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In vitro propagation of *Valeriana mooni* using nodal and leaf explants

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Abstract

Valeriana mooni (Family: Caprifoliaceae) (S:Lanka Thuwarala) is an endemic plant in Sri Lanka with economical and medicinal values. Previous studies revealed, that *V.mooni* shares pharmacogenetic properties with *Nardostachys jatamansi* (Family: Caprifoliaceae)which is extensively utilized in Ayurveda and traditional medicine in Sri Lanka. Since the exportation of *N.jatamansi* has been restricted in India, it is important to study an efficient propagation system for large- scale production of *V. mooni*. Therefore this study aimed to establish an efficient protocol for *in vitro* propagation of *V.mooni*. The best shoot induction (3.13±0.35) from nodal explants of *V.mooni* was observed after six week of incubation, in the presence of Indole-3-acetic acid (IAA)(1.0mg/L) and Kinetin (Kn) (5.0mg/L) with the maximum mean length of the shoots (3.21±0.10cm). The highest shoot multiplication (5.17±0.30) with maximum mean length resulted in MS medium supplemented with IAA (1.0mg/L) and Kn(5.0mg/L) during first and second sub culture level. Rooting *in vitro* shoots is successful in half-strength MS medium, supplemented with 1.0mg/L IAA, resulting in the highest mean roots per shoot(7.50±0.30). In the study of *in vitro* Callus induction using leaf explants. The best callus formation was achieved in MS media supplemented with 2,4- Dichlorophenoxyacetic acid (2,4-D) (1,5mg/L) and Kn (1.0 mg/L). Despite no significance improvement was observed in callus induction from leaf explants, utilizing nodal or axillary buds as explants can be a more promising approach for *in vitro propagation* of *V. mooni*.

Keywords: *Valeriana mooni*, *Nardostachys jatamansi, In vitro* propaogation , MS medium, Nodal explant

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Introduction

Valeriana mooni (Family :Caprifoliaceae) also known as "Lanka Thuwarala" is an endemic plant which has economical and medicinal value. It is critically endangered herb, finds in high altitude areas of Sri Lanka, and Tamilnadu, India (Jayaratne et al., 2017). Genus *Valeriana* which has more than 230 species. Plants of genus *Valeriana* play a major role in herbal medicine, they have sedative, anxiolytic antidepressant, antioxidant and antispasmodic properties, that is mainly because these plants naturally produce important valepotriates and other phytochemicals (flavones glycoside, lignans, sesquiterpenoids)(Bos et al.,2002).These phytochemicals are rich in plant roots and rhizomes. Therefore, the roots and rhizomes of this plant are used to treat a multitude of diseases and ailments (Patočka and Jakl, 2010).

Most of *Valeriana* species close to extinction in their natural habitats in worldwide, due to uncontrolled eradication and indiscriminate collection of plants at present (Samant et al., 1998). *Nardostachys jatamansi* is another species of the same family. It also has the same pharmacogenetic properties of *V. mooni*. *N.jatamansa* ,widely used Ayurveda and traditional medicine in Sri Lanka and it is imported from India. Currently, importation of this plant material has been restricted as this plant has become an endangered in India. However current studies show *V. mooni* could be used as a safe substitute to *N. jatamansi*. The Phytochemical screening, TLC and GCMS analyses studies reveal high similarity of pharmacognostic properties of these two species. Therefore *V.mooni* is recommended as a substitute for *N. jatamansi* and there is a need of conservation, development of suitable propagation systems and cultivation(Jayaratne et al., 2017).

Valeriana wallichi is another substitute for *N. jatamansi*. However, that species also does not grow naturally in Sri Lanka (Jayaratne et al., 2017). Genus *Valeriana,* usually propagate by seeds or root parts, yet propagation from the seeds and roots is not successful as seeds loose viability after one year and also fresh seeds have only 30% germination. Propaogation from roots is not successful because the multiplication of roots can affect root rot and white mold infections (Rao and Holt, 2005). Also, the percentage of important phytochemical constituents in plants could vary due to different growing conditions, processing methods and storage conditions. (Patočka and Jakl, 2010) Therefore, developing efficient propagation system of *V. mooni* is necessary for large scale production of quality valepotriates and other important phytochemicals for medicinal purposes.

The studies of *in vitro* propagation of *V. glechomifolia* shows, the levels of valepotriates in the tissue cultured plants are higher than those found in wild populations of *V. glechomifolia*(de Andrade Salles et al., 2002). *In vitro* propaogation or micropropagation could be a successful propaogation method of *V. mooni* for mass production which contain important biologically active compounds. When the optimum cultural conditions supply to the tissues under *in vitro*, it will be able to produce significant amount of important secondary metabolites.

Methodology

Types of explants

The explants used for *in vitro* propagation in this study, were nodes, axillary buds and young leaves of *V. mooni.* Mother plants were collected from herbal garden of the Department of Ayurveda, Pattipola, Sri Lanka. The root divisions were separated and planted in poly bags which contains coir dust, topsoil, and sand (2:2:1), and were maintained in the plant house. Mother plants were regularly watered and kept protected from pests and diseases. Additionally, 0.2% Albert solution was applied weekly for nourishment. Before collecting the explants (nodes segments, leaves), mother stock was treated with 0.2% fungicide solution (Mancozeb 80% (w/w)). This spraying process was carried out for seven consecutive days. Additionally, a final treatment was administered 24 h before the actual collection of explants. The nodes segments of *V. mooni* , initially cut to 2.0 cm in length.

Surface sterilization of explants

The collected nodal and leaf parts were rinsed under running tap water for 5 to 10 min. Subsequently, a diluted soap solution was used to wash them for 10 min, followed by immersing them in a 0.1% Captan for 45 min. All nodal/axillary buds and leaves explants were subjected according to four selected surface sterilization procedures (T1,T2,T3 and T4) in order to determine the most effective surface sterilization procedure with the highest survival rate. In T1 the explants were immersed in 0.1% $HgCl_2$ for 10 min and then treated 10% NaoCl with tween 20 for 15 min. Subsequently in T2= 0.1% $Hgcl_2$ for 10 min, 5% NaoCl with tween 20 for 10 min and 70% ethyl alcohol for 1 min, T3=0.1%$HgCl_2$ for 10 min, 5%NaOCl with Tween 20 for 15 min, 70% ethyl alchol for 1 min. T4= 0.1% $HgCl_2$ for 10 min, 10% NaoCl with tween 20 for 10 min and 70% ethyl alchol for 1 min. The explants, which were not subjected to any surface sterilization procedure and were cultured on PGR-free MS media, were taken as the control (T0). The treated plant segments underwent a triple wash with sterilized distilled water. Nodal segments were trimmed to 1.0 cm, leaves were cut into 1.0 cm × 1.0 cm pieces and then cultured in a plant growth regulators-free MS medium and placed in a culture room for a duration of 21 days at a controlled temperature of 25 ± 1°C and a relative humidity (RH) of 50 ± 5%, with a 16 photoperiod. After incubation period the number of survived, contaminated explants were recorded from each treatment.

Shoot induction

To achieve optimal shoot induction of *V. mooni*, a combination of Kn (3.0 mg/L, 4.0mg/L, 5.0mg/L ,6.0mg/L) with IAA (0.5 mg/L, 1.0 mg/L, 1.5 mg/L, 2.0mg/L) and BAP (1.0 mg/L, 2,0 mg/L, 3.0 mg/L) with IBA (1.0 mg/L, 1.5 mg/L) were selected as the plant growth regulators. There were 10/12 replicates were taken for each treatment. Surface sterilized 2.0 cm long nodal segments, were carefully trimmed to 1.0 cm using sterile forceps and blades. These nodal explants were then transferred to culture jar bottles containing full strength MS media supplemented with different combination of PGRs.The nodal explants cultured on PGR free full strength MS media were taken as control. The cultures were placed

in a growth room maintained at 24°C, following a 16 h light and 8 h dark photoperiod with a light intensity of 80 − 85$\mu mol/m^2/s^1$ provided by cool white fluorescent light. After the incubation period (6- 12 weeks) the number of shoots per nodal explant and the lengths of shoots were recorded.

Shoot multiplication

The generated shoots were further sub cultured after being trimmed into 1 cm in length, in MS medium supplemented with different combination of Kn(3.0mg/L, 5.0mg/L) and IAA(1.0 mg/L) PGRs to promote the multiplication. The MS media free from PGR were taken as control (M0). There were ten replicates taken for each treatment. Subculturing was performed twice, with a minimum interval of four weeks between each subculture, allowing sufficient time for shoot growth and development. After every incubation period (four weeks), the number of shoots per explants and length of shoots were recorded.

Root induction

Elongated *in vitro* shoots were taken for *in vitro* root induction and before transferring to the rooting medium, these shoots were introduced to PGR free MS medium and kept ten days to remove the residual effects of previously used PGRs. Two levels of IAA(1.0 mg/L and 2.0 mg/L) selected for *in vitro* root induction and *in vitro* rooting was tested for both full strength and half strength MS medium. Growth regulator free MS medium was used as the control. There were ten replicates were taken for each treatment. After five weeks of incubation period, the number of roots per shoot and length of generated roots were recorded.

Callus induction from leaf explants

Leaf discs 1.0 cm×1.0 cm was cultured in MS medium supplemented with different combinations of Kn (1.0 mg/L) with 2,4D (1.0 mg/L,1.5 mg/L, 3 mg/L) and Kn (1.0 mg/L) with NAA (1.0 mg/L,2.0 mg/L,3.0 mg/L). Growth regulators free MS media were taken as control. There were ten replicates for each treatment. Cultures were incubated at 24 °C under dark conditions and number of explants formed callus were recorded after 8 weeks of incubation.

Acclimatization of *in vitro* propagated *V. mooni*

Plantlets were transferred from the culture room to a laboratory for three days to acclimate to higher temperature (> 24°C) and humidity (50 ± 5% RH), exposed to natural light. Afterwards, plantlets with developed roots were washed, treated with fungicide, then planted in small pots with a 2:2:1 ratio of coco peat, topsoil, and sand. Pots were covered with polyethylene bags, gradually perforated for humidity. Acclimatization continued in a controlled greenhouse (24°C, 16 h light cycle). Regular irrigation ensured proper hydration.

Data analysis

A completely randomized design was employed for all the experiments conducted. To analyze the data, a two-way analysis of variance (ANOVA) was performed using MINITAB 17.0 software. Pairwise comparisons were conducted using Tukey's test at a confidence level of 5% to determine significant differences between groups.

Results and Discussion

Determination of best surface sterilization procedure for *V. mooni*

Microbial contamination is consistently associated with plant tissue culture. After 21 days of incubation, the tested surface sterilization protocols for explants, exhibited different levels of contaminations, dead explants and survival rates. The T3 (0.1%HgCl2 for 10 min, 5%NaOCl with Tween 20 for 15 min, 70% ethyl alcohol for 1 min) showed the most promising outcomes for *V.mooni* leaf explants showing highest survival and zero contamination. No contaminations were observed in this protocol and yielded the highest survival percentage of 80% indicating the successful elimination of microorganisms. The lowest survival rate (30%) was recorded in both T2 and T4. Only difference between T2 and T3 were duration of explants dipped in 5% NaOCl with drop of Tween 20. (explants were dipped in 5% NaOCl with drop of Tween 20 for 15 min in T3 and for 10 min in T2). Although, the 10% NaOCl in T4 may harm the cells of explants. Surface sterilant can also be toxic to the explants and the level of this toxicity is influenced by the concentration and duration of exposure to the disinfectants (Sivanesan et al., 2021).

T4 showed the highest survival rate (100%) of nodal explants with zero contamination. T1 exhibited minimum survival rate (16.6%) with highest percentage of contaminations (83.3%). This outcome could be attributed to the absence of washing in 70% Ethyl Alcohol which in adequately eliminate microorganisms present on surface. Over 90% of contaminations obtained from nodal and leaf explants due to fungi. These may be due to insufficient sterilization of the surface of explants.

Determination of the best shoot induction medium for *V.mooni*

Shoot initiation of *V. mooni* was observed across some shoot induction media after a period of three weeks of inoculation. During the first two weeks on shoot induction medium, there was noticeable swelling in the axillary bud area of the nodal segments. By the end of the third week, tiny shoots emerged from the axillary bud and became visible. Results revealed that, both Kn and IAA have significant individual effects ($p < 0.05$) on the number of shoots per explant. The interaction between Kn and IAA was also significant, ($P=0.024, P < 0.05$) indicating that the combined effect of Kn and IAA influence generating the number of shoots per explant.

The combined effect of auxins (IAA) and cytokinin (Kn), influencing different phases of the cell cycle. Auxins primarily affect DNA replication while cytokinins, have control over events leading to mitosis (Veselỳ et al., 1994). The highest mean number of shoots per explant and highest mean length of shoots were observed in MS medium supplemented with 5.0 mg/L Kn and 1.0

mg/L IAA. Tukey's pairwise comparison indicated that the resulted mean number of shoots per explants different between all other tested treatments (Table 1). The second maximum mean number of shoots per explant was observed in the medium presence with 1.0 mg/L IAA and 3.0mg/L Kn. There was no statistically significant difference between these two concentrations. (S7,S4).

Table 1: Effect of different concentration of IAA and Kn combination on mean number of shoots per explants and mean length of nodal/axillary buds per explants of *V.mooni* after six (6) weeks of inoculation. There were ten replicates in each treatment

Treatment code	PGR concentration		Mean number of shoots per explant± SE	Mean length of a shoot±SE (cm)
	Kn (mg/L)	IAA(mg/L)		
S0	0.0	0.0	0.00 ± 0.00^{e}	0.00 ± 0.00^{f}
S1	0.0	1.0	0.88 ± 0.23^{de}	0.53 ± 0.02^{ef}
S2	0.0	2.0	1.25 ± 0.25^{cd}	0.70 ± 0.08^{e}
S3	3.0	0.0	1.88 ± 0.13^{bcd}	2.91 ± 0.03^{ab}
S4	3.0	1.0	2.50 ± 0.27^{ab}	3.10 ± 0.11^{ab}
S5	3.0	2.0	2.00 ± 0.00^{bc}	2.37 ± 0.03^{cd}
S6	5.0	0.0	2.25 ± 0.25^{abc}	2.74 ± 0.03^{bc}
S7	5.0	1.0	3.13 ± 0.35^{a}	3.21 ± 0.10^{a}
S8	5.0	2.0	2.25 ± 0.25^{abc}	2.32 ± 0.09^{d}

(values are the means of data from contaminations free cultures with standard errors(±SE). Mean values having same letter(s) are not significantly different by Tukey's multiple comparison test ($p<0.05$. The distribution of variables obeys normal distribution to great extent)

Further studies on the induction of *V. mooni* , were involved testing additional combinations of Kn and IAA concentrations, along with increasing the number of replicates. After a 12-week incubation period, the highest mean number of shoots per explant and the greatest mean length of shoots were observed with 5.0 mg/L Kn and 1.0 mg/L IAA (Table 2). These results suggest that aligning the outcomes of the initial trial. Comparing the mean number of shoots per explants, the results showed that the combination of Kn with IAA(Table 1) was more effective than the combination of IBA with BAP.The highest number of shoots per explants were observed the presence of 1.5 mg/L IBA with 2.0mg/L BAP(0.88±0.29). This findings aligns on *in Vitro* clonal propagation of *N. jatamansi*, where the highest shoot induction occurred with BAP 2.0 mg/L and IBA 1.5 mg/L (7.50 ± 0.29)(Pant et al., 2021). *V. mooni* exhibited significantly lower mean number of shoots per explants compared to *N. jatamansi* although both belongs to same family. That indicates, different plant species may respond differently to the same PGR combinations, even within the same family.

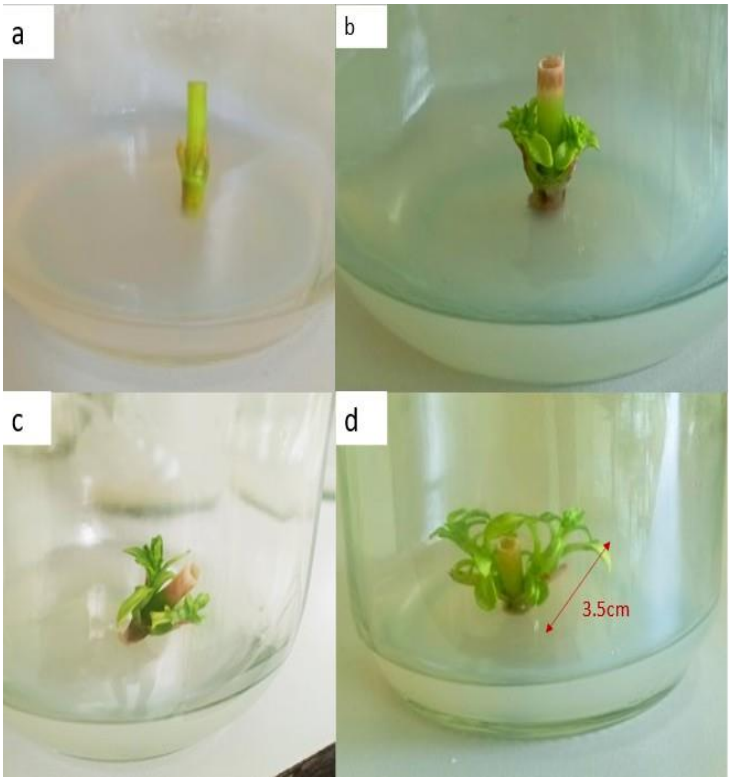

Figure 1: The growth of in vitro shoots of *V.mooni* within a span of 6 weeks cultured in MS media supplemented with 5.0mg/L Kn and 3.0mg/L IAA. Nodal explant cultured on media(a). Tiny in vitro shoots generated after 12 days(b). Increment in the length of in vitro shoots after 4 weeks (c). In vitro shoots reaching a length of 3.5 cm after 6 weeks(d)

Table 2: Effect of different concentration of IAA and Kn combination on mean number of shoots per explants and mean length of nodal/axillary buds explants of *V.mooni* after twelve weeks of inoculation. There were twelve replicates in each treatment

Treatment code	PGR concentrations		Mean number of shoots per explant± SE	Mean length of shoot± SE(Cm)
	IAA(mg/L)	Kn(mg/L)		
t0	0.0	0.0	0.8±0.33 ^{b}	1.2±1.31 ^{ab}
t1	0.0	4.0	1.9±0.79 ^{ab}	2.8±1.12 ^{ab}
t2	0.0	5.0	1.6±0.73^{b}	2.7±1.15 ^{ab}
t3	0.0	6.0	2.3±0.57 ^{ab}	4.2±1.02^{ab}
t4	0.5	0.0	0.9±0.37 ^{b}	2.5± 1.09 ^{ab}
t5	0.5	4.0	1.4±0.62 ^{b}	1.5± 0.80 ^{b}
t6	0.5	5.0	1.0±0.42 ^{b}	2.2± 0.9^{b}
t7	0.5	6.0	2.0±0.86 ^{ab}	3.1 ±1.29 ^{ab}
t8	1.0	0.0	0.9±0.41^{b}	2.6 ±0.88 ^{ab}
t9	1.0	4.0	0.4±0.30 ^{b}	1.9 ±0.9 ^{b}
t10	1.0	5.0	4.3±0.63^{a}	7.2 ±0.46 ^{a}
t11	1.0	6.0	1.8±0.59^{ab}	4.8 ±0.97^{ab}

t12	1.5	0.0	0.7±0.36^{b}	2.2±0.77^{b}
t13	1.5	4.0	1±0.51^{b}	2.5±1.05^{ab}
t14	1.5	5.0	0.9±0.41^{b}	2.4±0.83^{ab}
t15	1.5	6.0	0.1±0.1^{b}	1.3±0.89^{b}

(values are the means of data from contaminations free cultures with standard errors(±SE). Mean values having same letter(s) are not significantly different by Tukey's multiple comparison test ($p<0.05$. The distribution of variables obey normal distribution to great extent)

Determination of best PGRs combination for shoot multiplication

The aim of transferring *in vitro* shoots of *V. mooni* from shoot induction media to shoot multiplication media was to obtain high number of plants per explant. The highest mean number of shoots was observed in the shoot proliferation medium supplemented with 5.0mg/L of Kn and 1.0mg/L of IAA during both subcultures (Table 3). T here was a significance interaction ($P<0.05$) between treatments (M0,M1,M2,M3 and M4) used for multiplication and frequency of subculturing. Overall results revealed that the mean number of shoots per explant exhibited an increase, corresponding to the rate of subculturing. However, in M4 mean number of shoots per explant remained consistent (5.17±0.30) in both first and second subcultures. This consistency was further confirmed by the Tukey's pairwise test, which showed no significant difference between the first and second subcultures of the M4.

It was reported that a medium containing only cytokinins such as BAP and Kn was more effective in promoting *in vitro* shoot multiplication (Pan ,2021). The highest mean number of shoots per explant for *N jatamansi* was observed in a medium containing 2.0mg/L BAP and 1.5mg/L Kn(Pant et al., 2021). Contrastingly, present study demonstrated that the combination of cytokinin (Kn) and auxin (IAA) was more effective in promoting *in vitro* shoot multiplication than cytokinin (Kn) alone in the medium. M3 and M4 treatments promoted more shoot proliferation than M1 and M2(Table 3). This might be due to addition of auxins to the growth medium, along with cytokinins which stimulates cell division (Hasan et al., 2010). The combination of cytokinins and auxins had a significant impact on the number of shoots. Since auxins have ability to initiate cell division, they are involved in the development of meristems, causing either unorganized tissue or well-defined organs (George, 2008).

The lengths of the shoots were measured at the end of each subculturing period. It was observed that all the shoot multiplication media tested, stimulated increase in the mean shoot length with each subsequent subculturing (Figure 2). The highest mean length (6.32±0.13 cm) of shoots were recorded in M4. There was significant interaction ($p <0.05$) between different treatments and the frequency of subculturing for increment of mean shoot length. The mean length of shoots for each treatment (M1,M2,M3 and M4) showed an increase with each successive subculturing, except in the culture media without any Kn and IAA(Figure 2)

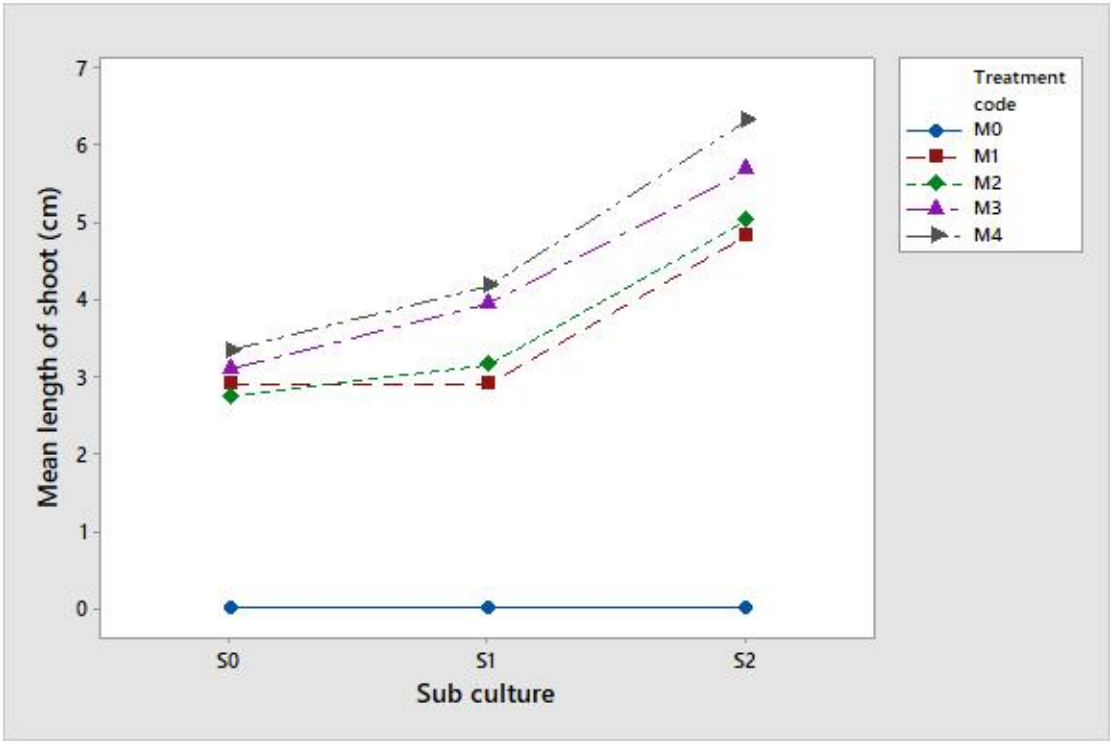

Figure 2: Interaction between different concentrations and combination of Kn,IAA treatments (M0,M1,M2,M3 and M4) on culture media and level of subculturing affect for mean length of shoot

Table 3: Mean number of shoots per explants at the end of each sub culture (S0,S1 and S3) in each shoot proliferation MS media containing different concentrations and combinations of IAA and Kn. There were ten replicates in each treatment.

Treatment code	PGR concentration		mean number of shoots per explant		
	Kn mg/L	IAA mg/L	S0	S1	S2
M0	0.0	0.0	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}
M1	3.0	0.0	1.83 ± 0.17^{d}	3.17 ± 0.70^{bcd}	4.00 ± 0.25^{abc}
M2	5.0	0.0	2.00 ± 0.00^{d}	2.50 ± 0.34^{cd}	4.33 ± 0.33^{ab}
M3	3.0	1.0	2.67 ± 0.33^{cd}	3.67 ± 0.33^{abc}	5.00 ± 0.25^{a}
M4	5.0	1.0	3.50 ± 0.34^{bcd}	5.17 ± 0.30^{a}	5.17 ± 0.30^{a}

(values are the means of data from contaminations free cultures with standard errors(±SE). Mean values having same letter(s) are not significantly different by Tukey's multiple comparison test ($p<0.05$)The distribution of variables obey normal distribution to great extent)

Determination of suitable growth medium for root induction

The results of the study investigated the effectiveness of different concentrations of IAA (0.0mg/L, 1.0mg/L, and 2.0mg/L) for root induction on both MS medium and half-strength MS medium using two parameters, the mean number of roots per shoot and the mean length of roots obtained are summarized in figure 3. Among the treatments use of half-strength MS media supplemented with 1.0mg/L IAA (R4), proved to be the most effective in promoting root formation. This treatment resulted in the highest mean number of roots (7.50±0.30) and the second highest mean length of roots (9.53±0.31cm). The highest mean length root was recorded in full strength MS media with no added PGRs (Table 4).

The results indicated that both the mean number of roots per shoot and the mean length of roots were relatively higher in the half- strength MS medium compared to the full- strength MS medium. This is because the successful *in vitro* rooting of shoots primarily relies on the concentration and duration of auxin treatment, as well as the salt strength of the basal medium. Although, high concentration of salts may inhibit root growth even when auxins are present in the culture medium.(Debergh, 1994) Therefore, reducing the concentration of macro and micronutrients to half of their usual levels during the rooting phase is crucial for most plant species. The findings of a previous study are in align with the results obtained in this study. It was reported that low levels of auxin resulted in the development of longer roots, whereas an increase in auxin concentration led to shorter roots (Kaur et al., 1999). This observation is consistent with the current study, where the highest mean length of roots (10.13±0.43 cm) was observed in IAA-free full- strength media, while the second-highest mean length of roots (9.18±0.30 cm) was recorded in IAA-free half-strength media. Additionally, both full strength and half strength MS media containing a higher concentration of IAA (2.0 mg/L) demonstrated the lowest mean length of roots

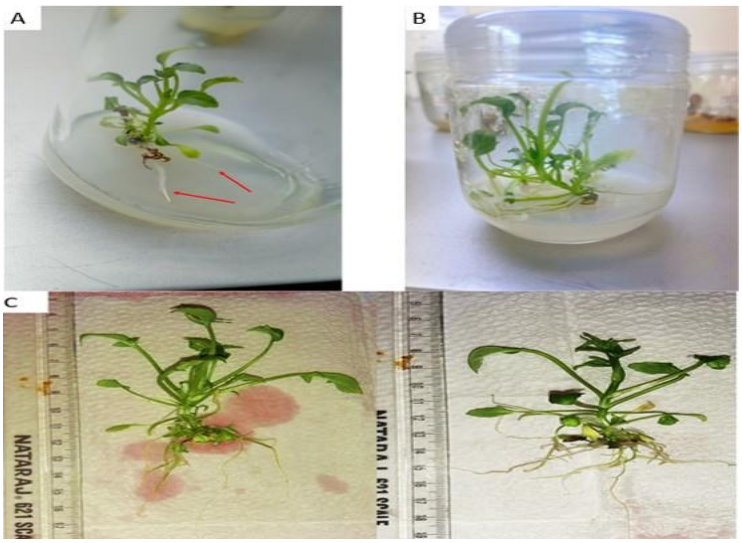

Figure 3: Root induction of in vitro shoots of V. mooni cultured on MS media supplemented with 2,4D 1.5mg/L and Kn 1.0mg/L. (a)Roots emerged from in vitro shoots after 17 days of inoculation. (b)Generated roots from in vitro shoots after 4 weeks of inoculation. (c)Plantlets of V.mooni removed from root induction culture medium after 5 weeks of inoculation.

Table 4: Effect of different concentration of IAA and strength of MS medium on mean number of roots per shoot and mean length of roots after 5 weeks of inoculation. There were ten replicates in each treatment.

Treatment code	Basel medium strength	IAA Concentration	mean roots per shoot	mean length of roots
R0	MS	0.0	6.75±0.48^{ab}	10.13±0.43^{a}
R1	MS	1.0	5.25±0.48^{bc}	8.00±1.05^{ab}
R2	MS	2.0	4.50±0.30^{c}	6.00±0.18^{b}
R3	½ strength MS	0.0	6.25±0.50^{abc}	9.18±0.30^{a}
R4	½ strength MS	1.0	7.50±0.30^{a}	9.53±0.31^{a}
R5	½ strength MS	2.0	5.25±0.25^{bc}	6.18±0.21^{b}

(values are the means of data from contaminations free cultures with standard errors(±SE). Mean values having same letter(s) are not significantly different by Tukey's multiple comparison test.) The distribution of variables obeys normal distribution to great extent)

Determination best culture media for callus induction

A study conducted Zamini (2016) revealed that the highest percentage of callus formation (95.83%) in *V. officinalis* was achieved when MS medium supplemented with 2,4-D (1.5 mg/L) and Kn (1.0 mg/L). The current study's findings are supported by the previous study, as callus formation was achieved in this study on MS media supplemented with 1.0 mg/L Kn and 1.5 mg/L 2,4-D (resulting in the highest callus formation of 66.6%). Additionally, callus formation was also observed with 1.0 mg/L Kn and 3.0 mg/L 2,4-D(33.33%). However, the callus formation in the present study was not as effective as reported in the previous study. While callus formation was observed after 6 weeks of incubation in the present study, the previous study reported callus initiation from explants within about 3 weeks(Zamini et al., 2016).

PGR combinations of NAA and Kn , showed no callus induction, indicating that NAA was not effective in promoting callus growth of *V.mooni*. In a study on callus induction from different explants in *Vigna radiata*, it was found that 2,4-D was more effective than NAA for callus formation. Additionally, the growth of callus was further enhanced by the addition of Kn(Rao and Holt, 2005). The combination of 2,4-D with a cytokinin resulted in faster and more effective stimulation of callogenesis and cell division in rapeseed cultivars(Afshari et al., 2011). Therefore using combination of 2,4D and Kn is comparatively effective for callus induction from leaf explants of *V.mooni*

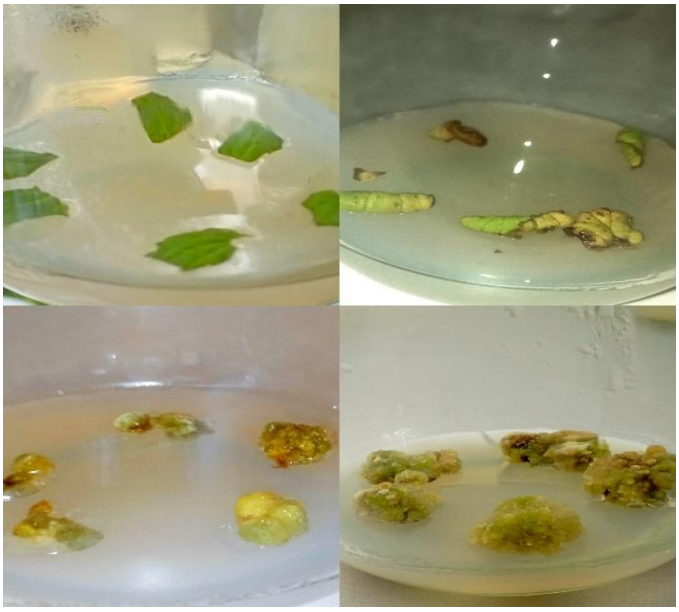

Figure 4: Different growth stages of callus from leaf explants of *V. mooni* cultured on MS media supplemented with 2,4D 1.5mg/L and Kn 1.0mg/L. (a)The day 1 of leaf explants cultured on PGR treated media, (b) 3 weeks after incubation, (c) 6 weeks after incubation, (d)8 weeks after incubation.

Acclimatization

During the acclimatization, it was observed that a significant percentage (90%) of the plantlets failed to survive and succumbed within 2-3 days. Therefore, it is necessary to improve the acclimatization procedure in the future. As this is a medicinal plant, it is necessary to evaluate the phytochemical composition of the plants obtained from tissue culture.

Conclusion

This study developed a reliable protocol for *in vitro* micropropagation of *Valeriana mooni*, enabling the production of homogeneous plants, which facilitate possibility for widespread cultivation and the synthesis of important secondary metabolites such as valepotriate, sesquiterpene, and jatamansone for medicinal purposes.

The best sterilization method for nodal explants was obtained from the treatment with 0.1% $HgCl_2$ for 10 min, 10% NaoCl with tween 20 for 10 min and 70% ethyl alcohol for 1 min, which resulted in the highest survival rate (100%) of explants without contamination. Additionally, the dipping the explants in 0.1%HgCl2 for 10 min, 5%NaOCl with Tween 20 for 15 min, 70% ethyl alcohol for 1 min showed the most promising outcomes for *V. mooni* leaf explants, achieving the highest survival rate (80%) with zero contamination indicating that the timing and concentration of the different components of the sterilization medium is critical for the survival of the explants. For shoot induction and multiplication from nodal explants, the optimal growth regulator combination was MS medium supplemented with 1.0 mg/L IAA and 5.0 mg/L Kn. This combination resulted in the highest mean number of shoots per explant and the greatest mean shoot length. For rooting, the best results were achieved with half-strength MS medium supplemented with 1.0 mg/L IAA, resulting in the highest mean number of roots per shoot.

Furthermore, the addition of 1.0 mg/L Kn and 1.5 mg/L 2,4-D to the MS medium resulted in the highest percentage of callus induction (66.6%) from leaf explants. This should be further improved by experimenting with different combinations of Kn and 2,4-D.

Future studies should prioritize the development of an enhanced acclimatization procedure for *V. mooni* plantlets to achieve a high survival rate and produce a substantial quantity of healthy and genetically uniform plants. Additionally, conducting comparative experiments between *V. mooni* plants obtained from tissue-culture and their mother plants is essential to identify potential variations in morphology or phytochemical composition.

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Conflict of Interest

The authors confirm that they have no conflict of interest.

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