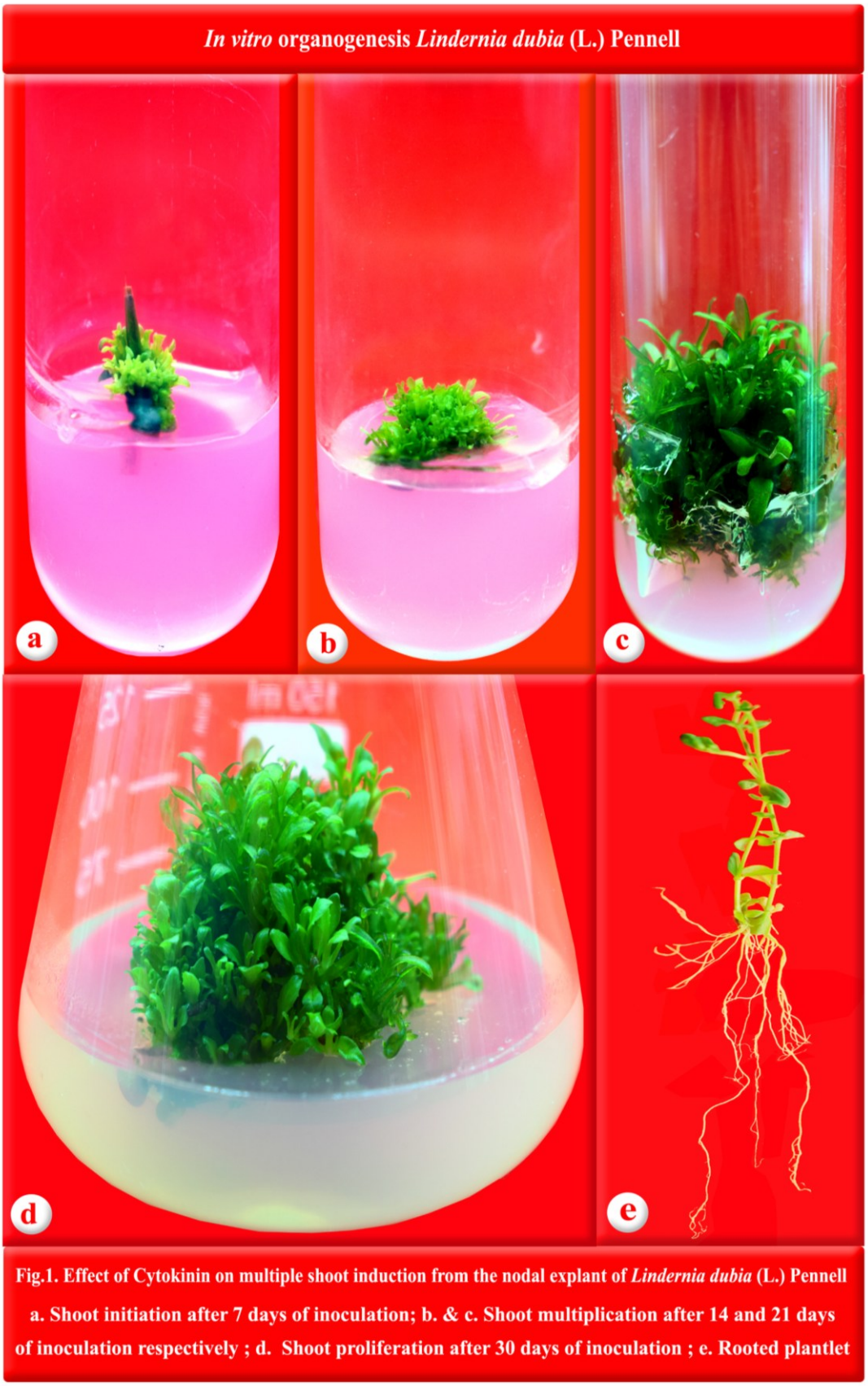

Fig.1. Effect of Cytokinin on multiple shoot induction from the nodal explant of *Lindernia dubia* (L.) Pennell

a. Shoot initiation after 7 days of inoculation; b. & c. Shoot multiplication after 14 and 21 days of inoculation respectively ; d. Shoot proliferation after 30 days of inoculation ; e. Rooted plantlet

Efficacy of Combinational Hormones

From individual hormonal regeneration it was confirmed half-strength MS media is the best regeneration media for *L. dubia* from nodal explants. So for the combination efficacy study of hormones only half-strength MS media is utilized to study the above mentioned morphological parameters. The results showed

BAP+KIN and KIN+GA3 as the best combinational hormones for its regeneration (Fig 2). Significant results are marked in the graphical representation of the result. The highest regeneration of 65 shoots and 63.9 shoots were observed in 2+1 and 8+4 micromolar concentrations of BAP+KIN and KIN+GA3 respectively. The shoot lengths were maximum (6.4 cm) at 8+4 µM of KIN+GA3 followed by BAP+KIN (6.2 cm), KIN +BAP (5.2 cm in 10+ µM) and finally 4.5 cm in 4+2 µM of BAP+GA3. For root regeneration only NAA+IAA in the same concentrations were used. The results were observed only in BAP+KIN combination and for other hormonal combinations the root lengths and numbers were less than 1 in mean average and for some repeats root formations were nil. The maximum numbers of roots were 2.4 and 1.2 cm length in 8+4 µM and 2+1 µM concentration respectively. Though, the shoot regeneration was successful root has been failed for some reasons. The rooted plantlet is then hardened in the hardening mixture and the survivality percentage was found to be 79% Which is low compared to Shaik *et al.*, (2010) which is 85% in *Lessertia frutescents*.

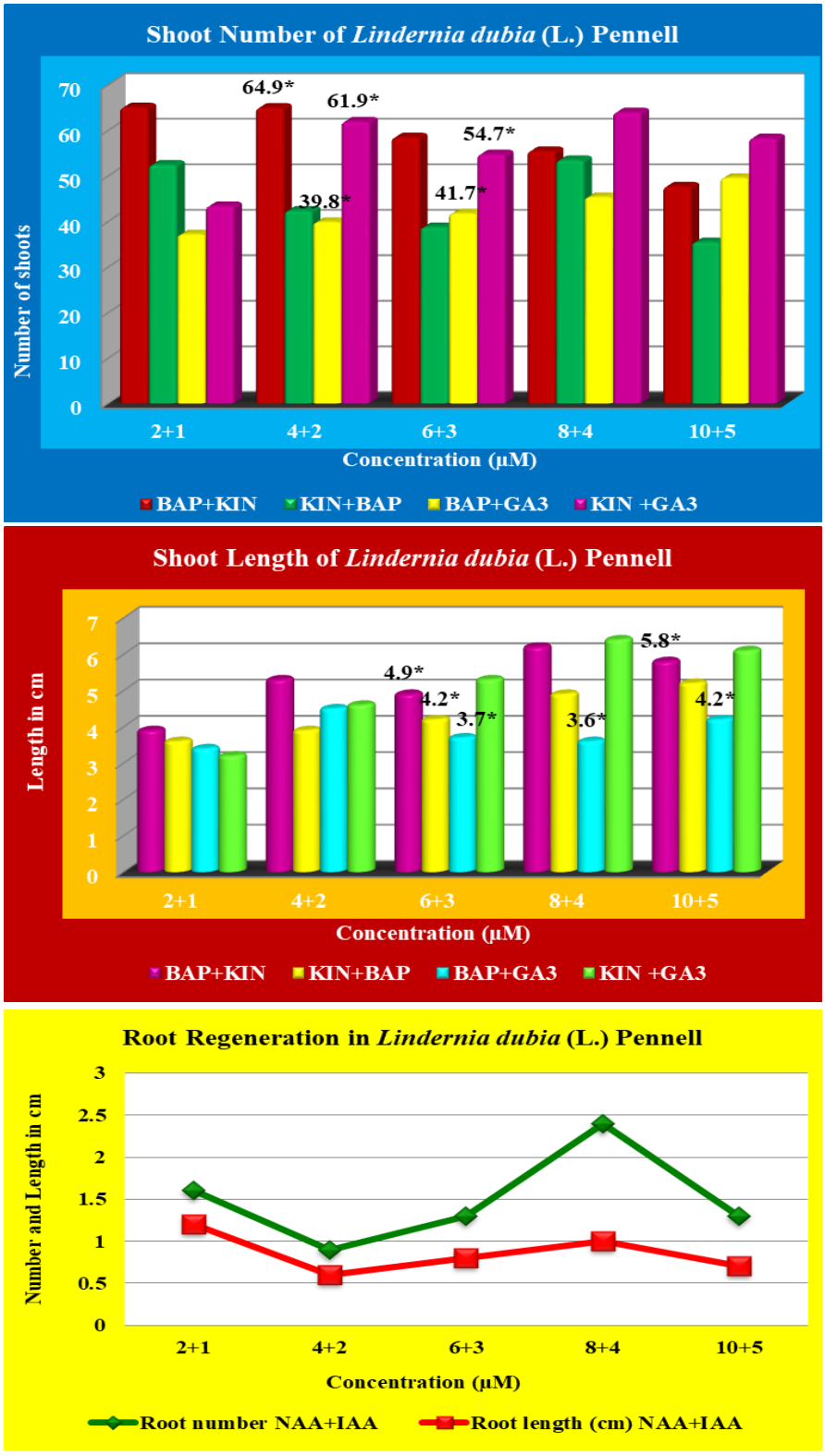

Fig 2: Hormonal efficiency on regeneration of *Lindernia dubia* (L.) Pennell in combinations of Hormones in half- strength MS medium

Our study reported shoots with maximum of 46 shoots while *Lessertia frutescents* had 12.9 shoots in FS media with half of the plantlets having hyperhydricity (Shaik *et al.*, 2010). Baskaran & Jayabalan, (2005) have obtained 94.3% rooting in IAA full strength medium. In *Turnera ulmifolia* (Manohari *et al.*, 2018) full-strength MS solid medium has given 8.3 ± 0.57 numbers of shoots in 8.88 mM of 6- BAP and 0.54 mM of NAA while it peeked with 59.5 ± 2.10 shoots found in BAP+ KIN+NAA semi solid media. Jabir et al., 2016 reported 55.6% of axillary bud growth in full MS medium, while it was 95.6% in half- strength media.

Hany A. El Shemy (2011) developed 14.3±0.9 shoots per explant at 3 mg/L BAP with longest shoots (3.5±0.4 cm). After third subculture, shoot multiplication significantly increased, averaging 86.5±3.6 shoots per explant in 3 mg/L BAP which is quite high compared to our regeneration result. de Jesús Romo-Paz et al. (2021) results demonstrated effective rooting 30.51 ± 0.94 with efficient proliferation with 6.57 ± 0.46 offshoots in auxin rich media of *Physalis angulata*. A Maximum of 16.60±0.25 shoots with length 6.5±0.23cm, having root of 5.56±0.65 and length 10.5±0.6 cm was obtained in MS media supplemented with 1mg/l BAP+0.2mg/l NAA (Jabir et al., 2016). The current result outperformed the above result. On the whole, BAP was reported to be the effective medium in the culture establishment (Kane and Gilman, 1991; Haw and Keng, 2003; Shrivastva and Rajani 1999). In combination BAP+KIN, KIN + GA3 were best for regeneration of *Lindernia dubia* while Karatas et al., 2013 reported combination with KIN and IBA as best in *Hygrophila polysperma*. From the study, it is found that half-strength media is highly efficient for regenerating *L .dubia* from nodal explants.

CONCLUSION

In conclusion, a successful development of a suitable micropropagation protocol for commercial multiplication of the wetland species *Lindernia dubia* has been developed to bridge the research gaps. The best medium for shoot and root multiplication was half-strength MS basal media augmented with 8 µM BAP and NAA. For combination of hormones, BAP+KIN, KIN+GA3 4+2 and 8+4 µM concentrations were best suited. In future, the plant species *L. dubia* can be made available throughout the year with no pathogens of similar trait like wild plant by this study. The medicinal potential of *Lindernia dubia* can be explored by pharmacological research, secondary metabolite formation, analysis, and quantification, all of which are made possible by this investigation.

CONFLICT OF INTEREST

The authors declare that **"They have no conflict of interest"**

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