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Optimization of direct shoot induction using cytokinin and auxin young leaf explants of *Enicostemma littorale* Blume.

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ABSTRACT

We report here a robust *in vitro* Reincarnation that is repeatable protocol for the medicinally important plant *Enicostemma littorale* Blume. Young leaf explants were employed to further develop direct shoot induction, focusing mainly on optimal plant growth regulator combinations and preferred concentrations. All experiments were performed in MS baseline substrate. Numerous meditations on the cytokinins 6-Benzylaminopurine (BAP) and kinetin (KN) were used singly or in concert to cause precocious shoot emergence. The highest number of shoot regeneration was observed over MS medium fortified with 2.22 µM BAP+2.32 µM KN with the highest frequency of shoot induction (92) and maximum number of shoots/explant (20). In contrast, calli production unusual was observed and formed with elevated levels of (BAP (8.88 µM) and KN (4.64 µM), which may be due to a threshold effect which restricted a direct shoot organogenesis. NAA: Induction of roots was tested at different concentrations of auxin naphthaleneacetic acid (NAA). Approximately fifteen roots per shoot were obtained on half-strength MS media containing 1.22 µM NAA (100 % rooting percentage). Downstream research involving the development of the propagation, conservation, and gene improvement of *E. littorale* at a large scale is expected to be expedited by the rapid and efficient regeneration process of the established protocol. These results are the basis for potential biotechnological applications of this medicinally pure species in the future and emphasize the importance of hormone profile in the context of positive direct organogenesis.

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INTRODUCTION

Several conventional medical systems such as Ayurveda, Siddha, and Unani, applied the amazing medicinal herb *Enicostemma littorale* Blume (Gentianaceae) since centuries (Rajamani *et al.*, 2013; Roy *et al.*, 2010). *E. littorale* apyrase is an apt molecule in this respect, in view of its toxicant inhibitory and enzyme inhibitory potential and possible role in precluding intervention (Bindhu, 2010; Katti *et al.*, 2010; Romi, 1994; Samanta *et al.*, 2000).Originated from tropical and subtropical zones of India and some other South Asian countries, *E. littorale* has a long tradition of use in medicine and folklore pharmacopoeia and is well-known to contain various pharmacological properties, including hepatoprotective, anti-inflammatory, antioxidant, antidiabetic and anti-ulcerogenic activities (Rajamani *et al.*, 2013; Roy *et al.*, 2010). These features have caused the plant to be included in traditional remedies for diseases and long-term medical conditions. Because traditional propagation method was not effective, so far the large-scale, sustainable culture and production of *E. littorale* has not been established though its application has become prevalent and its potential in the research community for treatment is great.

Stem cuttings, grafting and layering are the common methods for traditional propagation of *E. littorale* as described by (José and R, 2015). Such approaches are either limited by poor efficiency, seasonal restrictions and requirement for mature plant materials as a donor, or are simple and cost-effective by comparison. Besides, slow growth, susceptibility to diseases, and differences in the quality of the plant make it challenging to mass produce plants through conventional propagation methods (Swarts *et al.*, 2018). These constraints make it difficult to preserve genetically stable and medicinally potent lines of *E. littorale*, and to produce material on a large commercial scale.

Worldwide interest in plant-based pharmaceuticals, in particular those supported by scientific evidence, e.g., Ammi visnaga extract, has grown, which have made it necessary to develop fast and efficient growth protocols. Sustainable practices for cultivation of medicinal plants on a large scale are currently being discussed as the interest in herbal medicine and the phytomedical area is increasing. In this respect, tissue culture or *in vitro* plant propagation is one such method that is particularly appealing. With a basal medium containing plant growth regulators, this method ensures that the plant tissues grow and multiply in sterile environment (Lone *et al.*, 2020). Advantages of tissue culture are keeping genetic purity and freedom from diseases while in meristem culture as well as the quick production of a large number of plantlet.

One of the most important benefits of *in vitro* culture is that clonal plants ("clones") of the parent plants are produced. This is an especially favourable situation when dealing with medicinal plants such as *E. littorale* where phytochemical profile stability is a major contributor to therapeutic outcome. Tissue culture is one technique that may be used to produce uniform plant material, which is a major requirement for the standardisation of herbal products. Tissue culture is also a means of preserving their genetic resources and making them restorable to their native habitats.

For several species, including those with potential in medical use, the ability to culture whole plants from leaves- an explant source which is often used in tissue culture has proven to be of interest. Leaf explants are ease-to-handle explants and also they contain meristematic regions that can develop into roots or shoots under favourable condition. Type and concentration of plant growth regulators (PGRs) used, as well a s culture media play a key role with regard to the ability to induce shoots from leaf explants (Pathak and Joshi, 2015).

Among the regulators of plant development (PGRs), both auxins and cytokinins are the most frequently employed in tissue culture. Similarly, Different kinds of auxins include indole-3-acetic acid (IAA), naphthaleneacetic acid (NAA), and indole-3-butyric

acid (IBA) are believed to be involved in radical induction along with cell elongation, whereas cytokinins (like 6-benzylaminopurine or BAP and kinetin or KN) have been found to be significant inducers of cell division as well as shoot morphogenesis (Pathak and Joshi, 2015). The developmental fate of the explant is also much determined by the relative levels of Auxins and cytokinins in the raising medium; high cytokinin-to-auxin ratios usually lead to the inductive signal to form shoots, whereas high auxin with cytokinin ratios favor root development.

In tissue culture, direct organogenesis is a preferred route as it bypasses the callus phase and allows shoots to develop directly from explant tissue. This overcomes the problem of somaclonal variation and genetic instability in the regenerants. Direct regeneration is beneficial for mass multiplication of medicinal plants in terms of quality of bioactive compounds. In addition, direct organogenesis is generally faster and more efficient than indirect regeneration, which is associated with shorter culture duration and more rapid plantlet production.

Several investigations have successfully demonstrated the regeneration of different plants and explants via direct organogenesis. For example, Explants of shoot tips and leaves grown on medium enhanced with a high concentration of cytokinin could induce shoot directly in *G. jamesonii* (Cardoso and Silva, 2013). Other BAP and KN combinations have been used in distinct therapeutic plants to increase the quantity of subcultures and shoots per explant.

Additionally, the rooting phase, usually induced by low concentrations of exogenous auxins such as NAA, becomes important to produce healthy plantlets for acclimatisation and field transfer.

In vitro propagation of *E. littorale* has been poorly studied. Indirect regeneration via callus culture has been described in some reports, that is not so efficient because of its genetic instability resulting in chimeral plants. To promote the PGR needs for optimum shoot induction and rooting efficiency a direct organogenesis system from leaf explants must be developed promptly.

Earlier studies by Rajamani *et al.* (2013) and Roy *et al.* (2010) characterized the phytochemistry and medicinal properties of *E. littorale* in support of its medicinal value. For this reason, we maximise the amount of endogenous BAP, KN, and NAA in the *E. littorale* leaf explants in the current research so that shoots and roots may develop directly.

Hence through systematic study, we will be able to determine the best cytokinin to auxin ratio to give high roots and survival frequency, low callus number and good regeneration rate of the regenerated plantlets.

Several requirements need to be taken into account when setting up *in vitro* protocols: the basal media used, the physical state of the medium (either solid or liquid), the pH, light regime, temperature and relative humidity. The MS medium is the most commonly base media for plant culture systems for being rich in macronutrients, and for being sufficient for a variety of plant types to flourish.In the present study, MS medium is considered the basic medium for evaluation of several PGR combinations.

The age of the explant as well as the type of the explant has a major influence on the regeneration result. Nevertheless, meristematic cells are highly responsive to PGRs and are abundant in young tissue, thus a developing tissue at an early age should normally exhibit a faster regeneration rate than that in an older developing tissue as a young portion of young tissue. Therefore, young *E. littorale* leaves were chosen as explants for induction. Due to the ability for exogenous BAP or KN take up, an efficient shoot regeneration could be induced and with additional NAA treatment, vigorous roots should be ensured.

Direct organogenesis micropropagation is an ideal approach not only for the genetic transformation of *E. littorale* through biotechnological means but also for

its commercial production on a large scale. Further research in genetic transformation, induction of secondary metabolites and cryopreservation could be developed based on a stable tissue culture system. Furthermore, the methods developed could be useful in protection of this species in its natural habitat,which is threatened by overharvesting and habitat destruction. Induction of shoots directly from leaf explants for propagation and conservation of *Enicostemma littorale*, and development of an ex situ conservation protocol A significant gap in the distribution and preservation of medicinally important plant species is bridged by a simple, efficient, and reproducible protocol for large-scale direct shoot induction from leaf explants of *Enicostemma littorale* (Blume) Raynal using cytokinins and auxins. Shoot regeneration, root induction, and plantlet survival in the species may be improved by manipulation of hormonal balance and cultural conditions ensuring its conservation potential for times long storage as well as for the commercial culture. The knowledge gained from this study will be a prototype method for devising similar protocols of other medicinally potent and underutilised plant species as well as a field viable procedure for bulk cultivation of *E. littorale*.

In order to create an effective and direct shoot induction system from leaf explants of *Enicostemma littorale* Blume, several cytokinin and auxin concentrations and configurations were evaluated in the current study. Assess the morphogenic capacity of juvenile leaf explants and determine the ideal ratio of auxin and cytokinin for increased the regrowth harmonic and number of shoots per explants on various concentrations of various hormonal combination and develop a reliable protocol for *in vitro* direct organogenesis for mass multiplication and biodiversity conservation of medicinally important plant.

MATERIALS AND METHODS

Explant origin

Plant material collection: *Enicostemma littorale blume's* aerial section was gathered from a field in Tamil Nadu, India's Virudhunagar District. In this investigation, the leaf segments served as the explants.

Growth regulators and culture media variety

In the study plant were provided nutrition in the form of MS media basal (Murashige and Skoog, 1962), solidifying mediator combined with inhibitors of plant growth at various concentration for callusing, shoot initiation (BAP 2.22,4.44,8.88µM), KIN (1.16, 2.32, 4.64 µM) and rooting IAA (1.42, 2.85, 5.71, 8.56, 11.41,14.23 µM), NAA (1.22,2.60,4.37,8.05,10.74,13.4µMI), IBA (1.232.46,4.92,4.92,7.38,9.84,12.33µM), and NAA (1.22,2.60,4.37,8.05,10.74,13.4 µMI). They are thus soluble in water and subsequently in polar solvents to their final volume. It is also possible to find NAA and cytokinin as powders or crystals. After using 0.1N NaOH or 0.1 N HCl to bring the medium's pH down to 5.8, it was autoclaved for 20 minutes at (121°C and 1.06 kg cm^{-2}). Kinetin, which is also utilized to promoteshoots multiplication, increased shoot breaking as well as the regeneration quality of the shoots. Shoot regeneration was less frequent with kinetin when used alone compared to BAP, however its addition to BAP enhanced shoot elongation and vigour of the plants. Two PGRs kinetin and BAP synergistically enhance the quality of shoots and regeneration efficiency. The shoots, however, were shorter than 92% of juvenile leaf explants that directly regenerated shoots when only cytokinins were applied under the same conditions as Medium components and BAP as mentioned above. The cytokinins one of the most powerful hormones used in *in vitro* culture and propagation are definitely the cytokinins (endogenous plant stimuli that induce cell division and the meristematic activity of the shoot and development of adventitious shoots).

Sterilization and culture condition

Transplant sterilization

One of the crucial strategies in prevention of this contamination which could significantly hinder *in vitro* proliferation is to sterilize explants accordingly. *In vitro* culture is considered as a promising approach for *ex situ* conservation and fast

propagation of valuable plant species. Young leaves *Enicostemma littorale* plants were collected from a natural habitat. The explants were leaves. A rapid and effective protocol was applied to surface sterilize leaves and to remove any epiphytes without harming the plant material.

First, the leaves were soaked in tap water for 20-30 min under running water to remove dirt, dust, and other impurities. The leaves were washed in tap water and subsequently dipped in 70% ethanol for one to three minutes. This is so as to remove a variety of surface contaminants. Most endophyte isolation procedures also incorporate this step (Eevers *et al.*, 2015).

The leaves were then autoclaved with sodium hypochlorite. There are 2 important parameters, namely the quantity of NaOCl and the rate of the treatment. There is usually good response to 1-2% NaOCl for a period of 5–15 min in *Enicostemma littorale*, although standardization of the method may be appropriate. To remove the pollutants from the tissue without severe damage, particular amounts of time and concentration are required. In the context of NaOCl procedure, the mixture should be shaken during heating to achieve uniform sterilisation. Various plant species have been sterilised with success using sodium hypochlorite in the past (Eevers *et al.*, 2015).

Following the NaOCl treatment, the leaves were washed twice with sterile distilled water. Three to five replications of this process are required to clean any remaining NaOCl, which can be phytotoxic. A last wash in sterile distilled water solution diluted with 0.1% of fungicide captan or mancozeb for one or two minutes can also be included to reduce risk of fungal infection.

The cleaned leaves were cut into suitable explants (1.0–1.5 cm2) and sterilized in laminar air flow using sterile razor and surgical blade before culture. Thereafter, these explants were available for inoculation on the growth medium. While explant regrowth is not compromised, successful surface sterilisation is paramount (Talla *et al.*, 2018).

Culture media

An additional factor affecting in vitro proliferation is the selection of culture medium. The tissue culture techniques make the fast asexual propagation of plants possible, as your editor document illustrates. The combination of medium ingredients in MS medium is crucial for a successful plant tissue culture despite they consist of macro and microelements, vitamins and aminoacids (Achar and Vinjamuri, 2021; Qu *et al.*, 2002).

Sucrose is added to MS medium as a carbon source for *Enicostemma littorale* at varying levels (2-3%). An important limiting factor with respect to the energy required for cell expansion and differentiation is the optimum sucrose.

Agar is a rapid-setting medium. Concentrations of agar usually vary from 0.7 to 1.0%. It is important to determine the optimum agar level which will allow passage of water and nutrients and at the same time acts as a suitable support matrix for explants.

Regulators of growthlets are to be added to the culture media to stimulate shoot organogenesis. Cytokinins (like kinetin and 6-benzylaminopurine) trigger cell division and the growing of shoots. They can lead to chromosome elongation and dilatation of spindle fibers. Plus, as your board points out, adding phytohormones, such as the auxins and cytokinins that stimulate the induction of shoots from leaf explants, are commonly used to induce *in vitro* propagation.

Thus, auxin and cytokinin is typically used for shoot induction of EL. The genotypes and explant types are the key parameters for determining the best optimal concentrations of BAP and NAA. GAC A concentration of 0.5–2.0 g L–1 BAP in presence of 0.1–0.5 g L–1 NAA is often favorable. Based on the editor's document, fine-tuning of the auxin and cytokinin content of the growth media has leaded to

the optimisation of *in vitro* proliferation protocols, by controlling shoot development in planta from the explant.

Myo-inositol and thiamine are included in growth media to support plant growth and development. The two essential co-factors; thiamine (a cofactor for several enzymes responsible for the metabolism of carbohydrates) and myo-inositol (precursor to the formation of the cell wall).

Conditions of culture

As plants to grow and flourish *in vitro,* the culture environment's temperature, light, and humidity levels are crucial. A growth chamber with controlled temperature is used to cultivate the cultures. *Enicostemma littorale* bioassay 25 ± 2°C was the steady temperature that was maintained.

A regulated daylight hours of 16 hours of light and 8 hours of darkness governs the development of the shoots and roots. Growth lights (flourescent or LED) should be utilized to maintain the light levels between 30-50 µmol m-2 s-1.

In order to avoid rapid desiccation of the explants, humidity in the growth room should be maintained at 60-70%. Enough ventilation is also needed to avoid the accumulation of ethylene that can inhibit the outgrowth of shoots.

pH adjustment and sterilisation of media

The pH of the medium influences the enzyme activity and nutrient access. The pH of the MS medium must be adjusted to 5.7-5.8 before autoclaving. The simplest way of doing this is by adding a couple of drops of 1 N HCl or 1 N NaOH. The media is autoclaved at 121 °C, 15 lbs. for 20 minutes. When autoclaved, all living organisms and spores, including bacteria and fungal spores, are destroyed.

For substances to be added as vitamins or plant growth regulators, the culture medium is preferably cooled to 45°C after autoclaving. These needs to be filter sterilised using a 0.22 µm syringe filter and presented to autoclaved medium in their sterile state.

Culture: Creation and maintenance

The surface-sterilized explants are delicately positioned on the solidified medium with sterile Petri plates or culture inserts. To promote uptake, they must be placed so that the adaxial (upper) surface is next to the medium.

In the growth chamber, the cultures are incubated under the controlled environmental conditions previously described. Cultures should also be checked for contamination, or abnormality, at regular intervals.

To ensure an unimpeded supply of nutrients, and to minimize the formation of toxic metabolites, explants need to be regularly subcultured, or refreshed on fresh medium. The subculture regularly develops every three or four weeks. When aseptic cultures are established, new buds are generated (Debergh and Maene, 1981).

If there is any discoloured or necrotic tissue encountered when you are on the subculture transfer it instead to a sterile media. Aseptic transfer of transplant to new medium is also required.

Information gathering

The cultures were monitored at intervals during the course of the experiment. The period elapsed since culture initiation to shoot induction was reported as “days/shoot”. Percentage response (shoot development) was expressed as the number of responding explants divided by the total number of explants on that treatment. For shoot proliferation, the callusing, shooting and shoots per explant frequency were recorded.

The discriminatory value of statistical and experimental prediction of ASD. A statistical analysis was performed to ascertain the potential correctness and reliability of the findings. The triplicate assays are represented by the mean ± SD of

the data. Differences between groups was tested by one component analysis of variance (ANOVA). The Tukey's Duncan's Multiple range test (DMRT) was employed to perform post hoc comparisons for distinctive groups. Duncan reported that the R statistical software (1955) was used for statistical analysis. There was significance where the P value was less than 0.05. This approach enabled an extensive reconsideration of the strength and the variability of results.

RESULTS

Optimization of direct shoot induction with BAP and kinetin

To optimise a method for direct shoot induction from leaf explants of *Enicostemma littorale* Blume, the present study attempted to enhance the efficient combination use of kinetin and 6-benzylaminopurine (BAP) by maximising the application concentration. Explants were cultured in KI and BAP supplemented MS media. Days to the onset of shoots, intensity and type of callus formation, frequency of shoot induction (induced shoots/15 explants) and mean shoots explant −1 at the end of 30 days were recorded.

The results (Table 1) indicate that the composition and concentration of the two cytokinins used have a significant effect on shoot morphogenesis. For maximal induction percentage (92%) and mean number of shoots (20.00 ± 1.42 shoots per explant), the combination of 2.32 µM KI and 2.22 µM BAP (Fig. 1B) was found to be the best combination among other hormone combinations. At the cut surfaces, this treatment also caused a mild callusing response; the callus was light yellow and friable. The successful direct organogenesis was confirmed in view of the vigorous nature of the formed tubers and that they were formed directly from the explant with no callus-based development.

The best results for shoot induction (77%) were achieved when 2.32µM of KI and 4.44µ of BAP were used with 14.56±4.24 shoots per explant. This treatment also induced a delayed (>25 days) direct shoot formation at low level of callusing.

Interestingly, increasing KI to a low, constant level (1.16 µM) in combination with increasing BAP levels enhanced the number of shoots per explant. Shoot induction for example was 40% at 4.48 ± 1.73 shoots/explant while 1.16 µM KI + 2.22 µM BAP (Fig. 1A) was supplemented whereas it increased to 5% and average shoots per explant were 5.70 ± 1.01 when 4.44 µM BAP was applied. The induction frequency increased to 62% (average 6.46 ± 2.29 shoots per explant) with further increase in BAP level to 8.88 µM. The results indicate that BAP remarkably enhances the differentiation and proliferation of shoot buds when used with a lower concentration of KI.

However, the morphogenic efficiency was significantly decreased at higher concentration of both KI and BAP (e.g., KI 4.64 µM + BAP 8.88 µM). Besides retarding shoot regeneration up to 32 days, rather high level of callusing (+++), often overgrowing shoots, was induced. On an average, 0.56 ± 0.52 shoots per explant were formed and the percentage of shoot induction was reduced to 20%. The light yellow friable callus that formed under these conditions may in this case be resistant to a direct organogenic pathway.

Similarly, other high-hormone combinations like KI 4.64 µM + BAP 4.44 µM could induce an average of 2.44 ± 0.72 shoots per explant with slow initiation and excess callusing, which responded poorly (30% shooting). These results substantiate previous studies (de Greenham et al, Plant Physiology 35, 196-203 (1960)) that cell polarity or hormonal ratio disturbances may lead to toxic effects or other forms of tissue inhibition under *in vitro* culture supra-optimal cytokinin conditions.

Collectively, the results emphasize the importance of hormonal balance for direct shoot regeneration. Middle concentrations of KI and BAP (especially KI 2.32 µM + BAP 2.22 µM) showed the most positive organogenesis, thereafter, the efficiency was reduced due to both overproduction of callus and inhibition of morphogenesis.

Table 1. Effect of KI and BAP on direct shoot induction in *Enicostemma littorale* Blume

Conc. of growth regulators (µM)		Days required for shoot induction	Degrees of callusing	Nature of the callus	Shoot induction (%)	Average number of shoots per explants±SD
KI	BAP					
1.16	2.22	15	+	Light yellow callus	40	4.48±1.73^{a}
	4.44	17	+	Light yellow callus	55	5.70±1.01^{cde}
	8.88	20	+	Light yellow callus	62	6.46±2.29^{c}
2.32	2.22	22	+	Light yellow callus	92	20±1.42^{a}
	4.44	25	+	Light yellow callus	77	14.56±4.24^{a}
	8.88	27	++	Light yellow callus	50	8.78±1.64^{de}
4.64	2.22	29	++	Light yellow callus	67	6.44±0.52^{c}
	4.44	30	++	Light yellow callus	30	2.44±0.72^{f}
	8.88	32	+++	Light yellow callus	20	0.56±0.52^{bc}

Each value was recorded after 30 days of incubation. Data are given as mean (SD) of three independent experiments each with 9 replicates.

Auxins in root studies on root induction

Their effects on rooting and quality of rooting were studied, by placing elongated microshoots in a rooting medium containing different concentrations of three auxins, IBA, IAA and NAA (Table 2). Rooting physiology was assessed in terms of average number of roots per shoot after 30 days and the root induction %.

Table 2. Effect of NAA, IBA, and IAA rooting response on in *Enicostemma littorale* Blume

Auxin	Concentration (µM)	Average root (%)	Average number of root
NAA	1.22	100	12.44±0.88^{a}
	2.60	95	10.22±0.97^{b}
	4.37	85	8.78±1.48^{c}
	8.05	70	7.43±1.13^{d}
	10.74	60	6.43±1.32^{d}
	13.4	55	4.78±1.92^{e}
	1.23	45	3.89±0.97^{ef}
	2.46	30	3.78±0.97^{ef}
	4.92	28	3.56±1.50^{ef}
	7.38	26	2.78±1.85^{fghi}
	9.84	24	2.56±0.88^{fghi}
IBA	12.33	23	2.44±1.42^{fghi}
IAA	1.42	29	3.54±0.83^{efg}
	2.85	22	3.22±0.67^{efgh}
	5.71	18	2.32±1.09^{fghi}
	8.56	20	1.78±0. 67^{ghij}
	11.41	16	1.67±0.70^{hij}
	14.2	12	1.11±0.60^{ij}

Each value was recorded after 30 days of incubation. Data are given as mean (SD) of three independent experiments each with 9 replicates.

For bald cypress, with regards to rooting, NAA was more effective than the other two auxins. The rooting percentage (100%) and the number of roots per shoot (12.44 ± 0.88), (Fig. 1D) were the highest at 1.22 µM NAA. With the increase in concentration, the rooting rate decreased slowly. For instance, lower or higher concentration of 2.60 µM NAA was found to significantly reduced root numbers to an average of 10.22 ± 0.97 and reduced percent rooting to around 95 and above, respectively. The A B Lo W root number and rooting % decreased at 4.37, 8.05, and 10.74 NAA concentrations (8.78, 7.43, and 6.43, respectively). The most effective concentration for root formation was 13.4 µM of NAA among all concentrations tested, which becomes less effective or suppressive at higher concentrations (55% rooting, 4.78 ± 1.92 roots).

But compared to the others, IBA's root-forming performance was very poor. The highest IBA response was obtained at 1.23 µM, with 3.89± 0.97 roots per shoot and 45 % rooting percentage. The number of rootlets produced and rooting percentage were decreased with increasing concentrations and the minimum response (23 % rooting and 2.44 ± 1.42 roots) was recorded at 12.33 µM IBA. With IBA in the medium, roots were tenuity, less branching and had retarded rooting.

In this experiment, IAA was the relatively weaker auxin. At its best concentration of 1.42 µM, only 29% rooting was obtained and only 3.54 ± 0.83 roots per shoot. However, subsequent increasing of the IBM concentration up to 14.2 µM caused the further fall. Only 12% shoots rooted when exposed to 14.2 µM IAA, with an average number of 1.11 ± 0.60. Together, these results further implied that *in vitro*, IAA might be rapidly metabolised or degraded, possibly resulting in suboptimal rooting.

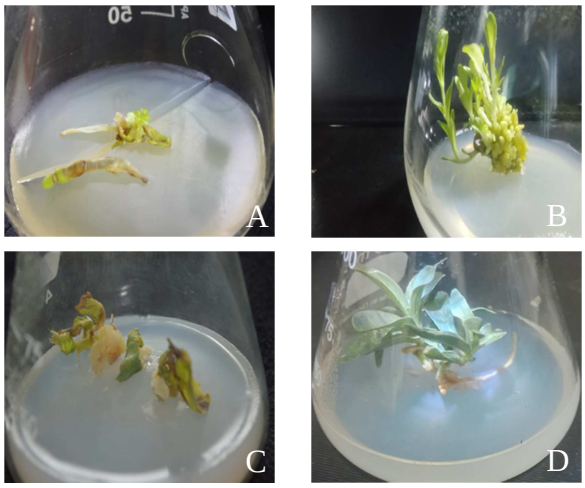

Fig. 1A-D. Directly using different auxin and cytokinin concentrations to induce growth in firable callus via immature leaf shoot and root induction

It was NAA > IBA > IAA in the rooting rate and root number; which was in accordance with the literature reported NAA was most efficient in inducing AR formation in gentianaceae plants. The thick, well-branched, and morphologically similar roots in the medium with NAA represented normal root system agriculture.

Callus attributes and morphogenic response

Callus formation was observed in all treatments during the study, though to differing degrees. The intensity of the callus ranged from + (mild) to +++ (profuse), and it was consistently described as light yellow and friable. Direct shoot emergence showed a positive correlation with mild to moderate callusing, particularly when cytokinin levels were optimized (e.g., 2.32 µM KI + 2.22 µM BAP). However, too much callus (especially in the high-cytokinin-containing treatments, e.g. 4.64 µM KI + 8.88 µM BAP) Light yellow friable callus (+++), (Fig. 1C), would apparently retard the outgrowth of shoot leaf, possibly due to the dedifferentiation of cells and deflection to the callogenic way. The stability of the optimized procedure was confirmed by the absence of somaclonal variation, vitrification or necrosis during culture. Roots were vigorous with clearly visible meristematic zones, and shoots were green, dense and structurally normal.

DISCUSSION

In this study, we showed the contribution of hormonal balance to direct shoot regeneration and root induction of your species. Results indicate that optimal concentrations for KI and BAP are related to the backcross embryogenic efficiences determined from the MS medium and deviating values resulted in relatively low performances due to excess callusing and/or retarded morphogenesis. Plant tissue culture has seen extensive applications in agribusiness and biotechnology (Twaij *et al.*, 2020).

Effect of cytokinins on the development of shoots

The highest amount of result was obtained using (2.32 µM KI and 2.22 µM BAP) from the MS medium showing about 92%, with 20.00 ± 1.42 shoots/explant, showing a promising respond to shits induction in your experiment. This finding is consistent with previous knowledge: cytokinins, including BAP, stimulate growth of new cells and shoots in plant tissue culture. Its essential role in differentiation and proliferation of shoot leaf is further complemented by the increasing trend of shoots observed at higher meditation of BAP (at the same low doses of KI).

In contrast, the large reduction in morphogenic potential at high concentrations of KI and BAP, underlines the importance of hormonal equilibria. Accordingly, tissues grown *in vitro* may exhibit toxin, polarisation or hormonal imbalance when supra-optimal levels of cytokinin are present. High levels of hormones were generally deleterious as, for example, in the combination KI 4.64 µM + BAP 4.44 µM they resulted in poor response (2.44±0.72 shoots per explant), delay in the beginning and in callusing, as you reported.

Effects of Auxin on Rooting Auxin had a significant effect on root formation; NAA was the best of all auxins tested for rooting, because it had previously been demonstrated that NAA is the most effective in stimulating the adventitious rooting of other plants. If rooting effectiveness declines with an increase in NAA concentration it would seem that auxins optima apply and that their over-concentration becomes inhibitory.

The instability or metabolism of IBA and IAA *in vitro* could contribute to their predominantly lower rooting response compared to NAA. [:] As a whole, the trend was NAA > IBA > IAA for rooting % and number of roots, which corroborates a previous study that NAA was superior to IBA in inducing the formation of AR in gentianaceae species.

Response to morphogeny and induction of callus

Callus was induced by all the treatments and there was an absolute association between callus formation and morphogeneic reaction. In the presence of optimal cytokinin concentrations, there was a good relationship between shoot increment and mild to moderate callusogenesis. On the other hand increased callusing inhibited the formation of shoot buds, presumably due to associated cell dedifferentiation and reprogramming to callogenic pathways.

Effect on micropropagation

In conclusion, your results could provide valuable data to optimize *in vitro* propagation protocols for your species. Efficient micropropagation methods for commercial purposes can be developed on the basis of the optima of hormone and the responses observed. Tissue culture of plants is a rapid multiplication method in case of some species with long generation time and poor germination of seeds (Lone *et al.*, 2020). There is also indirect role of micropropagation through manipulation of exogenous factors, those not possible in field, on endogenous ones (Hasnain *et al.*, 2022). By the initial stages, it was pioneered in plant tissue culture first real callus/tissue cultures and root and embryo cultures originated (Bhojwani and Dantu, 2013).

Prospects for the future

Further work could focus on:

1. Investigation of the molecular and physiological bases of the hormones described.
2. Optimizing other culture conditions (e.g., temperature, light quality, nutrient component) to enhance the regeneration efficiency.
3. Genetic studies- the assessment of the genetic homogeneity of micropropagated plants for true-to-type propagation. Commercial laboratories are no longer in business for early detection of off-types. Instead, the focus is on reducing somaclonal variances to facilitate precise screening and selection processes (Hasnain *et al.*, 2022).

Comparison with other studies

BAP's potential in shoot regeneration has been reported in other genera of the same family, Gentianaceae (José and R, 2015). Such tests frequently involve the evaluation of various PGRs necessary for the regeneration and proliferation phases (Cardoso and Silva, 2013).

BAP and NAA induced callus and shoot regeneration in nodal explants together which is in accordance with the previous report in *Enicostemma axillare* (Parvin *et al.*, 2018). As seen from hypocotyl *in vitro* shoots in Nigella damascena and similar results were obtained on the induction of shoot in *Swertia chirayita*, a member of the species of Gentianaceae. These results imply that BAP significantly influences shoot multiplication in Gentianaceae species.

Optimization experiments of tissue culture techniques frequently use Growth of plants enhancers added in varying doses to Murashige and Skoog medium. In the future, it may be worth considering the use of multiple sterilants without losing explant regenerability (Talla *et al.*, 2018). You results indicated that direct shoot regeneration from leaf explants could be efficiently induced at an optimum KI and BAP concentration.

CONCLUSION

The present study has developed a reliable and reproducible protocol of *in vitro* regenerations of *Enicostemma littorale* Blume, an important medicinal herb of family Gentianaceae of therapeutic importance. The objective was to enhance shoot induction from immature leaf explants, investigation into the influence of different amounts and combinations of auxin (NAA) and cytokinins (BAP and kinetin) on morphogenic response.

Medium MS (Murashige and Skoog`s) consisted of 2.22 µM of BAP and 2.32 µM KN resulted in the highest frequency (92% shoot organogenesis) and maximum average number (20) of shoots per explant, supporting the synergistic effect of BAP and KN in direct organogenesis. Instead, higher levels of cytokinins led to unwanted callus induction, demonstrating the need for a harmonious hormonal environment. The highest rooting (100%) with average number of roots (15-roots per shoot) was achieved on 1/2 strength MS medium supplemented with 1.22 µM NAA.

The large-scale clonal propagation of *E. littorale* for genetic conservation and the generation of biotechnological tools, such as novel treatments for biotechnology, would highly benefit from this efficient regeneration procedure. Results The results make a significant contribution to plant tissue culture, particularly for medicinal plants, and underline the importance of growth regulator concentrations in controlling morphogenic response.

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