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[1]Central Department of Botany, Tribhuvan University, Kathmandu, Nepal
[2]Plant Genetic Engineering Laboratory, Department of Biotechnology, Bharathiar University, Coimbatore, Tamil Nadu, India

Rapid clonal propagation and valepotriates accumulation in cultures of *Valeriana jatamansi* Jones, a high-value medicinal plant

Sushma Pandey[1], Sathish Sundararajan[2], Sathishkumar Ramalingam[2], Manju Kanu Baniya[1], Bijaya Pant[1*]

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Summary

Valeriana jatamansi, is well known for its medicinal and ethnobotanical values. An efficient and rapid *in vitro* propagation system for *V. jatamansi* is presented. The shoot bud explants from *V. jatamansi* plants were cultured on Murashige and Skoog (MS) media supplemented with different concentrations of plant growth regulators (PGR's). MS medium supplemented with 2 mg/L of benzyl amino purine (BAP) produced shoot bud regeneration. However, the proliferation rate was slow with fewer shoots. The nodal segments were excised from the *in vitro* plants raised on BAP (2 mg/L) and multiplied by supplementing MS medium with different PGR's at different concentrations. Among the tested growth regulators, supplementation of 10% coconut water resulted in maximum shoot length (6 cm), shoot number (13.0), root length (7.5 cm) and root numbers (19.6). The genetic fidelity of the *in vitro* raised plants was confirmed by analysis with RAPD and ISSR markers. GC-MS profiling of the root extract from *in vitro* raised plant revealed the presence of 21 compounds including valeric acid which is commercially and pharmaceutically important. The protocol developed here will be useful in the future towards large scale commercial production of valepotriates from *V. jatamansi*.

Keywords: Coconut water; GC-MS; genetic fidelity; shoot buds; *Valeriana jatamansi*

Introduction

Valeriana jatamansi Jones commonly known as Tagar or Sugandhawal, belonging to the family Valerianaceae is a native plant species of Himalayas but distributed worldwide in the tropical and subtropical parts (Prakash et al., 1999; Das et al., 2013). The genus *Valeriana* consists of about 250 species of which three species (*Valeriana jatamansi, Valeriana officinalis,* and *Valeriana edulis*) are considered important for the production of high-value aromatic oils. This plant species has numerous uses in traditional and modern systems of medicine. The essential oil and extracts from the roots and rhizomes are used in flavor, pharmaceutical and fragrance Industries and about 30 products are commercially available (Prakash, 1999). The *Valeriana* genus is used in several Ayurvedic formulations because of which this plant is of great demand in the pharmaceutical and drug industry (Das et al., 2013). The fresh rhizome and root of *Valeriana* species can yield 0.4-0.5% (w/v) essential oils (Mathela et al., 2005). Rhizomes and roots of the species are known to cure a multitude of disorders and diseases including obesity, skin diseases, epilepsy, insanity and skin poisoning (Prakash, 1999). The medicinal attributes of this plant can be owed to the presence of valepotriates, a group of monoterpenoids of iridoids having an epoxy group and beta-acetoxy isovaleric acids (Kaur et al., 1999). A crude drug named 'Valerian' is derived from the roots and rhizomes of Valerianaceae species (Houghton et al., 1999). The pharmacological activity of Valerian is attributed to valepotriates and sesquiterpenoids, two major chemical components (Mathela et al. 2005). Girgune et al. (1980) reported that the essential oil from rhizomes of *Valeriana* exhibited antifungal and antibacterial activities and later Wasowski et al. (2002) reported that two compounds 6-Methylapigenin and hesperidin isolated from the rhizome of Indian valerian showed anxiolytic and sedative properties. The antispasmodic, anticonvulsive and antidepressant properties of *V. jatamansi* are because of the presence of major valepotriates, valtrate, acevaltrate and didrovaltrate (Gupta et al., 1986). These compounds have been reported to have cytotoxic effects in rat hepatoma cells (Bounthanh et al., 1981) and antitumor activity (Marder et al., 2003).

In Nepal, this species has been banned for export and because of its high medicinal properties, the species has been prioritized for research and development (Chaudhary et al., 2016). Replenishment and conservation of this species are imperative to meet the increasing demands for the plant's roots and rhizomes since overexploitation of this plant resulted in the complete depletion of species and thus entered the list of endangered species in the Himalayan region (Kumar et al., 2012). Conventionally, the plant is propagated through seeds that have been reported to have limitations since the seeds remain dormant for a longer period in the natural environment (Kaur et al., 1999). However, with advances in plant tissue culture systems in recent decades, *in vitro* propagation on a large scale within a short period is a viable alternative. *In vitro* culture techniques also offer the advantages of rapid clonal propagation, production of important phytochemicals and germplasm conservation of rare, endangered and threatened medicinal and aromatic plants (Pant et al., 2014). There have been several reports on micropropagation of *Valeriana* species using different explants like leaves, stems, and roots. For instance, Chen et al. (2014) reported the multiplication of *V. jatamansi* through callus induction from leaf explants by supplementing 2,4-dichlrophenoxyacetic acid (2,4D) and shoots induction using the combination BA and NAA. While using callus, Zamini et al. (2016) reported that compared to 6 furfuryl aminopurine, kinetin (KIN) or TDZ, the combination of BAP (0.5 mg/L) + IBA (0.5 mg/L) supplementation resulted in a positive effect for plant regeneration and shoot induction frequencies in *V. officinalis*. Micropropagation has been reported in several other species in the genus *Valeriana*, such as *V. wallichii* (Mathur et al., 1988), *V. edulis* ssp. Procera (Enciso-Rodriguez, 1997), *V. jatamansi* (Kaur et al., 1999; Das et al., 2013) *V. glechomifolia* (De Carvalho et al., 2004;) and *V. officinalis* (Abdi et al., 2008; Reza et al., 2009). Zebarjadi et al. (2011), reported the high-frequency callus induction and shoot regeneration using leaf, stem and root explants of *V. officinalis*. There are only a few reports that demonstrate micropropagation in *V. jatamansi* and many of those protocols involve the callus cultures which could be time-consuming and involves rigorous subcultures (Purohit et al., 2015).

Whenever plant tissue culture system is used for the propagation of medicinally important species there is always a possibility of varia-

* Corresponding author

tions occurring within the *in vitro* raised plants for their genetic homogeneity. Somaclonal variations are often observed within the *in vitro* raised plants. To ascertain the genetic similarity between the *in vitro* grown plantlets and the mother plant, molecular markers are often used to study the variation within the germplasm (KUMAR et al., 2012). Among various markers used for such analysis, Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR) markers are very reliable and more often used by researchers. GC-MS analysis has been effectively used to determine the bioactive constituents of *V. jatamansi* roots and rhizomes. For instance, DAS et al. (2011) analyzed the volatile constituents of *V. jatamansi* from the Khasi Hills of north-east India by GC-MS and reported twenty-one compounds. Considerable variations in the essential oil yield and composition in roots and rhizomes of various populations of *V. jatamansi* was reported by VERMA et al. (2011). Though there are reports on the propagation of *V. jatamansi* (KAUR et al., 1999; DAS et al., 2013; JUGRAN et al., 2013a, b and 2015) an efficient, rapid and reproducible protocol is needed for mass propagation of *V. jatamansi* in a very short time. In the present investigation, a rapid and efficient *in vitro* protocol was established for *V. jatamansi*. Further, genetic homogeneity was confirmed for the *in vitro* regenerated plants, mother plants and the acclimatized plants using RAPD and ISSR markers followed by GC-MS analysis of the roots derived from *in vitro* plants for the presence of valuable bioactive compounds.

Material and methods

Plant material

V. jatamansi plants were collected from Dabur Nepal Pvt. Ltd., Kavre, Banepa and were planted in the departmental garden of Tribhuvan University (Fig. 1a).

In vitro propagation

Well established plants from the garden were used as a source for explants. The plants were harvested from the garden and rinsed in running tap water to remove all soil and debris followed by sterilization using few drops of liquid detergent Tween 20 (Himedia, India) and rinsing in sterile water for 30 min. The axillary shoot buds (1-2 cm) were excised from the mother plant and taken to a laminar airflow chamber and treated with 0.1% $HgCl_2$ (mercuric chloride) for 5 min followed by rinsing five times with sterile water to remove traces of $HgCl_2$. Afterward, the explants were blot dried using a Whatman filter paper placed onto sterile Petri plates and inoculated in test tubes containing Murashige and Skoog (MS) (MURASHIGE and SKOOG, 1962) medium with 3% sucrose (w/v), 0.1% myoinositol and supplemented with different combinations of 6-benzyl amino purine (BAP) and 1-naphthalene acetic acid (NAA). The following treatments has been employed: BAP: (0.5, 1.0, 1.5 or 2.0 mg/L), NAA (0.5, 1.0, 1.5, or 2 mg/L). The pH of the medium was adjusted to 5.8 with 1N NaOH or 1N HCL Prior to autoclaving (121 ºC, 20 min), and solidified with 0.8% plant tissue culture tested agar-agar (w/v) (Himedia, India). All cultures were incubated under 16 h photoperiod with a light intensity of 55 µmol m^{-2} s^{-2} provided by cool white fluorescent lamps (Phillips, India) at 25 ± 2 °C and 75% relative humidity (RH). The number of shoots and roots, as well as shoot and root length, were evaluated after 4 weeks of inoculation. Morphological changes were recorded based on visual observations.

The *in vitro* plants that were generated with axillary shoot bud explants on BAP supplemented media were used as explants for the shoot and root propagation. The nodal segments were excised from these *in vitro* grown plants and the explants were cultured on MS basal medium supplemented with different concentrations and combinations of PGR's. The following treatments were employed.

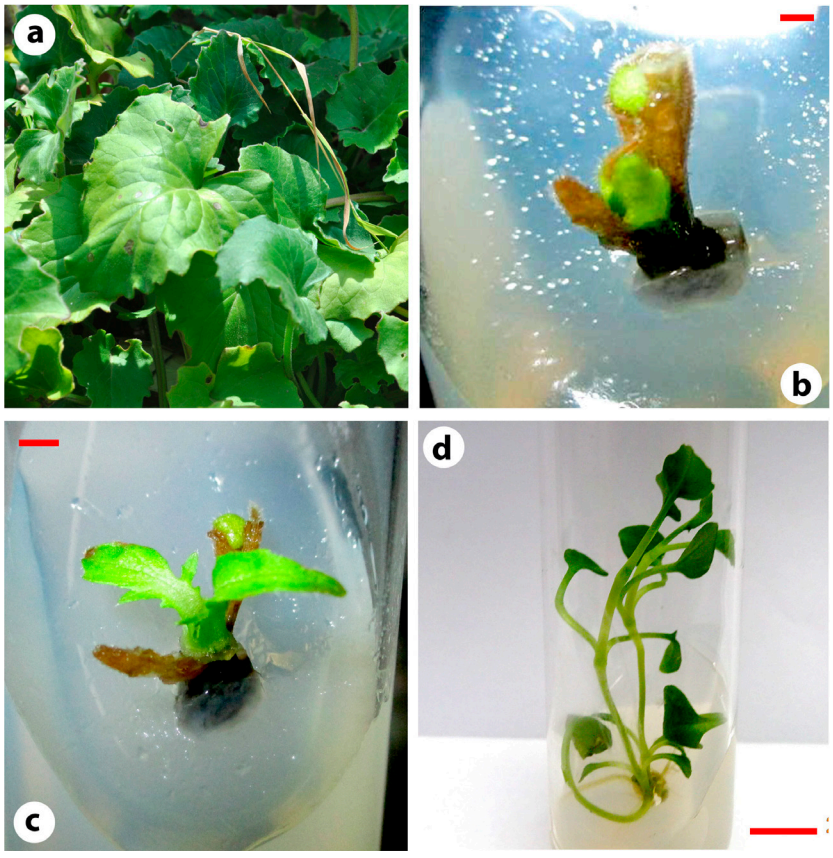

Fig. 1: *In vitro* regeneration of *V. jatamansi* shoot bud explants on MS+BAP (2 mg/L) (**a**) Mother plant of *V. jatamansi*; (**b**) shoot bud explants post 7 days incubation; (**c**) shoot bud initiation after 12 days of culture; (**d**) shoot elongation after 30 days of culture. Scale bar = 1cm (b, c); 2 cm (d)

MS+BAP (0.5-2.0 mg/L), MS+NAA (0.5-2.0 mg/L) and MS+ combination of BAP (0.5 mg/L) + NAA (0.5 mg/L), BAP (1.0 mg/L) + NAA (0.5 mg/L), BAP (2.0 mg/L) + NAA (1.5 mg/L) and BAP (2.0 mg/L) + NAA (2.0 mg/L) and MS+ coconut water (5, 10 and 15%). For the preparation of coconut water, young coconuts were collected from the agricultural farm, Kathmandu, Nepal and the coconut water was extracted in a sterile chamber and was filtered using Whatman filter paper and stored at -4 ºC before use. Coconut water was added before autoclaving in doses of 5, 10, and 15%, in the MS medium. All cultures were incubated under 16h photoperiod with a light intensity of 55 µmol m^{-2} s^{-2} provided by cool white fluorescent lamps (Phillips, India) at 25 ± 2 °C. Subcultures were carried out every 2-weeks. The number of shoots and roots formed was evaluated after 4 weeks of culture and the morphological changes were recorded based on visual observations.

Acclimatization and hardening

After 5-6 weeks, the *in vitro* raised plantlets were taken out from the culture medium, washed thoroughly in running tap water to remove excess agar and rinsed with distilled water. The plantlets were subsequently transplanted into plastic pots containing a mixture of coco peat: soil (3:1), and placed in a plant containment facility and watered on alternate days.

Genetic fidelity analysis

The genetic homogeneity of the *in vitro* raised plantlets were determined using RAPD and ISSR markers. Genomic DNA from randomly selected *in vitro* plants (five plants per treatment) and mother plants was extracted from fresh leaves following a modified CTAB method (Doyle and Doyle, 1990). The isolated DNA was checked in an agarose gel for integrity and purity using a Nanodrop (Nanodrop 2000, Thermo Scientific, USA). DNA was stored at -20 ºC until further use. Preliminary screenings of the samples were done for 35 RAPD primer sets and 10 ISSR primer sets. Primers used in the study were sourced from previous literature (Kumar et al., 2012; Purohit et al., 2015). Out of 35 primers screened for RAPD, only 10 primers showed a clear and reproducible banding pattern. Out of 10 ISSR primers screened, only 5 primers showed a clear and reproducible banding pattern, which were further used for the analysis. Polymerase chain reaction (PCR) was carried out in a 15 µL volume containing nuclease-free water, 1 µL Taq polymerase (0.5 U μL^{-1}), 0.5 µl dNTPs (0.2 mM), 1 µL primer and template DNA (50 ng) in a thermal cycler (Biorad, USA). The amplification cycle for RAPD primers is as follows. Initial denaturation 92 ºC for 5 min, 92 ºC for 1 min, 45 cycles of 35-60 ºC for 1 min, 72 ºC for 1min, 72 ºC for 5 min. The amplification cycle for ISSR markers is as follows. Initial denaturation 95 ºC, 94 ºC for 1 min, 35 cycles of 42 ºC- 60 ºC, 72 ºC for 2 min and 72 ºC for 7 min. The amplified products were separated on an agarose gel (1%) stained with ethidium bromide.

GC-MS analysis of *in vitro* plants

The gas chromatography and mass spectrometry (GC-MS) analysis of root extracts from *in vitro* plants (plants raised supplementing coconut water) were carried out using a GC-MS 4000 (Varian, USA) system with an HP-5MS Agilent column (30 m × 0.25 mm i.d., 0.25 µ film thickness). Injector temperature was set at 280 ºC and the oven temperature program used was as follows, holding at 5 ºC for 5 min, heating to 280 ºC at 3 ºC/min, and keeping the temperature constant at 280 ºC for 7 min. Helium was used as a carrier gas at a constant flow of 1.0 mL/min and an injection volume of 0.20 µL was employed. The MS scan parameters included electron impact ionization voltage of 70 eV, a mass range of 40-500 m/z. The compounds obtained were further studied referring to their biological properties.

Data Analysis

All experiments were repeated thrice, using three replicates each containing 20 explants. The values were expressed as means ± SD. All the data were further analyzed using analysis of variance (ANOVA) followed by Duncan's multiple range test ($p < 0.05$) with the aid of SPSS (version 17) statistical package program. $p < 0.05$ was considered as indicative of significance and the percentage of response was scored on the basis of DMRT analysis. The banding pattern in genetic fidelity analysis using RAPD and ISSR markers were scored manually.

Results and discussion

Establishment of shoot cultures and rooting

In vitro propagation studies on various *valeriana* species has been reported previously. For instance, in *V. wallichii* micropropagation, callus induction and shoot regeneration using shoot tips, auxillary buds and petiole explants have been reported (Mathur et al., 1988; Mathur, 1991). Similar *in vitro* propagation studies in *V. edulis* using seed, leaf, petiole, apical bud explants (Enciso 1997; Castillo et al., 2000), *V. officinalis* using leaf segments, petioles, terminal and axillary buds as explants (Reza et al., 2009; Chen et al., 2014), *V. glechomifolia* using shoot tips (De Carvalho et al., 2004) have also been reported. An efficient callus-mediated shoot regeneration system for the large-scale production of *V. jatamansi* Jones was reported by Das et al. (2013). However, there are only scarce reports on the use of nodal segments for the rapid propagation of *V. jatamansi*. In our study, the shoot bud explants were initially cultured on MS media supplemented with various concentrations of BAP, and/or NAA for axillary shoot initiation and proliferation. NAA had no effect on axillary shoot initiation from the shoot bud explants. Among the tested concentrations of BAP, shoot bud explants cultured on medium supplemented with BAP (2.0 mg/L) induced shoot initiation from the shoot bud explants 7 days post-inoculation (Fig. 1b). However, it was observed that the proliferation rate was slow (Fig. 1c) and only fewer shoots per explant were produced (Fig. 1d) at the end of 30 days. The proliferation rate reduced substantially after 10 days and even after 2 subcultures onto a fresh medium at a two-week interval did not increase the proliferation (i.e., number of shoots and roots).

The nodal segments were excised from the *in vitro* raised plants grown on BAP (2 mg/L) and were inoculated onto a medium supplemented with various concentrations of BAP, NAA, BAP+NAA and coconut water to test their efficacy on the proliferation of the nodal segment explants. Among the various concentrations of BAP tested, 2 mg/L induced shoot induction from the nodal explants after 7 days of culture (6.0 shoots per explant). The mean shoot length (3.0 cm), mean root numbers (6.3) and mean root length (1.4 cm) were also highest compared to other tested concentrations of BAP (Fig. 2a, b). Bhat and Sharma, (2015) reported efficient shoot induction frequencies using shoot tip explants by supplementing MS medium with BAP (1mg/L) +IAA (0.1 mg/L) in *V. officianalis*. In the present investigation, the combination of BAP and NAA also failed to increase the shoot and root numbers (Fig. 2c, d). Contrasting to our results, Purohit et al. (2015) achieved high number of shoots (2.33), number of leaves (15.33) number of roots (27.5) shoot length (3.20 cm), and root length (50 cm) using the combination of BAP (0.33 mg/L) along with NAA (0.93 mg/L), and GA_3 (0.035 mg/L). This particular combination of BAP and NAA has been used for high-frequency shoot multiplication for several species including *Valeriana*. Singh et al. (2015) reported that 0.675 mg/L BA was found optimal in inducing shoots from nodal explants in *V. wallichii* DC. In their study, compared to various growth regulators at different concentrations, BA (0.675 mg/L) was found to be the most effective for shoot induction recording 12.0 shoots per explant and 4.53 cm average shoot length. An earlier report by Kaur et al. (1999) revealed that shoot buds

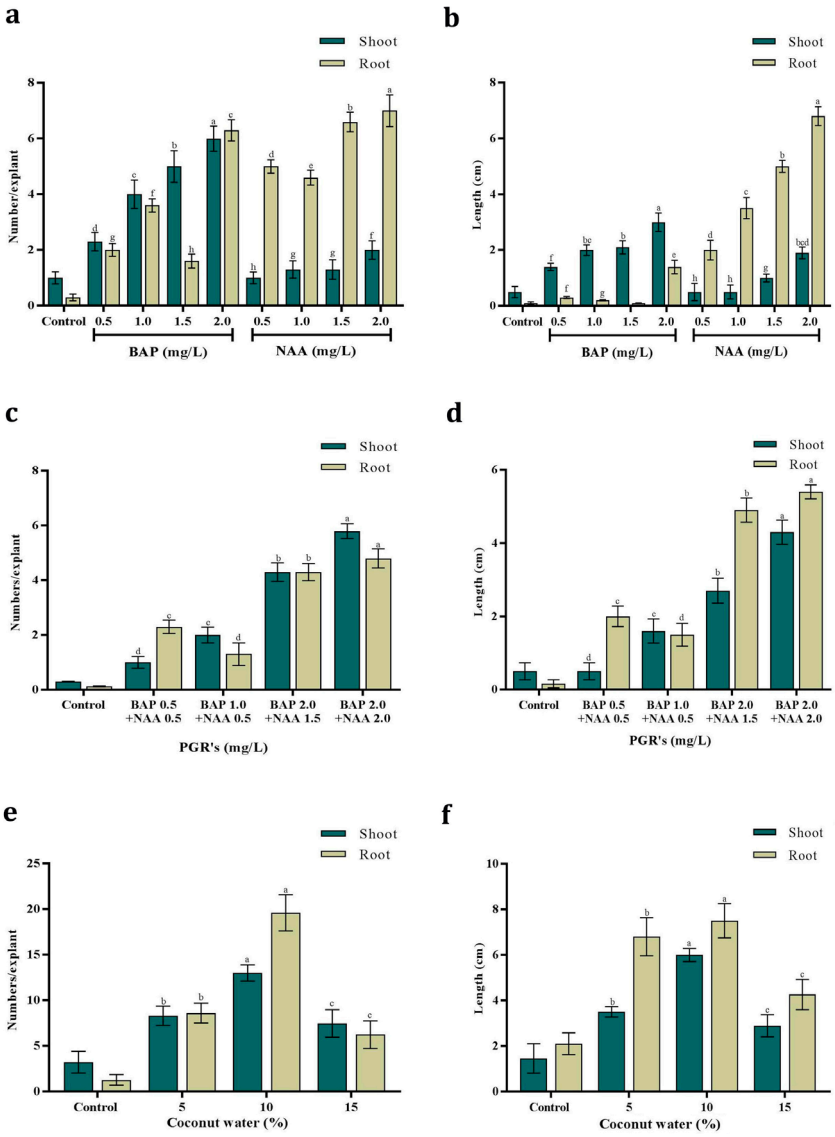

Fig. 2: Effect of PGR'S on shoot and root induction from nodal explants of *V. jatamansi* (**a**) mean number of shoots and roots per explant with different concentrations of BAP and NAA; (**b**) mean shoot and root lengths with different concentration of BAP and NAA. (**c**) mean number of shoots and roots per explant with different concentrations of BAP+NAA (**d**) mean shoot and root lengths with different concentration of BAP+NAA; (**e**) mean number of shoots and roots per explant with different concentrations of coconut water; (**f**) mean shoot and root lengths with different concentration of coconut water. Data taken after 5 weeks of culture. Experiments were repeated thrice, using three replicates each containing 20 explants. Data presented as mean±SD. Means following the same letter are not significantly different, according to Duncan's multiple range test (p<0.05).

cultured on medium fortified with BA and IAA or NAA induced shoots, and subsequently produced roots on the same medium. Interestingly, during our experiments with supplementation of NAA, the frequency of root formation increased and shoot formation decreased compared to treatments with BAP. The highest mean root numbers (7.0) and root length (6.8cm) was recorded with NAA at 2mg/L (Fig. 2a, b). There have been previous reports suggesting cytokinins BA, Thidiazuron (TDZ), zeatin or KIN had no significant effect on callus induction, somatic embryogenesis, or shoot organogenesis when added as a sole entity in the medium (Das et al., 2013). In their study 2,4D (0.5 mg/L) induced high-frequency callus induction with rhizome, petiole and leaf explants. However, for shoot organogenesis, the combination of 0.75 mg/L TDZ+0.5 mg/L NAA resulted in 88.6% regeneration frequency along with the highest shoot numbers per callus (15.20) and shoot lengths (3.60 cm).

To test the efficacy of coconut water in increasing the shoot proliferation of *V. jatamansi*, the nodal explants excised from *in vitro* developed shoots grown in the medium containing BAP (2 mg/L) were inoculated onto MS medium containing coconut water (5, 10 and 15%). The shoot proliferation increased significantly at 5 and 10% (Fig. 2e, f), however, the maximum response was attained with the supplementation of 10% coconut water in the medium (Fig. 3a-c). Coconut water (10%) supplementation resulted in maximum response with regard to shoot numbers (13.0), mean shoot length (6.0 cm), mean number of roots (19.6), and root lengths (7.5 cm). The results indicated that media supplemented with coconut water significantly increased the shoot induction of nodal explants from *V. jatamansi* (Fig. 3d, e). Coconut water is a complex additive that contains many nutritional and hormonal components and is widely used in the plant tissue culture media due to their growth regulatory properties that play vital roles in cell division and induction of rapid growth (Yong et al., 2009). In particular, coconut water contains PGR's (auxin, cytokinins, and gibberellins), natural inhibitors and regulators (ethylene, abscisic acid, phenols, and flavonols) in addition

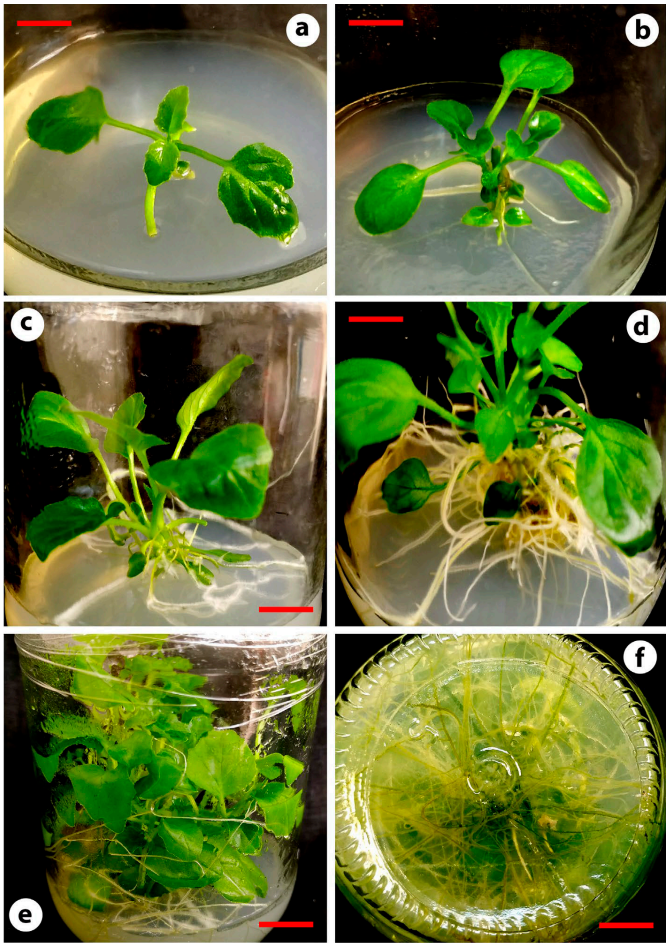

Fig. 3: *In vitro* regeneration of *V. jatamansi* nodal segments in MS+ 10% Coconut water (**a**) Nodal segment of *V. jatamansi*, after 5 days of culture; (**b**) multiplications of shoot after 8 days; (**c**) shoot multiplication after 15 days of culture; (**d**) adventitious root formation after 25 days of culture; (**e**) proficient shoot multiplication after 50 days of culture; (**f**) formation of root in MS+ 10% coconut water. Scale bar = 2 cm

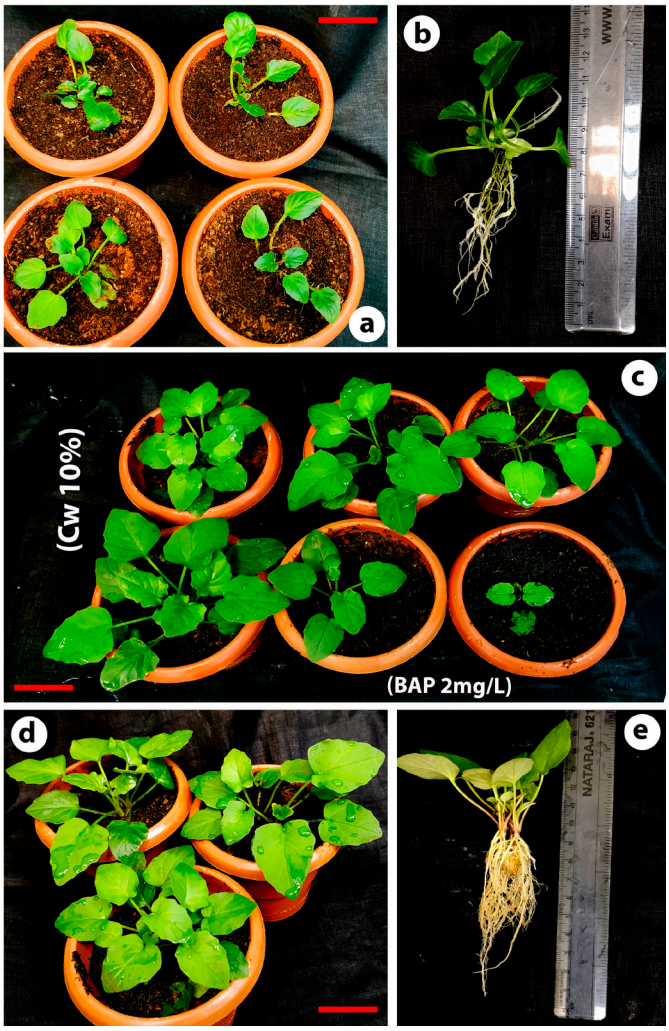

Fig. 4: Acclimatization of in vitro regenerated plantlets of *V. jatamansi* (**a**) Plants of *in vitro V. jatamansi* hardened on coco peat: soil (3:1); (**b**) roots formed prior to acclimatization; (**c**, **d**) acclimatized *in vitro* plantlets from BAP (2 mg/L) and coconut water (10%) treatment after 15 days; (**e**) formation of large clumps of roots along with rhizome after one month. Scale bar = 3cm

to sugars, vitamins, minerals and amino acids, organic acids (Yong et al., 2009). In the present investigation, supplementation of coconut water also enhanced the formation of roots. The increased dosage of coconut water (15%) reduced the shoot numbers, shoot length and root induction. Our results are in accordance with previous reports where coconut water was used to establish high-frequency shoot proliferation and rooting in many plant species. For instance, Prando et al. (2014) reported that the addition of coconut water (20%) increased the number of adventitious shoots produced per explants in *Corylus avellana*. Similarly, Swamy et al. (2014) reported that the addition of coconut water (10%) showed a better response in *in vitro* propagation of *Pogostemon cablin* Benth. Similar to our results Trevisan and Mendes, (2005) reported increased shoot elongation of *Passiflora edulis* with 10% coconut water. The enhanced morphogenetic potential of coconut water can be attributed to its growth regulator content specifically cytokinins (Chugh et al., 2009). The main advantage and significance of using coconut water extracts in tissue culture is that a natural source of growth promoter may eradicate harmful impurities that are present in the synthetic counterparts (Agampodi and Jayawardena, 2009).

In our study with the use of coconut water as a growth regulator also aided in reducing the time required for the propagation cycle (initiation to rooting) of *V. jatamansi* since no separate rooting experiments or addition of PGR's was required to induce proficient roots since supplementation with coconut water was sufficient to produce profuse rooting of the plants (Fig. 3f). Root induction in *Valeriana* species is very critical since the induction of roots at high frequency will aid in producing valuable phytochemicals rapidly to meet the demands of the drug industries. Chen et al. (2014) reported that some *Valeriana* species have been genetically transformed by *Agrobacterium rhizogenes* to induce hairy root cultures, (Zebarjadi et al., 2011). When leaf explants were used, IBA did not induce callus or adventitious shoots however it induced adventitious roots (Chen et al., 2014). The *in vitro* raised plants were hardened in the plant containment facility (Fig. 4a, b) and it was observed that the plants raised in media containing coconut water (10%) grew proficiently with high rooting capacity compared to that of plants raised in medium containing BAP (Fig 4c, d). Formation of rhizomes was also observed in the plants within 45 days of acclimatization which is a valuable outcome since the majority of bioactive constituents are reported to be present in the roots and rhizomes (Fig. 4e). The growth-promoting ability of coconut water was significantly witnessed both during the *in vitro* culturing of the explants in terms of shoot and root formation as well as after acclimatization of the plants in a containment facility.

Genetic fidelity analysis of *in vitro* plants

Ascertaining the genetic homogeneity between the mother plant, *in vitro* regenerated plants and the acclimatized plants are vital in the case of medicinal or plants that are commercially important. Especially if the aim of the research is towards commercialization of the particular plant species for its pharmaceutical importance. ISSR and RAPD primers have been successfully used to demonstrate clonal stability and to detect possible genetic variations within the *in vitro* regenerated plantlets in several *in vitro* propagated medicinal and commercially important plants species, like *Withania sominifera* (L.) Dunal (Nayak et al., 2012) *Alhagi maurorum* (Agarwal et al., 2015), and *Withania coagulans* Dunal (Tripathi et al., 2018). In the present study, 35 RAPD primers were analyzed initially out of which, ten primers (OPA O8, OPA 10, OPA 18, OPB 07, OPD 04, OPE 08, OPC 11, UBC 210, UBC 292 and UBC 465) gave similar and reproducible banding patterns between the *in vitro* raised plantlets and the mother plant (Tab. 1).

Tab. 1: List of RAPD primers used to confirm the genetic fidelity of *V. jatamansi*.

Primer Name	Sequence (5'-3')	Length (bp)	Annealing Temperature (˚C)	No. of Fragments
OPA 08	GTGACGTAGG	10	44.2	2
OPA 10	GTGATCGCAG	10	44.2	2
OPA 18	AGGTGACCGT	10	44.2	6
OPE 08	TCACCACGGT	10	44.2	3
OPC 11	AAAGCTGCGG	10	44.2	3
UBC 292	TGCCGAGCTG	10	44.2	2
OPB 07	GGTGACGCAG	10	41.4	5
OPD 04	TCTGGTGAGG	10	44.8	3
UBC 210	CCGGGGTTTT	10	40.4	2
UBC 465	AGCTGAAGAG	10	40.7	4

Further, the 2-month plants which were acclimatized in the plant containment facility were also checked for their genetic homogeneity and it was found that the banding pattern was similar to that of the mother plant (Fig. 5a-d). These results indicated that there was no variation in the genetic makeup among the mother, *in vitro* raised and subsequently hardened plantlets. Similarly, out of the 10 primer sets of ISSR markers used, 5 primers (HB 08, HB 09, HB 10, HB 11, and 17898 B) gave reproducible bands (Tab. 2). Similar to the results obtained from RAPD analysis 5 ISSR primers confirmed the genetic homogeneity of the *in vitro* regenerated and subsequently acclimatized plants with that of the mother plant (Fig. 5e-h). It has been reported that using nodal segments as explants results in the clonal propagation of true-to-type plants in *V. jatamansi* (Purohit et al., 2015). Similar to our results, Kumar et al. (2012) used RAPD markers to characterize the genetic diversity in a representative population of *V. jatamansi*. Jugran et al. (2013a, b, 2015) also reported the use of ISSR markers to identify the genetic similarity between *V. jatamansi* populations, however, most of these reports were based on the wild population in *V. jatamansi* Jones. Purohit et al. (2015) reported genetic fidelity analysis of the micropropagated *V. jatamansi* plants using ISSR markers. In the present research, both RAPD and ISSR markers were successfully used to confirm the genetic fidelity and absence of somaclonal variations among the *in vitro* regenerated plants.

Tab. 2: List of ISSR primers used to confirm the genetic fidelity of *V. jatamansi*.

Primer Name	Sequence (5'- 3')	Length	Annealing Temperature (˚C)	No. of Fragments
HB 08	GAGAGAGAGAGAGG	14	52.3	1
HB 09	GTGTGTGTGTGTGG	14	52.3	3
HB 10	GAGAGAGAGAGACC	14	52.3	4
HB 11	GTGTGTGTGTGTCC	14	52.3	3
17898 B	CACACACACACAGT	14	52.3	1

GC-MS analysis

The GC-MS analysis of *in vitro V. jatamansi* methanol extract fractions revealed the presence of 21 compounds which included six major bioactive compounds (Tab. 3). From the GC-MS spectra it was found that compounds 9,12-octadecadienoic acid, 9-octadecenoic acid (Z), methyl ester, Heptadecanoic acid 16 methyl, Hexadecanoic acid, Methyl ester (Fig. 6) was found to be in abundant along with the major compound of interest valeric acid also known as pentanoic acid in *V. jatamansi*. These bioactive compounds including Hexadecanoic acid and valeric acid have been reported to contain antioxidant, anti-infl mmatory, antihyperlipidemic, antimicrobial and neuroprotective effects (Ukwubile et al., 2019). Our results are in accordance with reports by Liu et al. (2013) who determined the chemical composition and insecticidal activities of essential oil from *V. jatamansi* roots against booklice, *Liposcelis bostrychophila* Badonnel. A total of 27 compounds were reported in their study using GC-MS whereas Das et al. (2011) analyzed the terpenoid compositions and antioxidant activities of two essential oils from Indian valerian essential oils and reported twenty-one compounds.

Conclusion

In vitro propagation methods can be effectively used to propagate medicinally important and commercially valuable plant species. *V. jatamansi*. is one such plant species where conventional propagation is hindered by various limitations including seed viability, poor rate

Tab. 3: List of major compound detected in GC-MS analysis of in vitro roots of *V. jatamansi*.

Name of compound	Retention Time (min)	Molecular Formula	MW (g/mol)	Area/Height ratio
Alpha.-d-glucopyranoside	22.027	$C_7H_{14}O_6$	194.1	15.98
1-Pentanol, 2-methyl-, acetate	22.384	$C_8H_{16}O_2$	144.2	6.82
9,12- Octadecadienoic acid	33.275	$C_{19}H_{34}O_2$	294.4	2.33
9-Octadecenoic acid	33.444	$C_{19}H_{36}O_2$	296.5	2.64
Hexadecanoic acid	30.199	$C_{17}H_{34}O_2$	270.5	2.42
Heptadecanoic acid	33.989	$C_{19}H_{38}O_2$	298.5	2.58
Pentanoic acid (Methyl Valeric Acid)	40.676	$C_6H_{12}O_2$	116.1	1.44

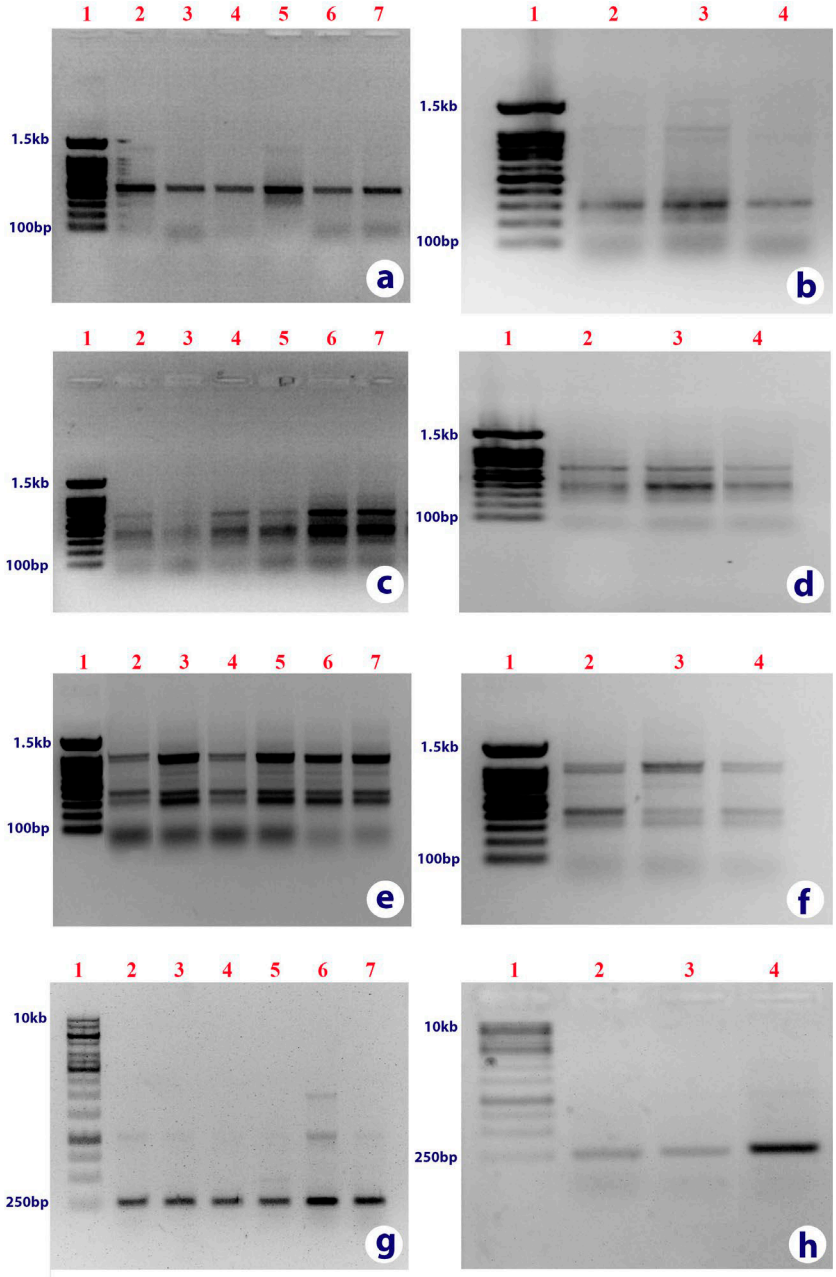

Fig. 5: Representative image depicting the genetic fidelity analysis in in vitro, mother and acclimatized plants of *V. jatamansi* using RAPD and ISSR primers (**a**) OPB 07 Primer **1**-marker, **2** to **6** *in vitro* plants; **7** mother plant; (**b**) OPA 07 Primer **1**-marker, **2** mother plant; **3** and **4** acclimatized plants; (**c**) OPA 08 Primer **1**-marker, **2** to **6** *in vitro* plants **7** mother plant; (**d**) OPA 08 Primer **1**-marker, **2** mother plant; **3** and **4** acclimatized plants (**e**) HB 08 Primer **1**-marker, **2** to **6** *in vitro* plants; **7** mother plant;(**f**) HB 08 Primer **1**-marker, **2** mother plant: **3** and **4** acclimatized plants; (**g**) HB 09 Primer **1**-marker, **2** to **6** *in vitro* plants **7** mother plant; (**h**) HB 08 Primer **1**-marker, **2** mother plant; **3** and **4** acclimatized plants

of germination and seed dormancy. The protocol developed here is highly reproducible, rapid and efficient for high-frequency micropropagation in *V. jatamansi*. The genetic fidelity analysis using two different markers to assess the similarity with the mother plant was carried out and no variations were detected in both tissue culture-derived plantlets and the subsequently acclimatized plants of *V. jatamansi*. Further, GC-MS analysis revealed the accumulation and detection of valuable bioactive compounds like valeric acid in the roots of *in vitro* raised plants. The whole process of plant regeneration to planting in the fields can be achieved within 4 months which is relatively rapid and this efficient protocol can be used for mass propagation of true to type *V. jatamansi* plants in order to meet the ever-increasing demands of the pharmaceutical industries.

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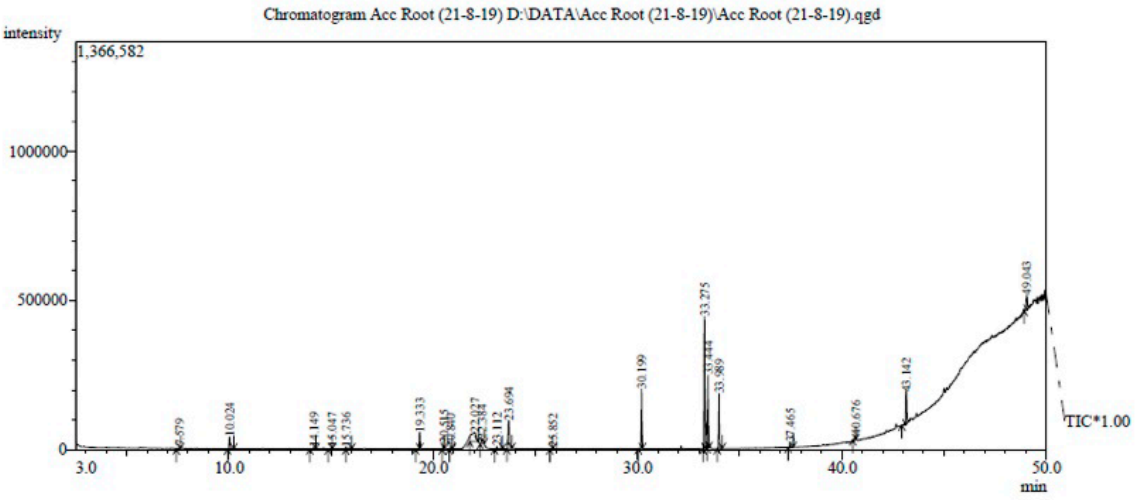
Conflict of interest

No potential conflict of interest was reported by the authors.



Peak Report TIC

Peak#	R.Time	I.Time	F.Time	Area	Area%	Height	Height%	A/H	Mark	Name
1	7.579	7.425	7.625	33990	0.73	8198	0.52	4.15	MI	Proline
2	10.024	9.933	10.233	136655	2.94	40864	2.60	3.34	MI	dl-Malic acid, dimethyl ester
3	14.149	13.958	14.258	11053	0.24	7312	0.47	1.51	MI	1-Naphthoic acid, tridec-2-ynyl ester
4	15.047	14.858	15.058	18074	0.39	7086	0.45	2.55	MI	1-PYRIDINEACETIC ACID, 4-(AMINOC
5	15.736	15.717	16.017	30390	0.65	7849	0.50	3.87	MI	2-PROPANOL, 1,3-DICHLORO-
6	19.333	19.133	19.333	89290	1.92	56041	3.57	1.59	MI	ENDO-3-HYDROXYMETHYLTRICYCL
7	20.515	20.433	20.533	47008	1.01	18513	1.18	2.54	MI	2-Nitrophenyl acetate
8	20.840	20.792	20.942	18091	0.39	5152	0.33	3.51	MI	TRANS-2-IODOCYCLOHEXYL ACETA
9	22.027	21.792	22.192	496639	10.68	31071	1.98	15.98	MI	.ALPHA.-D-GLUCOPYRANOSIDE, ME
10	22.384	22.292	22.492	115440	2.48	16934	1.08	6.82	MI	1-PENTANOL, 2-METHYL-
11	23.112	23.000	23.400	35402	0.76	8246	0.52	4.29	MI	ISOXAZOLE, 3-(3-BUTENYL)-5-METH
12	23.694	23.600	23.850	442676	9.52	93754	5.97	4.72	MI	3-Ethyl-5-methyl-1-heptyn-3-ol
13	25.852	25.708	25.858	13303	0.29	5336	0.34	2.49	MI	1H-TETRAAZOLE-1,5-DIAMINE #
14	30.199	30.033	30.233	483263	10.39	199677	12.71	2.42	MI	HEXADECANOIC ACID, METHYL EST
15	33.275	33.200	33.300	1118716	24.05	442534	28.17	2.53	MI	9,12-Octadecadienoic acid, methyl ester
16	33.444	33.400	33.500	639892	13.76	242796	15.45	2.64	MI	9-Octadecenoic acid (Z)-, methyl ester
17	33.989	33.900	34.150	476651	10.25	184830	11.76	2.58	MI	HEPTADECANOIC ACID, 16-METHYL-
18	37.465	37.367	37.617	48950	1.05	16737	1.07	2.92	MI	TETRADECANOIC ACID, 12-METHYL-
19	40.676	40.533	40.683	23233	0.50	16168	1.03	1.44	MI	3-HEPTANOL
20	43.142	42.942	43.142	176213	3.79	110930	7.06	1.59	MI	12-METHOXY-1-NITROBICYCLO[9.3.1
21	49.043	48.925	49.075	196029	4.21	51153	3.26	3.83	MI	Glaucyl alcohol

Fig. 6: GC-MS analysis of in vitro root extracts of *V. jatamansi* plants derived from media supplemented with coconut water.

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ORCID:
Bijaya Pant: https://orcid.org/0000-0001-5614-6031
Sushma Pandey: https://orcid.org/0000-0002-7653-7184
Sathish Sundararajan: https://orcid.org/0000-0001-6396-1341
Sathishkumar Ramalingam: http://orcid.org/0000-0002-3145-7324

Address of the corresponding author:
Prof. Bijaya Pant, Central Department of Botany, Tribhuvan University, Kathmandu, Nepal- 2642
E-mail: b.pant@cdbtu.edu.np