IN VITRO MASS PROPAGATION AND PRODUCTION OF BIOACTIVE METABOLITES FROM *VALERIANA JATAMANSI* JONES AND *TINOSPORA CORDIFOLIA* (WILD.) HOOK. F. & THOMS

A THESIS SUBMITTED TO THE

CENTRAL DEPARTMENT OF BOTANY

INSTITUTE OF SCIENCE AND TECHNOLOGY

TRIBHUVAN UNIVERSITY

NEPAL

FOR THE AWARD OF

DOCTOR OF PHILOSOPHY

IN BOTANY

BY

SUSHMA PANDEY

AUGUST 2023

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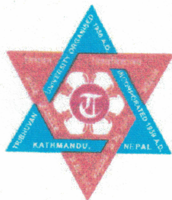

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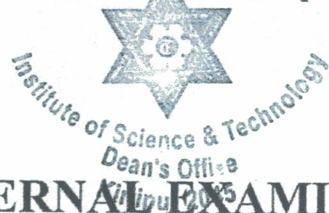

EXTERNAL EXAMINERS

The Title of Ph.D. Thesis: " *In Vitro* Mass Propagation and Production of Bioactive Metabolites from *Valeriana Jatamansi* Jones and *Tinospora Cordifolia* (Wild.) Hook. F. & Thoms "

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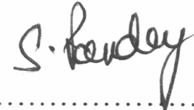
(Dr. Surendra Kumar Gautam)
Asst. Dean

DECLARATION

Thesis entitled "*In vitro* mass propagation and production of bioactive metabolites from *Valeriana jatamansi* Jones and *Tinospora cordifolia* (Wild.) Hook.F. & Thoms" which is being submitted to the Central Department of Botany, Institute of Science and Technology (IoST), Tribhuvan University, Nepal for the award of the degree of Doctor of Philosophy (Ph.D.), is a research work carried out by me under the supervision of Prof. Dr. Bijaya pant, Central Department of Botany, Tribhuvan University, Nepal.

This research is original and has not been submitted earlier in part or full in this or any other form to any university or institute, here or elsewhere, for the award of any degree.



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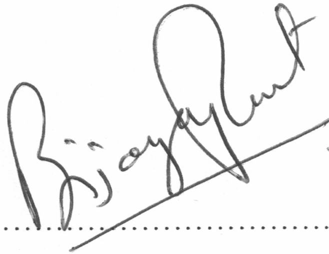
Sushma Pandey

RECOMMENDATION

This is to recommend that Mrs. SUSHMA PANDEY has carried out research entitled "*In vitro* mass propagation and production of bioactive metabolites from *Valeriana jatamansi* Jones and *Tinospora cordifolia* (Wild.) Hook.F. & Thoms" for the award of Doctor of Philosophy (Ph.D.) in Botany under my supervision. To my knowledge, this work has not been submitted for any other degree.

She has fulfilled all the requirements laid down by the Institute of Science and Technology (IoST), Tribhuvan University, Kirtipur for the submission of the thesis for the award of Ph.D. degree.



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LETTER OF APPROVAL

Date: 24/08/2023

On the recommendation of Prof. Dr. Bijaya Pant this Ph. D. thesis submitted by Sushma Pandey, entitled "*In vitro* mass propagation and production of bioactive metabolites from *Valeriana jatamansi* Jones and *Tinospora cordifolia* (Wild.) Hook.F. & Thoms" is forwarded by Central Department Research Committee (CDRC) to the Dean, Institute of Science and Technology (IoST), Tribhuvan University.

....

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……………………

Sushma Pandey

August, 2023

ABSTRACT

Valeriana jatamansi Jones (Sugandhawal) is a perennial herb, belonging to the Caprifoliaceae family known for its medicinal and ethnobotanical values. The species is threatened in its natural habitats as its rhizomes and roots are used for the production of medicines in the herbal and pharmaceutical industries due to the presence of valepotriates. Similarly, *Tinospora cordifolia* (Wild.) Hook. f. & Thoms. (Gurjo) is a climbing shrub belonging to the Menispermaceace family and has been shown to have a variety of ethnomedicinal, pharmacological and medicinal activities. *T. cordifolia* is also one of the most commercially used plants in pharmacy. The phytochemical and pharmacological properties of these two species have been extensively studied worldwide. However, in the context of Nepal, they are yet to be explored. In the case of *V. jatamansi* Jones, research has been carried out to optimize an efficient plant tissue culture system for mass propagation. The nodal segments taken from the *in vitro* plants developed on BAP (2 mg/L) with different PGRs at different concentrations and 10% coconut water produced the maximum shoot length (6 cm), shoot number (13.1), root length (7.5 cm), and root number (19.6). RAPD and ISSR markers were used to confirm the genetic integrity of the *in vitro* plants, acclimatised plants, and wild plants. The effects of sodium nitroprusside (SNP), a nitric oxide (NO) donor, and various growth regulators on callus induction and shoot organogenesis of *V. jatamansi* were investigated. 1.5 mg/L of 1-Naphthaleneacetic acid (NAA)+15 M SNP induced the highest callus frequency (91.18%). Maximum *in vitro* shoot multiplication was observed with 10% CW+15 µM SNP (89.32%). In addition, IAA, IBA, NAA and the elicitors methyl jasmonate (MeJA) and salicylic acid were added to adventitious root cultures of *V. jatamansi* Jones to analyse the production and growth kinetics (SA). Among the plant growth regulators studied, NAA (2.0 mg/L) induced maximum biomass (PGRs). MeJA, increased the biomass of the root cultures as compared to SA. Root cultures were analysed for their phytoconstituents and antioxidant potential. Methanolic in *vitro* extracts of the roots of *V. jatamansi* in NAA (2 mg/L) gave the highest content of phenols (55±1.00 mg GAE/g), flavonoids (219±1.00 mg QE/g) and DPPH activity (84.33±0.577%) as compared to the wild plants. Compared to NAA, the elicitor MeJA produced more biomass but less phytoconstituents. SA did not produce significant biomass. The GC-MS analysis showed that the roots treated with NAA

accumulated 50 bioactive compounds, similar to the roots of the wild plants, which contained 48 compounds. Valepotriates such as valeric anhydride, valeric acid, and derivatives were found in areas of the treated roots that had higher concentrations than those of the wild plants. The MeJA-treated cultures accumulated 21 compounds while the SA elicitation accumulated 23 compounds, including derivatives of valeric acid. The examination of gene expression using the sesquiterpene synthase genes revealed that the TPS2 gene expression was significantly higher in both samples (more than five fold increase) while TPS5 had the highest gene expression (16 fold) and TPS6 had a four-fold increase in the NAA treated *in vitro* root cultures compared to the *in vivo* root cultures. In *T.cordifolia*, the explants of nodal segments were first treated in MS with different doses of BAP and/or KIN, with MS+BAP (2.0 mg/L) and the nodal segments were treated with various concentrations of BAP, BAP+KIN, and coconut water for proliferation of which, 5 mg/L induced significant shoot induction (9.0 shoots per explant), and mean shoot length (8.0 cm). Nodal segments proliferation increased when the media were supplemented with nodal segments at 5% and 10%. However, the addition of MS BAP (5mg/L)+10% coconut water induced maximum response with shoot number (24.0) and mean shoot length (12 cm). The genetic fidelity of these plants was also confirmed by RAPD and ISSR marker analyses in wild and *in vitro* cultures. Total phenolic content (TPC) was highest in wild stem followed by *in vitro* plant extract and wild leaves. The TFC content, and antioxidant potential were highest in wild stems followed by the wild leaves and the *in vitro* plant. (TPC; wild stem 128.55±0.491 mg/g, *in vitro* plant 107.33±0.985, wild leaves 105.6847±1.060) (TFC wild stem; 83.61±0.2773 mg/g, *in vitro* plant 58.034±0.148, wild leaves 62.50±0.329). Antioxidant activity; wild stem with (IC50 193.229±0.013), *in vitro* plant with (IC50 219.83±0.468), wild leaves with (IC50 204.386±0.165). GC-MS Analysis of the wild stem revealed the presence of 40 compounds and 31 compounds in the *in vitro* plant detecting various bioactive compounds including palmitic acid. In the present study mass propagation through *in vitro* cultures of *V. jatamansi* and *T. cordifolia*, and phytochemical screening of both *V. jatamansi* and *T. cordifolia* were standardised which is the first of its kind with respect to these species in Nepal. Root cultures establishment, characterization of valepotriates in adventitious root cultures of *V. jatamansi* and expression of the sesquiterpene synthase gene was successfully developed

LIST OF ACRONYMS AND ABBREVIATIONS

°C	:	Degree centigrade
µM	:	Micromolar
2,4-D	:	2,4- Dichlorophenoxyacetic acid
Masl	:	Metre above sea level
A/H	:	Area/Height
AFLP	:	Amplified Fragment Length Polymorphism
ANOVA	:	Analysis of Variance
BAP	:	6-Benzylaminopurine
BM	:	Basal media
Cm	:	Centimeter
CW	:	Coconut water
CTAB	:	Hexadecyltrimethyl Ammonium Bromide
DPR	:	Department of Plant Resources
DPPH	:	2,2-diphenyl-1-picrylhydrazyl
DW/L	:	dry weight per liter
EDTA	:	Ethylene Diamino Tetra Acetate
et al.	:	and others
Etc	:	Etcetera
Eg	:	as an example,
Fig.	:	Figure
FW	:	Fresh weight
G	:	Gram

GC-MS	:	Gas chromatography–mass spectrometry
HCl	:	Hydrochloric acid
HPLC	:	High Pressure Liquid Chromatography
IAA	:	Indole-3-acetic acid
IBA	:	Indole-3-butyric acid
ISSR	:	Inter Simple Sequence Repeats
KN	:	Kinetin
L	:	Liter
Mg	:	Milligram
Ml	:	Milliliter
MS	:	Murashige and Skoog
MeJA	:	Methyl jasmonate
NAA	:	Naphthaleneacetic acid
NaOCl	:	Sodium hypochlorite
NaOH	:	Sodium hydroxide
PCR	:	Polymerase Chain Reaction
Ppm	:	Parts per million
Psi	:	Pound per square inch
PGRs	:	Plant growth regulators
RAPD	:	Random Amplified Polymorphic DNA
RAPD	:	Random Amplified Polymorphic DNA
RT	:	Room temperature
Sig.	:	Significance level
SPSS	:	Statistical Package for Social Sciences

SSR	:	Simple Sequence Repeats
SNP	:	Sodium nitroprusside
SA	:	Salicylic acid
T.U.	:	Tribhuvan University
viz.	:	Namely

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CHAPTER 1

INTRODUCTION

1.1 Medicinal Plants

Since the beginning of human civilization, food and medicine have been derived from plants and plant products. Traditional medical procedures make extensive use of medicinal herbs. Since plant-derived compounds are one of the key ingredients in allopathic treatments, more than 50% of medications are currently produced (Rao *et al*., 2004; Zahra *et al.,* 2020). The importance of medicinal plants provides clues to new areas of research and biodiversity conservation. Nepal Himalaya is a biologically varied ecosystem with global significance that is said to be rich in many types of medicinal plants. About 2000 of Nepal's 6500 blooming plants are regularly employed for traditional medical and healing purposes (Ambu *et al*., 2020). In Nepal, between 75 and 80 percent of the rural population uses these folk treatments. The traditions and culture of Nepalese people living in rural areas are strongly based in the knowledge of the usage of medicinal herbs (Pyakurel *et al*., 2019). There are over 1,624 medical plant species in Nepal, and about 100 species are traded each year according to the Medicinal and Aromatic Plants Database of Nepal (MAPDON), (Sawtee, 2015). According to the Department of Plant Resources (DPR), there are now 819 species of medicinal plants, up from 701 in 2007 (DPR, 2007, DPR, 2016). Till date 238 MAPs species have thus far undergone chemical testing to determine their therapeutic efficacy (Government of Nepal, 2004). There are 285 indigenous plant species from 43 groups in Nepal, and they are important biologically on a worldwide scale (Rajbhandari *et al*., 2011).

MAPs are more prevalent in forests and grasslands of hilly and mountainous terrain above 2,000m in Nepal (EPI, 2017). Before the introduction of chemical medicines, man relied on the healing properties of medicinal plants. Perhaps 90% of the world's population and developing countries still relies completely on raw herbs, shrubs, and unrefined extracts as medicines (Chen *et al*., 2016).

Medicinal plants are the "backbone" of traditional medicine, which means more than 3.3 billion people in less developed countries utilize medicinal plants regularly (Gupta *et al*., 2015). Nepal ranks 25th in global biodiversity richness and 11th among Asian countries (Ministry of Forests and Environment, 2018). The main cause of the decline in the wild population of many plant species, which has led to their inclusion on the rare and endangered list, is the harvesting of these plants for their therapeutic benefits (Pant, 2014). In light of this, *Valeriana jatamansi* Jones and *Tinospora cordifolia* (Willd.) Hook. f. & Thoms, two significant and valuable medicinal plants used traditionally as alternative medicines, were selected for this research investigation.

1.2 *Valeriana jatamansi* Jones

V. jatamansi Jones, also known as Tagar or Sugandhawal, is a perennial herb with a height of 20–70 cm, fibrous roots, and stems that are typically branching. It is a member of the Caprifoliaceae family (Ma *et al*., 2021). Although it is a Himalayan native plant species, it is also found in tropical and subtropical regions of China (Tibet, western and central China, Bhutan, Nepal, northern India, Pakistan, Afghanistan, and Myanmar) (Mabberley and Noltie, 2014). There are roughly 250 species in the genus *Valeriana*, two of which are found in Nepal: *V. barbulata* Diels and *V. hardwickii* Wall. *V. Stracheyi* and *V. Jatamansi* Jones Clarke, C.B. (Press *et al*., 2000; Government of Nepal, 2001). According to (Manandhar, 2002) and (Kanel *et al*., 2017), between 1500 m and 3600 masl, *V. jatamansi* can be found across Nepal. It grows well in a range of habitats, including tree shade, moist slopes, damp forests, stream banks, and the edges of torrents (Prakash *et al*., 1999; ANSAB, 2005; Das *et al*., 2013)

According to Jugran *et al*., (2013), *V. jatamansi* exhibits a variety of morphological and genetic characteristics and is reported to contain a lot of physiologically active components (Raina and Negi, 2015). It bears fruit from June to September and has white or pinkish-white flowers that bloom from April to July. It is made up of transverse, thick, lumpy rhizomes with internodes and nodes. It has short, strong rhizomes that can grow up to 4.5 cm long and typically range in diameter from 0.5-2 cm. Flowering is regarded as crucial for the development of valuable aromatic oils (Prakash *et al*., 1999). Valepotriates, a subclass of iridoids containing an epoxy group and beta-acetoxyisovaleric acids, may be the source of this plant's therapeutic benefits (Kaur *et al*., 1999). The roots and rhizomes of Caprifoliaceae species are used to make

the crude medicine “Valerian” (Houghton, 1999). Two important chemical components, valepotriates and sesquiterpenoids, are responsible for the pharmacological activity of valerian (Mathela *et al*., 2005). This plant species has several use in both conventional and contemporary medical practices. In the taste, medicinal and fragrance industries, extracts from the roots and rhizomes as well as essential oil are employed. The species rhizomes and roots are recognized to treat a wide range of conditions and illnesses, including obesity, skin conditions, epilepsy, insanity, and skin poisoning (Prakash *et al*., 1999).

Due to its significant medicinal value, Nepal has outlawed the export of this species, and research and development efforts have focused on it (Chaudhary *et al*., 2016). It is one of Nepal's most over-exploited species due to its great utility (Charmakar *et al*., 2021). Due to an increase in demand from the pharma and fragrance industries on both domestic and foreign markets, *V. jatamansi* commerce has seen a remarkable increase in both volume and value over the years.

In the meantime, unrestricted harvesting from the wild has led to an increase in overexploitation of the resource base (Charmakar *et al*., 2021). Understanding the distribution, production, use, and trade of medicinal plants is crucial because of unsustainable harvesting from the natural habitat and climate change. It is possible to employ these medicinal plants in a scientific manner, which will aid in their management, sustainable production and long-term preservation. The Government of Nepal (GoN) has designated *V. jatamansi* as one of the 30 most crucial medicinal and aromatic plants for the economic development of the nation and one of the 12 priority medicinal plants of Nepal for research and commercial cultivation in order to better conserve these highly beneficial plants (DPR, 2006). In order to prevent unsustainable harvesting, the Nepalese government has made it illegal to export raw *V. jatamansi* (MFSC, 2014).

Traditionally, *V. jatamansi* is spread through seeds, but it has been noted that this method has drawbacks because the seeds spend more time dormant in the natural environment (Kaur *et al*., 1999). *In vitro* propagation on a wide scale quickly is still an option, though, developments in plant tissue culture methods have increased in recent years. Rapid clonal multiplication, the generation of significant phytochemicals, and

the preservation of the germplasm of rare, endangered, and threatened medicinal and aromatic plants are additional benefits of *in vitro* culture techniques (Pant, 2014).

Recent years have seen a major decline in the plant due to unrestrained wild harvesting, necessitating its replenishment, culture, and research on *in vitro* growth as a replacement source of the plant and its medically and commercially important components. *V. jatamansi* is one of the Himalayan range's most heavily exploited plant species as a result of overexploitation by the drug industry for the lucrative compounds. It is necessary to replace and cultivate this plant because the indiscriminate gathering of it over the past ten years has resulted in its exhaustion in the wild. As of now, plants gathered from the wild help meet all the demands of different sectors (Gupta *et al*., 2006; Pant, 2014; Pant *et al*., 2021). *In vitro* culture techniques are in high demand right now since they not only provide a practical tool for rapid growth but also help to preserve medicinal plants that are in danger of extinction (Abraham *et al*., 2010).

1.3 *Tinospora cordifolia* (Wild.) Hook. F. & Thoms

The Menispermaceace family includes the widely spreading, glabrous, succulent, woody climbing shrub known as *Tinospora cordifolia* (Wild.) Hook. f. & Thoms. It grows up to an altitude of 1,200 masl and is dispersed throughout the tropical countries of Nepal, India, Sri Lanka, and China. In woods and other environments, it thrives in the tropical zone (DPR, 2007). About 32 distinct species of the genus *Tinospora* are found in tropical Africa, Madagascar, Asia to Australia, and the Pacific Islands (Forman, 1981; Mabberley, 2005).

Only two *Tinospora* species, *Tinospora cordifolia* (Wild.) Hook. f. & Thoms. and *Tinospora sinensis* (Lour.) Merr, have been identified in Nepal (Checklist of flowering plants of Nepal, 2000). Gurjo is the word for it in Nepali. *T. cordifolia* is a dioecious, perennial, glabrous, deciduous climber that grows on a variety of hedges and trees up to a height of 1000 m. It is often found growing in dry deciduous woods in tropical and subtropical countries. *T. cordifolia*, a well known herbal treatment in Nepal, India, Southeast Asia and other areas of the world has been shown to possess numerous ethnomedicinal, pharmacological, and medicinal actions. However, systematic current information on the plant's therapeutic efficiency is insufficient. One of the plants used most economically in pharmaceuticals is *T. cordifolia*. Other *Tinospora* species, including *T. sinensis* (Lour.) Merr, are replaced for or mixed together with *T. cordifolia*.

There are few characteristics that can be used to distinguish between *T. sinensis* and *T. cordifolia*, despite the similarities in their microscopic characteristics. The identifying characteristics are: Sclerenchymatous sheaths in the cortical sections of *T. cordifolia* dissolve into sporadic, irregular patches, whereas in *T. sinensis*, they are fragmented into parts that seal the vascular bundle and endure even after additional secondary growth. *T. cordifolia* lacks crystals, whereas *T. sinensis* has a sizable calcium oxalate crystal inside the lumen of every cork cell. In comparison to *T. sinensis*, *T. cordifolia* has more mucilaginous cells. *T. cordifolia* has fewer vascular strands, but *T. sinensis* has more. In comparison to *T. sinensis*, Xylem is well developed in each strip of vascular strand in *T. cordifolia*. When compared to *T. sinensis*, *T. cordifolia* pith is large and made up of cells with thin walls. *T. cordifolia* contains more starch than *T. sinensis* does (Ragunathan and Mitra, 1982). Approximately 10,000 tonnes are thought to be required annually for this species to prepare crude herbal medications under the Indian system of medicine (ISM) (Singh, 2004). The presence of many bioactive substances, including glucosides and alkaloids like berberine, is what gives this plant its medicinal importance (Singh *et al.*, 2003). Particularly in Ayurveda, the stems, roots, leaves, and starch extracted from the stems and roots are used for therapeutic purposes (Singh *et al.*, 2003). The water extract from the root is used to treat leprosy and is a potent emetic for visceral blockages (Nayampalli *et al.*, 1982). Additionally, the root has anti-diabetic properties (Gupta *et al.,* 1967). The stem, leaves, barks, and roots extracts exhibit potent antioxidant properties (Stanley *et al.*, 1999). Decoction of the leaves is used to cure gout, while the bitter component found in the stem is used to treat debility, dyspepsia, fever, and urinary illness (Singh *et al.*, 2003). The leaves, bark, and roots of this plant are primarily responsible for its pharmaceutical significance because they contain a variety of bioactive substances, including alkaloids, glycosides, lactones, saponins, tannins, steroids, polysaccharides, and aliphatic compounds with various medicinal uses. Immune modulatory or immunostimulatory, anti-neoplastic, anti-oxidant, anti-hyperglycemia, anti-hyperlipidemia, anti-tuberculosis, anti-osteoporotic, anti-angiogenic, anti-malarial, and anti-cancer action are a few of the key applications (Nasreen *et al.*, 2010; Sinha, 2015). Intense interest in the plant has been sparked by the identification of its active ingredients and their biological significance in the prevention of disease (DPR, 2007). Numerous chemical substances have been identified from this plant, including aporphine, alkaloids, diterpenes, berberine, palmatine, tembertarine, magniflorine, choline, and tinosporin (Forman *et al.*, 1981).

Methanol stem extracts of *T. cordifolia* are efficient in treating microbiological infections (Mabberley *et al*., 2005). According to reports, urinary pathogens like *Klebsiella pneumonia* and *Pseudomonas aeruginosa* were inhibited by an aqueous, ethanol, and acetone extract of the leaves and stem of *T. cordifolia* (Raghunathan, 1982). The various members of this family are a great source of polysaccharides, terpenes, and alkaloids that have shown anti-inflammatory (Tiwari *et al*., 2014), immune-stimulatory (including phagocytosis) (Sharma *et al*., 2012), anti-diabetic (Sharma *et al*., 2013), and anti-oxidant (Sharma *et al*., 2013) activity (Khan *et al*., 2011). From various water and solvent fractions, compounds from several classes, including sesquiterpenes, phenylpropanoids, and alkaloids, were extracted and identified (Kapil and Sharma, 1997). It has been demonstrated that a commercially available vaccination for infectious bursal illness enhances the immune protection response in chicks when the stems of *T. cordifolia* are extracted (Sachan *et al*., 2019). The surface glycoprotein, receptor binding domain, RNA dependent RNA polymerase, and major protease of the SARS-CoV-2 virus had strong binding affinities for the *in vitro* compounds berberine, isocolumbin, magnoflorine, and tinocordiside (Sagar and Kumar, 2020). Because *T. cordifolia* has a significant amount of phenolics, the development of plant tissue culture technology offers a huge potential for the quick replication of plant germplasm. It is a potent strategy for the immediate to medium-term preservation of significant plant species. The return of plants into their original habitats and large-scale plant propagation are made possible through tissue culture technologies (Leander and Rosen, 1988; Lernda and Svensson, 2000).

1.4 Importance of plant tissue culture in secondary metabolites production

Plant tissue culture methods can be viewed as a developing technology for large-scale reproduction, research on germplasm conservation, the manufacture of physiologically active chemicals, and the enhancement of genetic resources (Mulabagal and Tsay, 2004). Plant tissue culture technology creates homogenous, sterile, and compatible *in vitro* regenerated plants, which are then utilized to characterize biochemical processes and synthesize bioactive chemicals. Due to simple extraction procedures and the absence of significant pigment amounts, the chemicals isolated from tissue cultures are simple to purify, which likely helps to lower production and processing costs. These significant developments have caused research in this field to exceed expectations

(Fajili *et al*., 2022). Due to their dependence on by the majority of the pharmaceutical businesses, medicinal plants are now becoming endangered. These organic molecules are challenging to chemically synthesise due to their complexity. An alternate remedy in this regard is the *in vitro* generation of plant secondary metabolites (Fajili *et al*., 2022).

The uses of secondary metabolites is highly significant in the field of agrochemicals, pharmaceuticals, fragrance, food additives and pesticides. Besides these secondary metabolites are performing a potent role in fighting COVID-19 (Khan *et al*., 2021). Plants are a rich source of bioactive secondary metabolites such as phenolics and flavonoid compounds (Dai & Mumper, 2010). The identification of active compounds is needed for the validation of plants in the discovery of therapeutic drugs (Proestos *et al*., 2006). Gas chromatography and mass spectrometry (GC-MS) has become an established technological platform for the detection and identification of secondary metabolites in the plants. According to the World Health Organization (WHO), medicinal plants are the major resources of medicines for the prevention and treatment of several diseases. Approximately 25% of all prescriptions contain one or more active ingredients from plants (Fabricant & Farnsworth, 2001; Hossain, 2011). Cancer chemoprevention by phytochemicals derived from plants has shown promising results against various malignancies. Therefore, they potentially represent an inexhaustible source of chemicals for the discovery of new drugs. Plants have a wide variety of natural compounds such as phenols, flavonoids, vitamins, anthocyanins, carotenoids, and bibenzyls (Panche *et al*., 2016; Khoo *et al*., 2017). Plant-derived compounds are rich in antioxidant properties. Naturally occurring antioxidants have been reported to function as oxygen quenchers, peroxide decomposers, enzyme inhibitors, and synergists (Birben *et al*., 2012).

Plants naturally occurring antioxidants are thought to be the source of the health benefits (Lobo *et al*., 2010). Plant phenolics, which are significant phytochemicals, have been thought to have a high antioxidant capacity by blocking the enzymes that produce ROS and decreasing highly oxidized ROS (Dai & Mumper, 2010). As a result, phenolic compounds have drawn increased interest as potential treatments and preventative measures for a variety of disorders linked to oxidative stress. The primary sources of biologically active metabolites that are frequently utilized to treat a wide range of ailments, particularly in impoverished countries where access to healthcare is

limited, are traditionally used medicinal plants. Despite the long history of use of many plants, little scientific research has been done on them.

1.5 Role of plant growth regulators in *in vitro* cultures

Plant growth regulators (PGR'S) are frequently used in the production of numerous secondary metabolites, such as medicinal substances (Vanisree and Tsay, 2004). The ability of the plant cells to regenerate plant tissues from somatic differentiated cells also called as organogenesis is stimulated by Plant Growth Regulators. These PGR'S when added exogeneously, interact with other endogenously produced phytohormones to modulate developmental processes in plants (Lee *et al*., 2019). Out of various used PGR'S, auxins and cytokinins are the most frequently used PGR'S for most applications (Philips *et al*., 2019). The importance of PGR'S in cell culture may also be helpful in triggering production of specialized metabolites which elicit cells in adapting and surviving in *in vitro* conditions (Ramabulana *et al*., 2021).

Signalling molecules such as sodium nitroprusside (SNP), a nitric oxide (NO) donor, have been successfully exploited to produce high *in vitro* proliferation rates in a variety of plant species in addition to conventional PGRs that are used in plant tissue culture systems. Exogenous SNP supplementation has been shown to improve *in vitro* propagation in a number of plant species, including Arabidopsis, tobacco, and parsley (Tun *et al*., 2001), cucumber (Pagnussat *et al*., 2002). According to all of these investigations, NO and/or SNP contribute to cell division, shoot regeneration, and multiplication.

1.6 Adventitious root cultures

One of the most important systems for increasing biomass and producing metabolites with high commercial value is adventitious root cultures (Baque *et al*., 2012; Wang *et al*., 2013). Due to the quick development of biotechnology, adventitious root cultures are now the primary method for producing secondary metabolites from medicinal plants (Rahmat *et al*., 2019). Utilizing hairy roots and adventitious roots to their full potential might have beneficial synergistic effects that boost biomass yield and the accumulation of bioactive chemicals from medicinal plants (Masooma *et al.,* 2022). Due to their great efficiency and ease of scaling up to bioreactors, hairy and adventitious root cultures have been the most frequently used for this purpose (Cui *et al*., 2013). According to

(Sivanandhan *et al*., 2012) manipulating biotic and abiotic elicitors can encourage the accumulation of medicinally significant metabolites in plant culture systems, indicating that elicitation is a useful method for enhancing the production of secondary metabolites.

1.7 Genetic clonal fidelity using molecular markers

There is always a potential of differences arising within the *in vitro* produced plants for their genetic homogeneity whenever a plant tissue culture technology is used for plant multiplication. Variations in somaclonal structure are frequently seen in *in vitro* produced plants. Among the several markers, RAPD and ISSR applications are more prevalent due to their excellent reproducibility, dependability, simplicity, cost effectiveness, and more frequent use by researchers (Sultana *et al*., 2022). Molecular markers are frequently employed to examine the variation within the germplasm in order to determine the genetic similarity between the *in vitro* generated plantlets and the mother plant (Mittal and Sharma, 2017).

The current research workout to advance the technology of plant tissue culture using different biotechnological strategies for both these plant species. This research focuses on using different explants for *in vitro* propagation of *V. jatamansi* and *T. cordifolia* collected from Nepal. Phytochemical analysis for the total phenolics, flavonoids, and antioxidant activity of the wild roots and also the *in vitro* roots was determined and compared followed by GC MS of the wild plants to characterize the valuable secondary metabolites. Additionally, using RAPD and ISSR markers for both the plants, genetic homogeneity was established for the mother plants, *in vitro* regenerated plants, and the acclimatized plants. Since roots contain promising medicinal properties in *V. jatamansi* Jones, adventitious root cultures were established and further GC MS analysis of the roots was performed to characterize the valuable bioactive compounds. An investigation was made to find out how SNP and other growth hormones interact with each other to affect the callus induction and shoot proliferation in the medicinally significant and critically endangered plant *V. jatamansi*, since NO and/or SNP plays a role in cell division as well as in shoot regeneration and multiplication.

In light of this background, attempts were made to determine how PGRs and elicitors MeJA and SA affected adventitious root cultures and the subsequent accumulation of high-value valepotriates in *V. jatamansi*. Further gene expression studies using

sesquiterpene gene synthase, which is responsible for the production of valepotriates were carried out to study the gene expression in wild and *in vitro* roots of *V.* jatamansi. This research will not only help in innovation for drug discovery, health, and economic benefit but also will help in the conservation of the two important medicinal plants and their products, and their sustainable utilization of the resources for the benefit of society.

1.8 Rationale

Since *V. jatamansi* Jones and *T. cordifolia* (Willd.) Hook. f. & Thoms. which are selected for the present research are in the threatened category, there is an urgent need for their conservation (Charmakar *et al*., 2021; Singh *et al*., 2010; Singh *et al.,* 2013). Tissue culture is the only technology that can preserve the germplasm as well as mass propagation is possible without disturbing the natural habitat (Pant *et al*., 2014; Pradhan *et al*., 2014). *V. jatamansi and T. cordifolia* is an important plant of the Ayurvedic System of Medicine and found in various classical texts for the treatment of many diseases. At present times, this drug has been subjected to numerous chemicals, pharmacological, pre-clinical, and clinical investigations and many new therapeutic applications have been indicated (Ma *et al*., 2021).

Micropropagation of *in vitro V. jatamansi* and *T. cordifolia* is very important as we can produce large number of plants in a short duration of time irrespective of seasonal variation. Micropropagation through nodal segments is very significant as exact clones can be obtained. Plant regeneration *in vitro* is very productive as the whole plant can be regenerated in a very short time. With increasing demand, these species are over exploited by people and even the pharmaceutical company. Thus optimizing a standardized protocol, every portion of the plants can be utilized as explants using different combinations of growth regulator (Moraes *et al*., 2021).

Flavonoids, valepotriates, flavone glycosides, essential oils, lignans, phenolic compounds, sesquiterpenoids, sesquiterpenoid glycosides, and other phytochemicals are present in the crude plant of *V. jatamansi* Jones. The two main substances that were predominantly separated from the roots and rhizomes of these plants are valepotriates and flavonoids. They have a wide range of biochemical abilities, including digestive, neuroprotective, antiviral, antidepressant, anti-tumor, and anti-oxidant capabilities. The most effective ones among them for treating conditions like irritable bowel syndrome

include valtrate, acevaltrate, baldrinal, didrovaltrate, and 11-ethoxyvi burtinal. They also have antispasmodic and analgesic effects (Wang *et al*., 2017; Ma *et al*., 2021). This plant's roots and rhizomes have long been used in traditional medicine to treat a variety of conditions, including anxiety disorders, hypnotic sedation, epilepsy, snake poisoning, and hyperlipidemia (Prakash 1999; Jugran *et al*., 2017). This herb is a key raw ingredient in the health medication tagara and is therapeutically effective at treating depressive insomnia (Li *et al*., 2020).

In *T. cordifolia* the various metabolites present are in the form of alkaloids and terpenoids which are berberine, palmitine, tinosporide, tinosporaside, tinocordioside, tinocordifolioside, sesquiterpene, tinocordifolin. The chemical components of the plant, such as diterpenoid lactones, glycosides, steroids, sesquiterpenoids, phenols, aliphatic compounds, essential oils, a combination of fatty acids, and polysaccharides, contributes to the pharmacological effects of the plant. These can be found in various plant body parts, such as the root, stem, and entire section (Sharma *et al*., 2019). The ayurvedic practice has several potent herbal alternatives for chronic cough, inflammation, and respiratory distress which are often seen in the SARS-CoV-2 infection. *T. cordifolia* (wild.) Hook. f. & Thoms formulated in the product form of Giloy Ghanvati, was used as a means of treatment to the SARS-CoV-2 spike-protein inducing disease phenotype in a humanized zebrafish model suggesting that *T. cordifolia* is a highly valuable and useful plant in the pharmaceutical sector (Balkrishna *et al*., 2021). Due to their medicinal values they are being highly collected from the natural habitats thus leading to the depletion of wild natural resources.

To overcome such problem, plant tissue culture technique is being used which will conserve the germplasm of such species and also help to produce the secondary metabolites (Vanisree *et al*., 2004; Gueven, 2012). Secondary metabolites synthesized in the cells of *in vitro* developed plant materials are used for their bioactivity (Lo *et al*., 2004). Biotechnological means for the production of both plant products using plant cell and organ cultures has been considered as an attractive alternative. Genetic fidelity can also be tested between the *in vivo* and *in vitro* obtain plants.

One of the better methods is phytochemical analysis, which is used to isolate many chemical compounds present in the plants. Finally, the *in vitro* regenerated plants can be exported to different parts of the world. Also, gene expression studies of

sesquiterpene synthase responsible for the production of valepotriates using Real-Time PCR and enhancement of the bioactive compounds were done using different elicitors and plant growth regulators in case of *V. jatamansi* Jones. Thus the plant products from the field scale to the lab-scale can be accomplished, as well as the production of significant secondary metabolites that may be advantageous to society.

1.9 Hypothesis

Current research hypothesizes that all the plant cells are totipotent in nature which can produce identical mass of regenerants when provided with suitable environmental conditions. The *in vitro* regenerated plants genetic integrity is also crucial for the establishment of clonal multiplication. Moreover, this technology is useful for the mass production of commercially important species. Extracts of *in vivo* plant material of *V. jatamansi* Jones and *T. cordifolia* are the sources of highly effective phenolics, flavonoids, and antioxidants, as they have bioactive compounds. Similarly, extracts of *in vitro* plant materials of two of the above mentioned species are also the sources of effective phenolics, flavonoids, and antioxidants and they have bioactive secondary metabolites. Plants possess metabolites that can be synthesized in their different tissues or specific cells/tissues which can be produced in culture by optimizing the culture condition. Enhancement of the identified metabolites and gene expression studies using different strategies is possible in the plant cell culture system.

1.10 Objectives

The general objectives of this research is mass propagation of *V. jatamansi* Jones and *T. cordifolia* (Wild.) Hook. f. & Thoms. from different explants in culture medium under aseptic conditions and screening of the bioactive metabolites present *in vitro* callus/suspension cultures by developing enhancement strategies.

1.11 Specific objectives

1. To establish the culture condition and culture type for mass propagation of *V. jatamansi* Jones and *T. cordifolia* (Wild.) Hook. f. & Thoms.
2. To determine the genetic homogeneity between *in vivo* mother plants and *in vitro* grown plants by using molecular markers for *V. jatamansi* Jones and *T. cordifolia* (Wild.) Hook. f. & Thoms.

3. To determine the total phenolics, flavonoids, and antioxidant activity of *in vitro* and *in vivo* plant extract and characterization of bioactive secondary metabolites of *V. jatamansi* Jones and *T. cordifolia* (Wild.) Hook. f. & Thoms.
4. To establish adventitious roots cultures of *V. jatamansi* Jones and enhancement strategies for the production of valepotriates (bioactive secondary metabolites)
5. To study the gene expression of sesquiterpene synthase gene in *V. jatamansi* by Real-time PCR

CHAPTER 2

REVIEW OF LITERATURE

This chapter focuses on the literature which have been reviewed during the present research work.

2.1 Plant Tissue Culture

In vitro plant tissue culture technique is one of the most prominent biotechnological tools conducted in the well sophisticated lab under the aseptic condition. *In vitro* technology is highly applicable to maintain the same clones as mother stock and production of superior clone. The tissue culture techniques have high economic value and are widely used for the improvement of field crops, forest, horticulture and plantation crops for increased agricultural and forestry production (Arditti, 1977).

The Valeriana genus, often called tagar, Indian valerian, or sugandhawal, contains over 250 species. For the creation of valuable aromatic oils, three of these species—*Valeriana jatamansi*, *Valeriana officinalis*, and *Valeriana edulis* are crucial. The plant is polygamous or occasionally polygamo-monoecious, dioecious, perennial, and tetraploid (Prakash, 1999; Rajkumar *et al*., 2011). The species typically grows in muddy, wet areas such as ditches, damp woodlands, and near streams. The species is known to thrive especially in the understory of Qurecus leucotricophora-Pinus roxburghii mixed forests and on grassy environments in the Himalayan region. The species has a wide range of physical and genetic characteristics that impact not only where it appears geographically but also how many active components it contains. The species flowering and fruiting season lasts from March to June. The species reproduces asexually (rhizome) and sexually (seeds) (Jugran *et al*., 2013). The wild *Valeriana jatamansi* Jones plant thrives in the temperate Himalayan region between 1000 and 3000 m altitude. It is also known as Indian valeriana (English), Mushkibala (Kashmiri/Hindi), Sugandhwala, or Tagar (Sanskrit) (Rani *et al*., 2015). Currently, unchecked harvesting of wild plants, primarily for medicinal uses, has led to a rapid decrease of the stock in natural environments. Therefore, it is crucial to preserve this species and use it sustainably (Purohit *et al*., 2015). Traditionally, seeds are used to

spread *V. jatamansi*, but in nature, seeds germinate slowly and are latent for a long time. Root suckers provide an alternative, although their small population size typically makes the process difficult.

Therefore, *in vitro* methods for large-scale multiplication would be an effective choice and have been reported for several Himalayan medicinal herbs (Giri *et al*., 2012; Chandra *et al*., 2006; Pandey *et al*., 2004). These methods are thought to be an effective way to multiply species that are hard to propagate, rare or endangered, and useful for both commercial cultivation and conservation (Nandi *et al*., 2002). Numerous studies have examined the micropropagation of various *Valeriana* species employing explants such leaves, stems, and roots. For instance, (Chen *et al*., 2014) reported that *V. jatamansi* was multiplied through the production of calluses from leaf explants with the addition of 2, 4-dichlorophenoxyacetic acid (2, 4-D) and the induction of shoots with the combination of BA and NAA. In comparison to 6 Furfuryl aminopurine, Kinetin (KIN), or TDZ, the combination of BAP (0.5mg/L)+IBA (0.5mg/L) supplementation had a good impact on plant regeneration and shoot induction rates in *V. officinalis*, according to research done utilizing callus (Zamini *et al*., 2016). Other species in the genus *Valeriana* that have been found to micropropagate include *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* (Bello *et al*., 2004; Abdi *et al*., 2008; Reza *et al*., 2009). Using *V. officinalis* leaf, stem, and root explants, (Zebarjadi *et al*., 2011) observed high-frequency callus induction and shoot regeneration. There are very few reports that show successful micropropagation of *V. jatamansi*, and many of those papers use callus cultures, which can take a lot of time and require frequent subcultures (Purohit *et al*., 2015).

Similar to *T. cordifolia*, which grows on a variety of hedges and trees, it is a rather large, widely-spreading, glabrous, dioecious, perennial deciduous climber. They normally grow up to an altitude of 1000 m in tropical and subtropical dry deciduous woods. It produces flowers that are clearly male and female. While the dry stem shrinks and the bark separates from the wood, the fresh stem has a green succulent bark that is coated in a thin layer of brown bark that is flecked with warty lenticels. The slender branches have parallel ridges and grooves, pendulous fleshy roots, and glabrous bark that is either pale or shiny (Handique *et al*., 2014). There have only been a few studies about the micropropagation of *T. cordifolia* through nodal segments, however the

regeneration techniques they used revealed just a few unstable shoots (Mittal *et al.*, 2017). Nodal explants have been used in the majority of studies in MS basal media with varied combinations and concentrations of plant growth hormones (Handique *et al.*, 2014). An earlier micropropagation procedure was created utilizing North East India's *Tinospora cordifolia* (Handique *et al.*, 2009). This experiment used MS basal medium with various combinations and quantities of BAP, Kinetin, and IAA. Various explants (shoot tip, axillary bud, and cotyledonary node) were cultivated on MS media supplemented with varying amounts of plant growth hormone according to a procedure for micropropagating *T. cordifolia* (Handique *et al.*, 2009). They stated that shoot elongation was greatest in MS medium containing Kinetin (3.0 mg/L) and Gibberellic Acid (0.5 mg/L), while kinetin at 3.0 mg/L showed to be the best for shoot induction. Polyvinylpyrrolidone (PVP) was additionally employed to inhibit phenol exudation in the culture. For shoot proliferation from nodal explants, the effects of various auxins and their combination with cytokinin, however, were ineffective. According to (Handique *et al.*, 2014), callus production was seen in *Tinospora tissue* culture when nodal segments, leaves, and inter-node explants were planted on various hormone combinations in MS Medium. While roots were grown in the medium containing 1.0 mg/L BAP (1.0 mg) and 2.5 mg/L Naphthaleneacetic Acid, only nodal explants demonstrated improved shoot growth in MS medium containing kinetin (1.5 mg/L) (NAA). Production of active chemical constituents through *in vitro* culture is an emerging technology nowadays. In cell suspension cultures generated from leaf explants of *T. cordifolia* (Rao *et al.*, 2008) reported the generation of Berberine, an isoquinolene alkaloid, along with its related analogs protoberberine and palmatine. It has been found that Tinospora cell suspensions produce 5 to 14 times more berberine than the full plant does.

Four week old *Tinospora* leaf, petiole, and stem derived calli were subcultured on MS media and supplemented with various growth regulators in an effort to increase the concentration of berberine in the plant. The basal production medium for berberine *in vitro* production was found to be MS medium with NAA (2 mg/L) supplemented with BA or kinetin, each at 2 mg/L, providing 7.55 and 7.36 g of berberine per gram of calli, respectively. In comparison to callus cultures made from leaves and petioles, those made from stem segments had the highest levels of berberine (Kalimuthu *et al.*, 2007). According to (Verma *et al.*, 2006), hairy roots of *T. cordifolia* were created from shoot

cultures using *Agrobacterium rhizogenes* transformation on a solid yeast mannitol broth medium, while roots were sub-cultured on liquid MS medium containing B5 vitamins and 3% sucrose without hormone under optimal growth conditions. They found that cultures treated with 500 mg/L of L-Tyrosine as a precursor produced more berberine (0.034%) than the control. According to (Singh *et al*., 2003) much chemical research has been done on *T. cordifolia*, and so far, significant chemical compounds have been identified. The majority of the separated ingredients are from a variety of classes, including alkaloids, glycosides, steroids, sesquiterpenoids, phenolics, aliphatic chemicals, and polysaccharides. The return of plants into their original habitats and large-scale plant propagation are made possible through tissue culture technologies (Bhojwani *et al*., 1989). Plant production is scaled up and made more effective through *in vitro* propagation. Additionally, plant cell and tissue culture, genetic engineering, and alternatives to the traditional method for improving medicinal plants may be used. The *in vitro* cultures could be maintained throughout time and expanded as needed. The flow of genetic material both inside and between nations is facilitated through tissue culture (Sehrawat *et al.,* 2002).

2.2 Genetic clonal fidelity

A number of DNA based reliable molecular markers have proven to be the most effective means to study the genetic fidelity in various plant species. Use of available molecular markers such as Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Restriction Fragment Length Polymorphism (RFLP), Inter Simple Sequence Repeats (ISSR), Simple Sequence Repeats (SSR) are frequently used to differentiate between the species and the cultivars and to determine the relationship between the species within the genus and other related groups. RAPD and ISSR are two simple and quick techniques; where the former detects nucleotide sequence and the latter permits detection of polymorphism in inter microsatellite loci using a primer designed from dinucleotide or trinucleotide simple sequence repeats (Parab and Krishnan, 2008).

By demonstrating 100% monomorphism, a study using inter simple sequence repeat (ISSR) markers showed genetic stability for in vitro produced plants. The current *in vitro* clonal production method of this significant medicinal plant is effective because of its high multiplication rate and genetic stability (Purohit *et al*., 2015). Using ISSR

markers, numerous researchers have already shown genetic stability in a number of *in vitro* produced medicinal plants, including *Rosa hybrida* (Senapati *et al*., 2012), *Habenaria edgeworthii* (Giri *et al*., 2012), and *Saussurea involucrata* (Yuan *et al*., 2009). Systematic germplasm sampling and molecular status assessments using DNA marker technology have become standard procedures in recent years.

Among the several DNA polymorphism detection methods (such as RFLP, AFLP and ISSR), RAPD has been extensively utilized to examine clonal integrity and find genetic and somaclonal differences (Jokipii *et al*., 2004). ISSR markers are generally used because this method has comparative advantage over RAPD, SSR and AFLP markers. Because they are quick, simple, and don't require radioactive materials, molecular techniques like random amplified polymorphic DNA (RAPD) analysis are the most often used approach for determining the patterns of genetic variations that already exist in *in vitro* cultures (Mishra and Nishani, 2011). Random amplified polymorphic DNA (RAPD) analysis indicated no evidence of genetic variation in the tissue culture raised plants in *Valeriana officinalis* (Ghaderi and Jafari, 2014).

The Rayalseema region of Andhra Pradesh showed genetic diversity in *T. cordifolia* using random amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSR) markers (Nanzenn *et al*., 2019). When the clonal fidelity of micropropagated plantlets was examined in *T. cordifolia* using the inter simple sequence repeat (ISSR) marker, it was discovered that these plantlets were identical to the mother plant (Mittal *et al*., 2017)

2.3 Role of sodium nitroprusside in enhancing growth of *in vitro* cultures

Nitric oxide (NO) is a small, pervasive, gaseous, and biologically active molecule. Numerous physiological processes, including stomatal closure, photosynthesis, fruit ripening, organ senescence, floral control, and the growth and development of plants, depend on NO (Khan *et al*., 2012; Dong *et al*., 2014; Khan *et al*., 2017). It also activates the plant's defense mechanisms to reduce the harm from oxidative stress.

Nitric oxide has recently been employed to create numerous procedures for plant tissue culture (Rico-Lemus and Rodr'guez-Garay 2014). Due to its characteristics of being a relatively inexpensive, well-documented application, and sustained NO generation, SNP is the most popular NO donor (Zandonadi *et al*., 2010). Exogenous NO and/or SNP supplementation has been demonstrated to improve *in vitro* propagation in a

number of plant species, including Arabidopsis, tobacco, and parsley (Tun *et al*., 2001), cucumber (Pagnussat *et al*., 2002), mung bean (Huang and She, 2003), tomato (Correa-Aragunde *et al*., 2004), Chinese crab apple (Han *et al*., 2009), Di (Sarropoulou *et al*., 2015). Based on these investigations, NO and/or SNP contribute to cell division, shoot regeneration, and multiplication. According to (Kalra and Babbar, 2010), nitric oxide could improve the regeneration response by increasing the number of meristems, and they hypothesized that NO might be crucial in controlling the gene expression responsible for meristem differentiation. Similar findings were made in Glycine max by (Karthik *et al*., 2019) and (Kazemi *et al*., 2019), where exogenous SNP addition improved *in vitro* responses.

2.4 Ethnomedicinal uses of *Valeriana jatamansi* Jones and *Tinospora cordifolia*

One of the sources of raw materials for traditional medicines in the pharmaceutical and fragrance industries is the well-known sub-tropical and temperate traditional medicinal plant known as sugandhawal (*V. jatamansi* Jones). Usefulness of *V. jatamansi* was prominent because of its multiple values. It has long been used for four use categories: herbal remedy, subsistence use, household economy, and religious/ritual use. Subsistence use as tea, incense, food, insect repellent and home economics as marketed for livelihood are categories that show its socioeconomic potentials. Of the use reports on *V. jatamansi*, over 55% were meant for primary health care (Charmakar *et al*., 2021). *V. jatamansi* is revered as a sacred plant in India and used there to treat blood pressure, mental illnesses, and laxative, carminative, and aphrodisiac conditions (Kamboj, 2000). (CSIR, 1976). It has been used to treat dermatitis, epilepsy, insanity, snake poisoning, sleeplessness, obesity, mental problems, and other conditions in China (Zhang and Ding, 2015). In Pakistan, it is used to lessen tension, anxiety, and sadness. The plant's rhizomes and roots were also harvested and exchanged for additional household revenue in addition to being used in households and the local economy. According to (Schippmann *et al*., 2002), as human demands, populations, and commercial trade rise, there is a growing demand for wild species. This has a negative impact on the ecosystem, reduces genetic diversity, and removes incentives to protect wild populations (IUCN, 2002).

T. cordifolia is widely used as a tonic in the Indian Ayurvedic medical system and also has anti-periodic, antispasmodic, anti-inflammatory, anti-arthritic, and anti-allergic

effects. It is also highly used for treating diabetes (Handique, 2014). Additionally, it is utilized in numerous ayurvedic preparations for the treatment of various ailments (Shanti and Nelson, 2013). *T. cordifolia* exhibits effective immunological modulation. The stem of *T. cordifolia* being used as medicine is reported in Ayurvedic pharmacopoeia of India. The extract of its stem is useful in skin diseases. The root and stem of *T. cordifolia* are prescribed in combination with other drugs as an antidote to snake bite and scorpion sting (Handique, 2014). The name amrita refers to a substance that promotes youth and lifespan. Due to its anti-inflammatory and anti-diabetic properties, it is an essential component of several ayurvedic formulations, including Amritharishtam and Dhanwanthara thailam (Pillai and Siril, 2019). *T. cordifolia* is used in tribal or folk medicine in different parts of the country. Almost all the parts of the plant are documented to be useful in ethnobotanical surveys conducted by ethnobotanists.

2.5 Medicinal uses

About 30 commercially available products use the essential oil and extracts from the roots and rhizomes in the flavor, medicinal, and fragrance industries (Prakash, 1999). *Valeriana* species fresh rhizome and root can produce 0.4-0.5% (w/v) essential oils (Mathela *et al*., 2005). The species rhizomes and roots are recognized to treat a wide range of conditions and illnesses, including obesity, skin conditions, epilepsy, insanity, and skin poisoning (Prakash, 1999).

The presence of valepotriates, a class of monoterpenoids of iridoids with an epoxy group and beta-acetoxy isovaleric acids, may be responsible for this plant's medicinal properties (Kaur *et al*., 1999). Two important chemical components, valepotriates and sesquiterpenoids, are responsible for the pharmacological activity of valerian (Mathela *et al*., 2005). Wasowski *et al*., (2002) later found that two substances (6-Methylapigenin and hesperidin) isolated from the rhizome of Indian valerian demonstrated sedative and anxiolytic effects. According to (Girgune *et al*., 1980), the essential oil from Valeriana rhizomes had antifungal and antibacterial properties. The main valepotriates valtrate, acevaltrate, and didrovaltrate are the major compounds in *V. jatamansi* which is responsible for its antispasmodic, anticonvulsive, and antidepressant activities (Gupta *et al*., 1986). According to (Bounthanh *et al*., 1981), these substances exhibit cytotoxic effects on rat hepatoma cells and anti-tumor activity (Marder *et al*., 2003).

Essential oil, a priceless aromatic oil that is widely utilized around the world, is found in the plant's rhizome and roots (Mathela *et al*., 2005). Ayurvedic, Unani, and contemporary medical systems all include *V. jatamansi* in their treatment regimens (Das *et al*., 2013). Patchouli alcohol and bornyl isovaltrate are among the compounds found in the essential oil extracted from the roots and rhizomes (Bos *et al*., 1998). Numerous pharmacological investigations into the *Valeriana* species essential oil components indicated that they have sedative, anxiolytic, antifungal, and antibacterial activities (Wasowski *et al*., 2002). The plant sedative qualities are thought to be caused by the presence of valepotriates, nonglycosidic iridoid esters. The primary valepotriates in *V. jatamansi* such as valtrate, acevaltrate, and didrovaltrate, are responsible for the plant's varied therapeutic effects (Gupta *et al*., 1986).

Major medicinal properties of this plant along with their parts used are depicted in figure 1.

Figure 1: Medicinal uses of *Valeriana jatamansi* Jones

The leaves, bark and roots of *T. cordifolia* are primarily responsible for its pharmaceutical significance because they contain a variety of bioactive substances, including alkaloids, glycosides, lactones, saponins, tannins, steroids, polysaccharides and aliphatic compounds with a range of therapeutic applications. Immune modulatory or immune stimulatory, anti-neoplastic, antioxidant, anti-hyperglycemia, anti-hyperlipidemia, anti-tuberculosis, anti-osteoporotic, anti-angiogenic, anti-malarial, and anti-cancer action are a few of the key applications (Sinha and Sharma, 2015; Nasreen *et al*., 2010). *T. cordifolia* is widely used in traditional medicine due to its biological characteristics, which include anti-periodic, anti-inflammatory, immunomodulatory, anti-neoplastic activities, anti-oxidant, anti-diabetic, anti-spasmodic, anti-stress, anti-leprotic, anti-malarial, anti-allergic, anti-arthritic activity, and hepatoprotective

properties. *T. cordifolia* is used to treat a number of illnesses, including fevers, diabetes, asthma, dyspepsia, jaundice, skin conditions, urinary issues, chronic diarrhea, and dysentery. Along with these conditions, it is used to treat rheumatoid arthritis, leprosy, and helminthiasis.

The juice of the plant stem is beneficial for conditions like diabetes, dyspepsia, vaginal and urethral discharges, radioprotective activity, jaundice, and regulates blood sugar levels. The bark of the plant stem has anti-inflammatory, allergic, anti-spasmodic, anti-pyretic, and anti-leprotic properties. The entire plant is utilized for analgesic and neuropharmacological purposes, as well as an antidote to snakebite and scorpion sting (Handique *et al*., 2014). The medicinal uses of this plant is depicted in figure 2.

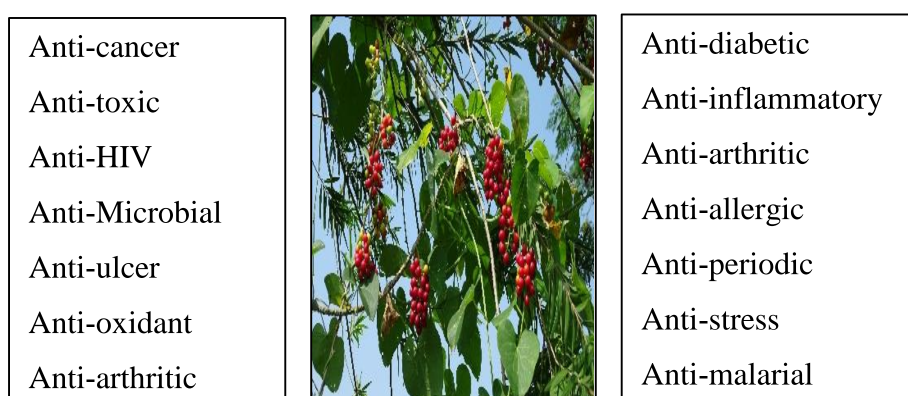


Figure 2: Medicinal uses of *Tinospora cordifolia*

2.6 Phytochemistry and pharmacology

There is a number of marketable high-value secondary metabolites currently used in the pharmaceutical, food and agricultural industries produced in natural plants and tissue culture raised plants (Jiang *et al*., 2015; Pant, 2014; Rao & Ravishankar, 2002). Various research initiatives are geared towards maintaining reliable sources of these and other useful primary and secondary metabolites. Crude plant of *V. jatamansi* Jones contains flavonoids (Jugran *et al*., 2016), valepotriates (Wang *et al*., 2017), flavone glycosides (Tang *et al*., 2003), essential oils (Verma *et al*., 2011), lignans (Lin *et al*., 2010), phenolic compounds (Tewary *et al*., 2005), sesquiterpenoids (Ming *et al*., 1997), bakkenollide type sesquiterpenoids (Xu *et al*., 2011), sesquiterpenoid glycosides (Tan *et al.,* 2016) and other phytochemicals. The two main substances that were predominantly isolated from the roots and rhizomes of these plants are valepotriates and flavonoids. (Fig 2) summarizes respectively, the bioactivities of the herb extracts

and main bioactive components. Among them, valtrate acevaltrate baldrinal didrovaltrate and 11-ethoxyviburtinal are the most potent ingredients in treatment of various diseases like irritable bowel syndrome, antis-pasmodic, analgesic, etc (Ma *et al*., 2021).

Numerous substances, including alkaloids, diterpenoid lactones, glycosides, steroids, sesquiterpenoids, phenolic compounds, aliphatic compounds, and polysaccharides, have been identified from *T. cordifolia.* This plant's leaves contain high levels of protein (11.2%), calcium, and phosphorus (Khosla *et al*., 1971). They have isolated clerodane furano diterpene glucosides as their acetates from stems. By using Gas Chromatography-Mass Spectrometry (GC-MS) research. (Jahfar, 2003, Jahfar *et al*., 2004) reported the characterisation of the glycosyl component from *T. cordifolia*. It has been reported that callus and cell suspension cultures were created from plant stem explants where buildup of berberine and jatrorrhizine (protoberberine alkaloids) was seen (Chintalwar *et al*., 2003). The major pharmacological effect of this plant includes anti-inflammatory, anti-arthritic, anti-osteoporotic activities, anti-allergic activity, antioxidant activity, antineoplastic, radio-protective activity, antipyretic, anti-infective activity, anti-hyperglycemic activity, immunomodulatory activity and hepato-protective activity (Upadhyay *et al*., 2010).

2.7 Adventitious root cultures

The cell culture system might be a beneficial and trustworthy method for producing significant amounts of high-value secondary metabolites (Fazal *et al*., 2014). One of the most important systems for increasing biomass and producing metabolites with high commercial value is adventitious root cultures (Baque *et al*., 2012; Wang *et al*., 2013). Due to their great efficiency and ease of scaling up to bioreactors, hairy and adventitious root cultures have been the most frequently used for this purpose (Cui *et al*., 2013). According to Sivanandhan *et al*., (2012), adventitious root cultures development conditions can be more easily managed than those of other culture methods. The adventitious roots can acquire significant amounts of secondary metabolites and are generally more stable. Additionally, compared to field grown plants, it is possible to produce a comparatively high amount of biomass using *in vitro* cultures in a short amount of time (Murthy *et al*., 2008). The ability to express recombinant proteins, high genetic and biochemical stability, rapid growth rates, and generation of secondary

metabolites on a wide scale are main benefits of growing hairy roots (Petrova *et al*., 2013).

When a plant is developing normally or in reaction to environmental challenges like wounding, flooding, or mineral deficiency, adventitious roots can grow from any organ other than the root itself. They often sprout from leaves, stem nodes, and internodes and can grow both beneath and above ground. Adventitious roots play a variety of significant responsibilities for the plant and aid in its ability to endure harsh environmental circumstances. There are various endogenous and external physiological variables involved in the complex molecular process of adventitious root development (Sorin *et al*., 2005). The four phases of adventitious root creation, according to Zhang *et al*., (2017), include root pre-emergence, early root development, huge root growth, and final root configuration phase. The root pre-emergence phase involves molecular and biochemical process alterations that take place prior to any cytological occurrence until the emergence of primordial roots. The level of endogenous substances undergoes several modifications during the adventitious root development. This is primarily related to the regulation of hormone levels which might play a great role in it. Auxin and ethylene in this situation predominantly function as activators, whereas cytokinin and ethylene primarily function as inhibitors (Pop *et al*., 2011). These roots exhibit excellent stability and growth rates, creating large amounts of alkaloids, terpenoids, and phenols in their cell and tissue spaces, which are simple to develop in an appropriate hormone-supplemented medium with little inoculum (Sivakumar *et al*., 2006).

2.8 Enhancement of plant cell cultures using plant growth regulators and elicitors

PGRs are frequently used in the production of numerous secondary metabolites, such as medicinal substances (Vanisree and Tsay, 2004). In plant culture systems, the manipulation of biotic and abiotic elicitors can promote the accumulation of medicinally significant metabolites (Sivanandhan *et al*., 2012; Sivakumar *et al*., 2019). Elicitation is a useful technique for enhancing the manufacture of secondary metabolites, according to (Radman *et al*., 2003). Elicitors can be primarily divided into biotic and abiotic chemicals based on their makeup and shape. The use of the abiotic elicitors SA and MeJA to successfully increase isoflavones in Glycine max was reported by Saini *et al*., (2013). MeJA was successfully employed by Wang *et al*., (2015) in liquid Hypericum perforatum cells to increase flavonoid production.

Psoralea corylifolia suspension cultures have successfully used biotic elicitors to accumulate high psoralen content (Ahmed and Baig, 2014). The targeted metabolite and plant species are thought to affect the right kind and amount of elicitors (Ho *et al*., 2018). Elicitors, including yeast extract and methyl jasmonate, operate as signaling chemicals that are detected by elicitor-specific receptors on the plant cell membrane and cause transcriptional activation of genes involved in the manufacture of secondary metabolites. The primary criteria, involved are the optimization of many parameters, including as the kind, concentration, exposure length, and treatment schedule of elicitors, for efficient and effective elicitation processes (Halder *et al*., 2019).

2.9 Gene expression studies of valepotriates

Isoprenes (C5), monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20) and triterpenes are few of the many volatile representatives of terpenes, one of the main secondary metabolites in medicinal plants (C30). Members of this family perform numerous crucial tasks, such as defending plants from a variety of insects, pests, herbivores, and microbiological pathogens like bacteria and fungi (Park *et al*., 2016). According to Yeo *et al*., (2013) valerenic acid and its probable biosynthetic precursor valerenadiene, sesquiterpenes discovered in the roots of *V. officinalis*, are responsible for the biological actions of valerian. Seven *V. officinalis* terpene synthase genes (VoTPSs) were also discovered by Yeo *et al*., (2013), of which two were functionally classified as monoterpene synthases and the remaining three served as substrates for sesquiterpene synthases (Yeo *et al*., 2013). Houghton, (1999); Takemoto, (2009) reported the significance of sesquiterpene derivatives valerenic acid and valeranone to possess potent biological activities. Additionally, they reported that valerena-1, 10-diene is thought to be the source of valerenic acid, but valeranone may be derived from a germacrene precursor.

CHAPTER 3

MATERIALS AND METHODS

3.1 Plant materials

V. jatamansi (Fig. 3) and *T.cordifolia* (Fig. 4) plants were collected from Banepa, Kavrepalanchok, Nepal at an altitude of 1500 m (27.6332° N, 85.5277° E), and established in the departmental garden of Central Department of Botany, Tribhuvan University. Well-established plants from the garden were used as a source for explants.

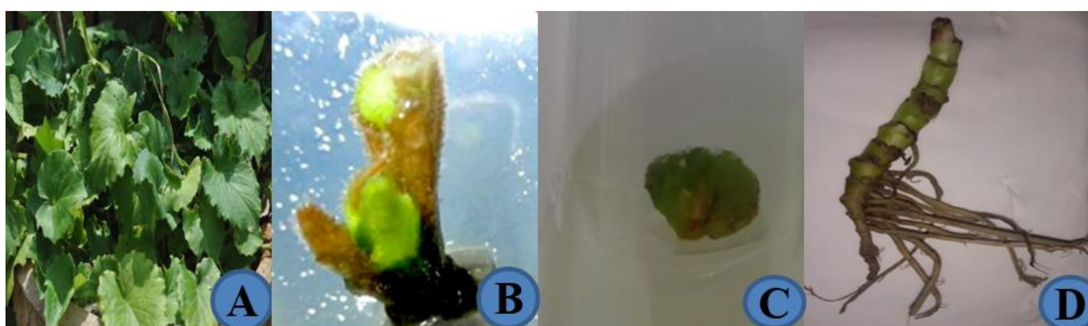


Figure 3: (A) Wild Plants of *V.jatamansi* Jones; (B) Shoot bud explants used for Plant regeneration; (C) Leaf explant used for callus induction; (D) Dried underground rhizome with roots of *V.jatamansi* Jones.

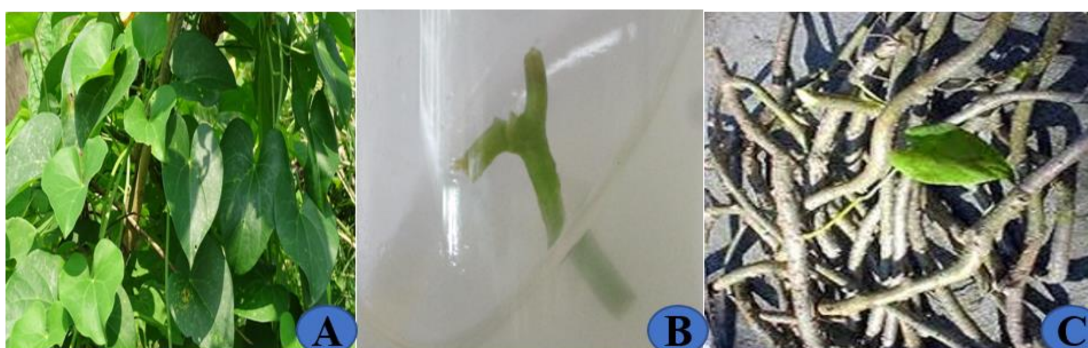


Figure 4: (A) Wild Plants of *T.cordifolia*; (B) Nodal segment of *T. cordifolia* for inoculation; (C) Dried stems of *T. cordifolia*.

The explants used for the present investigation are as follows:

- Shoot bud were used as an explant for plant regeneration in *V. jatamansi* Jones and nodal segment as an explant for *T. cordifolia.*

- Young medium sized three weeks old nodal segments derived from *in vitro* culture of shoot buds were used as explants for *in vitro* mass propagation of plants and large scale production for *V. jatamansi* and *T. cordifolia.*
- Roots from *in vitro* plants were used to establish adventitious root cultures in *V. jatamansi.*
- Young, healthy leaves derived from wild plants and tissue cultured plants were used as explants for DNA (Genetic homogeneity) in *V. jatamansi* and *T. cordifolia* and RNA isolation (Real-Time PCR) in *V. jatamansi*.
- The *in vitro* cultures and wild plants were used to extract the phytochemical constituents and its analysis in *V. jatamansi* and *T. cordifolia*.

3.2 Methods

The methods applied for the *in vitro* plant regeneration, micropropagation, biochemical analysis, adventitious root culture, elicitor treatment, characterization of secondary metabolites, and test of genetic homogeneity, gene expression and acclimatization in *V.jatamansi*. Whereas *in vitro* plant regeneration, micropropagation, biochemical analysis, characterization of secondary metabolites, and test of genetic homogeneity in *T. cordifolia* are described under the following headings:

3.2.1 *In vitro* plant regeneration

The method for *in vitro* plant regeneration of *V. jatamansi* and *T. cordifolia* is described as under:

3.2.2 Preparation of stock solution

The MS (Murashige & Skoog, 1962) medium were used as the basal medium for the present investigation along and in combination with different concentration of hormones (BAP, NAA, 2, 4-D, KIN). The composition of the MS media is as follows.

3.2.3 Preparation of Murashige and Skoog's medium (MS)

The Murashige and Skoog (1962) medium consists of macronutrients (stock A), micronutrients (stock B), iron source (stock C), vitamins (stock D), sucrose, and agar (a gelling agent). The composition of the MS medium is as follows (Table 1).

Table 1: Preparation of stock solution for MS medium

Components	mg/l	(10X) gm/l Stock	Working Vol. for 1 L medium
Stock A (macro-nutrient)-10X (g/lit)			
Macronutrients			
Potassium nitrate (KNO_3)	1900	19.0	100 ml
Ammonium nitrate (NH_4NO_3)	1650	16.5	
Calcium chloride ($CaCl_2.2H_2O$)	440	4.4	
Magnesium sulphate ($MgSO_47H_2O$)	370	3.7	
Potassium dihydrogen phosphate (KH_2PO_4)	170	1.7	
Stock B (micro-nutrient)-100X (mg/100 ml)			
Boric acid (H_3BO_3)	6.2	620	1 ml
Manganese-sulphate ($MnSo_4.4H_2O$)	22.3	2230	
Zinc sulphate ($ZnSO_4.7H_2O$)	8.6	860	
Sodium-molybdate ($Na_2MoO_4.2H_2O$)	0.25	25	
Cobalt Chloride ($CoCl_2.6H_2O$)	0.025	2.5	
Copper sulphate ($CuSO_4.5H_2O$)	0.025	2.5	
Potassium Iodine* (KI)	0.83	83	
Stock C (Iron source)-10X (mg/100 ml)			
Sodium ethylene diamine tetra acetate (Na_2EDTA)	37.3	373	10 ml
Ferrous sulphate ($FeSO_4$)	27.8	278	
Stock D (Vitamins)-100X (mg/100 ml)			
Glycine	2.0	200	1 ml
Nicotinic acid	0.5	50	
Pyridoxin HCL	0.5	50	
Thiamin HCL	0.1	10	
Myo inositol**	100	10,000	

* 100 ml stock solution of KI was made separately and used as 1ml/l in MS medium.

** Myo-inositol was freshly made at the time of media preparation (100 mg/l).

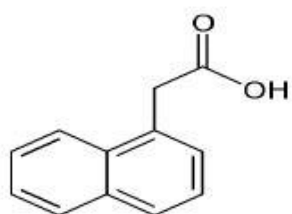
Chemical		g/l
Carbon source	Sucrose	30 g
Gelling agent	Agar	8 g

During the preparation of each stock solution, the above chemicals were weighed accurately and dissolved completely in distilled water. Myo inositol was added directly

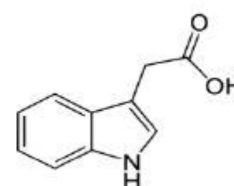
while preparing the medium (100 mg/l). To dissolve the chemicals more readily, the solutions were stirred with a magnetic stirrer. The final volume was made upto 1 liter for stock A and 100 ml for stock B, C, and D. Due to light sensitivity, all stock solutions were kept in clean brown bottles and stored in the refrigerator at 4ºC.

3.2.4 Hormones used for the experiment

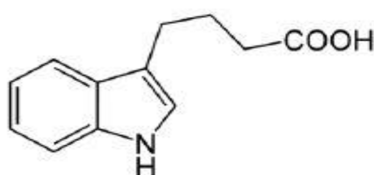
Plant hormones or phytohormones are organic substances thatare naturally produced in plants and also found in synthetic form. These are of various types and differ in their activity but most of them stimulate the growth and development of plants.



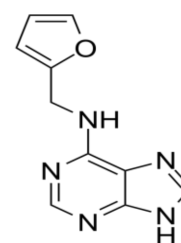
α-Naphthalene Acetic Acid



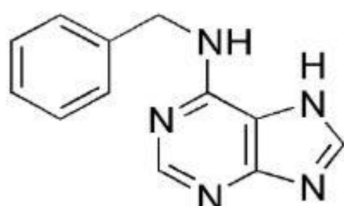
Indole-3-Acetic Acid



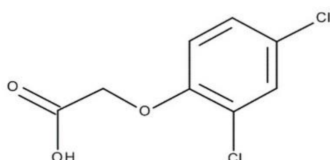
Indole-3-Butyric Acid



Kinetin



6-Benzylaminopurine



2, 4-Dichloro phenoxy acetic acid

Figure 5: Structure of plant growth hormones

The growth hormones used in the present investigation for Plant germination and Plantlet development are auxins, [NAA (α-Naphthalene Acetic Acid), IAA (Indole-3-Acetic Acid, 2, 4-Dichlorophenoxy acetic acid), and IBA (Indole-3-Butyric Acid)] and cytokinins [BAP (6-Benzyl Amino Purine)]

3.2.5 Preparation of hormones

In the present investigation, two plant growth hormones viz. cytokinin and auxins were used. For the preparation of cytokinin, i.e., BAP and Kinetin, 10 mg of it was dissolved in 2.5 ml of 0.5N NaOH and for the preparation of auxins, i.e., (NAA, IAA, 2,4-D) and IBA, 10 mg of each was dissolved in 2.5 ml of 1N NaOH. After that, the final volume was made 100 ml by the addition of sterile water in each hormone separately. 1 ml of this hormone stock was used for 100 ml of media to make 1 mg/l of hormone concentration in the media.

3.2.6 Preparation of 1 liter media

The following protocol was applied for the preparation of 1 litre liquid (suspension) and solid media (static) of both MS. One litre sterilized conical flask was taken.

- 100 ml of Stock A, 1 ml Stock B, 10 ml of Stock C, and 1 ml of Stock D (only for MS) were added one by one in about 400 ml sterile distilled water in the sterilized conical flask.
- 30 g of sucrose was weighed and dissolved in a stock solution.
- Sterile distilled water was added up to 900 ml and the solution was stirred by a magnetic stirrer to mix the stock and sucrose solution more homogenously.
- The pH of the media was maintained at 5.8 by using 0.1 N NaOH or HCl.
- Then, finally, the volume was made 1000 ml by adding sterile distilled water.
- For the preparation of the hormone medium, hormone stocks were added according to the media requirement in separate beakers to make 100 ml media in it.
- For the solid media, it was solidified by adding 0.8 g (0.8%) agar in each beaker containing 100 ml. It was heated with the help of a heater up to boiling to melt the agar. When the solution become clear, about 16 ml was poured in each of the 6 to 8 sterilized culture tubes. Then, each culture tube was enclosed by an aluminum foil cap. Whereas in case of suspension or liquid media preparation, agar which is used as a solidifying agent was not used.

- The culture tubes containing media were sterilized in an autoclave at the temperature of 121℃ and pressure of 15 lb /sq inch for 20 minutes. After cooling down, the tubes were taken out and kept in a slanting position (only solid) inside the culture room (25±2℃).

3.2.7 Sterilization Procedures

Sterilization is the process of making a contamination-free environment for the culture and growth of plant tissues. During *in vitro* culture, maintenance of an aseptic environment is the most difficult task as the cultures are easily contaminated by microbes like fungi, bacteria, etc. The contaminants produce toxic metabolites which inhibit the growth of cultured plant tissues. Therefore, each part that is used for culture is necessary to handle aseptically and carefully.

3.2.8 Sterilization of glassware's and metal instruments

The required glassware and metal instruments underwent wet and dry heat sterilization while the media were being prepared. Glass and metal objects like forceps, scalpels, needles, and scissors were submerged in detergent solution for 24 hours before being rinsed with tap water. Glass items like beakers, Petri dishes, culture tubes, pipettes, conical flasks, glass rods, and measuring cylinders were also used. They were then dried in a hot air oven at 150°C for two hours after being steam sterilized in an autoclave for 20 minutes at 15 lb pressure. Aluminum foil was used to wrap metal instruments before storing them in the hot air oven to sterilize them. Before utilizing, flame sterilization was carried out in a laminar airflow.

3.2.9 Surface sterilization of plant material

The plant materials used for the present investigation were axillary shoot buds of *V. jatamansi* Jones and nodal segments of *T. cordifolia*.

The freshly collected plants were first thoroughly washed under running tap water for at least 30 minutes to remove the soil and other external particles attached to their surface. The axillary shoot buds and nodal segments were cut from the whole plant. The axillary shoot buds and nodal segments were dipped in detergent water and Tween 20 (0.1%) for 15-20 minutes, shaken well, and again washed in running tap water until all the detergents were washed off clearly. After that, distilled water was used to rinse both explants. They were then surface sterilized for five minutes with 0.1% Mercuric

chloride ($HgCl_2$), and any remaining $HgCl_2$ was washed away by rinsing them five times in sterile water. The explants were then placed onto sterile Petri plates and blot dried using a Whatman filter paper. In a laminar air flow cabinet, the chemical surface sterilizing procedure was carried out.

3.2.10 Inoculation of explants

The inoculation of axillary shoot buds of *V. jatamansi* Jones and nodal segments explants of *T. cordifolia* was carried out in the laminar airflow cabinet. Before inoculation, the laminar airflow cabinet was cleaned with spirit or cotton soaked with 70% ethyl alcohol. The culture tubes containing media, sterile instruments, and glassware were exposed under ultraviolet (UV) radiation for 45 minutes to remove the possible contaminants presenting and around the transfer area. After turning off the UV light, the blower was kept running during the process of inoculation. The surface-sterilized shoot buds of *V. jatamansi* Jones and nodal segments of *T. cordifolia* were put on a sterile Petri dish containing sterile filter paper for soaking the surface moisture of explants. Both the explants were inoculated on different strengths of MS. Afterward, the explants were blot dried using a Whatmanfilter paper placed onto sterile Petri plates and inoculated in test tubes containing Murashige and Skoog (MS) (Murashige andSkoog, 1962) (full, half, and quarter strength) medium with 3% sucrose (w/v), 0.1% myoinositol and supplemented with or without the combination of BAP or NAA for *V. jatamansi* Jones and BAP or Kinetin for *T.cordifolia.* All cultures were incubated under 16/8 h (light/dark) photoperiods using white fluorescent tubes (Phillips, India) at 25±2 °C and 75% relative humidity (RH). The initiation and rate of plant regeneration and development were observed and recorded every week. Morphological changes were recorded based on visual observations.

3.3 Establishment of *in vitro* cultures for *V. jatamansi* and *T. cordifolia*

3.3.1 *In vitro* propagation of *V jatamansi* Jones

The appropriate developmental stage of cultures, obtained from *in vitro* culture of *V. jatamansi* were aseptically taken out from the culture vessels and cultured individually on different strengths of MS solid media supplemented with or without BAP and NAA. Only the media showing positive response for the cultures were further considered for the experimental research, which consists of BAP: (0.5, 1.0, 1.5 or 2.0 mg/L), NAA

(0.5, 1.0, 1.5, or 2 mg/L). The number of shoots and roots, as well as shoot and root length, were evaluated after 4 weeks of inoculation.

Explants from the *in vitro* plants that were produced using axillary shoot bud explants on media supplemented with BAP were used for the propagation of shoots and roots. These *in vitro* grown plants nodal segments were removed, and the explants were then cultivated on an MS basal medium that had various PGR concentrations and mixtures added to it. The following treatments on the basis of positive response to the cultures were employed which was 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%). Subcultures took place every four weeks. After 4 weeks of culture, the quantity of shoots and roots developed was assessed, and the morphological changes were noted based on visual observations.

3.3.2 Preparation of Coconut Water

Young coconuts were collected from Kathmandu, Nepal, for the production of coconut water. The coconut water was then extracted in a sterile chamber, filtered using Whatman filter paper, and stored at -40 C before usage. In the MS medium, coconut water was added in dosages of 5, 10, and 15% before to autoclaving.

3.3.3 Acclimatization of plantlets

Acclimatization is a natural phenomenon in which an individual organism adjusts to a gradual change environment. The fully grown *in vitro* plantlets (4-5 cm long) derived from *in vitro* inoculated shoot buds and nodal segments with well-developed roots of *V. jatamansi* Jones were acclimatized in various substrates. The following steps were carried out for acclimatization:

- At first, the culture tubes containing rooted plantlets were opened and kept at room temperature for 1 week.
- The plantlets grown in cultured conditions were picked out and washed with running water to remove the entire agar attached to it.
- The plantlets were dipped in fungicide, Bavistine (0.1%) for 5 minutes to minimize the chances of infection.

- Then, the plantlets were washed with distilled water for a few minutes and kept in blotting paper for drying.
- The plantlets were then transferred to the cleaned earthen pots for sand rooting containing the appropriate potting mixture.
- After one week of transplantation, the 5% Hyponex solution [Nitrogen, Phosphorus, and potassium (NPK)] was sprayed once a week regularly for fastening their growth.
- The transferred plantlets were covered with transparent polythene sheets to control the humidity. The small holes were made into a polythene sheet for aeration.
- The plants were kept in the green house for several weeks and finally, they were transferred to the natural environment to check their growth.
- The potting mixture played an important role in successful acclimatization. Therefore following potting coco peat along with soil in the ratio of 2:1 mixture was used in the present investigation for the acclimatization process:

3.3.4 Establishment of *in vitro* cultures of *T. cordifolia*

The *in vitro* cultures were obtained using nodal segment as explant using the following protocol

3.3.5 Inoculation in culture medium

Explants of nodal segments were grown on MS basal medium that also included various quantities and combinations of PGR's, which were choosen on the basis of primary positive response of the cultures which were BAP: (0.5-2 mg/L), Kinetin (KIN) (0.5-2 mg/L) and combination of BAP (0.5 mg/L)+KIN (0.5mg/L), BAP (1.0 mg/L)+KIN (0.5 mg/L), BAP (2 mg/L)+KIN (1.5 mg/L) and BAP (2 mg/L)+KIN (2 mg/L), MS+BAP (5mg/L) and MS+BAP (5mg/L)+Coconut water (5%, 10). After the proliferation of nodal segments, the shoots were inoculated in MS+IBA (0.5-2 mg/l) for rooting. All cultures were incubated under 16/8 h photoperiod using white fluorescent tubes (Phillips, India) at 25±2°C. Subcultures were conducted four weeks later. After 4 weeks of inoculation, the frequency and number of shoots developed were counted. Visual observations were used to record morphological changes.

3.4 Marker analysis for genetic variation and homogeneity of *V. jatamansi* and *T. cordifolia*

The genetic fidelity analysis of *V.jatamansi* and *T.cordifolia* is described using the following protocol

3.4.1 Extraction and quantification of genomic DNA

Young and healthy leaves harvested from wild (non-tissue cultured mother plant collected from natural habitat) and five explants, each set derived from *in vitro* culture of shoot buds, and nodal segments grown on MS media with 10% Coconut Water were used as explants for DNA extraction of *V. jatamansi* Jones and *T. cordifolia*. In order to sterilize the surface of young leaves harvested from the wild mother plant, they were washed under running water and rinsed with 70% ethyl alcohol. Leaves derived from *in vitro* culture were not subjected to surface sterilization. Total genomic DNA of the leaf (100 mg) was extracted using Cetyl trimethyl ammonium bromide (CTAB) method. The quality and quantity of extracted DNA were determined by gel electrophoresis on 1% agarose gel and UV spectrophotometer. The DNA content of the isolate was adjusted to 10-20 ng/µl by TE buffer or DNA rehydration solution and stored at -20°C for further study.

3.4.2 Extraction of DNA from CTAB method

Genomic DNA of *in vitro* acclimatized plant leaves, *in vitro* leaves and wild leaves of *V. jatamansi* Jones was extracted by using modified CTAB method (Doyle, 1990). Approximately 0.2 gm of leaf samples were taken and ground to fine powder by using motor and pestle in liquid nitrogen. 750 µl of CTAB buffer (2% CTAB, 0.5M EDTA, 5M NaCl, 1M Tris-HCl, pH 8.0, 0.2% β-mercaptoethanol) was added to make the fine paste and transferred into clean sterilized micro-centrifuge tube (vol. 2.0 ml). Samples were incubated at 65^0 C for 45 minutes in a recirculating water bath. After incubation, samples were centrifuged at 10,000 rpm for 5 minutes to spin down cell debris. Then supernatant was then transferred to a clean sterilized micro-centrifuge tube and an equal volume of Chloroform: Isoamyl alcohol in the ratio of 24:1 was added and mixed gently by inversion several times (5-8 minutes). It was again centrifuged for 10 minutes at12, 000 rpm and the upper aqueous phase was transferred to sterilize the micro-centrifuge tube. An approximately equal volume of ice-cold propanol was added to each sample.

The tube was slowly inverted several times to precipitate the DNA. The tube was kept at -20^0 C for one hour. After that, DNA was spinned at 12,000 rpm for 5 minutes to form the pellet. The supernatant was discarded and the pellet was washed with ice-cold 70% ethanol (400 µl volumes). Again, it was centrifuged at 10,000 rpm for 5 minutes to get rid of salt. Then, ethanol was discarded out and the pellet was left to dry for 30 minutes till all the ethanol evaporates out. Finally, it was re-suspended in T.E. buffer (40 µl) and stored at 4oC. The quality and quantity of extracted DNA were estimated by electrophoresis on agarose gel (1%) and by UV spectrophotometry respectively. The concentration of extracted DNA was adjusted to 10-20 ng and finally, DNA samples were stored at – 20ºC.

3.4.3 DNA amplification

Screening of 35 set of RAPD primers and 10 ISSR primers was done, after which the primer showing amplification were selected. Out of this a set of ten 10 decamer RAPD (Table 2) and five 14 decamer ISSR primers, (Table 3) were used to amplify DNA fragments of the selected explants. In a thermal cycler, a 15-l reaction volume containing nuclease-free water, 1 LTaq polymerase (0.5 U l-1), 0.5 ldNTPs (0.2 mM), 1 l primer, and 50 ng of template DNA was used for the Polymerase Chain Reaction (PCR) reaction for the RAPD and ISSR assays (Biorad, USA). Purohit *et al*., (2015) and Kumar *et al*., (2012) both used cycling conditions that were optimized for finding the optimal cycling conditions. The following describes the RAPD primer amplification cycle. Initial denaturation at 92 °C for five minutes, 92 °C for one minute, 45 cycles at 35 °C to 60 °C for one minute, 72 °C for one minute, and 72 °C for five minutes. For ISSR markers, the amplification cycle is as follows. Initial denaturation at 95 °C, followed by 94 °C for 1 min, 35 cycles between 42 °C and 60 °C, 72 °C for 2 min, and 72 °C for 7 min.

Table 2: List of RAPD primers with total no of bands amplified

Primer Name	Sequence (5'-3')	Length (bp)	Annealing Temperature	No of Fragments	
				V.jatamansi	*T.cordifolia*
OPA08	GTGACGTAGG	10	44.2	2	2
OPA 10	GTGATCGCAG	10	44.2	3	1
OPA 18	AGGTGACCGT	10	44.2	5	2
OPE-08	TCACCACGGT	10	44.2	3	2
OPC 11	AAAGCTGCGG	10	44.2	3	2
UBC 292	TGCCGAGCTG	10	44.2	2	1
OPB 07	GGTGACGCAG	10	45.0	1	3
OPD 04	TCTGGTGAGG	10	46.0	3	4
UBC 210	CCGGGGTTTT	10	46.0	3	2
UBC 465	AGCTGAAGAG	10	44.2	3	3

Table 3: List of ISSR primers along with bands amplified

Primer Name	Sequence (5'-3')	Length (bp)	Annealing Temperaure	No of Fragments	
				V.jatamansi	*T.cordifolia*
HB8	GAGAGAGAGAGAGG	14	52.3	3	1
HB9	GTGTGTGTGTGTGG	14	52.3	1	3
HB10	GAGAGAGAGAGACC	14	52.3	2	2
HB11	GTGTGTGTGTGTCC	14	52.3	3	1
17898B	CACACACACACAGT	14	52.3	1	2

3.4.4 Gel electrophoresis

The PCR amplified fragments were separated on 1% agarose gel using 1X TAE (Tris Acetic acid and EDTA) buffer and stained with ethidium bromide (1 µg/ml). The gel was runned at 90 V constant for 1.5 to 2 hrs and then visualized and photographed under UV light in a gel documentation system (UVITEC, Cambridge). The sizes of the

amplification fragments were determined by comparisons with the 100bp DNA ladder marker (BioLabs). The PCR reactions were repeated thrice and only the clear bands were considered.

3.5 Callus induction and shoot regeneration in *V. jatamansi*

The leaves of *V. jatamansi* Jones from *in vitro* grown plants were cut off and inoculated onto MS medium with several plant growth regulators, including NAA, BAP, KIN, and 2,4-D at varying concentrations. Different SNP concentrations (5, 10, 15, 20, and 30 µM) were supplied in the media in a separate experiment to optimize the SNP (Himedia, India) concentration. The cultures were kept at 25±2 °C and exposed to light at a light intensity of 40–50 mol m–2 s–1 during a 16-hour photoperiod. The subcultures were conducted every seven days, and the cultures were occasionally observed.

3.5.1 Shoot induction

The calli were transferred to regeneration medium supplemented with varied concentrations of NAA, BAP (0.5, 1.0, 1.5, 2.5 mg/L), and 1, 5, 10, 15 and 20% coconut water after 20 to 25 days of culture (CW). Following growth assessments, SNP in the various concentrations previously described was added to the best responsive growth regulator. The cultures were evaluated on a regular basis, and the average number of shoots and explants as well as the percentage of produced shoots were recorded. With 50 explants in each experiment, each treatment was performed three times. After 4 weeks of cultivation, the healthy shoots were transplanted to pots with a soil and vermicompost mixture in the ratio of (2:1) and allowed to acclimatised in a plant containment facility with continuous lighting, 26±2 °C, and 75% relative humidity.

3.6 Adventitious root cultures of *V. jatamansi* Jones

Extraction of sterile roots (1.0 g) from mature plants was used to perform *in vitro* root multiplication. These roots were then inoculated into culture flasks containing MS liquid media supplemented with various PGRs at varying concentrations, including NAA [0.5, 1.0, 1.5, and 2.0 mg/L], IBA [0.5, 1.0, 1.5, and 2.0 mg/L], and IAA [0.5, 1.0, 1.5, and 2.0 mg/L]. The control used was liquid MS media without PGRs. Every culture was raised in an orbital shaker with an 80 rpm setting at 25±2°C and a 16-hour photoperiod.

3.6.1 Growth kinetics of adventitious root cultures

The adventitious root cultures growth was evaluated using an initial inoculum concentration of 0.1 g fresh weight per liter (FW/L) in 100 mL of MS medium supplemented with various hormones under the previously specified growth conditions. Every ten days, fresh weight (FW) of biomass was measured and injected in the fresh media till day 40. Further, the biomass was removed, three times rinsed with sterile distilled water, then shade dried to record the dry weight (DW) of the samples.

3.7 Elicitor treatment

MeJA and SA were produced in an aqueous solution of 50% ethanol (v/v) and filter-sterilized via a 0.22 m Millipore filter for the elicitation tests (Sartorius, Germany). The culture conditions and initial inoculum were kept exactly as stated above. MeJA was employed at concentrations of 0, 1, 3, 5, and 10 µM, and SA was used at 0, 60, 100, and 300 µM. Different elicitor concentrations were introduced to the cultures, and the cultures responses to the treatments were monitored intermittently for up to 40 days. After this, the root cultures were harvested and used for the biochemical analysis.

3.8 Biochemical analysis of *V. jatamansi* Jones

After the initial growth assessments, one gram of each of the *in vitro* root cultures from various hormonal combinations as well as the wild plant from all of the growth hormonal combinations were extracted with methanol for 48 hours while being constantly shaken, and were then centrifuged at 4000 g for 15 minutes. In order to measure the total phenolics, flavonoids, and antioxidant activity, the supernatant was combined and kept at 40 ^{0}C.

3.8.1 Determination of extract yield

The mass of powder of dried stems was measured. The total dry weight of the extract was measured after the removal of the solvent. The percentage yield of extract was calculated by the following formula:

Percentage yield of extract = weight of extract/weight of powder x 100%

3.8.2 Total Phenolic Content (TPC)

Using the Folin-Ciocalteu technique and gallic acid as the reference, the TPC of the extracts was calculated (Qiu *et al*., 2010). 2. 5 mL of the Folin-Ciocalteu reagent and 10% (v/v) of the extracts were combined. 7.5% (p/v) Na_2CO_3 (2 mL) was added after 2 minutes, and then the mixture was incubated for 10 minutes at 50 ºC. The results are presented as milligrams of gallic acid equivalent per gram of dry biomass weight (mg GAE/g DW) using a standard calibration of gallic acid solution. The absorbance was determined at 755 nm.

3.8.3 Total Flavonoid Content (TFC)

Using quercetin as a reference, the flavonoid-aluminum ($AlCl_3$) complexation method (Ghasemi *et al*., 2009) was used to calculate the TFC. 5% (p/v) NaNO2 (0.3 mL) was combined with the extract (1 mL) and incubated at room temperature for 5 minutes (RT). After that, 0.5 mL of 2% (p/v) $AlCl_3$ was added, and the sample was gently agitated before being neutralized with 1 N NaOH after 6 minutes (0.5 mL). Samples devoid of $AlCl_3$ were used as a blank when the absorbance was measured at 425 nm. TFC was determined using the quercetin alcoholic solution standard calibration method and represented as milligrams of quercetin equivalent per gram of dry biomass weight (mg QE/g DW).

3.8.4 DPPH radical scavenging assay

The DPPH radical activity (2, 2-diphenyl-1-picrylhydrazyl) was calculated using (Liyana and Shahidi, 2006). The methanolic extract was combined with 1.0 mL of DPPH in methanol (0.135 mM) (1.0 mL). The reaction mixture was completely vortexed at RT for 30 minutes in the dark before the absorbance at 517 nm was determined. Triplicates of each sample were used for analysis. The calculation of the radical scavenging activity was done as follows:

DPPH radical scavenging activity (%) = [(Abs control – Abs sample)]/ (Abs control)] x 100 where Abs control is the absorbance of DPPH radical+methanol

Abs sample is the absorbance of DPPH radical+sample extract /standard.

3.9 Biochemical analysis for *T. cordifolia*

The wild stem and leaf extracts from the *in vitro* plants were extracted with methanol for 48 hours while being continuously stirred, and they were centrifuged at 4000g for 15 minutes. In order to measure the total phenolics, flavonoids, and antioxidant activity, the supernatant was combined and kept at 4 ^{0}C.

3.9.1 Total Phenolic Content (TPC)

TPC in Methanolic extract of *T. cordifolia* was determined using Folin- Ciocalteau phenol reagent colorimetric method (Zhang *et al*., 2006). For this, 96 well plate was used and 75 µL of distilled water, followed by 25 µL of either sample (1mg/mL) or standard (25-200mg/mL) and 25 µL of F–C reagent (diluted 1: 1 (v/v) with distilled water) were added in each well. Then all the reaction mixture was thoroughly mixed by repetitive pipetting. After that solution was mixed and left for 6 min, followed by the addition of 100 µL of 1M Na_2CO_3 to each well. Then the plate was covered and incubated in the dark for 90 min. The absorbance at 765nm was measured with a microplate reader (Azure biosystems Microplate Reader). The blank or control was prepared by replacing the same volume of plant sample with absolute methanol. Each standard and sample solution were taken in triplicate. Gallic acid was used as a standard at 25, 50 100, and 200 µg/mL to obtain a calibration curve. Total phenolic content was expressed in terms of a milligram of Gallic acid equivalent per gram of plant extract (mgGAE/g).

3.9.2 Total Flavonoid Content (TFC)

TFC in methanolic extract of *T. cordifolia* was determined using Aluminium Chloride ($AlCl_3$) colorimetric method Chang *et al*., (2002) with slight modification. Firstly 1 mg/ml of plant extracts were prepared from 100 mg/ml stock solutions. From these 1 mg/ml stocks 250 µl of the plant was taken in a test tube. Then 750 µl of 10% $AlCl_3$ was added to the test tubes followed by 50µl (1M) potassium acetate. Then the solutions were diluted by adding 1.4 ml distilled water and incubated at room temperature for 30 minutes. The blank was prepared by replacing the same volume of absolute methanol. Then, 200 µl reaction mixtures were transferred from the test tube to the 96 well plates in triplicate. The absorbance at 415 nm was measured with a microplate reader (Azure biosystems Micro-Plate reader). The calibration curve was obtained using Quercetin

was used as a standard at 25, 50 100, and 200 µg/mL to obtain a calibration curve. The total flavonoid content was expressed in terms of milligram of Quercetin equivalent per gram of plant extract (mg QE/g).

3.9.3 DPPH radical scavenging assay

The antioxidant activity was assessed through free radical activity by methanolic extra*ct of T. Cordifolia* using DPPH. The activity of crude extract of *T. cordifolia* was determined by following Ben (Mansour *et al*., 2016). The stock solution of each extract and ascorbic acid (Standard or positive control) was prepared (1 mg/mL) using methanol. Then, a serial dilution was carried out to obtain a solution at concentrations of 200, 100, 50, and 25 µg/ml. A fresh DPPH solution of 0.2 mM was prepared by dissolving 7.88 mg of DPPH powder (molecular weight 394.32 gm/mol) in 100 ml of methanol away from direct light. Then, a volume of 50 µL of each sample (25–200 µg/mL) was mixed with 150 µL of DPPH in a 96-well plate in triplicate. The blank or control was prepared by replacing the same volume of plant sample with absolute methanol. After 30 minutes of incubation at room temperature, the absorbance was measured at 517 nm using a microplate reader (Azure bio systems Microplate reader). A lower absorbance value indicates the higher antioxidant activity of the sample. The free radical scavenging activity of the plant samples was calculated in percentage using the formula:

Radical scavenging activity(%) = Ab-Ab2/Ab1 x 100

3.10 Gene expression analysis in *V.jatamansi*

Using Trizol reagent, total RNA was extracted from the *in vitro* roots and wild roots of *V. jatamansi* Jones (Ambion, USA). Diethylpyrocarbonate (DEPC) was utilized to pretreat all the materials used in the RNA separation process before they were autoclaved. Liquid Nitrogen was used to grind the samples into a fine powder in a clean mortar and pestle. A leaf sample (around 100 mg) was ground with 1 ml of trizol, and it was let to sit for 10 minutes at room temperature. After adding 200 µL of chloroform to the suspension, the tubes were incubated at room temperature for an additional 5 minutes. The samples were then spun down for 15 minutes at 4 ^{0}C at 12000 rpm, where the upper aqueous layers were transferred to a new tube, and half of the volume of ice cold isopropanol was then added. Following 15 minutes at room temperature, the tubes

were centrifuged at 12000 rpm for 10 minutes at 4 ^{0}C. After discarding the supernatant, 500 µl of ice-cold 75% ethanol (v/v) was poured to the pellet, which was then spun at 7500 rpm for five minutes. 50 µL of RNase-free water was added to the pellet, which was then incubated at 55 °C in a water bath for 10 minutes. Using a Nanodrop spectrophotometer 2000 (Thermo Scientific, USA), the RNA concentrations (ng/L) and purity ratios (260/280 nm and 260/230 nm) were examined.

Table 4: List of RT-PCR primers with annealing temperature

Primer Name	Sequence (5'-3')	Length (bp)	Annealing Temperature
Tps1-1	F-GCTTCATCAAAGGGAGCTTG R-TTGACTGCATCGGTGAAGAG	20	55
TPS 1-2	F-CTTCATCAAAGGGAGCTTGC R-TTGACTGCATCGGTGAAGAG	20	55
TPS-2-1	F-CACGAGAAAGGTGCATCTGA R-GCCACTTGTTCCTTGCTCTC	20	55
TPS-2-2	F-CACGAGAAAGGTGCATCTGA R-CCACTTGTTCCTTGCTCTCC	20	55
TPS-3-1	F-CAAGATTTCGCAAATGAGCA R-TGTCCTCGCCTAATGATTCC	20	55
TPS-3-2	F-GGTTGATCGACTCCCTTCAA R-GGCGTGACATTAAAGCCATT	20	55
TPS-4-1	F-CAAGATTTCGCAAATGAGCA R-TGTCCTCGCCTAATGATTCC	20	55
TPS-4-2	F-GGTTGATCGACTCCCTTCAA R-GGCGTGACATTAAAGCCATT	20	55
TPS-5-1	F-CTATGCGAAACAAGCCATGA R-GATCAAAGGAGTGCCGGATA	20	55
TPS-5-2	F-CTATGCGAAACAAGCCATGA R-AATACACCCATTCCGACCAA	20	55
TPS-6-1	F-CCTTTATGGACGACCAAGGA R-TACAACTTTCCAACGCAACG	20	55
TPS-6-2	F-CGTTGCGTTGGAAAGTTGTA R-ACTCTTCCACCACCTTGACG	20	55

A high capacity RNA to cDNA kit (PrimeScript TM 1st strand cDNA Synthesis Kit, Takara, Japan) was used to create first-strand cDNA from 1 µg of total RNA.

Semiquantitative pcr was carried out using primer listed in (Table 4). The reaction was then loaded into gel and products were viewed in the detection unit. Real-Time PCR (Applied Biosystems, USA) was used to investigate quantitative gene expression in a 20 μL reaction volume containing 1 μL of cDNA template, 5μM of each primer, and 10 μL of SYBR Green real-time PCR master mix (SYBR® Premix Ex TaqTM, Takara, Japan) was used for the qRT-PCR. For each sample, the studies were carried out in biological triplicates. The $2^{-\Delta\Delta Ct}$ technique was utilized to determine the relative expression ratio using sesquiterpene synthases (TPS) as a reference gene (Schmittgen and Livak, 2008). The 40 cycles of annealing and extension at 60 ^{0}C for 34 s were used in the qRT-PCR reactions, which started with initial denaturation at 95 ^{0}C for 30 s, followed by denaturation at 95 ^{0}C for 5s. At the conclusion of each extension cycle, data was acquired. The list of gene-specific PCR primers is in (Table 4). Using the Primer 3 programme, primers were created based on multiple sequence alignment between different Valeriana species that express the sesquiterpene synthase genes.

3.11 Identification of compounds in the extract of *in vivo* and *in vitro* plant materials by GC-MS Analysis

To identify the chemicals in the extracts of in vitro cultures and wild samples of *V.jatamansi* Jones and *T. cordifolia,* gas chromatography- mass spectrometry (GC MS) analysis was done. Gas chromatography coupled with mass spectrometry (GC-MS 4000, Varian, USA) system analysis was used to determine the best responding PGR and elicitor treatment among the tested concentrations based on root suspension growth kinetics and phytochemical analysis (Pandey *et al*., 2020a). 0.20 μL volume of sample was injected into HP-5MS Agilent column (30 m × 0.25 mm, 0.25 μ film thickness). The temperatureof the oven was set to rise from 70 ^{0}C at a rate of 5 ^{0}C/min to 280 ^{0}C with hold time of 4 and 8 minutes, respectively. As a carrier gas, helium was employed with a split flow rate of 1.0 Ml/min.

3.12 Statistical analysis

All experiments were repeated thrice, and the data is completely randomized using three replicates. As means SD, the values were expressed. With the aid of the SPSS (version 17) statistical package program, all the data were further examined using analysis of variance (ANOVA), followed by Duncan's multiple range test ($p< 0.05$). $P<0.05$ was

regarded as indicative of significance, and the DMRT analysis was used to score the proportion of response. Manual scoring was done on the banding pattern in the genetic fidelity analysis utilizing the RAPD and ISSR markers.

CHAPTER 4

RESULTS

4.1 RESULTS

The results of the given objective are discussed below

4.2 Establishment of the culture condition and culture type for mass propagation of *V. jatamansi*

4.2.1 *In vitro* culture of Shoot buds of *V. jatamansi* in different media

In this study, axillary shoot initiation and proliferation were achieved by first culturing the shoot bud explants on MS media supplemented with varying concentration of BAP and/or NAA. For the experiment, several concentrations were chosen based on the culture's response. Axillary shoot initiation from the shoot bud explants was however unaffected by NAA. Shoot bud explants cultured on media enriched with BAP (2.0 mg/L) triggered shoot initiation from the shoot bud explants 7 days after inoculation among the evaluated BAP concentrations (Fig 6b). However, it was found that the rate of proliferation was slow (Fig 6c) and at the conclusion of 30 days, only fewer shoots (6 shoots) per explant were produced (Fig 6d). After 10 days, the proliferation rate significantly decreased, and even two subcultures onto a new medium at intervals of four weeks did not increase the proliferation (i.e., number of shoots and roots). Therefore, the shoots from (Fig 6d) were inoculated in MS media with various concentrations of hormones and other growth promoters in order to further boost the proliferation rate of nodal segments.

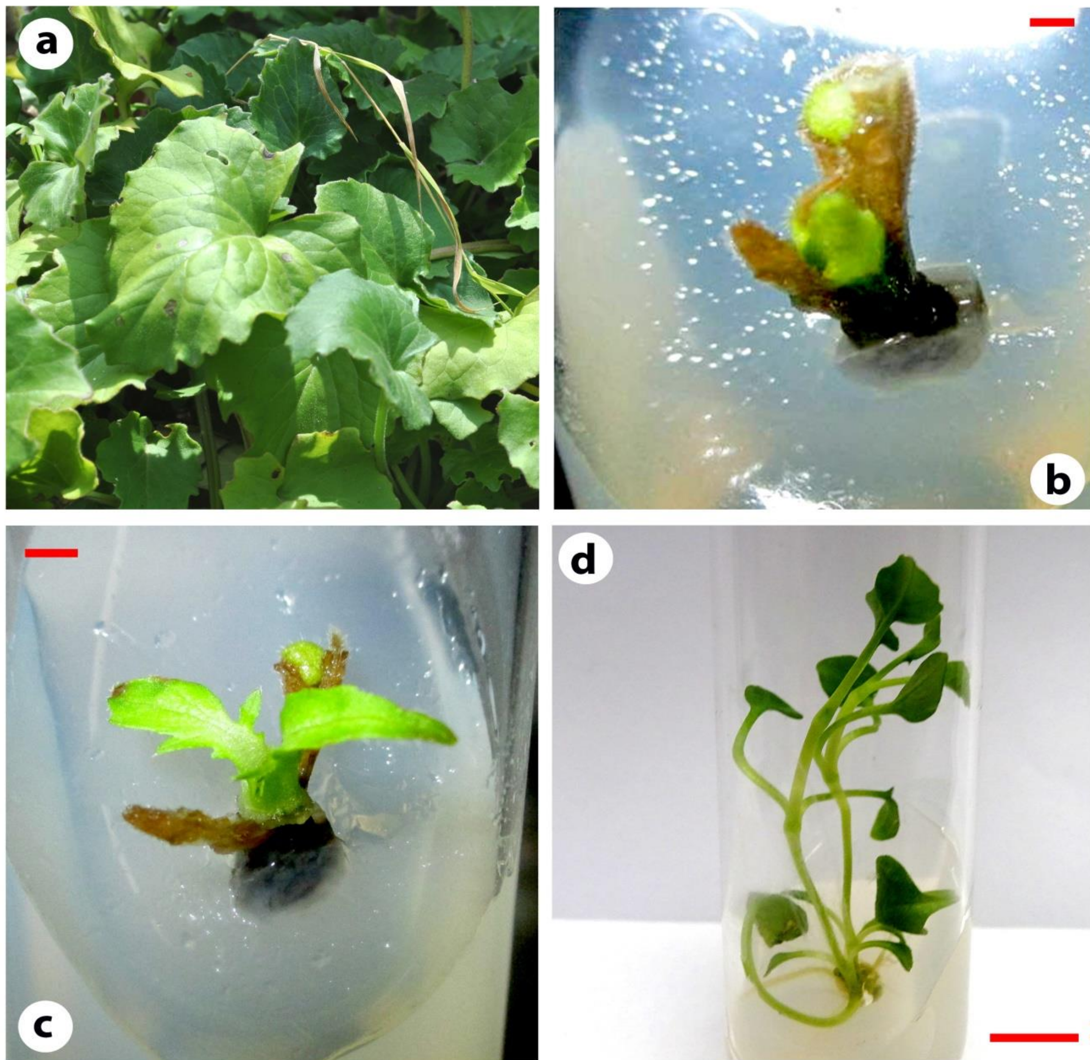


Figure 6: *In vitro* regeneration of *V. jatamansi* on MS+BAP (2 mg/L) (a) Mother plant of *V. jatamansi*; (b) shoot bud explants post 3 days incubation; (c) shoot bud initiation after 10 days of culture; (d) shoot elongation after 30 days of culture. Scale bar = 1cm (b, c); 2 cm

4.2.2 Proliferation of Nodal Segments in *V. jatamansi*

To investigate the effectiveness of BAP, NAA, BAP+NAA and coconut water on the proliferation of the nodal segment explants, the nodal segments were removed from the *in vitro* produced plants cultured on BAP (2mg/L). After seven days of culture, 2 mg/L of the BAP concentrations caused the induction of shoots from the nodal explants (6.0 shoots per explant), while the mean shoot length (3.0 cm), mean root numbers (6.3) and mean root length (1.4 cm) were also the maximum (Fig 7a, b). With NAA at 2 mg/L, the highest mean root numbers (7.0) and root length (6.8 cm) were observed (Fig 7a, b). Nodal segments that had been inoculated in MS and supplemented with various concentrations of coconut water underwent further mass multiplication.

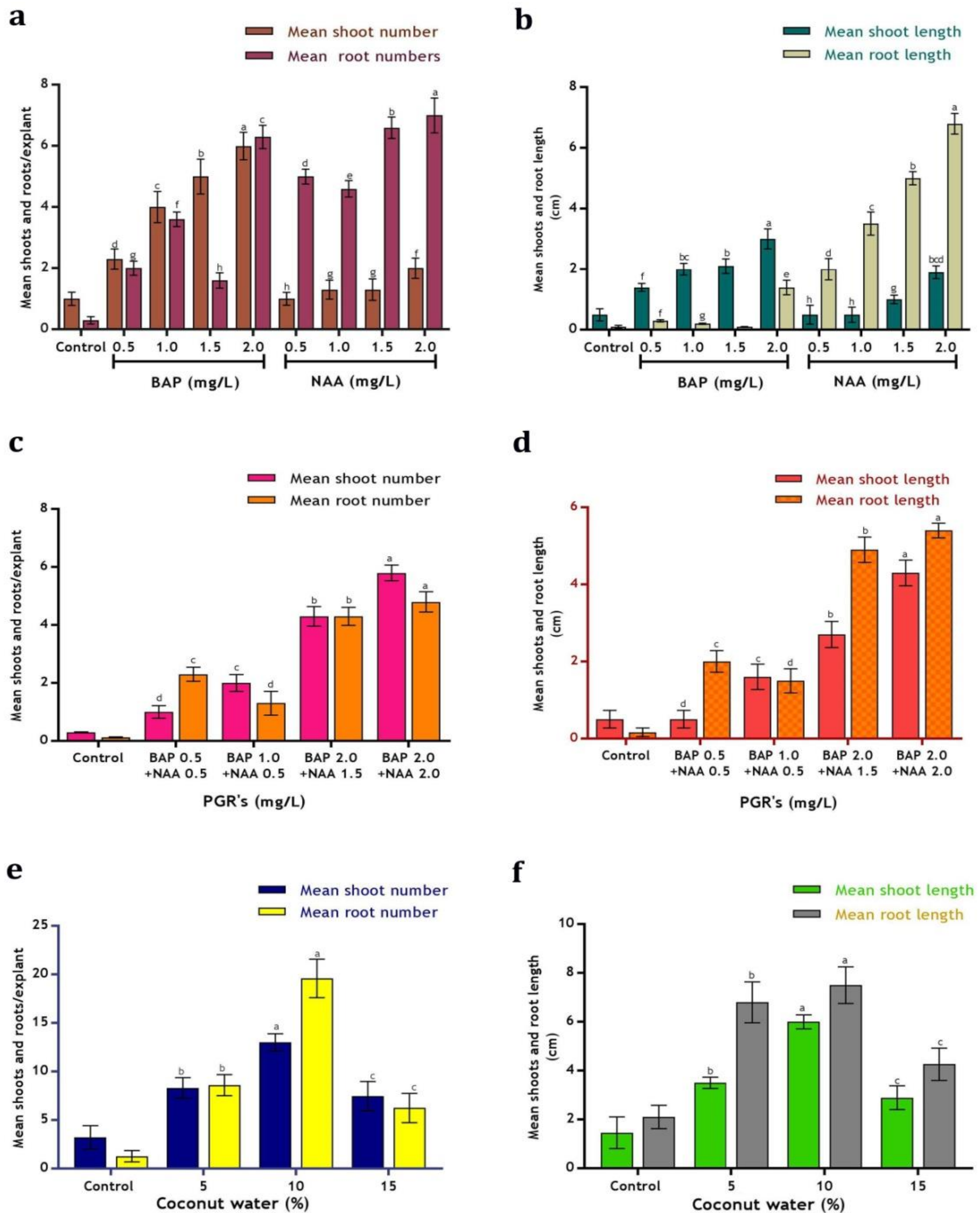


Figure 7: Effect of PGR'S on shoot and root induction from nodal explants of *V. jatamansi* (a) mean number of shoots and roots per explant with different concentrations of BAP and NAA; (b) mean shoot and root lengths with different concentration of BAP and NAA; (c) mean number of shoots and roots per 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. Data presented as mean±SD (*n*=). Means following the same letter are not significantly different, according to Duncan's multiple range test ($p<0.05$).

4.2.3 Mass Multiplication of the Nodal segments Using Coconut Water in *V. jatamansi*

The nodal explants removed from *in vitro* generated shoots cultured in medium containing BAP (2mg/L) were inoculated into MS media with coconut water (5, 10, and 15%) to investigate the effectiveness of coconut water in promoting the shoot proliferation of *V. jatamansi*. The shoot proliferation greatly enhanced at 5 and 10% (Fig 7e, f), but the medium supplemented with 10% coconut water produced the greatest response (Fig 8 a-c). 10% coconut water supplement produced the best results in terms of mean number of roots (19.6), mean number of shoots (13.0), mean shoot length (6.0 cm), and root lengths (7.5 cm) (Fig 8d, e). The results made it abundantly evident that adding coconut water to the media considerably improved the induction of shoots in nodal explants from *V. jatamansi* (Fig. 8a, b, c, d, e, f). In this study, the use of coconut water as a growth regulator also helped to shorten the time needed for the propagation cycle (from initiation to rooting) of *V. jatamansi* because coconut water supplementation alone was sufficient to produce profuse rooting in the plants without the need for additional rooting experiments or PGR addition (Fig 8f).

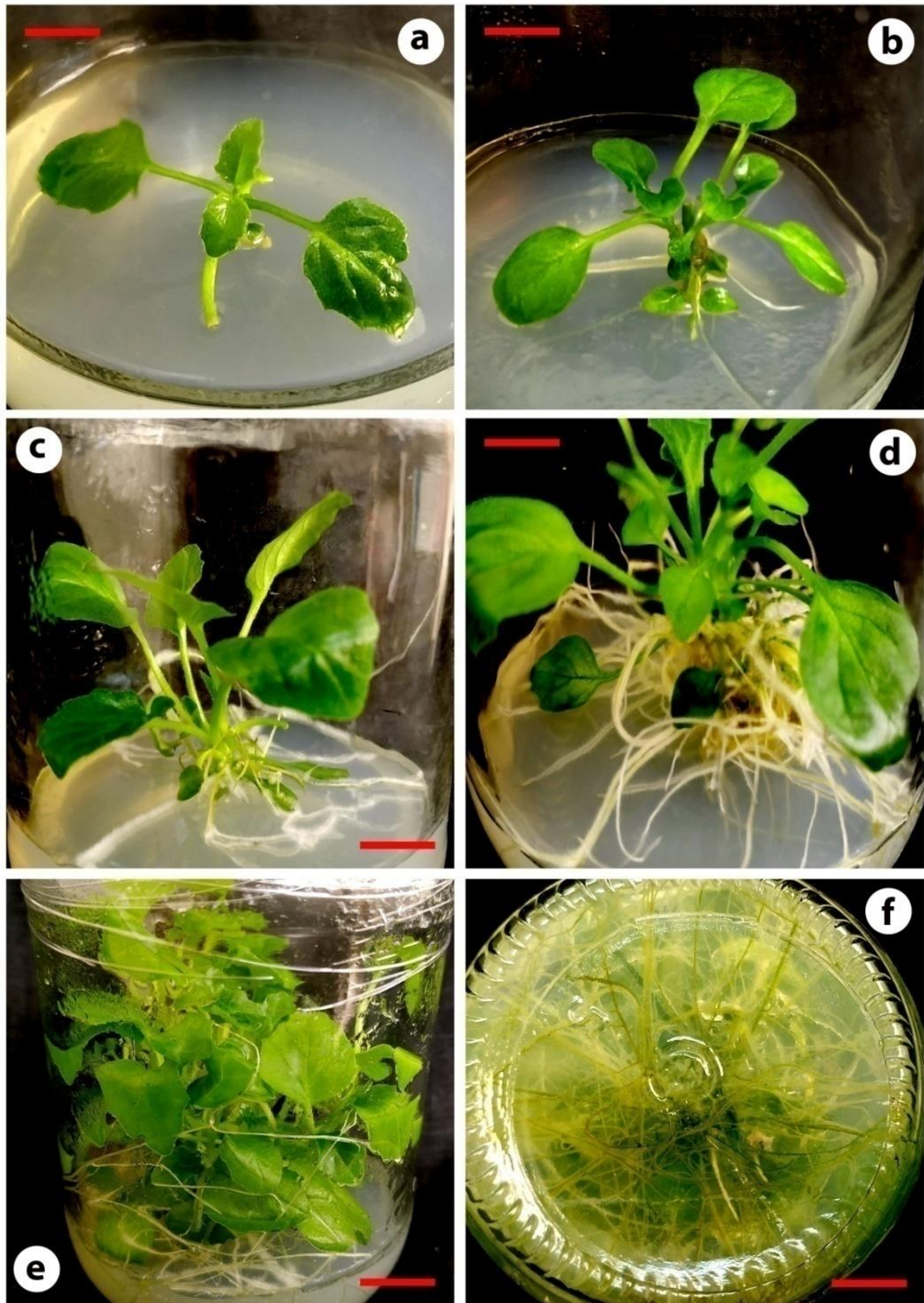


Figure 8: *In vitro* regeneration of *V. jatamansi* on MS+ 10% Coconut Water (a) Nodal segment of *V. jatamansi*, after 3 days of culture; (b) multiplications of shoot after 8 days; (c) shoot Multiplication after 15days of culture; (d) adventitious root formation after 25 days of culture; (e) proficient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

4.2.4 Acclimatization in *V.jatamansi*

In the plant containment facility, the *in vitro* grown plants were hardened. It was found that the plants raised in media containing coconut water (10%) grew well and had a higher rooting capability than the plants raised in media containing BAP (Fig 9c, d). Within 45 days of acclimatization, rhizome formation was also seen in the plants, which is an important finding because the majority of bioactive components are thought to be concentrated in the roots and rhizomes (Fig 9e)

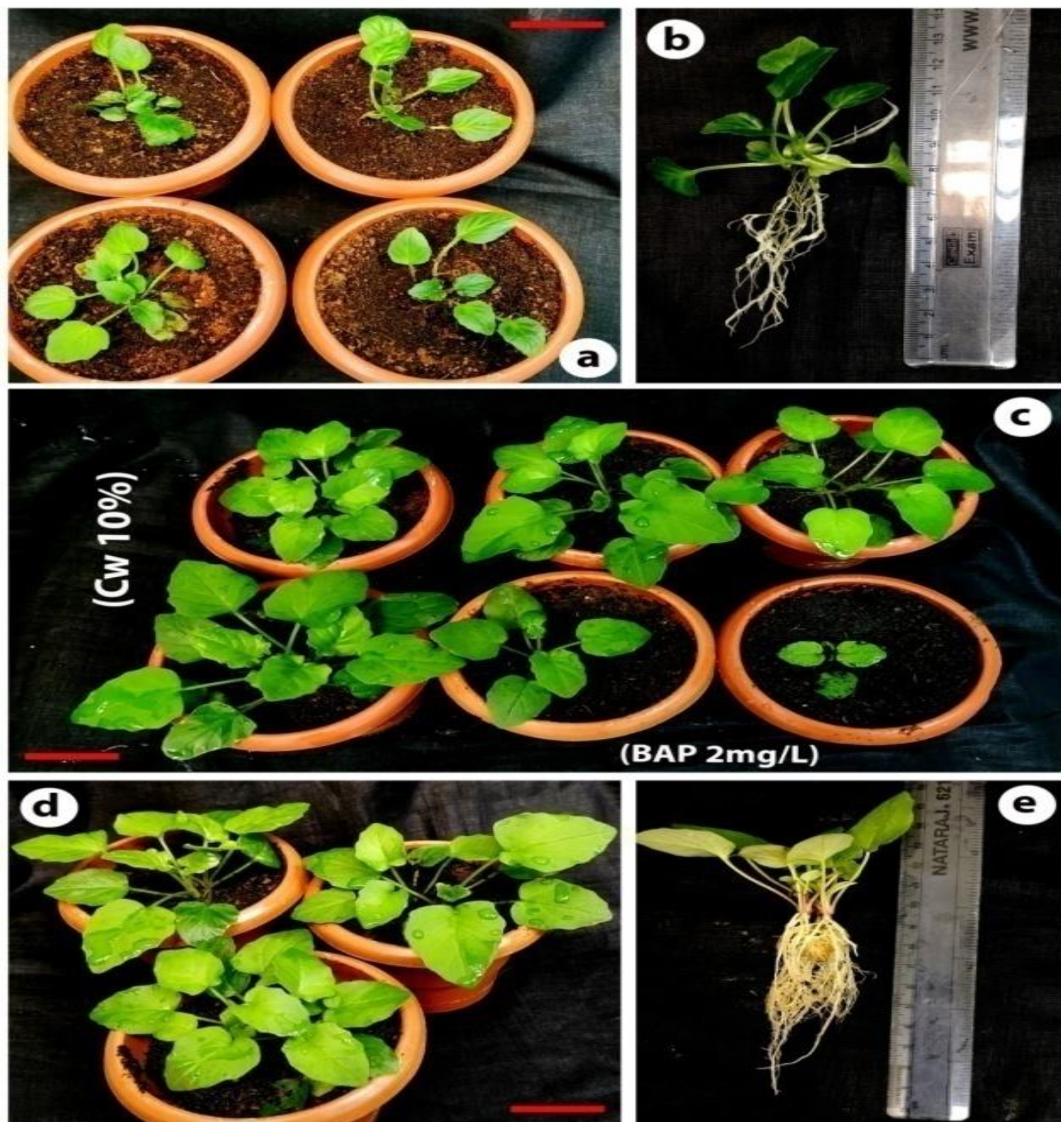


Figure 9: Acclimatization of *in vitro* regenerated plantlets of *V. jatamansi* (a) Plants of *V. jatamansi*, hardened on coco peat: soil (3:1); (b) roots formed prior to acclimatization; (c, d) acclimatized *in vitro* plantlets from BAP (2mg/L) and coconut water (10%) treatment after 15 days; (e) formation of large clumps of roots along with rhizome after one month of planting. Scale bar = 3cm

4.2.5 Establishment of Callus and regeneration of *in vitro* plants in *V. jatamansi*

Different concentrations of NAA, BAP, KN, and 2, 4-D were investigated for their callus induction effectiveness with the leaf explants of *V. jatamansi* in order to induce callus. BAP produced a callus induction response of 51.21% at concentration of 0.5 mg/L. The KN concentration at 2.0 mg/L produced the highest reaction (58.18%) among the various concentrations. A concentration of 1.5 mg/L of 2, 4-D caused a significant frequency of callus induction (70.18%). Of the four growth regulators tested, NAA (1.5 mg/L) generated the highest callus formation, with 76.17% of the explants developing calli (Table 5). After 4 days, the leaf explants inoculated onto the callus induction medium swelled, and after 10–12 days of incubation, callus formation was seen (Fig 10a). Since the media started to brown after seven days, the callus was frequently subcultured onto a new medium. BAP supplementation caused the calli to develop root hair-like structures (Fig 10b). However, neither the 2, 4-D (Fig 10c) nor NAA (Fig 10d) or NAA-supplemented medium-grown calli displayed any roots or hairy structures from the calli. All of the studied growth hormones' callus induction rates significantly decreased as growth regulator concentrations (2.0 and 2.5 mg/L) were raised.

Table 5: Effects of plant growth regulators on callus induction from leaf explants of *V. jatamansi*.

Plant growth regulators (mg/L)				Callus induction in leaf explants (%)
Control	-	-	-	0.00±00
BAP	NAA	KN	2,4D	
0.5	-	-	-	51.21±0.21^{fg}
1.0	-	-	-	29.02±0.23^{p}
1.5	-	-	-	38.24±1.16^{l}
2.0	-	-	-	22.02±0.22^{sr}
2.5	-	-	-	12.35±1.23^{t}
-	0.5	-	-	42.15±0.21^{j}
-	1.0	-	-	48.28±0.22^{i}
-	1.5	-	-	76.17±0.20^{a}
-	2.0	-	-	68.22±0.21^{c}
-	2.5	-		21.35±0.33^{s}
-	-	0.5	-	36.23±0.19^{m}
-	-	1.0	-	34.31±0.20^{n}
-	-	1.5	-	52.10±0.26^{f}
-	-	2.0	-	58.18±0.24^{e}

		2.5		30.24±0.38[o]		
-	-	-	0.5	40.18±0.32[k]		
-	-	-	1.0	49.18±0.33[h]		
-	-	-	1.5	70.18±0.31[b]		
-	-	-	2.0	67.18±0.23[d]		
-		-		-	2.5	24.00±0.35[q]

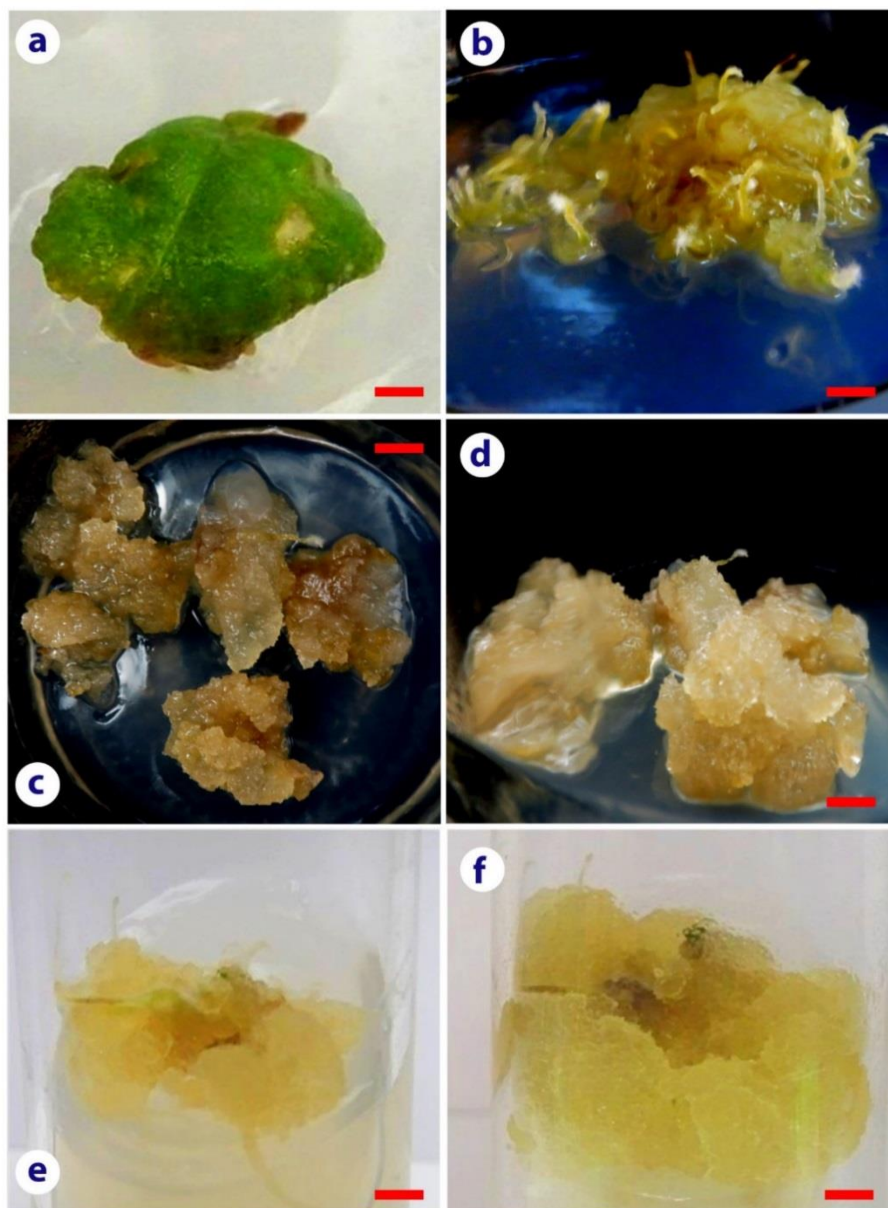
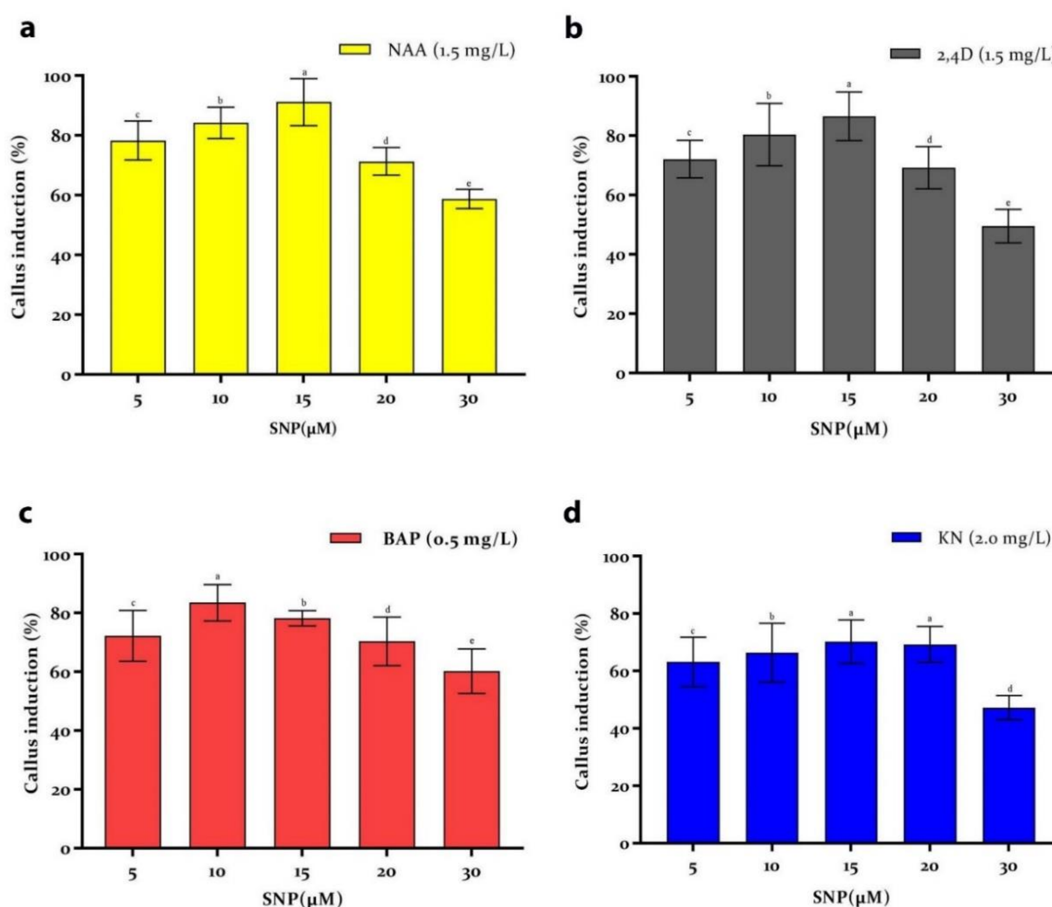


Figure 10: Callus induction of *V. jatamansi* leaf explants with different plant growth regulators and SNP combinations (a) Leaf explant 7 days post inoculation onto callus induction medium; (b) callus induced from leaf explants supplemented with 0.5mg/LBAP; (c) callus induction from leaf explants with 1.0mg/L2,4-D; (d) callus induction with 1.5mg/LNAA; (e) callus induced from leaf explants after 14 days of inoculation onto medium supplemented with 1.5 mg/LNAA+10 µM SNP; (f) callus induced from leaf explants after 14 days of inoculation onto medium supplemented with 1.5 mg/LNAA+15µM SNP. Scale bar = 1cm.

4.2.6 Effect of Sodium nitroprusside (SNP) and growth regulators on callus, multiple shoot induction and tissue browning of *V.jatamansi* Jones

The callus induction frequency was considerably increased by exogenous SNP supplementation. In contrast to calli developed in media with growth regulators and no SNP, those induced in medium supplemented with SNP displayed a friable and compact character (Fig 10c, d) (Fig 10e, f). The outcomes of SNP supplementation on the formation of calluses using four various growth regulators are shown in (Fig 11).



Values represent the mean ±SD. Means following the same letter within columns are not significantly different, according to Duncan's multiple range test ($p < 0.05$).

Figure 11: Callus induction experiments with leaf explants of *V. jatamansi* supplemented with different hormone choice, concentration and SNP treatments (a) Effects of SNP supplementation on callus induction percentages with 1.5 mg/LNAA; (b) effects of SNP supplementation on callus induction percentages with 1.5 mg/L2,4-D; (c) effects of SNP supplementation on callus induction percentages with 0.5 mg/LBAP; (d) effects of SNP supplementation on callus induction percentages with 2.0 mg/L KN.

The combination of NAA (1.5 mg/L)+15 M SNP caused the highest callus induction (91.18%), and consistently, the combination of NAA (1.5 mg/L) with all tested SNP

treatments provided a better callusing response (Fig 11a). The medium supplemented with 2, 4-D (1.5 mg/L)+15 M SNP produced the second-best response (86.55%) (Fig 11b). Callus induction was induced by the medium containing 0.5 mg/L (BAP)+10 M SNP at a rate of 83.50% (Fig 11c). Compared to the other tested growth regulators along with SNP supplementation, medium supplemented with KN responded with lower callusing percentages (Fig 11d, Table 5). The medium devoid of growth regulators and SNP was used as a control for this experiment (Table 5).

4.2.7 Effect of Coconut Water with SNP in Shoot Induction

CW proved to be the most effective growth regulator for producing shoots from the *V. jatamansi in vitro* calluses (Table 6). The 10% CW produced 81.22% shoot induction with the most shoots per explant, yielding the highest shoot induction frequency (34.31). Since this medium produced the best response when compared to the others, different concentrations of SNP were added to it in order to test its impact on the multiple shoot induction efficiency (Table 6). After being transferred to BAP+SNP medium, the calli began to produce green shoot buds (Fig 12a) and noticeable hairy root-like structures (Fig 12b), and after 10 days, the shoot buds developed into separate shoots. In contrast, green-colored buds began to show up in the calli inoculated onto the medium containing CW and SNP early on day three of the inoculation (Fig 12c, d) and the shoots became longer within 6-7 days after inoculation. When compared to BAP+SNP treatments, the CW+SNP combination had earlier shoot development and elongation (Fig 12e, f). Compared to the response obtained with the medium added with BAP+SNP, CW+15M SNP generated the greatest response (89.32%) with 35.42 mean shoots (Fig 13a, b).

The addition of a higher concentration of SNP (30 M) decreased the shoot induction percentages in both the BAP and CW cases, and a sharp decline in the quantity of shoots was also seen. Individual shoots from the calli inoculated onto the BAP+SNP (Fig 14a) and CW+SNP treatments (Fig 14b,c) treatments were excised and allowed to proliferate, and the shoots from the CW+SNP supplementation showed rapid proliferation (Fig 14d, e) and the leaves of the plants were relatively broader and greener compared to BAP treatments (Fig 14f).

Table 6: *In vitro* multiple shoots induction from calluses of *V. jatamansi.*

BAP (mg/L)	NAA (mg/L)	CW (%)	Percentage of explants forming shoots (%)	Mean number of shoots per explant
0.5	-	-	48.01±0.23^{g}	21.02±0.14^{f}
1.0	-	-	55.21±0.12^{e}	20.01±0.22^{fg}
1.5	-	-	66.10±0.32^{c}	26.32±0.18^{d}
2.0	-	-	54.20±0.56^{ef}	19.62±0.23^{h}
-	0.5	-	23.31±0.21^{jk}	10.12±0.35^{j}
-	1.0	-	27.32±0.22^{i}	12.17±0.42^{i}
-	1.5	-	24.17±0.18^{j}	9.04±0.28^{k}
-	2.0	-	14.37±0.14^{h}	6.08±0.20^{l}
-	-	1	62.22±0.51^{d}	28.18±0.23^{c}
-	-	5	72.28±0.38^{b}	31.45±0.24^{b}
-	-	10	81.22±0.54^{a}	34.30±0.30^{a}
		15	72.23±0.28^{b}	28.32±0.32^{c}
-	-	20	72.66±0.23^{b}	24.28±0.12^{e}

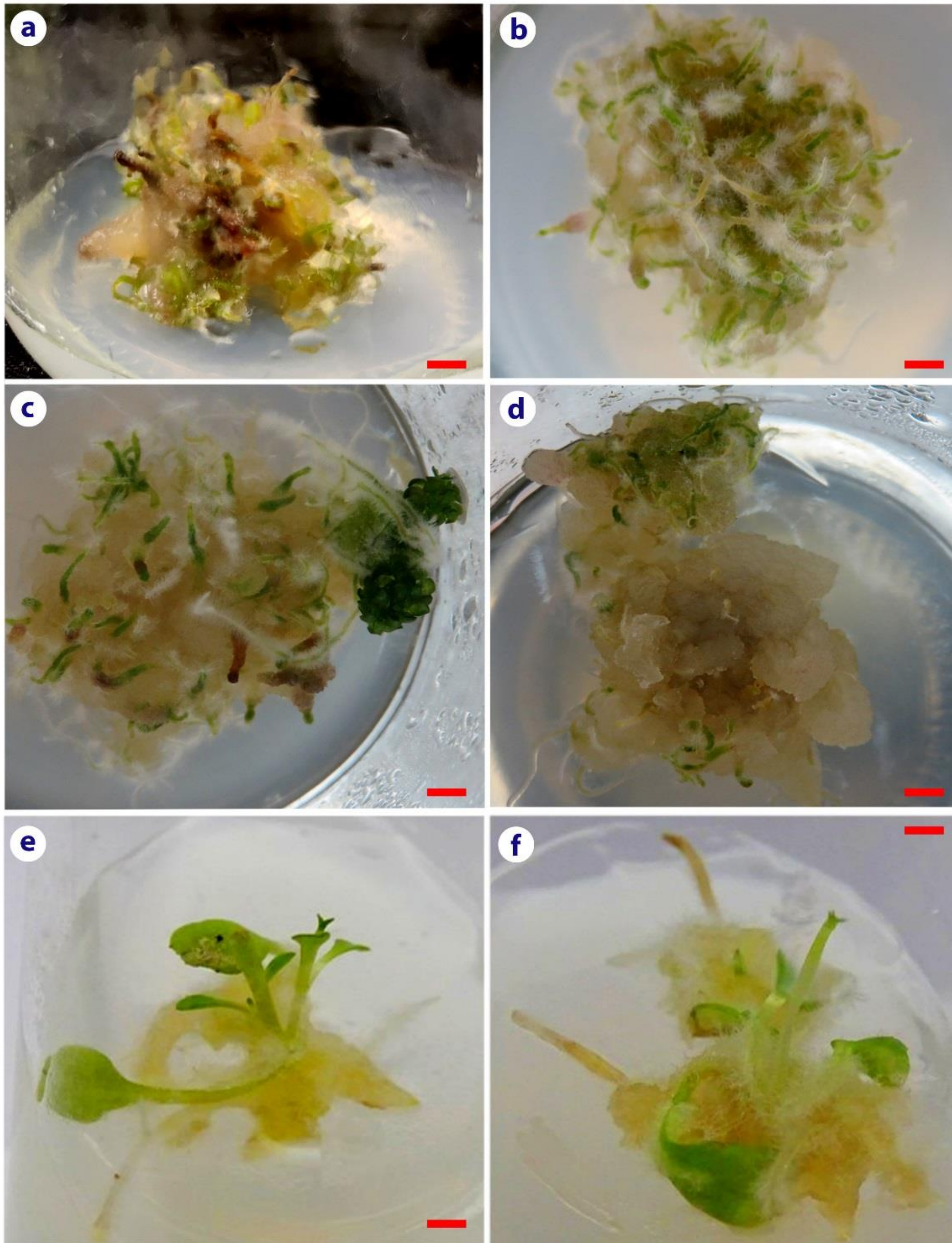


Figure 12: Effect of growth regulators and SNP on shoot induction of callus from *V. jatamansi* Shoot formation from callus supplemented with (a) 1.5 mg/LBAP+SNP 10 µM; (b) 1.5 mg/L BAP+SNP 15 µM; (c) 10% CW+15 µM SNP; (d) 10% CW+10 µM SNP; (e, f) rapid shoot initiation and formation of distinct individual shoots from two individual callus explants supplemented with 10% CW+15 µM SNP. Scale bar = 1cm.

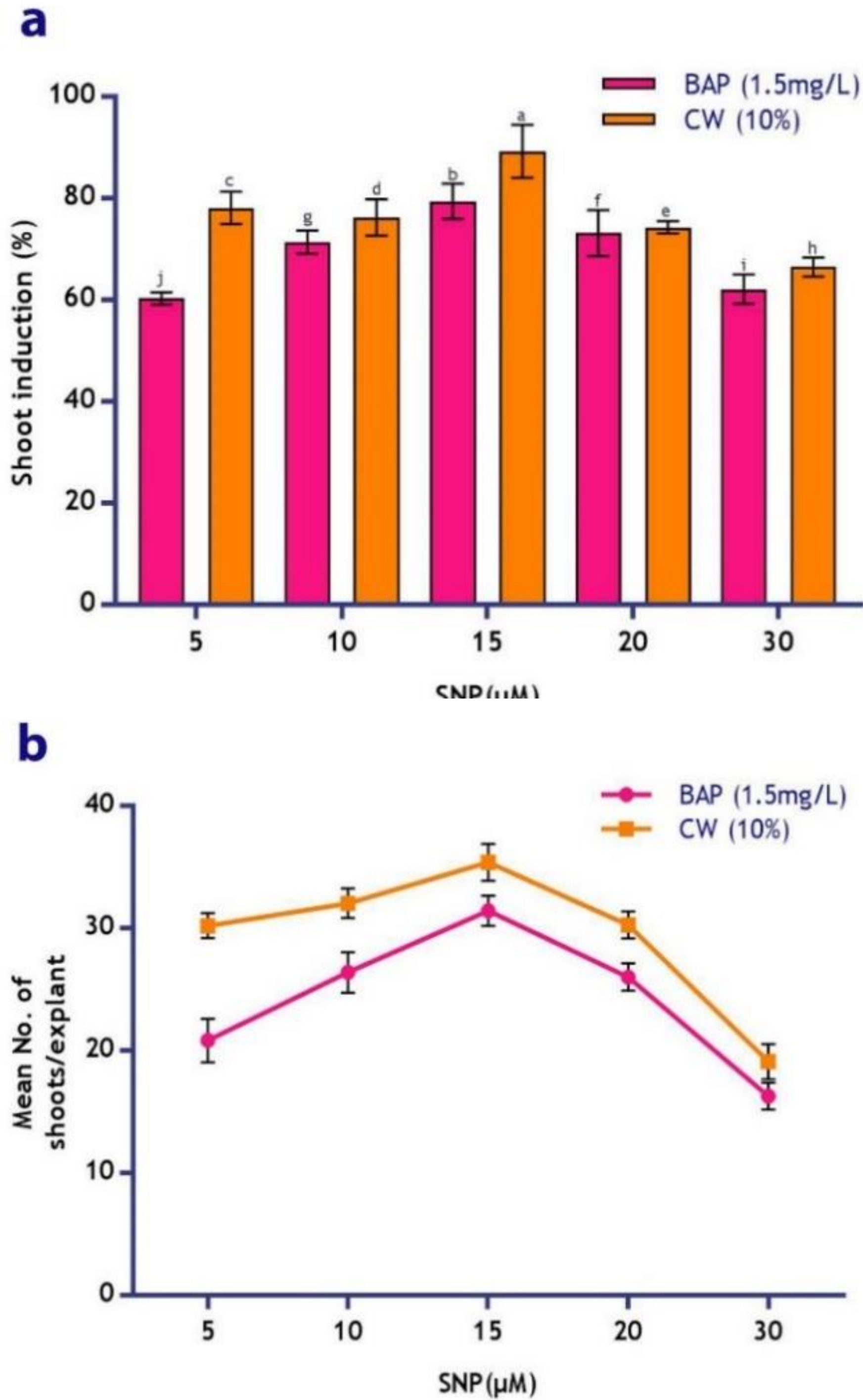


Figure 13: Effect of SNP on multiple shoot inductions from *in vitro* calli of *V. jatamansi* (a) Shoot induction frequencies of calli inoculated onto medium supplemented with 1.5 mg/L BAP, 10% CW and different concentrations of SNP; (b) mean shoots formed per explant with different concentrations of SNP. Values represent the mean±SD. Means following the same letter within columns are not significantly different, according to Duncan's multiple range test ($p < 0.05$).

Figure 14: Influence of SNP on shoot and root induction of *V. jatamansi.* (a) Shoot proliferation from callus explants supplemented with 1.5 mg/LBAP+15 µM SNP; (b,c) individual shoots excised and allowed to proliferate in 1.5 mg/LBAP+15 µM SNP fresh medium; (d) shoot proliferation from callus explants supplemented with 10% CW + 15 µM SNP; (e,f) individual shoots excised and allowed to proliferate in 10% CW+15 µM SNP fresh medium showing rapid proliferation and broader leaf structures upon transfer to fresh medium; (g) root formation with 10% CW and 10% CW+15 µM SNP. Scale bar = 2cm.

4.2.8 Effect of SNP on Callus browning

Increased growth regulator concentration led to callus browning and hyperhydricity (Fig 15a). All of the growth regulators that were evaluated had callus browning signs when used at higher dosages, especially NAA and BAP (Fig 15b). When the calli browning was left in the media without SNP, the plant eventually died as a result of stunted shoots, shoot necrosis, and other effects (Fig 15c, d). However, callus browning was lessened when the calluses were transplanted to media containing SNP, which later permitted plants to recover (Fig 15e, f).

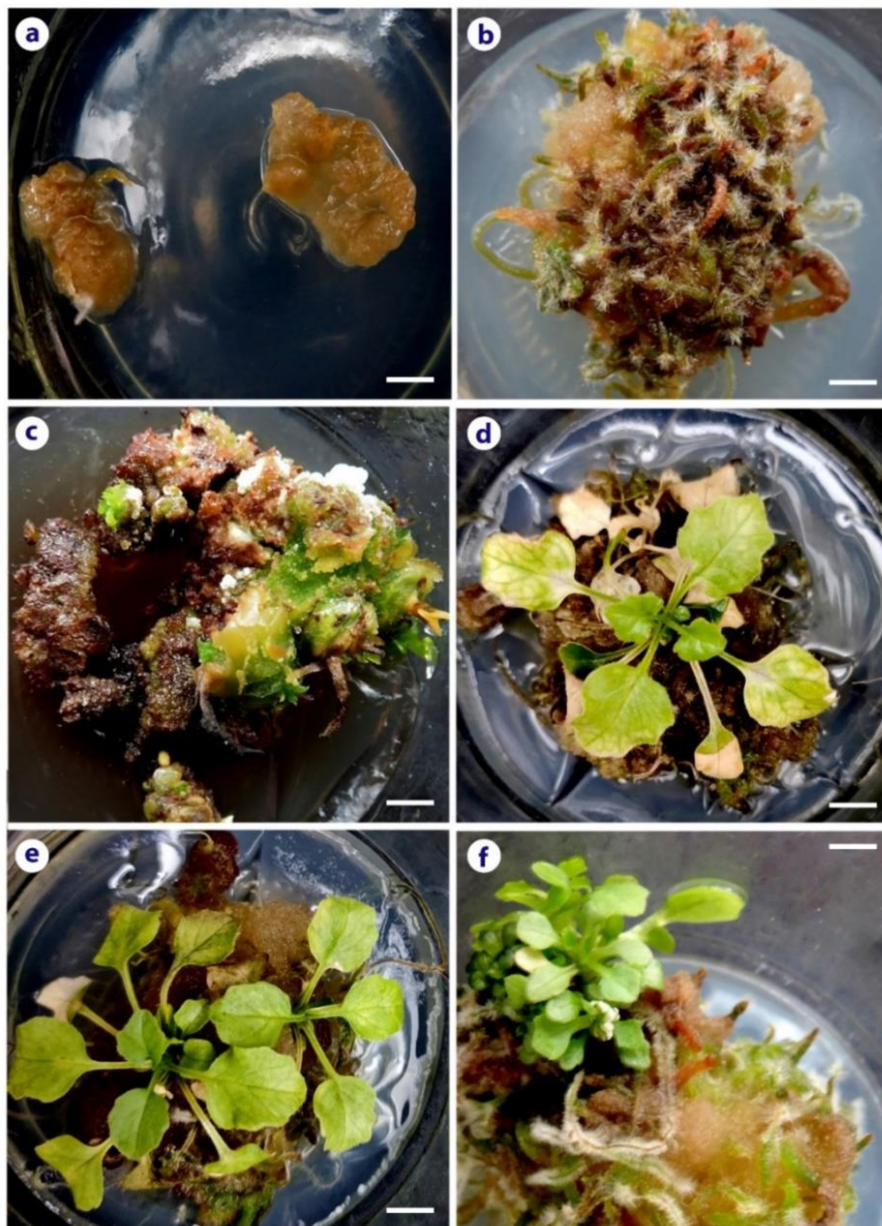


Fig 15. Effects of SNP on *in vitro* callus browning and shoot regeneration of *V. jatamansi*
(a) Callus browning and hyperhydricity symptoms with medium supplemented with BAP 2.5 mg/L; (b) calli turning brown after 7 days of culture on shoot induction medium

supplemented with 2.0 mg/LNAA; (c) callus browning and shoot necrosis during culture of explants in medium supplemented with NAA 2.0 mg/L; (d) proliferating shoot withering after 12 days of incubation in medium supplemented with 2.0 mg/LBAP; (e) calli initially cultured in 2.0 mg/L BAP transferred to BAP 2.0 mg/L+15 µM SNP showing recovery and shoot proliferation after 12 days of incubation; (f) calli initially cultured in 2.0 mg/L NAA transferred to NAA 2.0 mg/L+10 µM SNP showing recovery with initiation of multiple shoots after 6 days of incubation. Scale bars=1 cm

4.3 Establishment of culture condition and culture type for mass propagation of *Tinospora Cordifolia*

In the current investigation, axillary shoot initiation and proliferation were achieved by first cultivating the nodal segment explants on MS media supplemented with different concentrations (Table 7) of BAP and/or KIN. This course of treatment was selected in light of the nodal segment's favorable response to various concentrations of plant growth hormones. Nodal segment explants cultivated on media supplemented with BAP (2.0 mg/L) caused shoot initiation from the nodal segment explants 7 days after inoculation, among the evaluated BAP doses (Fig 16b). When transferred to MS+KIN (2 mg/L), it was discovered that the proliferation rate was modest (Fig 16c) and that only two shoots per explant were formed at the end of 30 days (Fig 16d). After 10 days, the proliferation rate significantly decreased, and even two subcultures onto new media at intervals of two weeks did not increase the proliferation (i.e., number of shoots and roots). However, as compared to other BAP concentrations, MS+KIN (2mg/L) was successful in developing shoots with more nodal segments (7.33±0.577) nodal segment no) and a shorter shoot length (3.06±0.0577 cm) (Fig 16f, 18, Table 7).

Table 7: Effect of different concentrations and combinations of PGR's on induction and proliferation of nodal segments of *T. cordifolia*

PGR's			Average shoot number	Average shoot length (cm)
BAP (mg/L)	Kinetin (mg/L)	Coconut water (%)		
0	0	0	0.66±0.577	0.233±0.0577
1.5	-	-	2.66±0.577	0.6±0.100
2	-	-	1.66±0.577	1.16±0.115
0	1.5	-	4.33±0.577	2.06±0.115
0	2	-	7.33±0.577	3.06±0.0577
0.5	0.5	-	2.3±0.577	2.133±0.1527
1	0.5	-	3.66±0.577	3.233±0.152
2	1.5	-	2.33±1.154	2.100±0.173
2	2	-	5.66±1.154	3.033±0.0577
5	0	-	9.33±0.577	8.633±0.55
5	-	5	13.33±1.52	9.433±0.378
5	-	10	20.33±0.577	12.10±0.10

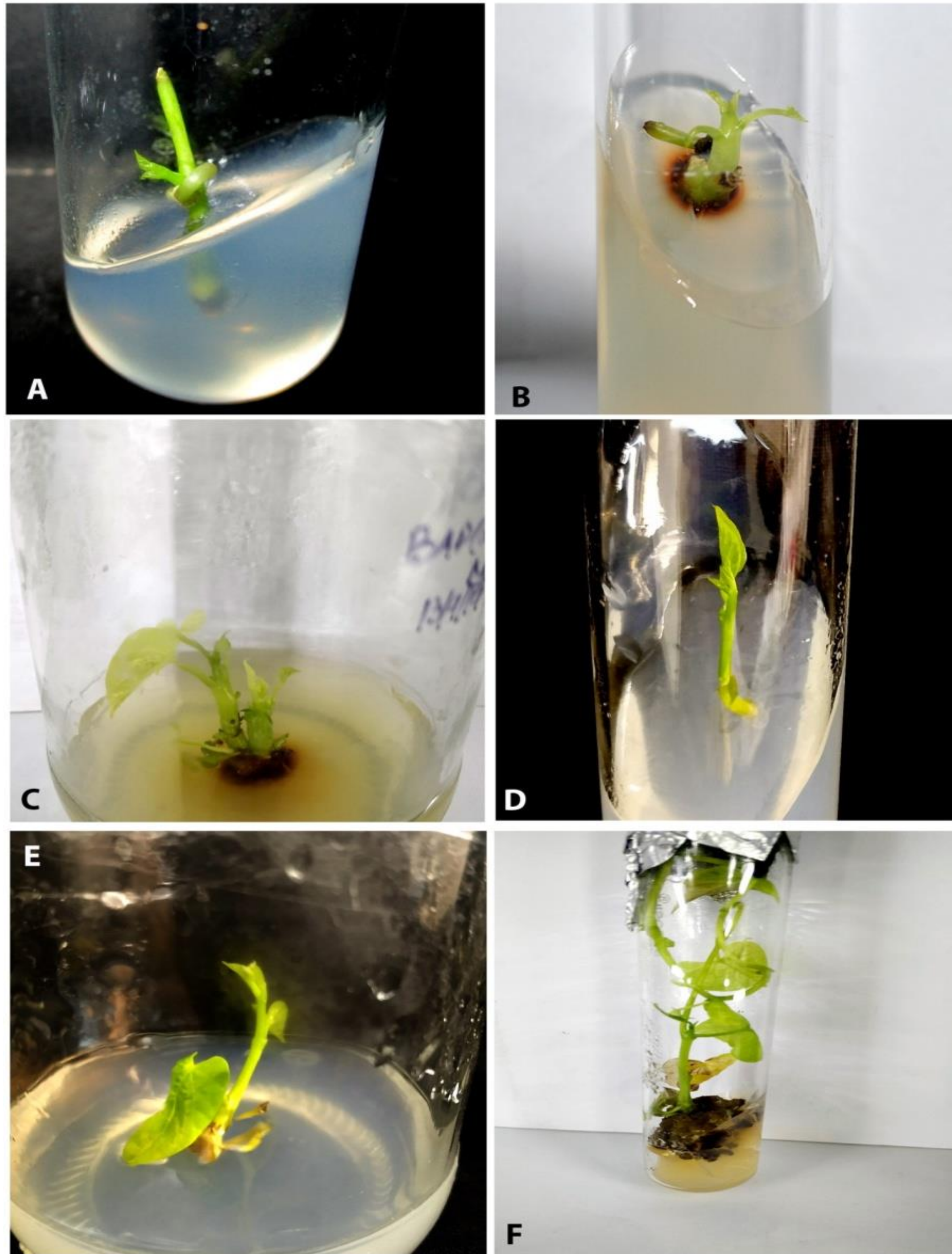


Figure 16: *In vitro* regeneration of *Tinospora cordifolia* (a) Nodal segment of *Tinospora cordifolia* inoculated *in* MS+BAP(2mg/L); (b) nodal segment explant post 7 days incubation in BAP (2mg/L)*;* (c) nodal segment explant post 25 days incubation in BAP (2mg/L); (d) Nodal segment of *Tinospora cordifolia inoculation in* MS+KIN (2mg/L)*;* (e) Nodal segment of *Tinospora cordifolia* explant post 7 days incubation in MS+KIN (2mg/L); (f) Nodal segment of *Tinospora cordifolia* explant post 25 days incubation in MS+KIN (2mg/L).

4.3.2 Mass Multiplication and Rooting of the Nodal segments in *T. cordifolia*

The nodal explants taken from *in vitro* generated shoots grown in media containing BAP (2mg/L) were inoculated onto MS+BAP (5mg/L) alone and supplemented with coconut water (5%, 10%) in order to investigate the effectiveness of coconut water in promoting the shoot proliferation of *T. cordifolia*. However, the maximum response was attained with the addition of 10% coconut water in the medium, producing more shoots (20.33±0.577 no of shoots) and (12.10±0.10 cm length) as compared to other concentrations of coconut water (Fig 17H, 18). The shoot proliferation increased significantly at 5 and 10% (Fig 17D). All the experiment was performed using control where only MS was used without different plant growth regulators.

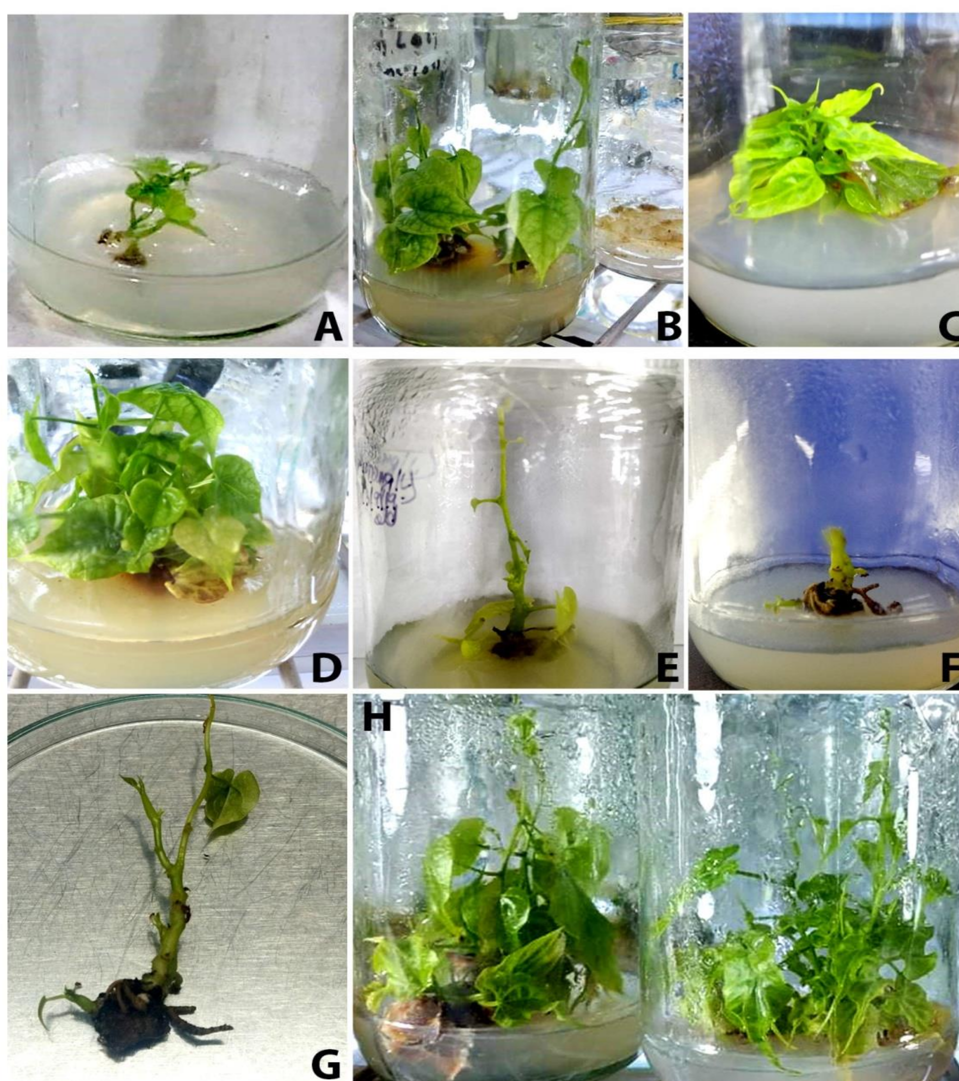


Figure 17: *Mass multiplication of T. cordifolia* (A); Nodal segment of *T. cordifolia* inoculated in MS+BAP (5mg/L); (B) nodal segment explant post 10 days incubation in BAP (5mg/L); (C) nodal segment explant post 25 days incubation in BAP (5mg/L); (D) Nodal segment of *T. cordifolia*

inoculated in MS+BAP (5mg/L)+5% CW; (E) Nodal segment of *T.cordifolia* explant post 15 days incubation in MS Basal Medium; (F,G) Rooting in *T.cordifolia* in MS+BAP (0.5mg/L); (H) Nodal segment of *T.cordifolia* explant post 25 days incubation in MS+BAP (5mg/L)+10% CW.

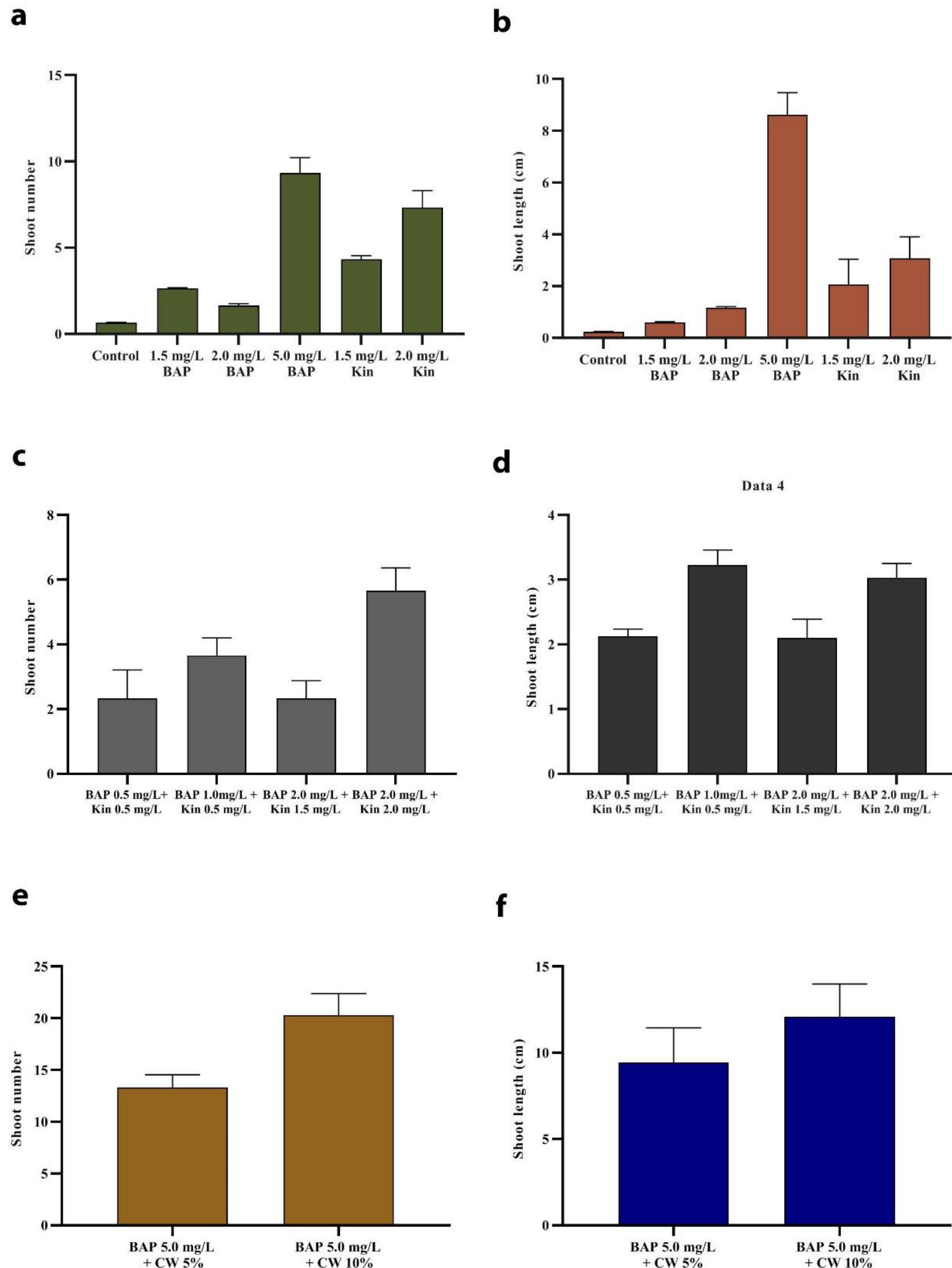


Figure 18: Effect of PGR'S on shoot induction from nodal explants of *T. cordifolia* (a) mean number of shoots per explant with different concentrations of BAP and Kin (b) mean shoot lengths with different concentration of BAP and Kin (c) mean number of shoots per explant with different concentrations of BAP+Kin (d) mean lengths with different concentration of BAP+Kin (e) mean

number of shoots per explant with different concentrations of BAP and coconut water (f) mean shoot lengths with different concentration of BAP and coconut water. Data taken after 5 weeks of culture. Data presented as mean ± SD (*n*=). Means following the same letter are not significantly different, according to Duncan's multiple range test ($p<0.05$).

Supplementing with coconut water (10%) resulted the best response in terms of shoot numbers (20.0) and mean shoot length (12.0 cm). The outcomes proved that in comparison to the control MS Basal Medium, media enriched with coconut water greatly improved the shoot induction of nodal explants from *T. cordifolia* (Fig 17H) (Table 7, Fig 17E, Fig 18). Similar to this, rooting was also seen when MS+BAP (5mg/L)+10% CW-containing medium shoots were infected with MS+IBA (0.5mg/L) (Fig 17 F, G).

4.4 Genetic fidelity analysis between *in vivo* mother plants and *in vitro* grown plants by using molecular markers in *V. jatamansi* and *T. cordifolia*

4.4.1 Extraction of genomic DNA

Total genomic DNA of *in vitro* and *in vivo* mother plant material (leaves) of *V. jatamansi* Jones was extracted by CTAB method .CTAB method gave rise to highly concentrated DNA (50 ng/ul). DNA samples were diluted in TE buffer (to make 25 ng) and subjected to gel electrophoresis in 1% agarose gel (w/v). After sometime, clear smear of DNA bands were observed. Extracted DNA thus obtained was then used for PCR amplification

4.4.2 Polymerase chain reaction

In the present investigation, PCR-based molecular technique of RAPD and ISSR markers were used to assess the genetic variations among the five randomly chosen tissue cultured plantlets and compared with the non-tissue cultured source plant of *V. jatamansi* Jones collected from the wild (mother plant), as well as genetic homogeneity was analyzed among the acclimatized *in vitro* plant and mother (wild) plant.

RAPD, ISSR analysis of *V. jatamansi*

Ten of the 35 RAPD primers used in the current investigation (OPA O8, OPA 10, OPA 18, OPB 07, OPD 04, OPE 08, OPC 11, UBC 210, UBC 292 and UBC 465) produced similar and repeatable banding patterns between the *in vitro* generated plantlets and the

mother plant (Table 2). The total number of bands for the RAPD primer ranged from 1 to 6, and the band sizes ranged from 200 to 1100 bp. In a similar manner, 5 primers (HB8, HB9, HB10, HB11 and 17898B) from the 10 primer sets of ISSR markers employed provided reproducible bands patterns between the *in vitro* produced plantlets and the mother plant (Table 3). The number of bands for the ISSR primer ranged from 1 to 3, while the band sizes ranged from 250 to 1000 bp.

4.4.3 Genetic fidelity analysis of *V. jatamansi*

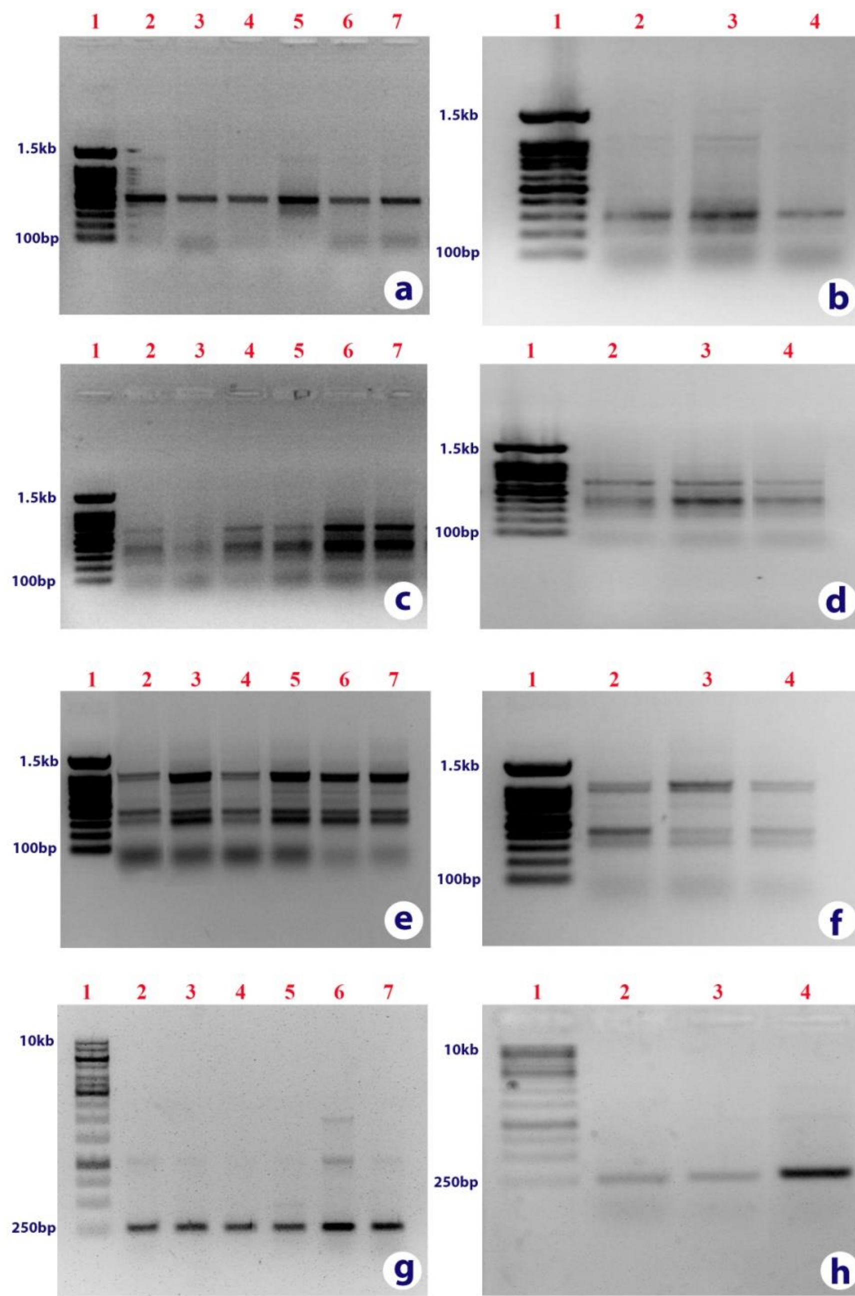


Figure 19 Representative images depicting the genetic fidelity analysis of *in vitro*, mother and acclimatized plants of *V. jatamansi* using RAPD and ISSR primers (a) OPB 07 Primer 1- marker, 2 to 6

in vitro plants; 7 mother plant; (b) OPB 07 Primer 1- marker, 2 mother plant; 3 and 4 acclimatized plants; (c) OPA08 Primer 1- marker, 2 to 6 *in vitro* plants 7 mother plant; (d) OPA08 Primer 1- marker, 2 mother plant; 3 and 4 acclimatized plants (e) HB08 Primer 1- marker, 2 to 6 *in vitro* plants; 7 mother plant; (f) HB08 Primer 1- marker, 2 mother plant: 3 and 4 acclimatized plants; (g) HB09 Primer 1- marker, 2 to 6 *in vitro* plants 7 mother plant; (h) HB09 Primer 1- marker, 2 mother plant; 3 and 4 acclimatized plants

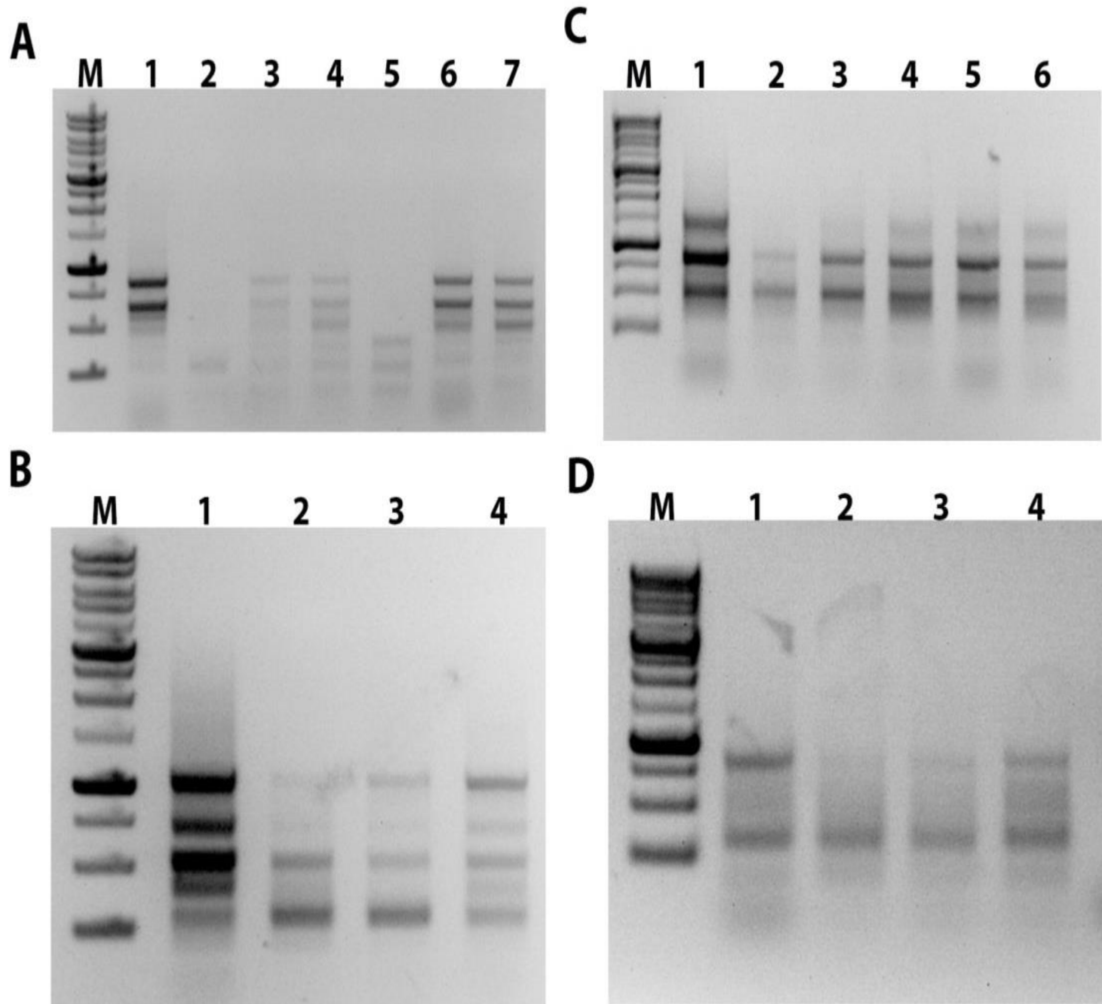


Figure 20: Representative images depicting the genetic fidelity analysis of *in vitro*, mother plant of *V. jatamansi* using RAPD, M depicting the molecular ladder of 100 bp-1.5 kb (A) OPA 10 Primer -1 to 6 *in vitro* plants; 7 mother plant; (B) OPA 18 Primer 1- to 3 *in vitro* plants and 4 mother plant; (C) OPC11 Primer 1 to 5 *in vitro* plants, 6 mother plant; (D) UBC292 Primer 1 to 3 *in vitro* plants; 4 mother plant.

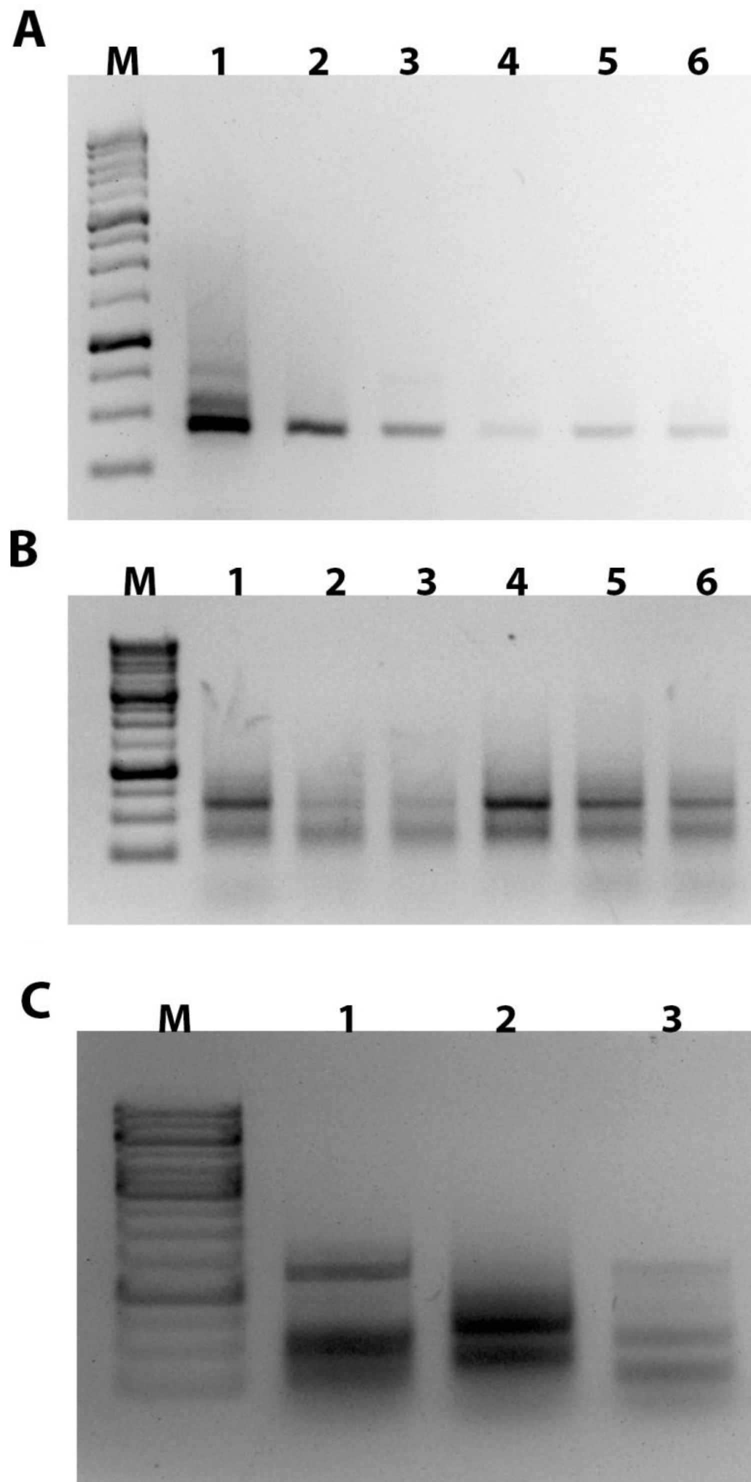


Figure 21: Representative images depicting the genetic fidelity analysis of *in vitro*, mother plant of *V. jatamansi* using ISSR, M depicting the molecular ladder of 100 bp-1.5 kb**;** (A) 17898 B Primer 1 mother plant, 2 to 6 *in vitro* plants; (B) HB10 Primer, 1 mother plant , 2 to 6 *in vitro* plants; (C) HB11 Primer, 1 mother plant, 2 to3 *in vitro* plants

RAPD, ISSR analysis of *T.cordifolia*

In the current study, 35 RAPD primers were first tested, and ten primers (OPA O8, OPA 10, OPA 18, OPB 07, OPD 04, OPE 08, OPC 11, UBC 210, UBC 292 and UBC 465) provided identical and repeatable banding patterns between the *in vitro* produced plantlets and the mother plant (Table 2). The number of bands varied from 1 to 4 with total no of 22 bands for RAPD primer with band size between 100-1200 bp (Fig 22). Similarly, out of the 10 primer sets of ISSR markers used, 5 primers (HB8, HB9, HB10, HB11 and 17898B) gave reproducible bands patterns between the *in vitro* raised plantlets and the mother plant (Table 3). The number of bands varied from 1 to 3 with total no of bands for ISSR primer with band size between 100-400 bp (Fig 23)

4.4.4 Genetic Fidelity analysis of *T. cordifolia*

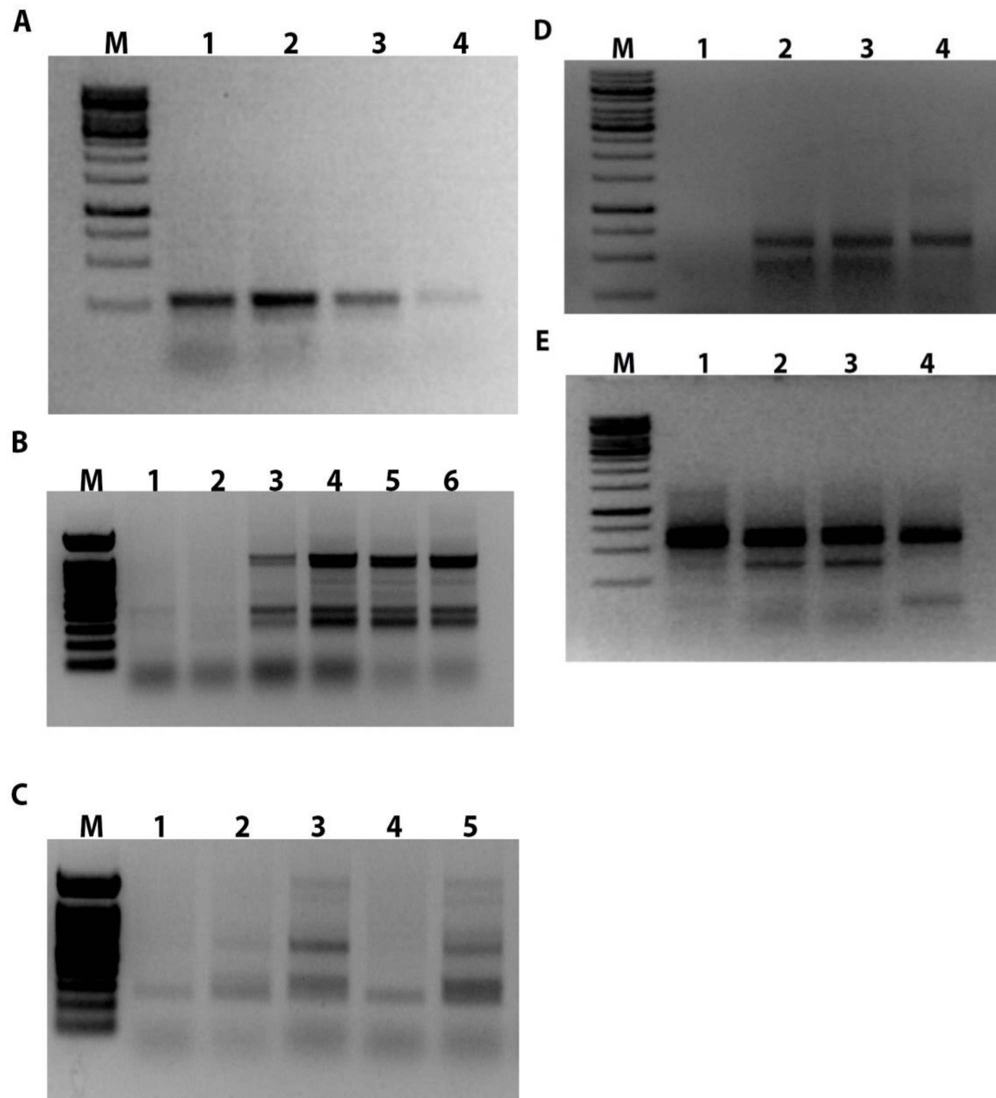


Figure 22 Representative images depicting the genetic fidelity analysis of *in vitro*, mother plant of *T. cordifolia* using RAPD , M depicting the molecular ladder of 100 bp-1.5 kb (A) OPA 10 Primer , 1

mother plant , 2 to 4 *in vitro* plants; (B) OPB07 Primer , 3 mother plant ,4 to 6 *in vitro* plants; (C) OPD 04 Primer, 1 to 4 *in vitro* plants, 5 mother plant; (D) OPE 08 Primer , 2 mother plant , 3, 4 *in vitro* plants; (E) OPA 18 Primer, 1 mother plant, 2 to 4 *in vitro* plants.

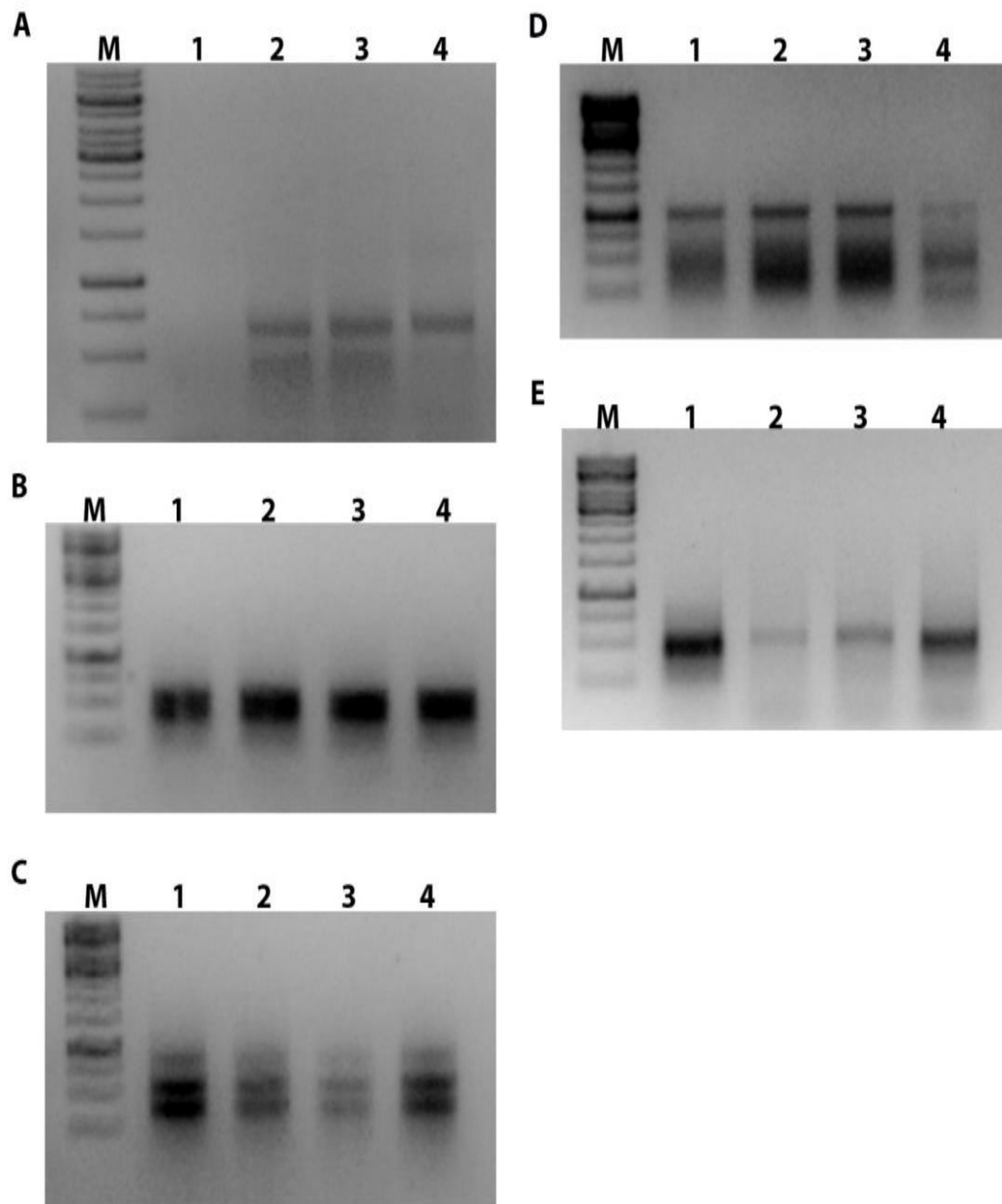


Figure 23 Representative images depicting the genetic fidelity analysis of *in vitro*, mother plant of *T. cordifolia* using ISSR M depicting the molecular ladder of 100 bp-1.5 kb (A) 17898 B Primer 1 to 3 *in vitro plants* , 4 *in* mother plants; (B) HB08 Primer, 1to 3 *in vitro* plants ,4 mothe*r* plant; (C) HB09 Primer, 1to 3 in vitro plants, 4 mothe*r* plant; (D) HB10 Primer , 1-Mother plant, 2-4 *in vitro* plants; (E) HB11 Primer, 1-Mother plant, 2-4 *in vitro* plants

4.5 *In vitro* adventitious root multiplication of *V. jatamansi*

4.5.1 Growth kinetics of adventitious root cultures

V. jatamansi plant nodal explants were inoculated into the MS media and allowed to grow (Fig 24 a). The roots were harvested from the well-proliferated *in vitro* plants (Fig 24b, c) and inoculated in the liquid MS medium that had been treated with various PGRs at various concentrations. Based on the FW and DW analysis, the adventitious root cultures growth rate from the original inoculum was evaluated. The cultures grown on a medium enriched with IAA (Fig 24e) and IBA (Fig 24f) demonstrated greater root proliferation compared to the control cultures (basal media devoid of PGRs), which exhibited a slower growth response (Fig 24d). However, NAA (2.0 mg/L) caused extensive rooting and the production of huge clumps of roots (Fig 24g).

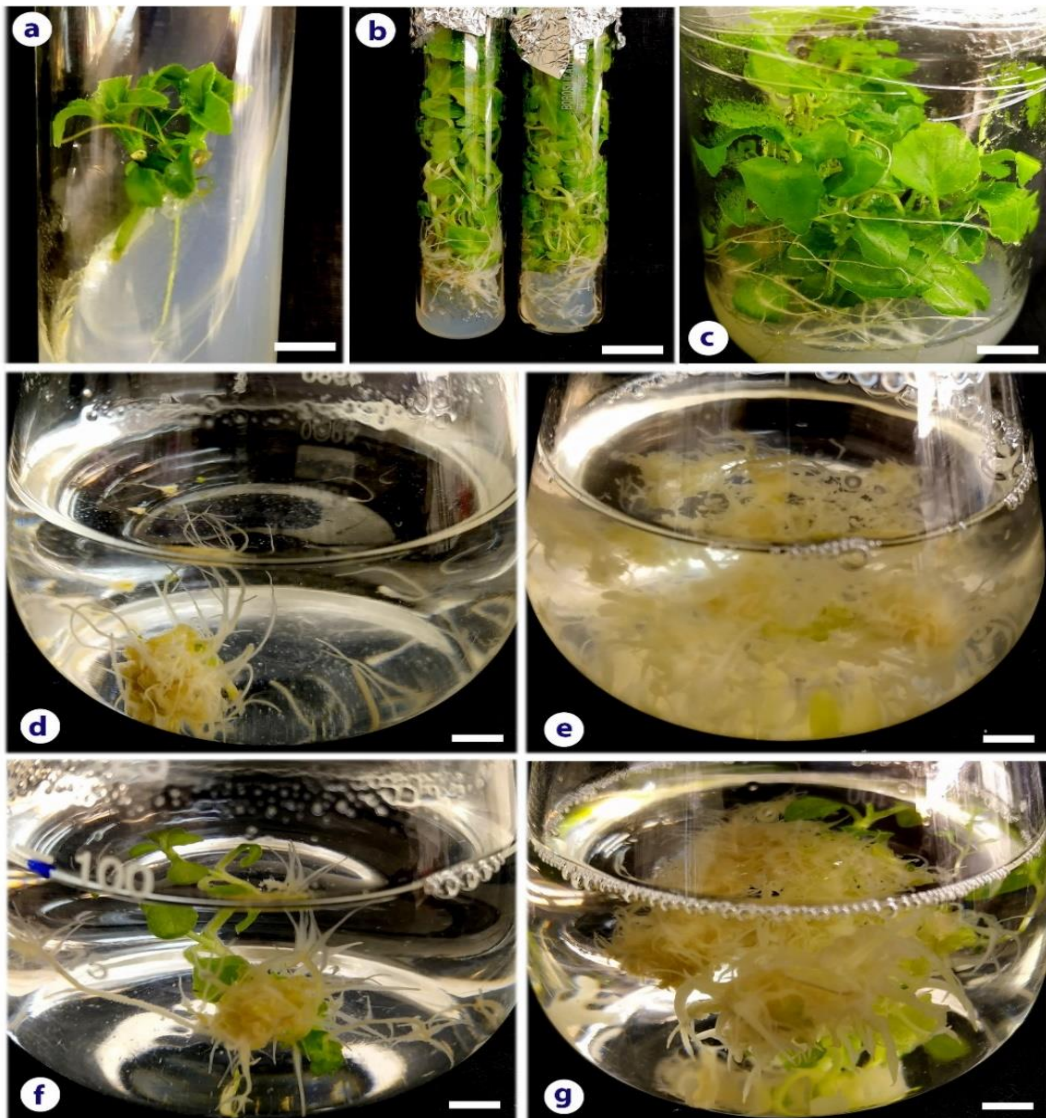


Figure 24: Establishment of *in vitro V.jatamansi* plants and adventitious root cultures (a) shoot proliferation from nodal segment explant of *V.jatamansi*; (b and c) establishment of *in vitro* plants during routine subculture; (d) adventitious root formation in MS basal media after 25 days; (e) adventitious root formation in medium supplemented with IAA (2.0 mg/L) after 25 days; (f) adventitious root and prominent shoot formation in medium supplemented with IBA(1.5 mg/L) after 25 days; (g) adventitious root cultures established in medium supplemented with NAA (2.0 mg/L) after 25 days. Scale bar = 2cm

In comparison to the control, adding NAA (2.0 mg/L) caused the largest biomass accumulation among the investigated PGRs producing a FW of 7.43g and a DW of 3.06g, comparated to the control respectively (2.78g of FW and 1.0g DW). In general, liquid cultures with NAA supplementation demonstrated a comparatively superior increase of root biomass.

Adventitious roots treated with various PGR concentrations showed growth progression up until day 30, after which the proliferation decreased (Table 8). IAA, on the other hand, produced 4.23g when the medium was supplemented with 1.5 mg/L and 5.13g when 2.0 mg/L was used (Fig 25c, d). With a maximum FW of 4.01g and 4.11g with 2.0 mg/L on days 30 and 40, respectively, the IBA-treated cultures did not respond well relative to the other two tested PGRs (Fig 25e, f). However, compared to NAA, neither IAA nor IBA were able to boost root biomass. Additionally, it was found that the start of the shoot parts produced all of the PGRs examined in the liquid cultures. Although less root growth was seen in media supplemented with IBA, which in turn decreased the ultimate biomass levels, the most obvious growth of shoots was seen in cultures treated with IBA (Fig 25f).

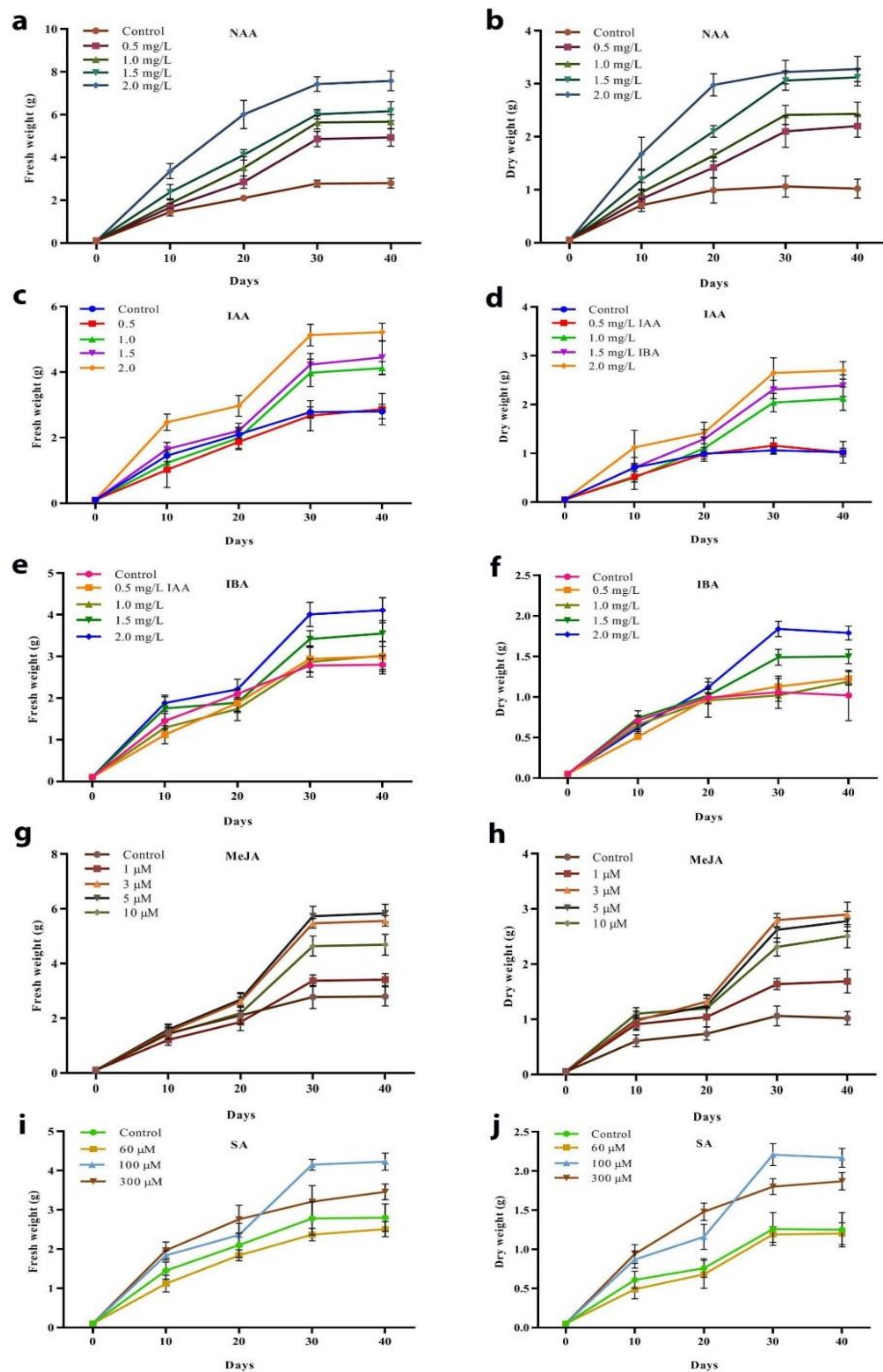


Figure 25. Fresh and dry weight analysis of adventitious root cultures grown in medium supplemented with different growth hormones and elicitors at various concentrations. (a) FW; (b) DW analysis of root cultures grown in medium containing NAA; (c) FW; (d) DW analysis of root cultures grown in medium containing IAA; (e) FW; (f) DW analysis of root cultures grown in medium containing IBA; (g) FW; (h) DW analysis of root cultures grown in medium containing MeJA; (i) FW; (j) DW analysis of root cultures grown in medium containing SA. Data collected till 40 days of incubation and the values are presented as mean±SD of three replicates.

Table 8: Fresh and dry weight analysis of adventitious root cultures grown in medium supplemented with different growth hormones at various concentrations.

Treatments	Day 0		Day 10		Day 20		Day 30		Day 40	
	FW (g)	DW (g)	FW(g)	DW(g)	FW(g)	DW(g)	FW(g)	DW(g)	FW(g)	DW(g)
Control MS basal	0.1	0.05	1.45±0.18	0.71±0.12	2.10±0.12	0.99±0.24	2.78±0.16	1.06±0.20	2.80±0.22	1.02±0.31
NAA (mg/L)										
0.5	0.1	0.05	1.66±0.19^{g}	0.83±0.18^{d}	2.85±0.29^{d}	1.42±0.20^{d}	4.86±0.36^{e}	2.10±0.30^{e}	4.94±0.41^{e}	2.20±0.24^{e}
1.0	0.1	0.05	1.85±0.22^{e}	0.95±0.19^{c}	3.51±0.52^{c}	1.65±0.11^{c}	5.64±0.31^{c}	2.41±0.18^{d}	5.67±0.33^{c}	2.43±0.26^{d}
1.5	0.1	0.05	2.38±0.36^{c}	1.19±0.20^{b}	4.12±0.24^{b}	2.10±0.11^{b}	6.02±0.22^{b}	3.06±0.18^{b}	6.16±0.45^{b}	3.12±0.16^{b}
2.0	0.1	0.05	3.36±0.35^{a}	1.68±0.31^{a}	6.01±0.66^{a}	2.98±0.21^{a}	7.43±0.34^{a}	3.22±0.22^{a}	7.58±0.46^{a}	3.28±0.24^{a}
IAA (mg/L)										
0.5	0.1	0.05	1.02±0.54^{i}	0.52±0.11^{g}	1.87±0.24^{g}	0.98±0.14^{h}	2.67±0.46^{i}	1.06±0.16^{h}	2.87±0.48^{i}	1.02±0.22^{i}
1.0	0.1	0.05	1.23±0.26^{h}	0.50±0.24^{g}	1.99±0.32^{f}	0.97±0.22^{h}	3.98±0.42^{f}	2.04±0.19^{e}	4.12±0.20^{f}	2.12±0.24^{e}
1.5	0.1	0.05	1.65±0.21^{g}	0.71±0.21^{e}	2.01±0.23^{f}	1.29±0.20^{f}	4.03±0.34^{f}	2.31±0.19^{de}	4.12±0.51^{f}	2.39±0.22^{d}
2.0	0.1	0.05	2.97±0.25^{b}	1.12±0.35^{b}	2.47±0.32^{e}	1.32±0.22^{e}	5.13±0.33^{d}	2.65±0.31^{c}	5.22±027^{d}	2.70±0.18^{c}
IBA (mg/L)										
0.5	0.1	0.05	1.12±0.22^{i}	0.51±0.01^{g}	1.88±0.20^{g}	0.98±0.07^{h}	2.94±0.31^{h}	1.13±0.10^{h}	3.0±0.36^{h}	1.23±0.08^{h}
1.0	0.1	0.05	1.29±0.20^{h}	0.66±0.11^{f}	1.74±0.28^{h}	0.96±0.05^{h}	2.87±0.36^{h}	1.02±0.07^{h}	3.12±0.33^{h}	1.19±0.02^{h}
1.5	0.1	0.05	1.75±0.28^{f}	0.74±0.02^{e}	1.89±0.24^{g}	1.02±0.07^{g}	3.42±0.20^{g}	1.49±0.10^{g}	3.55±0.31^{g}	1.50±0.09^{g}
2.0	0.1	0.05	1.98±0.19^{d}	0.62±0.05^{f}	2.06±0.24^{f}	1.12±0.07^{g}	4.01±0.29^{f}	1.84±0.10^{f}	4.11±0.30^{f}	1.79±0.08^{f}

The data was analysed using one-way analysis of variance (ANOVA). Means following the same letter within columns are not significantly different, according to Duncan's multiple rangtest ($p<0.05$).

Data presented as mean±SD of three replications. The data was analysed using one-way analysis of variance (ANOVA). Means following the same letter within columns are not significantly different, according to Duncan's multiple range test (p<0.05).

4.6 Effect of elicitor on adventitious root cultures of *V. Jatamansi*

The FW and DW of the elicitor-treated root cultures were also evaluated on a regular basis, and it was discovered that the MeJA-treated cultures had the highest levels of biomass accumulation after 30 days (Table 9). MeJA at 3µM, 5µM, and 10µM all demonstrated increased root proliferation when compared to MeJA at 1µM (Fig. 26a). MeJA at 3 µM and 5 µM indicated respective FW of 5.47g and 5.73g (Fig 25g, h). The growth hormone treatments and MeJA both showed significant biomass accumulation in SA, whereas SA did not produce significant biomass (Fig 25i, j). At the 30 days interval, the FW varied between 2.37g (60 µM) and 4.15 (300 µM) for the various concentration examined (Fig 25i, j). It was found that the PGR NAA (2.0 mg/L) was the optimum choice to produce adventitious roots in the liquid cultures of *V. jatamansi* when compared to the elicitation studies.

Table 9: Fresh and dry weight analysis of adventitious root cultures grown in medium supplemented with elicitors MeJA and SA at various concentrations. As mean±SD of three replications. The data was analysed using one-way analysis of variance (ANOVA). Means following the same letter within columns are not significantly different, according to Duncan's multiple range test ($p<0.05$)

Treatments	Day 0		Day 10		Day 20		Day 30		Day 40	
	FW(g)	DW(g)	FW(g)	DW(g)	FW(g)	DW(g)	FW(g)	DW(g)	FW(g)	DW(g)
Control	0.1	0.05	1.45±0.22	0.061±0.11	2.10±0.31	0.074±0.12	2.78±0.42	1.06±0.18	2.80±0.35	1.02±0.12
Elicitors (µM)										
MeJA										
1	0.1	0.05	1.21±0.20[f]	0.91±0.11[bc]	1.86±0.31[e]	1.04±0.18[e]	3.37±0.21[e]	1.64±0.10[f]	3.41±0.22[e]	1.69±0.21[f]
3	0.1	0.05	1.51±0.24[cd]	0.97±0.10[b]	2.61±0.33[b]	1.32±0.11[b]	5.47±0.18[b]	2.80±0.12[a]	5.56±0.19[b]	2.90±0.22[a]
5	0.1	0.05	1.58±0.21[c]	0.99±0.16[b]	2.68±0.22[b]	1.24±0.21[c]	5.73±0.36[a]	2.62±0.22[b]	5.84±0.32[a]	2.78±0.18[b]
10	0.1	0.05	1.42±0.19[e]	1.10±0.11[a]	2.18±0.22[d]	1.20±0.18[cd]	4.64±0.32[c]	2.31±0.16[c]	4.69±0.38[c]	2.51±0.21[c]
SA										
60	0.1	0.05	1.12±0.21[f]	0.49±0.12[d]	1.84±0.14[e]	0.68±0.18[f]	2.37±0.16[g]	1.19±0.10[g]	2.51±0.19[f]	1.20±0.14[g]
100	0.1	0.05	1.84±0.12[b]	0.87±0.10[c]	2.36±0.30[c]	1.16±0.16[d]	4.15±0.14[d]	2.21±0.14[d]	4.23±0.22[d]	2.17±0.12[d]
300	0.1	0.05	1.97±0.21[a]	0.94±0.12[b]	2.76±0.36[a]	1.48±0.11[a]	3.21±0.41[f]	1.80±0.10[e]	3.46±0.20[e]	1.87±0.11[e]

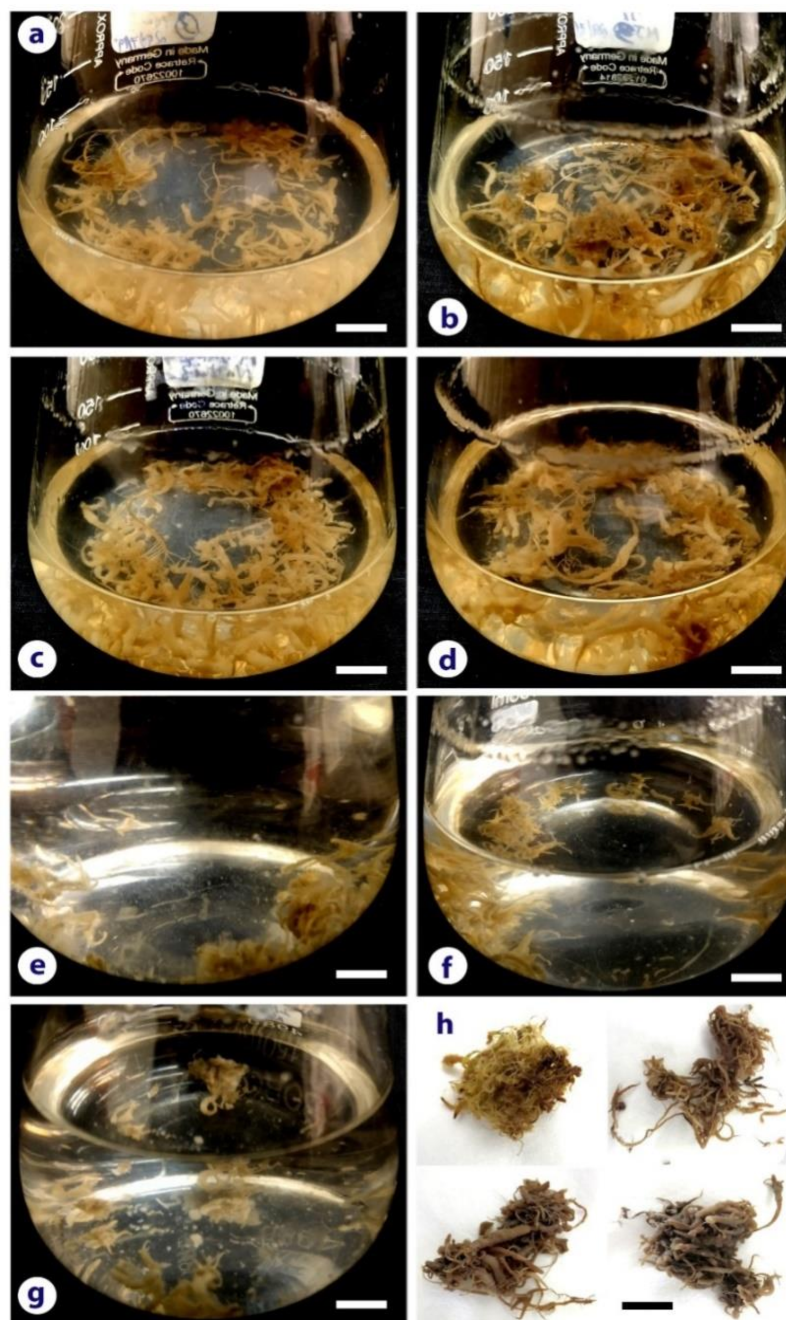


Figure 26: Elicitation experiments with *V.jatamansi* adventitious root cultures. Root cultures after 8 days of elicitation with MeJA and SA (a) MeJA 1µM; (b)MeJA 3µM; (c) MeJA 5µM; (d)MeJA10µM; (e)SA 60 µM; (f) SA 100 µM; (g) SA 300 µM; (h) representative images of part of adventitious roots grown in NAA 2 mg/L (top left), MeJA 3µM (top right), MeJA 5µM (bottom left) and SA 100µM (bottom right) harvested for extract preparation. Scale bar= 1 cm

4.7 Phytoconstituent and antioxidant potential of different cultures in *V. Jatamansi*

The results of the phytochemical and antioxidant potential of different *in vitro* cultures along with the wild mother plant is discussed below:

The methanol *in vitro* root extracts of *V. jatamansi* plants grown in solid medium containing MS+10% CW possessed the highest phenolics (55±1.00 mg/ g^{-1}), flavanoid content (219±1.00 mg/g^{-1}) and antioxidant activity (84.33±0.577%) when compared with wild extracts (Phenolics, 49±1.00 mg/ g^{-1}, flavanoids, 124±1.00 mg/ g^{-1}, and antioxidant 78.33±0.569%) (Table 10, 11)

Table 10: Contents of total phenolic and flavonoid of in vitro and wild extract. Values are expressed as mean ± SD (n=3).

Sample	Extract	Total phenol (mg gallic acid g^{-1})	Total flavonoids (mg quercetin g^{-1})
In vitro	Methanol	55± 1.00	219±1.00
Wild	Methanol	49±1.00	124±1.00

Table 11: Contents of total phenolic and flavonoid of *in vitro* and wild extract. Values are expressed as mean±SD (n=3).

Sample	Extract	DPPH (%)
In vitro	Methanol	84.33±0.577
Wild	Methanol	78.33±0.569

After growth kinetic analysis, root cultures treated with PGRs and elicitors had their total phenolic and flavonoid contents analyzed, and the antioxidant capacity of the root cultures was evaluated using the DPPH assay. The best responding cultures with PGR treatments (2.0 mg/L NAA, 1.5 mg/L IBA, and 1.0 mg/L IAA) based on FW and DW were taken for the analysis along with the roots collected from plants that were cultivated in the wild (Fig 26).

The roots cultivated in NAA (2.0 mg/L) had the highest phenolic content (58.69 mg GAE/g), which was substantially greater than the phenolic content of the other PGRs-induced roots and the wild roots (42.0 mg GAE/g) (Fig 27a). Compared to roots grown in the basal medium without PGRs, the IBA (1.5mg/L) and IAA (1.0mg/L) generated root cultures revealed 46.78 and 46.00 mg GAE/g of phenolics and 28.66 mg GAE/g of GAE respectively. Interesting findings came from the TPC studies with the elicitor-treated roots cultures. As previously mentioned, it was discovered that MeJA-incorporated roots resulted in greater FW and biomass accumulation. However, it was discovered that the TPC was higher in the SA treated root cultures since 100µM SA

produced a TPC of 50.75mg GAE/g. As opposed to SA treated root culture extracts, MeJA-treated roots displayed TPC that ranged between 41.00 to 48.56mg GAE/g (Fig 27b). The highest antioxidant potential was found in wild roots (79.04%), and cultures grown in NAA (2.0 mg/L) showed DPPH radical scavenging percentages comparable to (78.51%) those of the wild roots. The antioxidant potential of the hormonal and elicitor treated root cultures also revealed that the highest antioxidant potential was observed in the wild roots (79.04%) (Fig 27e). With 100μ M and 300 μM, respectively, the SA-treated roots displayed antioxidant potentials of 73.88% and 71.11% (Fig 27f).

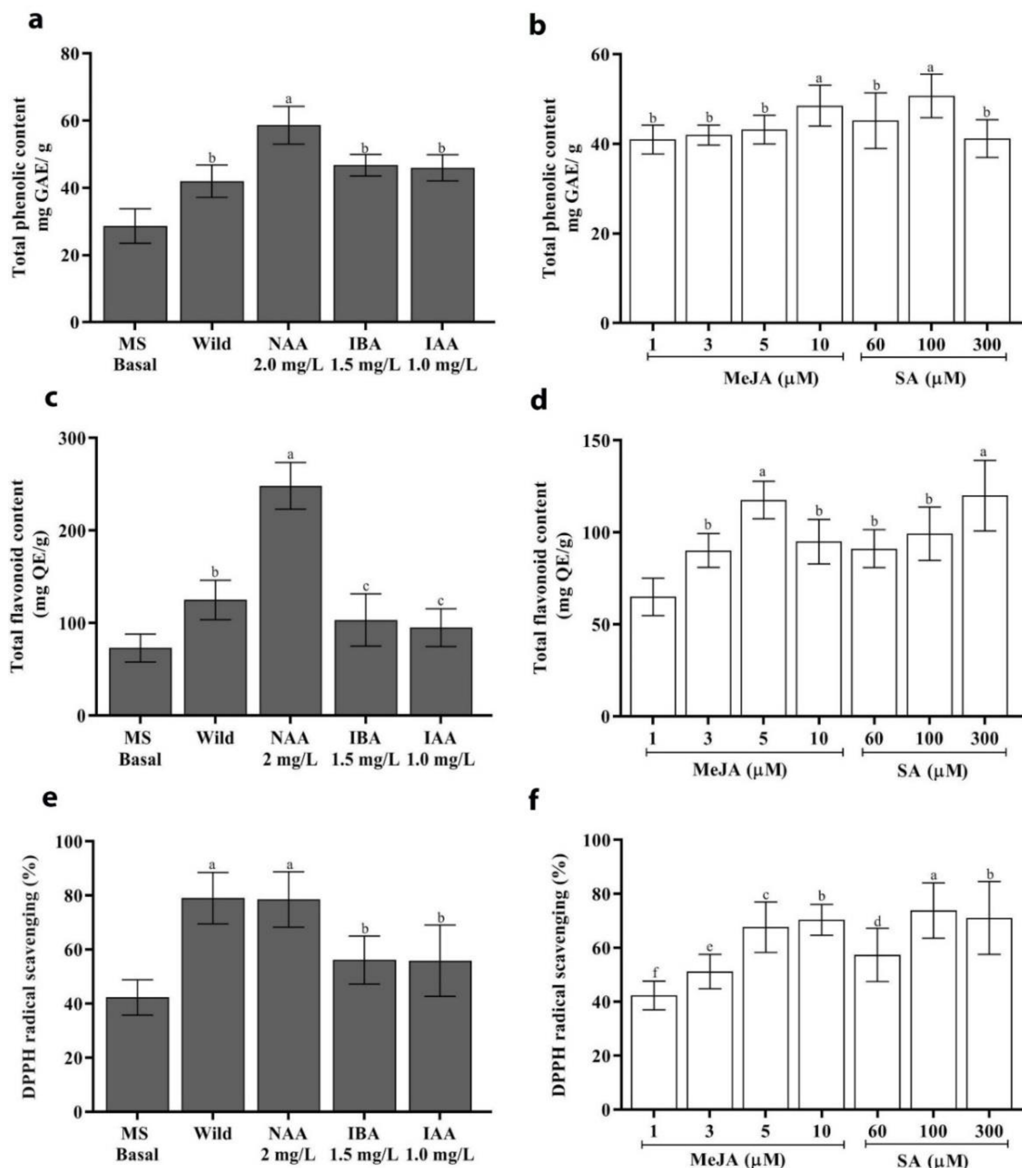


Figure 27: Biochemical analysis of the adventitious root cultures under different treatments (a)TPC of adventitious root extracts from cultures grown supplementing different PGRs; (b) TPC of adventitious root extracts from cultures grown supplementing different elicitors; (c) TFC of adventitious root

extracts from cultures grown supplemented with different growth hormones; (d) TFC of adventitious root extracts from cultures grown supplemented with different elicitors; (e) antioxidant potential of root extracts from cultures grown in different growth hormones; (f) antioxidant potential of root extracts from cultures grown in different elicitors; Values represents the mean±SD.Same superscriptsindicate means not significantly different, according to Duncan's multiple range test (p<0.05).

4.8 Phytoconstituent and antioxidant potential of different cultures in *T. cordifolia*

The Total Phenolic Content (TPC), were found to be highest with the extracts of wild stem followed by *in vitro* plant extract followed by wild leaves. The wild stem, followed by the wild leaves, and *in vitro* plant, had the highest total flavonoid content (TFC) and antioxidant potential. (TPC Wild stem 128.55±0.491 mg/g, *in vitro* plant 107.33±0.985, Wild leaves 105.6847±1.060) (TFC Wild stem 83.61±0.2773 mg/g, *in vitro* plant 58.034±0.148, Wild leaves 62.50±0.329) Antioxidant activity wild stem with IC50 193.229±0.013, *in vitro* plant with IC50 219.83±0.468 (Table 12, 13).

Table 12: Contents of total phenolic and flavonoid of *in vitro* and wild extract. Values are expressed as mean ± SD (n=3).

Sample	Extract	Total phenol (mg gallic acid g-1)	Total flavonoids (mg quercetin g-1)
***In vitro* (plant)**	Methanol	107.33±0.985	58.034±0.148
Wild (Stem)	Methanol	128.55±0.491	83.61±0.2773
Wild (Leaves)	Methanol	105.6847±1.060	62.50±0.329

The absorbance against the reagent blank was determined at 710 nm and 510 nm with an UV Visible spectrometer for phenolics and flavonoids, respectively. Total phenolics content was expressed as mg gallic acid equivalents (GAE) and total flavonoid content expressed as mg quercetin equivalents (QE).

Table 13: Total antioxidant capacity of the extracts. Values are expressed as mean ± SD (n=3).

Sample	Extract	DPPH(IC50)
***In vitro* (Plant)**	Methanol	219.83±0.468
Wild (Stem)	Methanol	193.229±0.013
Wild (leaves)	Methanol	204.386±0.165

The absorbance against the reagent blank was determined at 765 nm with an UV-Visible spectrometer. Total antioxidant capacity was expressed in percentage of DPPH activity.

4.9 GC-MS analysis for characterization of bioactive compounds in *V. Jatamansi*

Six significant bioactive chemicals, among 21 discovered by GC-MS analysis of *in vitro V. jatamansi* methanol root extract fractions from plants raised on MS+10% CW containing medium (Table 14). In addition to the main compound of interest valeric acid, also known as pentanoic acid, in *V. jatamansi*, it was discovered from the GC-MS spectra that the compounds 9, 12-octadecadienoic acid, 9, octadecenoic acid (Z), methyl ester, Heptadecanoic acid 16 methyl, Hexadecanoic acid, and Methyl ester (Fig 28). Similarly, 51 chemical constituents were found in a methanolic root extract from a wild plant by GC-MS (Table 15, 16). The important bioactive compounds were detectedin root tissues of wild plantsas mentioned in (Table 17).

Table 14: List of major compound detected in GC-MS analysis of *in vitro* plant extracts of *V. jatamansi* grown in MS+10% CW

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

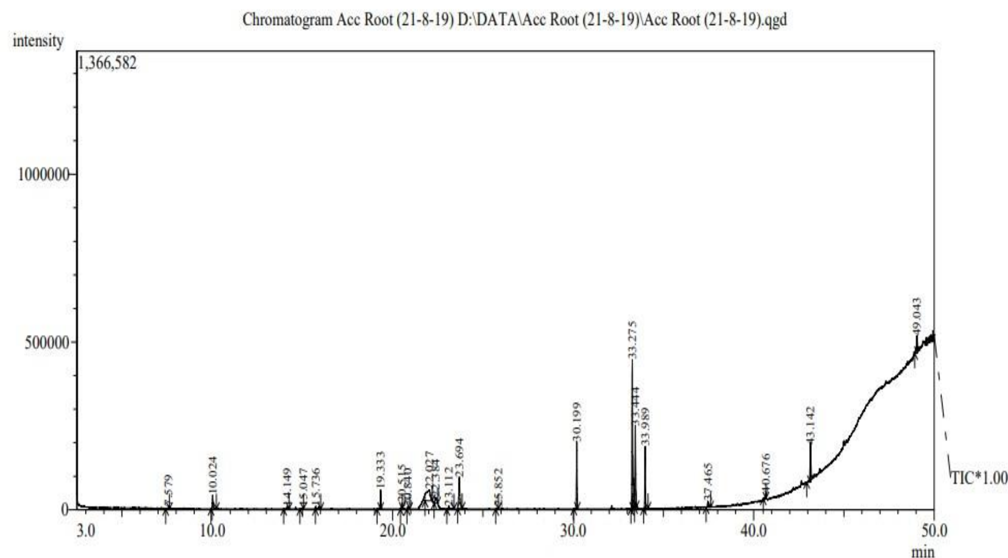


Figure 28: GC- MS analysis of *in vitro* root extracts of *V. jatamansi* plants derived from media supplemented with coconut water

Table 15: GC-MS analysis of in vitro plant plant extracts of *V. jatamansi* grown in MS+10% CW

Peak #	Retention time	A/H	Compound name
1	7.579	4.15	Proline
2	10.024	3.34	dl-Malic acid, dimethyl ester
3	14.149	1.51	1-Naphthoic acid, tridec-2-ynyl ester
4	15.047	2.55	1-PYRIDINEACETIC ACID, 4-(AMINOC
5	15.736	3.87	2-PROPANOL, 1,3-DICHLORO-
6	19.333	1.59	ENDO-3-HYDROXYMETHYLTRICYCL
7	20.515	2.54	2-Nitrophenyl acetate
8	20.840	3.51	TRANS-2-IODOCYCLOHEXYL ACETA
9	22.027	15.98	ALPHA.-D-GLUCOPYRANOSIDE, MET
10	22.384	6.82	1-PENTANOL, 2-METHYL-
11	23.112	4.29	ISOXAZOLE, 3-(3-BUTENYL)-5-METHY
12	23.694	4.72	3-Ethyl-5-methyl-1-heptyn-3-ol
13	25.852	2.49	1H-TETRAAZOLE-1,5-DIAMINE #
14	30.199	2.42	HEXADECANOIC ACID, METHYL EST
15	33.275	2.53	9,12-Octadecadienoic acid, methyl ester
16	33.444	2.64	9-Octadecenoic acid (Z)-, methyl ester
17	33.989	2.58	HEPTADECANOIC ACID, 16-METHYL
18	37.465	2.92	TETRADECANOIC ACID, 12-METHYL-
19	40.676	1.44	3-HEPTANOL
20	43.142	1.59	12-METHOXY-1-NITROBICYCLO[9.3.1
21	49.043	3.83	Glaucyl alcohol

Table 16: List of major compounds detected in GC-MS analysis of in wild roots of *V. jatamansi* with biological importance.

Name of compound	Retention Time (min)	Molecular Formula	MW (g/mol)
Valeric anhydride	22.727	$C_{10}H_{18}O_3$	186
Valerenol	27.276	$C_{15}H_{24}O$	220
Hexadecanoic acid	34.360	$C_{17}H_{34}O_2$	270
DL-Proline	20.939	$C_6H_9NO_3$	143
Octadecanoic acid	38.158	$C_{19}H_{36}O_2$	296

The methanolic extracts of wild samples, along with root cultures in NAA (2.0 mg/L), MeJA (5µM), and SA (100µM), were subjected to GC-MS analysis. The results showed that the wild *V. Jatamansi* contained 48 components, including six main bioactive compounds (Fig 29a, Table17). The root extracts from NAA (2.0 mg/L) treatment contained 50 compounds, including significant bioactive compounds like valeric anhydride, propanoic acid, hexadecanoic acid, octadecanoic acid, butanoic acid, proline, and valeric acid, which were present in higher area concentrations compared to the wild extracts (Fig 29b, Table 18). Interestingly, compared to the wild and NAA-treated samples, the root fractions treated with elicitor displayed considerably fewer compounds. 23 bioactive compounds were found in the extracts of SA (100 µM), including the significant bioactive substances divalerate, butanoic acid, pentanoic acid, valeric acid, furaldehyde, and palmitic acid (Fig 29c, Table 19). There were 21 chemical compounds found in the root extracts of MeJA, but the valepotriates could not be found, only various classes of fatty acids, alcohols, and esters were found (Fig 29d, Table 20) in supplements of *V. jatamansi*. Chromatograms of extracts from (a) wild roots (b) NAA (2 mg/L) (c) MeJA 5µM (d) SA 100µM.

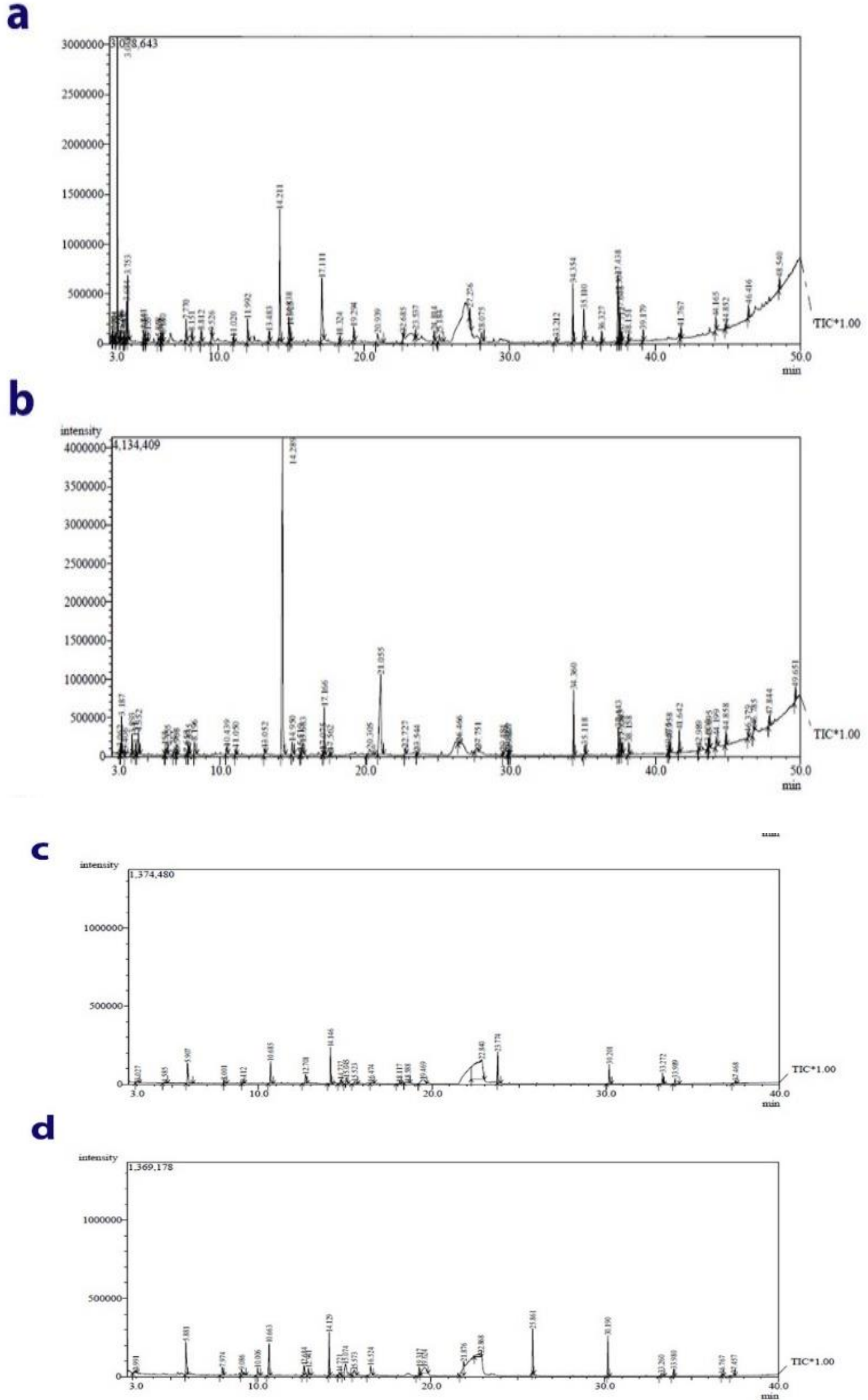


Figure 29: GC-MS analysis of extracts derived from adventitious root cultures grown in different supplements. Chromatograms of extracts from (a) wild roots; (b) NAA (2mg/L); (c) MeJA 5 µM; (d) SA 100 µM

Table 17: List of compounds detected in GC-MS analysis with wild root samples of *V. jatamansi*

Peak#	Retention time	A/H	Compound name
1	2.701	1.60	acetic acid, hydroxy-, methyl e
2	2.792	3.18	1,4-dioxin, 2,3-dihydro-
3	2.935	1.89	propanoic acid, 2-oxo-, methyl e
4	3.053	1.93	Glycerin
5	3.225	2.06	Isopropyl Alcohol
6	3.266	1.73	2-propyn-1-ol
7	3.472	2.25	2-Propanone,
8	3.533	3.01	2-propanone,
9	3.684	2.56	n-methyl-n-(methyl-d3)-aminohe
10	3.753	2.38	methyl 2-oxopropanoate
11	4.841	2.18	Propane, 2-fluoro-2-methyl-
12	4.910	4.86	2-furancarboxaldehyde
13	5.133	3.37	butane, 2,2'-thiobis-
14	5.858	4.16	Ethanone, 1-(7-oxabicyclo-[4.1.0]-hept-1-yl)
15	6.042	4.33	p-Dioxane-
16	6.180	5.42	butanoic acid,
17	7.770	3.06	2-Cyclopenten-1-one, 2-hydroxy-
18	8.151	3.84	Butanedioic acid, 2,3-dihydroxy- [R-(R*,R
19	8.812	4.24	2-furancarboxaldehyde
20	9.526	3.25	2-Hydroxy-gamma-butyrolactone
21	11.020	2.84	2-Butenedioic acid (E)-, dimethyl ester
22	11.992	3.59	2,5-Furandicarboxaldehyde
23	13.483	3.61	1,3,5-Triazine-2,4,6-triamine
24	14.211	3.19	3-acetoxy-3-hydroxy-propionic
25	14.838	3.15	hepta-2,4-dienoic acid methyl e
26	14.925	4.00	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy
27	17.111	4.99	2-furancarboxaldehyde
28	18.324	2.44	1,2-Cyclohexanedicarboxylic acid, 3-fluoro
29	19.294	3.27	5-(Hydroxymethyl)-2-(dimethoxymethyl)fu

30	20.939	7.56	DL-Proline, 5-oxo-, methyl ester
31	22.685	2.83	Valeric anhydride
32	23.537	2.59	Citric acid, trimethyl ester
33	24.814	3.64	4,6-methanocyclopenta[b]pyran
34	25.184	4.34	ethanone, 1-(1-cyclohexen-1-yl)
35	27.276	3.16	Valerenol
36	28.075	6.11	Cyclohexanone, 2-acetyl-
37	33.212	7.11	Cyclohexasiloxane, dodecamethyl-
38	34.354	2.97	Hexadecanoic acid, methyl ester
39	35.110	4.29	n-Hexadecanoic acid
40	37.438	3.03	Linoleic acid ethyl ester
41	37.504	2.95	9,12,15-Octadecatrienoic acid, methyl ester
42	37.608	2.91	9-octadecenoic acid (z)-
43	37.712	3.02	cis-10-Heptadecenoic acid, methyl ester
44	38.151	2.79	octadecanoic acid, methyl est
45	39.179	2.71	Cyclododecasiloxane
46	41.767	2.81	Heptasiloxane, hexadecamethyl-
47	44.852	3.38	Tricosanoic acid, methyl ester
48	48.540	3.24	1h-purin-6-amine, 2-fluorophen

Table 18: List of compounds detected in GC-MS analysis with root cultures grown in medium supplemented with NAA (2mg/L)

Peak#	Retention time	A/H	Compound name
1	3.062	1.77	propanoic acid
2	3.187	1.84	Glycerin
3	3.406	1.89	2-propyn-1-ol
4	3.893	2.13	propanoic acid
5	4.151	2.41	2,3-Butanediol
6	4.352	2.50	2,3-butanediol
7	6.158	3.22	Hexanenitrile
8	6.355	7.74	butanoic acid
9	6.742	6.26	2(3h)-furanone

10	6.968	2.70	methyl 4-oxobutanoate
11	7.658	4.22	Butane
12	7.835	3.21	2-Cyclopenten-1-one
13	8.196	5.02	Butanedioic acid
14	10.439	4.83	2-methyloxazole
15	11.050	2.57	2-Butenedioic acid (E)-, dimethyl ester
16	13.052	5.12	4-methyloxazole
17	14.289	4.98	3-acetoxy-3-hydroxy-propionic
18	14.950	4.57	2,3-dihydro-3,5-dihydroxy-6-met
19	15.519	2.95	6-Methyl-1,5-diazabicyclo [3.1.0] hexane
20	15.683	6.24	5-Methoxypyrrolidin-2-one
21	17.075	3.73	Pentanedioic acid, 2-oxo-, dimethyl ester
22	17.166	2.92	dimethyl ester
23	17.562	2.97	5-Hydroxymethyldihydrofuran-2-one
24	20.305	11.49	MI 3-Hydroxy-2-methylglutaric acid dimethyl
25	21.055	8.31	DL-Proline, 5-oxo-, methyl ester
26	22.727	3.88	Valeric anhydride
27	23.544	2.79	Citric acid, trimethyl ester
28	26.466	3.27	Ethanone
29	27.751	13.45	D-Allose
30	29.484	2.91	Cyclodecanol
31	29.825	4.15	4-((1E)-3-Hydroxy-1-propenyl)-2-methoxy
32	29.897	2.63	V Pentadecanal
33	34.360	2.89	Hexadecanoic acid, methyl ester
34	35.118	3.76	n-Hexadecanoic acid
35	37.443	2.92	9,12-Octadecadienoic acid (Z, Z)-, methyl e
36	37.583	4.48	cis-11,14-Eicosadienoic acid, methyl ester
37	37.708	3.98	cyclopropaneoctanoic acid
38	38.158	2.90	octadecanoic acid, methyl est
39	40.875	3.51	hexadecanoic acid
40	40.958	3.28	Behenic alcohol
41	41.642	2.82	eicosanoic acid, methyl ester
42	42.989	2.91	Octacosyl acetate
43	43.600	2.69	(6z,9z)-6,9-pentadecadien-1-ol #
44	43.695	4.34	5h-benzo[a]cycloheptene #

45	44.199	4.56	Hexadecanoic acid
46	44.858	3.28	docosanoic acid, methyl ester
47	46.379	3.36	Tricosanoic acid, methyl ester
48	46.785	4.47	E, E,Z-1,3,12-Nonadecatriene
49	47.844	3.14	Tetracosanoic acid, methyl ester
50	49.651	3.68	Docosanoic acid, 2-hydroxy-, methyl ester

Table 19: List of compounds detected in GC-MS analysis with root cultures grown in medium supplemented with SA (100µM)

Peak#	R.Time	A/H	Compound name
1	2.991	6.09	2-furaldehyde
2	5.881	3.47	Valeric acid
3	7.974	3.98	2,5-Furandicarboxaldehyde
4	9.086	5.11	Levoglucosenone
5	10.006	2.85	butanedioic acid, hydroxy-, dim
6	10.663	3.3	hepta-2,4-dienoic acid methyl e
7	12.684	2.74	4-Hepten-3-one, 4-methyl-
8	12.941	2.75	dimethyl 3-hydroxy-3-methylpe
9	14.129	2.48	3,3,3-Trifluoro-2-(4-hydroxy-2-methoxy-ph
10	14.721	3.75	1,3-isobenzofurandione
11	15.074	7.52	beta.-D-Ribopyranoside, methyl
12	15.573	6.92	beta.-Methyl xyloside
13	16.524	4.56	L-Proline, 5-oxo-, methyl ester
14	19.317	3.36	1,2,3-propanetricarboxylic acid
15	19.624	15.43	.beta.-D-Glucopyranose, 1,6-anhydro-
16	21.876	5.71	l-glutamic acid, n-acetyl
17	22.868	14.89	Butanoic acid
18	25.861	2.93	1-tetradecanamine
19	30.190	2.55	Hexadecanoic acid, methyl ester
20	33.260	3.21	1-undecyne
21	33.980	2.54	heptadecanoic acid, methyl est
22	36.767	2.99	eicosyl acetate
23	37.457	3.12	tetradecanoic acid

Table 20: List of compounds detected in GC-MS analysis with root cultures grown in medium supplemented with MeJA (5µM)

Peak#	R.Time	A/H	Compound name
1	3.027	6.1	3-Furaldehyde
2	4.585	7.28	cyclohexanone
3	5.907	3.71	Pentanoic acid, 4-oxo-, methyl ester
4	8.001	2.96	2,5-Furandicarboxaldehyde
5	9.112	2.77	1H-Imidazole
6	10.685	3.15	hepta-2,4-dienoic acid methyl e
7	12.701	4.90	4-Hepten-3-one, 4-methyl-
8	14.146	2.47	3,3,3-Trifluoro-2-(4-hydroxy-2-methoxy-ph
9	14.737	4.46	1,2-benzenedicarboxylic acid
10	15.045	4.78	beta.-Methyl xyloside
11	15.523	5.72	butanoic acid
12	16.474	6.38	Butanoic acid, anhydride
13	18.117	2.01	2,6-methano-1h-pyrrolo[1,2-b]py
14	18.588	10.41	RS-2,3-hexanediol
15	19.469	18.15	butanoic acid
16	22.840	33.43	alpha.-d-glucopyranoside, met
17	23.774	2.34	methyl (3-oxo-2-[(2z)-2-pentenyl
18	30.201	2.49	hexadecanoic acid, methyl est
19	33.272	2.49	11,14-Eicosadienoic acid, methyl ester
20	33.989	2.67	heptadecanoic acid, methyl est
21	37.468	2.62	tetradecanoic acid

4.10 GC-MS analysis for characterization of bioactive compounds in *T. cordifolia*

The GC-MS analysis of *in vitro T. cordifolia* methanol stem extract fractions from wild plants revealed the presence of 40 compounds including palmitoine (Table 21, Fig 30). Similarly, GC-MS methanolic *in vitro* plant extract grown in MS+BAP (5mg/L)+10% CW containing medium revealed the presence of 32 compounds (Table 22, Fig 31).

Table 21: List of compounds detected in GC-MS analysis with wild stem plants of *T. cordifolia*

Peak #	R. Time	Area%	Name	Base m/z
1	5.517	0.18	Propanal, 2,3-dihydroxy-	43.00
2	10.073	0.23	E-14-Hexadecenal	41.05
3	11.561	0.93	Tetradecanoic acid	43.05
4	11.936	0.45	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	68.05
5	12.007	0.20	2-Pentadecanone, 6,10,14-trimethyl-	43.05
6	12.257	0.77	Pentadecanoic acid	43.05
7	12.417	0.44	10-Nonadecenoic acid, methyl ester	55.05
8	12.550	5.88	Hexadecanoic acid, methyl ester	74.05
9	12.833	0.69	Cyclopentadecanone, 2-hydroxy-	41.05
10	13.011	20.78	Pentadecanoic acid	43.05
11	13.209	0.16	Heptadecanoic acid, methyl ester	74.05
12	13.587	0.72	Pentadecanoic acid	43.05
13	13.727	11.79	7-Octadecenoic acid, methyl ester	55.05
14	13.871	3.32	Octadecanoic acid, methyl ester	74.05
15	14.235	24.41	cis-9-Hexadecenal	55.05
16	14.350	5.89	Octadecanoic acid	43.05
17	15.071	0.59	n-Nonadecanol-1	83.10
18	15.249	0.30	Methyl 18-methylnonadecanoate	74.05
19	15.306	0.74	Octadecanoic acid, 12-hydroxy-, methyl ester	55.05
20	16.180	0.31	1,2-15,16-Diepoxyhexadecane	55.05
21	16.274	1.42	n-Nonadecanol-1	43.05
22	16.447	0.81	Docosanoic acid, methyl ester	74.05
23	16.536	1.30	Hexadecanoic acid, 2-hydroxy-1-(hydroxy	43.5
24	16.663	0.87	1,2-Benzenedicarboxylic acid, diisooctyl ester	149.10
25	17.071	0.68	Tricosanoic acid, methyl ester	74.05
26	17.153	0.47	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	43.05
27	17.311	0.30	2-Dodecen-1-yl(-)succinic anhydride	55.05
28	17.447	1.09	1,3-Dioxolane, 4-[[(2-methoxy-4-octadecenyl)oxy]me	57.05
29	17.669	2.51	6-Octadecenoic acid, methyl ester	55.05
30	17.786	2.59	9,19-Cycloergost-24(28)-en-3-ol, 4,14-dimethyl)	218.25
31	18.027	0.29	Ergost-25-ene-3,5,6,12-tetrol, (3.beta.,5.alpha.,6	69.05
32	18.133	1.71	8-Methyl-6-nonenamide	59.05
33	18.273	1.20	2,6,10,14,18,22-Tetracosahexaene,	69.05
34	18.407	1.38	9,19-Cycloergost-24(28)-en-3-ol,	55.05

35	18.547	0.68	Hexadecanoic acid, 2-hydroxy-, methyl ester	57.05
36	18.927	0.15	Hexacosanoic acid, methyl ester	74.05
37	19.103	1.45	1,6,10,14,18,22-Tetracosahexaen-3-ol	69.05
38	19.458	1.30	Stigmast-4-en-3-one	124.15
39	19.547	0.75	Cholest-7-en-3-one, 4,4-dimethyl	55.05
40	19.701	0.28	9(11)-Dehydroergosteryl benzoate	251.25
		100		

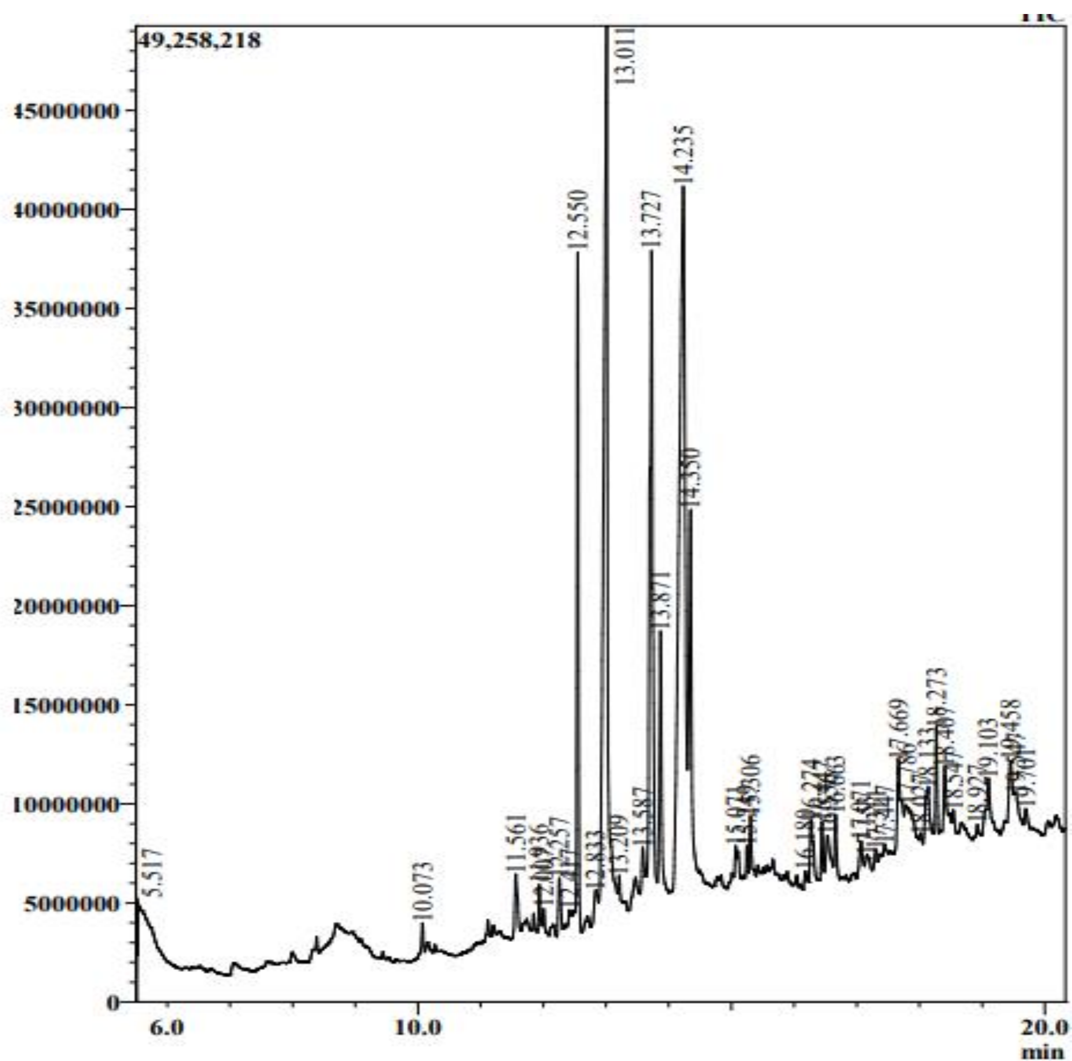


Figure 30: GC-MS chromatogram of extracts derived from wild stem extracts of *T. cordifolia* plants

Table 22: List of compounds detected in GC-MS analysis in *in vitro* plants of *T. cordifolia.*

Peak	R. Time	Area%	Name	Base m/z
1	3.523	0.40	3-Decyn-2-ol	43.05
2	8.684	2.36	4-Chloro-3-n-butyltetrahydropyran	41.05
3	8.797	0.76	9-Octadecenoic acid (Z)-, 2-hydroxy-1 (hydroxymethyl)	55.05
4	8.885	1.29	Cyclopropane, (3-chloropropyl) methylene	41.05
5	9.216	1.13	Pentadecanoic acid, 14-methyl-, methyl ester	74.05
6	9.367	0.52	Cyclopropane, (3 chloropropyl) methylene-	41.05
7	9.495	1.07	495 9-Hexadecenoic acid	55.05
8	9.609	17.04	Pentadecanoic acid	43.05
9	9.748	5.18	(2E,4E)-5-Chloro-3,4-dimethyl-2,4-heptadiene	41.05
10	9. 830	3.30	830 (2E,4E)-5-Chloro-3,4-dimethyl-2,4-heptadiene	63.00
11	9.913	1.40	1-Propene, 3,3'-oxybis	67.10
12	9.965	1.68	Cyclopropane, (3-chloropropyl) methylene	41.05
13	10.255	0.61	2-Cyclopentene-1-undecanoic acid, (+)-	41.05
14	10.390	4.21	8-Octadecenoic acid, methyl ester	55.05
15	10.421	6.01	(2E,4E)-5-Chloro-3,4-dimethyl-2,4-heptadiene	79.10
16	10.540	4.97	Heptadecanoic acid, 10-methyl-, methyl ester	74.05
17	10.627	3.04	5,5,10,10 Tetrachlorotricyclo [7.1.0.0(4,6)]decane	75.05
18	10.703	1.43	Pentane, 1,1,1,5-tetrachloro-	89.05
19	10.818	21.69	cis-Vaccenic acid	55.05
20	10.942	7.10	Octadecanoic acid	43.05
21	11.300	1.32	E-11-Tetradecenol,trimethylsilyl ether	41.05
22	11.508	5.16	1,1-Dichloro-2,3-dimethylcyclopropane	75.05
23	12.535	0.58	Octadecanal	43.05
24	12.850	0.50	Cyclopentadecanone, 2-hydroxy-	57.10
25	12.932	0.54	Trichloroacetic acid, hexadecyl ester	43.05
26	13.078	0.72	17-Pentatriacontene	57.10
27	13.169	0.52	Pentadecanal-	57.05
28	13.330	0.75	1,2-Benzenedicarboxylic acid, diisooctyl ester	149.15
29	14.950	2.09	2,6,10,14,18,22-23Tetracosahexaene	69.10
30	15.028	2.19	Heptasiloxane, hexadecamethyl	73.10
31	16.382	0.25	9(11)-Dehydroergosteryl benzoate	69.10
32	16.707	0.20	3-t-Butyl-7a dimethylaminomethyltetrahydr-pyrrolo	58.05

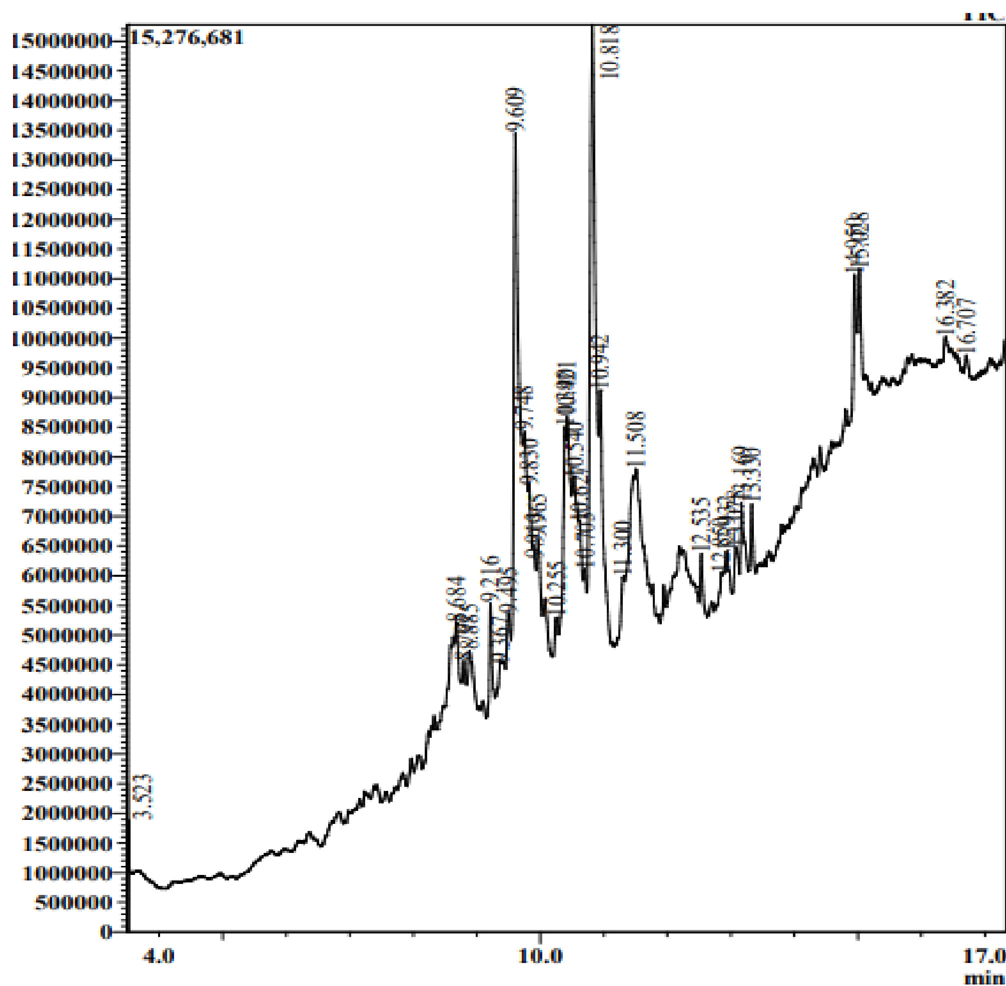


Figure 31: GC-MS chromatogram of *in vitro* plant extracts of *T. cordifolia* plants

4.11 Gene expression analysis of sesquiterpene synthase gene in *V. Jatamansi*

mRNA was isolated from the root extracts of *in vitro* grown *V. jatamansi* (Fig 32) and subsequently Cdna conversion was done for RT-PCR to calculate the gene expression level. Semiquantitative PCR was also performed which revealed the expression of (TPS) Sesquiterpene synthase gene in roots of *V. jatamansi* producing bands of approximately 200 bp. The relative gene expression analysis using sesquiterpene synthase genes was carried out. Primers TPS1, TPS2, TPS3, TPS4, TPS5, TPS6 were tested for their expression. Results revealed that out of five primers used the expression of TPS2 genes were significantly higher (more than fivefold increase) in both the acclimatized and *in vitro* root extracts whereas TPS5 showed the highest of gene expression (16 fold) with the *in vitro* roots as compared to the *in vivo* roots, TPS6 showed about the expression of four-fold increase in the root samples of the *in vitro* cultures as compared to the *in vivo* plant roots (Fig 33).

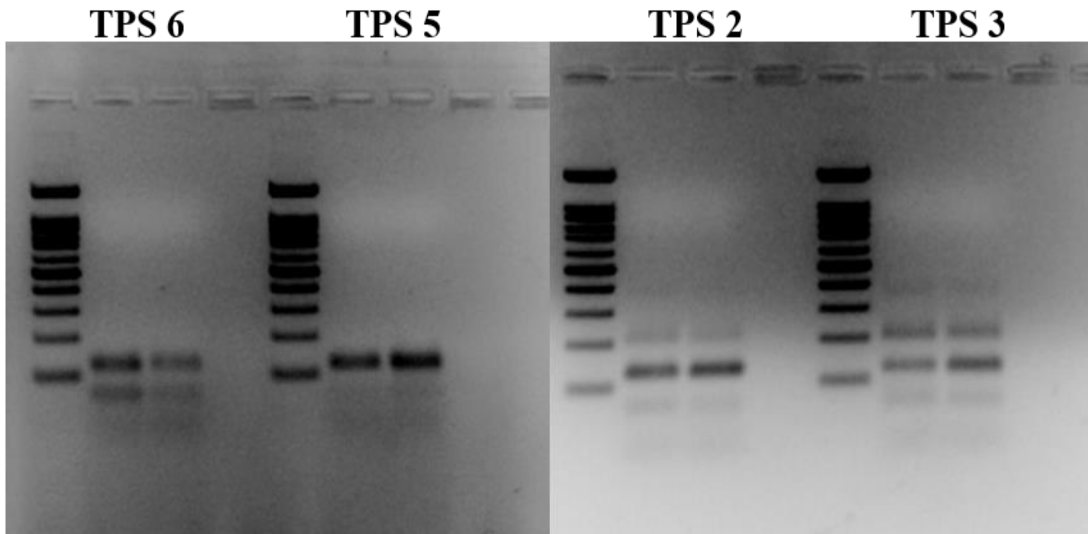


Figure 32: Semiquantitative PCR OF TPS6, TPS5, TPS2, TPS3 Gene with molecular ladder of 100 bp-150 kb.

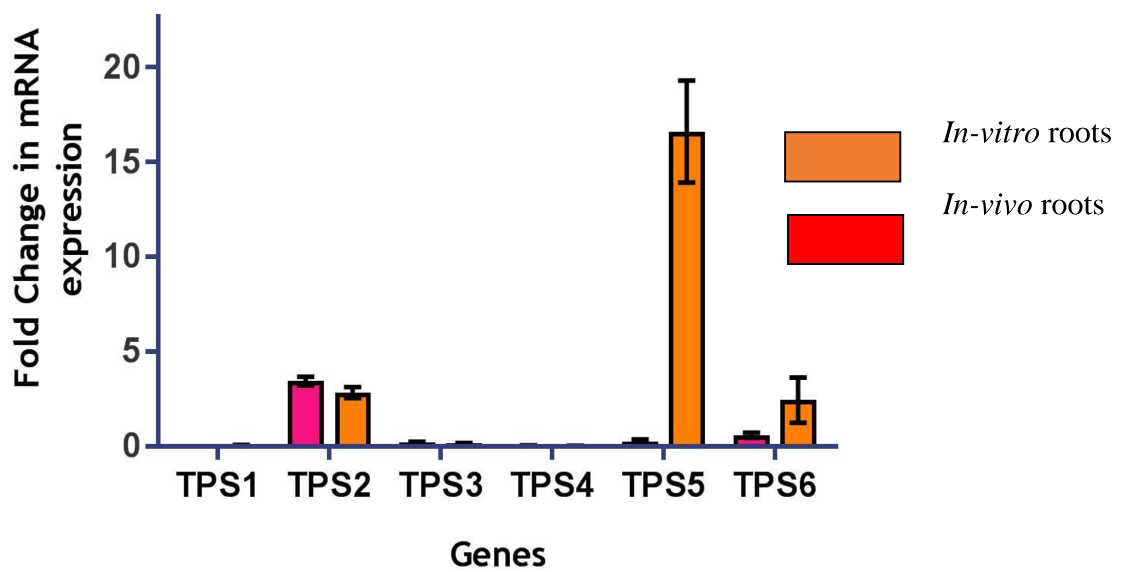


Figure 33: Gene Expression Analysis using RT-PCR in *in vitro* and in vivo roots of *V. jatamansi*

CHAPTER 5

DISCUSSION

5.1 Establishment of shoot cultures, rooting and acclimatization in *V. jatamansi* Jones

Studies on *in vitro* propagation of different *Valeriana* species have been published in the past. A study using shoot tips, auxillary buds and petiole explants to study the micropropagation of *V. wallichii*, callus induction and shoot regeneration has been published (Mathur *et al*., 1988; Mathur, 1991). Similar *in vitro* propagation investigations have been reported for using explants of seeds, leaves, petioles and apical buds for *V. officinalis* using explants of leaf segments, petioles, terminal and axillary buds and for *V. glechomifolia* using shoot tips (de Carvalho *et al*., 2004). However, there aren't many reports on *V. jatamansi* using nodal segments. By adding BAP (1 mg/L) and IAA (0.1 mg/L) to MS medium (Bhat and Sharma, 2015) reported effective shoot induction frequencies using shoot tip explants in *V. officianalis*. The combination of BAP and NAA in the current experiment likewise failed to increase the quantity of shoots and roots (Fig 7c, d). In this research, (Purohit *et al*., 2015) used a combination of BAP (0.33 mg/L) combined with NAA (0.93 mg/L) and GA_3 (0.035 mg/L) to obtain high numbers of shoots (2.33), leaves (15.33), roots (27.5), shoot length (3.20 cm), and roots (50 cm). Several species, including *Valeriana*, have used this specific BAP and NAA combination for high-frequency shoot multiplication. According to (Singh *et al*., 2015), 0.675 mg/L BA was the most effective dose for stimulating shoots from nodal explants in *V. wallichii* DC. In their investigation, BA (0.675 mg/L) was shown to be the most efficient for shoot induction, recording 12.0 shoots per explant and 4.53 cm average shoot length when compared to several growth regulators at different doses. According to an earlier study by (Kaur *et al*., 1999), shoot buds cultivated on medium enriched with BA and IAA or NAA stimulated shoots and then formed 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 7a, b). Previous studies suggested that adding

cytokinins BA, Thidiazuron (TDZ), zeatin, or KIN alone to the media had no appreciable impact on callus induction, somatic embryogenesis, or shoot organogenesis (Das *et al*., 2013). They used explants of rhizome, petiole, and leaf to generate high frequency callus formation in their study using 2,4D (0.5 mg/L). However, for shoot organogenesis, 0.75 mg/L TDZ + 0.5 mg/L NAA produced the highest shoot numbers per callus (15.20) and shot lengths, as well as an 88.6% regeneration frequency (3.60 cm). Due to its growth-regulating qualities, which are important for cell division and the stimulation of rapid growth, coconut water is a complex additive that contains a variety of nutritional and hormonal components (Yong *et al*., 2009). In addition to carbohydrates, vitamins, minerals, and amino acids, organic acids, and PGRs (auxin, cytokinins, and gibberellins), coconut water also contains naturally occurring inhibitors and regulators (ethylene, abscisic acid, phenols, and flavonols) (Yong *et al*., 2009). A very interesting finding in my study was that adding coconut water to the current experiment also improved the growth of roots in addition to shoots (Fig 8a, b, c, d, e). In a recent study Hamdeni *et al*., (2022) discussed the use of natural additives, such as coconut water, to improve *in vitro* tissue culture and produce genetically stable plants. The number of shoots, shoot length, and root induction were all lowered by the 15% increase in coconut water dosage. Our findings are consistent with other studies that established high frequency shoot multiplication and roots in numerous plant species using coconut water. For instance (Prando *et al*., 2014) showed that *Corylus avellana* generated more adventitious shoots per explant when coconut water (20%) was added. Similar to this, (Swamy *et al*., 2014) reported that *Pogostemon cablin* Benth's *in vitro* growth responded better when coconut water (10%) was added. Similar to the findings in this work Trevisan and Mendes, (2005) showed that 10% coconut water improved the shoot elongation of *Passiflora edulis*.

In contrast to earlier research, which only noted the production of shoots, this study documented the rapid elongation of shoots as well as the formation of many roots. Coconut water's increased morphogentic potential is due to the presence of growth regulators, notably cytokinins (Chugh *et al*., 2009). The fundamental benefit and importance of employing coconut water extracts in tissue culture is that growth hormones may function considerably better when separated from a natural organic source than synthetic hormones do (Jayawardena and Agampodi, 2009). The production of valuable phytochemicals quickly will help to meet the demands of the

pharmaceutical sectors, hence root induction in *Valeriana* species is crucial. According to Chen *et al*., (2014), *Agrobacterium rhizogenes* genetically modified several *Valeriana* species to produce hairy root cultures (Zebarjadi *et al*., 2011). In our investigation, MS+10% CW containing medium with root induction was also noted (Fig 8f).

In the plant containment facility, the *in vitro* grown plants were hardened. It was found that the plants raised in media containing coconut water (10%) grew well and had a higher rooting capability than the plants raised in media containing BAP (Fig 9c, d). Within 45 days of acclimatization, rhizome formation was also seen in the plants, which is an important finding because the majority of bioactive components are thought to be concentrated in the roots and rhizomes (Fig 9 e). Both throughout the *in vitro* culturing of the explants in terms of shoot and root production as well as following the acclimation of the plants in a containment facility, the growth-promoting effect of coconut water was clearly observed (Pandey *et al*., 2020a, b). The plants were then moved to the field for extensive *V. jatamansi* proliferation. (Pandey *et al*., 2020a, 2020b, 2020c).

5.2 Establishment of callus and regeneration of *in vitro* plants of *V. jatamansi* using SNP and plant growth regulators

Maximum callus induction frequencies were recorded in *V. jatamansi* Jones by (Das *et al*., 2013) utilizing rhizome explants on media supplemented with 2, 4-D, while the maximum regeneration frequencies were attained by thidiazuron and NAA addition to MS medium. According to Chen *et al*., (2014) adding 2, 4-D and NAA to the media exclusively caused leaf explants to develop embryogenic calli, which in turn differentiated into adventitious shoots and somatic embryos in *V. officinalis*. The callus induction percentages vary depending on the explant type and plant species, which is why in the current investigation NAA alone was sufficient to elicit the maximum callusing response (Fig 10). The best responsive concentration in each of the four tested growth regulators was selected and SNP at four different concentrations was supplemented in order to determine the optimal dose of SNP needed to increase the callus induction potential. The callus induction frequency was considerably increased by exogenous SNP supplementation. In contrast to calli developed in medium with growth regulators and without SNP, those that were induced in medium supplemented

with SNP had a friable and compact character (Fig 10e, f). According to the results of this study, 10% CW is the ideal concentration of growth regulator needed to induce multiple shoot induction in *V. jatamansi* callus explants (Table 6, Fig 14). High concentrations of zeatin and related compounds, kinetin, and precursors similar to N6-(D2-isopentenyl)-adenine (iP) can be found in CW, a natural material (Tan *et al.*, 2011). For the *in vitro* growth and development of many plant species, the benefits of CW supplementation alone or in combination with growth regulators have been reported in the past (Maharjan *et al.*, 2019).

Nitric oxide (NO) has been shown to improve the regeneration response by increasing the number of meristems, according to Kalra and Babbar, (2010), who also hypothesized that NO may be crucial in controlling the gene expression responsible for meristem differentiation. Both Karthik *et al.*, (2019) and Kazemi *et al.*, (2019) found similar outcomes in Glycine max, where exogenous SNP supplementation improved *in vitro* responses. However, during the rooting experiments in *V. jatamansi*, there was no need for an additional medium for root induction since the supplementation of coconut water itself induced roots and CW+SNP treatments further enhanced the formation of roots in the medium. Zebarjadi *et al.*, (2011) used an additional rooting medium MS+IBA (2mg/L) for rooting of *in vitro* derived *V. officianlis* plantlets (Fig 14g).

Plant growth is inhibited and cell proliferation is decreased as a result of callus browning (Mondal *et al.*, 2014). It was discovered that callus browning and hyperhydricity were caused by a higher concentration of growth regulators (Fig 15a). Some of the calli produced in the presence of SNP supplementation exhibited hyperhyrdicity symptoms, particularly at SNP concentrations of 20 and 30 µM. Similar to our findings (Fig. 15), Subiramani *et al.*, (2019) reported reduced callus browning and hyper acidity in *Canscora diffusa*, *Dioscorea opposita* tuber explants by Xu *et al.*, (2009) and *Ficus religiosa* by Hesami *et al.*, (2018) (Pandey *et al.*, 2020a).

5.3 Establishment of *in vitro* culture of nodal segments in different media in *T. cordifolia*

There have been reports of various protocol for *T. cordifolia in vitro* propagation. Sultana *et al.*, 2013) observed that nodal explant culture of *T. cordifolia* resulted in the formation of numerous shoots frequently. Benzyl Amino Purine (BAP), Kinetin, and Thidiazuron (TDZ) were utilized in various combinations and quantities in MS basal

medium throughout this experiment. Within 30 days of inoculation, the combination of BAP (2 mg/l), Kinetin (4 mg/l), and TDZ (0.20 mg/l) in MS medium produced a maximum average of 10.29 shoots per explant, and roots were formed days later in half-strength MS medium supplemented with IBA (2 mg/l). An earlier micropropagation protocol was created utilizing North East India's *Tinospora cordifolia* (Handique *et al.*, 2009). This experiment used MS basal medium with various combinations and quantities of BAP, Kinetin, and IAA. In this experiment, MS+ BAP (2 mg/L) and KIN (2 mg/L) nodal segments produced fewer shoots (Fig 16). The plant's growth is enhanced by increasing the concentration and adding coconut water to the MS+BAP (5mg/L) containing medium, which produced more nodal segments (20.33±0.577) from shoots with long shoots (12.10 ±0.10) (Table 7, Fig 17 H). This is due to the fact that cytokinins, which have been identified in *V. jatamansi*, are responsible for the increased growth activity of coconut water (Chugh *et al.*, 2009; Pandey *et al.*, 2020b). Comparatively to MS+IBA (0.5mg/L), which produces noticeable roots in *T. cordifolia*, similar high concentrations of MS+IBA (1, 1.5 and 2mg/L) did not proved good in production of roots (Fig 17g). This is the first account of *in vitro* propagation of this species in Nepal using coconut water as an additional supplement.

5.4 Genetic fidelity analysis Using RAPD and ISSR markers in *V. jatamansi*

In the case of therapeutic plants or plants that are important commercially, determining the genetic homogeneity between the mother plant, *in vitro* regenerated plants, and the acclimatized plants is crucial. There are numerous variables, including dietary content, stress, hormonal balance, and culture time, which is a significant contributor to *in vitro* variability (Khan *et al.*, 2011). It is crucial to determine if the *in vitro* regenerants are genetically identical to the mother plant or not, particularly if the research's goal is to commercialize the specific plant species for its medicinal value. Several *in vitro* propagated medicinal and commercially significant plant species, including *Withania sominifera* (L.) Dunal, *Alhagi maurorum*, and *Withania coagulans* Dunal, have successfully used ISSR and RAPD primers to demonstrate clonal stability and to detect potential genetic variations within the *in vitro* regenerated plantlets (Tripathi *et al.*, 2018). Ten primers (OPA O8, OPA 10, OPA 18, OPB 07, OPD 04, OPE 08, OPC 11, UBC 210, UBC 292 and UBC 465) produced 28 bands with sizes ranging from 200 to 800 bp when used to create the banding patterns between the *in vitro* cultivated plantlets

and the mother plant in the current study (Table 2). Additionally, the genetic homogeneity of the 2-month plants that had been acclimatised in the plant containment facility was examined, and it was discovered that the banding pattern was comparable to that of the mother plant, which produced three bands with sizes between 400 and 600 bp (Fig 19 a-d). There was no genetic variation among the mother, *in vitro* grown, and acclimatized plantlets, according to the genetic homogeneity results with all the RAPD primers (Fig 19 a–d, 20). Similar to this, out of the 10 primer sets of ISSR markers used, 5 primers (HB8, HB9, HB10, HB11, and 17898B) produced reproducible bands that generated 10 bands with sizes between 250 and 1100 base pairs (Table 3, Fig 19 e-h, 21), demonstrating the genetic homogeneity of the *in vitro* regenerated and subsequently acclimatised plants. These plants also produced 4 bands with sizes between 250 and 1300 base pairs with (Fig 19 e- h). It has been claimed that clonal multiplication of true-to-type plants of *V. jatamansi* occurs when nodal segments are used as explants (Purohit *et al*., 2015). Greater polymorphisms are indicative of greater genetic variations. However the present investigation revealed the presence of higher genetic similarity than polymorphism. The reason for some variation may be associated with the intrinsic factors associated with biological materials and some of the culture conditions as shown in different species (Smith *et al*., 1997; Lakshmanan *et al*., 2006). Kumar *et al*., (2012) employed RAPD markers to characterize the genetic diversity in a representative population of *V. jatamansi* Jones. Their findings were similar to ours in that there was significantly less variance. Polymorphism was established with the presence of either new bands in genotypes or loss of original bands in mother plants (Chandrika & Rai, 2010; Ramesh *et al*., 2011). The banding profile created by various molecular marker systems can indicate genetic diversity modified by genetic and epigenetic mechanisms (Phillips *et al*., 1994). This system helps to detect the reliability of the plant tissue culture protocol for large scale exploitation and species restoration programme. ISSR markers are considered as effective and reliable genetic markers for assessing the degree of genetic variation of many crop plants. As compared to other molecular markers, ISSR could reveal high polymorphism and found to be potentially useful for studying genetic variation, diversity, introgression analysis and identification of germplasm. High genetic similarity and variation was also reported by RAPD markers in different crop and cultivated populations (Khaled *et al*., 2015; Singh & Sengar, 2015). However, sometimes RAPD markers fail to reveal changes in the repetitive regions of the genome of some species (Palombi & Damiano, 2002).

Therefore, the two marker systems employed in the present investigation is used to crosscheck and validate the degree of genetic variability of test genotypes (Bhattacharyya & Kumaria, 2015). The use of ISSR markers to determine genetic similarity between *V. jatamansi* populations was also reported by Jugran *et al*., (2013a, b, 2015), although the majority of these results were based on the wild population in *V. jatamansi* Jones. The genetic fidelity investigation of the micropropagated *V. jatamansi* plants using ISSR markers was reported by (Purohit *et al*., 2015). In the current study, the genetic fidelity and lack of somaclonal differences among the *in vitro* regenerated plants were successfully verified using both RAPD and ISSR markers (Pandey *et al*., 2020b).

5.5 Genetic fidelity analysis using RAPD and ISSR markers in *T. cordifolia*

It is well recognized that micropropagated tissues, particularly during long-term cultures, are easily susceptible to somaclonal changes (Larkin and Scowcroft, 1981). Therefore, the preferred method for assessing the genetic stability of both regenerated plants and wild *T. cordifolia* plants uses RAPD and ISSR markers (Mittal *et al*., 2017). The RAPD study showed that the *T. cordifolia in vitro* derived plants displayed identical banding patterns as those of mother plants, which formed 22 bands of about 100–1400 bp, demonstrating that there was no genetic diversity in the DNA of *in vitro* regenerated plantlets (Fig 22, 23). Similarly ISSR analysis also revealed that the *in vitro* derived plants of *T. cordifolia* exhibited same banding patterns as that of mother plants which produced 9 bands of approximately 100-500 bp. There have been a number of RAPD based investigations on *T. cordifolia* that were limited to wild populations, however the results of these research could not be as trusted because of the low repeatability and low amount of RAPD marker polymorphism. The ISSR markers, on the other hand, showed increased heterozygosity and can therefore be linked to the discovery of high polymorphism, making them the most favoured DNA marker (Lade *et al*., 2020). This study reports confirmation of genetic homogeneity with RAPD and ISSR markers (Table 1-2, Fig 22-23) of the *in vitro* and mother plant from plants collected in Nepal.

5.6 *In vitro* root multiplication and Growth kinetics of *V. Jatamansi* Jones

One practical alternative to traditional ways for generating bioactive chemicals to suit the ever-growing industrial demands is *in vitro* plant cell cultures (Khan *et al*., 2017). Adventitious root cultures are the most popular of the many plant cell cultures used to produce high biomass, and subsequently significant bioactive chemicals, in a constrained amount of time and space (Saeed *et al*., 2017). According to Rani *et al*., (2017), a variety of techniques, such as elicitation, temperature stress, and PGR modification, can be used to produce commercially and pharmaceutically significant compounds through adventitious roots. Maximum adventitious root biomass accumulation was seen in *Prunella vulgaris* L. callus immersed in a liquid medium containing 1.0mg/L of NAA (Fazal *et al*., 2014). NAA demonstrated more biomass accumulation than cultures treated with IAA and IBA. Similar to our findings Lee *et al*., (2011) and *A. paniculata* showed that the addition of NAA enhanced adventitious root induction rate, the number of roots per explant, and root length (Sharma *et al*., 2013). Lower NAA concentrations (1.0 and 2.0 mg/L) studied with different plant species showed a quick increase in adventitious root production, and higher NAA concentrations led to a decrease in the number of roots (Raju and Prasad 2010; Sun and Hong, 2010). According to Peeters *et al*., (1991), NAA is absorbed six times quicker in tobacco than IAA, which is the reason for consideration of NAA as most effective inducer of adventitious roots. Under *in vitro* circumstances, NAA is also more rapidly absorbed and used than other growth hormones. According to the findings of this investigation, 2.0 mg/L NAA, which was relatively less than IAA and IBA, is the optimum PGR to assure high frequency rooting in *V. jatamansi* liquid cultures (Fig, 24 g). NAA has been cited in numerous researches employing adventitious root suspension cultures as the most efficient growth regulator for increased biomass accumulation (Yan *et al*., 2014; Khan *et al*., 2015; Saeed *et al*., 2017). Khan *et al*., (2015) hypothesized that liquid media fortified with NAA produced efficient adventitious root production in comparison to the solid medium. Furthermore, because of the simple accessibility of nutrients, PGRs, and water, liquid cultures are more likely to support plant cell growth and development. Our results are consistent with those of Saeed *et al*., (2017) who discovered that NAA stimulated the growth of root biomass in *Ajuga bracteosa* Wall ex Benth and proposed that this was because NAA has a longer half life in plant cell culture than other PGRs.

5.7 Phytoconstituent and antioxidant potential in *V. jatamansi*

The phenolic content of all the methanolic extracts from MS+10% CW containing medium was considerably high as compared with the wild (Table 10,11), which could be a major contributing factor to the strong antioxidant activity of this plant extracts. According to Afolayan *et al*., (2007), high phenolic content of plant extracts could be responsible for their enhanced antioxidant activity. These phyto constituents of *V. jatamansi* when isolated and administered may reduce the risk of cardiovascular disease, cancer and protect against urinary tract infections (Howell *et al*., 2002). Phenolic compounds are known for their antioxidant activity. This activity is related to their redox properties in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides (Zheng and Wang, 2001). They are also certained to have inhibitory effect on carcinogenesis. The study of flavonoids which are a groups of natural compounds are also classified as natural phenolics (Agrawal, 1989). There are great interest for therapeutic uses of valepotriates and valerenic acids in *Valeriana* species. The therapeutically used plants for the drug preparation are collected from the field, which means that supply will be limited and uncertain.

The greatest class of phytochemicals with the highest antioxidant activity is the polyphenols, which are also the most potent inhibitors of free radicals, immune system stimulators, regulators of gene expression, and other cellular activities (Ahmad *et al*., 2013). Enhancing the production of commercially important metabolites through manipulation of the plant tissue culture conditions, including the selection and concentration of PGRs, is a sustainable method (Palacio *et al*., 2012). In contrast to the findings of our research, Fazal *et al*., (2014) found that cultures treated with NAA at lower concentrations resulted in higher TPC levels in the dry biomass of adventitious roots of *P. vulgaris* L. The root cultures grown in a medium supplemented with 0.5 and 1.5mg/L NAA showed the greatest TPC, TFC, and DPPH radical scavenging capabilities in their investigation. In adventitious root cultures of *Fagonia indica*, Khan *et al*., (2017) reported the beneficial effects of NAA in comparison to other hormones like IAA and IBA. They hypothesized that the superiority of NAA towards adventitious root formation in comparison to other hormones can be attributed to the irreversible conjugation and oxidation of those PGRs in the plant cell. The *in vitro* root extracts of

V. jatamansi showed greater TPC, TFC, and antioxidant potential as a result of the favorable effects of NAA at 2.0 mg/L on biomass production (Fig 27).

5.8 Effect of elicitor on adventitious root cultures and phtyochemical constituents of *V. jatamansi*

The most used method for producing and high-level accumulation of medicinally significant metabolites is elicitation, which is also seen to be an effective strategy (Yue *et al*., 2016). MeJA causes the production of numerous types of defense-related substances such phenolic acids, flavonoids, and alkaloids by activating a number of stress signaling pathways (Saeed *et al*. 2017). The TPC, TFC, and antioxidant potential were found to be high with that of the cultures grown with PGRs, but could not exceed the TPC, TFC contents of root cultures grown in NAA (2mg/L) in the cultures grown with elicitors MeJA and SA. The biomass accumulation was minimal in the case of SA. The cultures that was treated with MeJA produced more biomass but lacked TPC and TFC (Fig 26, 27). Numerous research indicate that adding exogenous MeJA to plant cell cultures of different plant species increases the generation of beneficial therapeutic substances (Faizah *et al*., 2018). Anthraquinone and flavonoid accumulation in adventitious and hairy root cultures may have been enhanced by MeJA elicitation, according to prior studies in a variety of plant species (Lee *et al*., 2015; Perassolo *et al*., 2017; Han *et al*., 2019). MeJA was found to inhibit root growth and favor the production of metabolites with cell and adventitious root cultures of *Oplopana xelatus* and *Talinum paniculatum*, which is in contrast to the findings of our study where the addition of MeJA increased the biomass but decreased the accumulation of phytoconstituents (Jiang *et al*,. 2017; Faizal and Sari, 2019). The TPC, TFC, and antioxidant potential of roots treated with elicitors may be independent of biomass, according to these results. These kinds of antioxidant activities based on DPPH that are both biomass dependent and PGRs independent, as well as biomass dependent and PGRs dependent, have previously been observed in a variety of plant species (Ali and Abbasi, 2013; Tariq *et al*., 2014). The phyto constituent composition and antioxidant activity were dependent on the biomass accumulation with different PGRs and their concentrations in the present inquiry, thus the same biomass and PGRs dependant/independence could not be applied to the root cultures grown in different PGRs (Pandey *et al*., 2022).

5.9 Phytoconstituent and antioxidant potential in *T. cordifolia*

Some research work on *T. cordifolia* has been reported from some areas of the world like Angat, Bulacan, Philippines and some from different regions of India (Upadhyay *et al*., 2021). However, comparative total phenolics, flavanoids and antioxidant reports on himalayan *T. cordifolia* stem and leaf extracts as well as with *in vitro* plant extracts so far have not been investigated with modified protocol. Methanol was choosen as an extraction solvent surveying many literatures because it is a better solvent for the extraction of chemicals then other solvents (Sahoo *et al*., 2013). Recently Upadhayay *et al*., (2021) reported high phenolic activity, flavonoid and antioxidant activity in the methanolic stem extract then the methanolic leaf extract of *T. cordifolia,* but there is no report on *in vitro* plant extracts. In this study the same was observed where it was found that the TPC of wild stem was higher (128.55±0.491 mg GAE/g) then the methanolic leaf extracts (105.6847±1.060), whereas the *in vitro* plant extracts showed the total phenolics (107.33±0.985 mg GAE/g). The TFC was also higher in the methanolic stem extracts (83.61±0.2773 mg GAE/g), as compared to methanolic leaf extracts (62.50±0.329 GAE/g), whereas the *in vitro* plant extracts showed the total flavonoids (58.034± 0.148 mg GAE/g). The antioxidant activity was higher in wild stem with IC50 (193.229±0.0013) as compared to wild leaves with IC 50 (204.386±0.165), whereas the *in- vitro* plant extract showed the antioxidant activity with IC50 (219.83±0.468) (Table 12, 13). The TPC of methanolic stem extract was less than the TPC in the methanolic leaf extract (51.86 mg GAE /g) as reported by (Upadhyay *et al*., 2013). Similar results were reported by IIaiyaraja and Khanum, (2011) with total phenolic content of 33.93 mg GAE /g and 52.17 mg GAE /g in the methanolic stem and methanolic leaf extracts of *T. cordifolia*, respectively. The total phenolic content of methanolic leaf extract of *T. cordifolia* was reported to be 2.00 mg GAE /g by (Shwetha *et al*., 2016). Also Upadhayay *et al*., (2021) also reported the total phenolics of 17.48 mg which was less than the present findings. The *in vitro* extracts also produced similar results comparatively little lesser then the wild in this study. The TFC in the methanolic leaf extract of *T. cordifolia* was 0.7 mg QE/g reported by Shwetha *et al*., (2016) which was less than the present findings. The methanolic stem extract showed higher antioxidant activity and higher phenol and flavonoid content as compared to the Methanolic leaf extract. The similar results were observed by Garg and Garg, (2018) showing lesser flavonoid content in methanolic stem extract (2.14 mg QE/g) as compared to the

methanolic leaf extract (4.53 mg QE/g) extracts. ABTS scavenging assay of leaf and stem extracts of the methanolic solvent of *T. cordifolia* from Mysore showed greater inhibition as compared to the present study with the least IC50 values of 95µg/mL and 107 µg/Ml (IIaiyaraja and Khanum, 2011). This is the first report which is based on comparative study in wild and *in vitro* extracts of *T. cordifolia.* The assay supported that there was a positive relationship between total phenolic content, flavonoid and antioxidant activity Upadhayay *et al*., (2021), which was observed in this study. The concentration in *in vitro* cultures can be increased using different elicitors based enhancement strategies (Kumar *et al*., 2017).

5.10 GC-MS analysis for characterization of bioactive compounds in *V. jatamansi*

According to earlier studies and laboratory experiments with species of a similar one, it was discovered that methanolic extracts produced more reliable outcomes than water extraction. Six significant bioactive compounds, identified by GC-MS analysis of the *in vitro V. jatamansi* methanol extract fractions, were among the 21 compounds present (Table 14). In addition to the main compound of interest valeric acid, also known as pentanoic acid, in *V. jatamansi*, the GC-MS spectra revealed the presence of other compounds 9,12-octadecadienoic acid, 9, octadecenoic acid (Z), methyl ester, Heptadecanoic acid 16 methyl, Hexadecanoic acid, and methy ester (Fig 29). Hexadecanoic acid and valeric acid are two bioactive chemicals that have been linked to antioxidant, anti-inflammatory, anti-hyperlipidemic, antibacterial, and neuroprotective properties (Ukwubile *et al*., 2019). The findings of this study are consistent with studies by Liu *et al*., (2013) who identified the chemical makeup and insecticidal properties of essential oil from *V. jatamansi* roots against the booklice, *Liposcelis bostrychophila* Badonnel. Das *et al*., (2011) in their paper examined the terpenoid compositions and antioxidant activity of two essential oils from Indian valerian essential oils and reported twenty-one compounds. When profiled by GC-MS, the quantities of both metabolites were found in the root tissues of wild plants (Table 15, 16). When the root cultures were grown in NAA (2.0 mg/L), the GC-MS analysis similarly produced results that were identical to those of the phytoconstituent study and showed the accumulation of 50 compounds, compared to the presence of 48 compounds in the wild roots. However, less bioactive compounds were discovered in root cultures that had been exposed to elicitors. In root cultures cultivated in NAA and SA, the

existence of economically and pharmaceutically significant valepotriates, such as divalerate and valeric acid, was found. Ascorbic acid serves as a cofactor in photosynthesis, behenic alcohol is used in the cosmetic business, valeric acid acts as an anticonvulsant, and palmatine, an alkaloid, participates in plant metabolism. Unusually, no such chemical constituents were found in cultures that had received MeJA treatment. When NAA was added to the *in vitro* callus cultures of *V. jatamansi*, as was the case in this work, Das *et al*., (2013) found greater valepotriates content. Other than this study, several studies have examined the antioxidant and phytochemical contents of the essential oils from the wild plants of *V. jatamansi* alone to date (Thusoo *et al*., 2014). However, the therapeutic value of *V. officianalis* and *V. walichhi* has been thoroughly investigated, and the valepotriates present in their *in vitro* adventitious and hairy root cultures have been evaluated (Nikolova *et al*., 2017; Nandhini *et al*., 2018). To our knowledge, however, this is the first account of the accumulation of valepotriates in *in vitro* root cultures of *V. Jatamansi*, which was caused by PGRs and elicitation (Pandey *et al*., 2022).

5.11 GC-MS analysis for characterization of bioactive compounds in *T. cordifolia*

Hexadecanoic acid, methyl ester, pentadecanoic acid, 7-octadecanoic acid, cis-9-hexadecenal, which was present in ratio covering high area concentration, and 10-nonadecenoic acid were among the major active compounds identified by GC-MS analysis of *in vitro T. cordifolia* methanolic wild extract fractions of stem. In the wild stem and in an *in vitro* extract of *T. cordifolia*, methyl ester, also known as palmitine, was found (Table. 22, 23). GC MS Studies of *T. cordifolia* indicates that the different compounds were the presence of various bioactive components which justifies the use of the whole plant for various ailments by traditional practitioners. The active principles with their retention time (RT), molecular formula, molecular weight (MW) and concentration (%) are presented. The compound 3, 7, 11, 15-Tetramethyl-2-Hexadecen-1-ol present is reported to have analgesic, anti-inflammatory and antipyretic activities (Tu *et al*., 2021). Similarly Pentadecanoic acid, cis-9-Hexadecenal was also present (Table 22, 23, Fig 30, 31). Similarly in *in vitro* extracts, 32 compounds was detected including palmitine, Pentadecanoic acid, cis-Vaccenic acid. Palmitine is a terpenoid which is an antioxidant compound and also an anticancer compound (Saha and Ghosh, 2012). Even though the *in vitro* extract produced little less compounds as compared to the wild, but the *in vitro* plant extracts contained many important

compounds of biological importance. Both these compounds along with other important biological compounds in both wild and *in vitro* have been detected in *T. cordifolia* which have not been reported in species from Nepal.

5.12 Gene expression analysis of sesquiterpene synthase gene in *V. jatamansi*

The genes responsible for the production of Valerian, a class of Valepotriates commonly known as Sesquiterpene synthase (TPS) gene have been studied. In this study, 5 set of primers were used to perform RT PCR for studying the gene expression level of (TPS) in different in vitro root produced in NAA medium and wild root extracts of *V. jatamansi* Jones. When semiquantitative PCR of cDNA isolated from *in vitro* roots and in vivo roots was performed 4 out of 5 primers produced bands. TPS5 and TPS6 produced band of high intensity of approximately 200 bp, whereas TPS2 and TPS3 produced band of low intensity of approximately 200bp. These bands are sesquiterpene synthase gene. This signifies that sesquiterpene synthase gene which is responsible for the production of valepotriates is found in roots. Simultaneously RT-PCR was performed to study the level of gene expression with five primers (TPS1, TPS2, TPS3, TPS4, TPS5, TPS6). RT-PCR resulted in the higher gene expression (approximately five fold increase in the *in vitro* and *in vivo* extract. Whereas TPS5 showed the highest increase of gene expression (16 fold) in case of *in vitro* root extracts as compared to *in vivo* roots. TPS6 showed about the expression of four fold increase in the *in vitro* root samples as compared to the *in vivo* roots. In this case, the high level of expression of TPS6 (Fig 32, 33) in case of *in vitro* roots and *in vivo* root production medium is because of the high level accumulation of valepotriates (Yeo *et al*., 2012). This results can be correlated with the GC-MS result where NAA containing root extracts produced more compounds as compared to the wild and valeric anhydride was present in higher area ratio in NAA treated *in vitro* roots as compared to the wild roots.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The *in vitro* regeneration and production of important bioactive metabolites of *V. jatamansi and T. cordifolia* using different plant tissue culture techniques can successfully be utilized to cultivate valuable plant species, both commercially and medicinally. Hence the present investigation was focused on *in vitro* mass propagation and ex-situ conservation of *V. jatamansi* and *T. cordifolia*. Since *V. jatamansi* and *T. cordifolia* contains important bioactive metabolites, production of metabolites *in vitro* and *in vivo* plants were studied using different biotechnological strategies. The present finding offers an opportunity to local growers, commercial nurseries for large scale propagation of plants and valuable important secondary metabolites in a short span of time.

One of these plant species, *V. jatamansi*, has limits in its conventional propagation due to factors like seed viability, slow germination, and seed dormancy. A rapid, highly reproducible and efficient regeneration protocol was developed for large scale micropropagation in *V. jatamansi*. It was found that full strength of MS medium supplemented with 10 % CW was found to be more effective with respect to other concentrations of MS supplemented with different plant growth regulators. No differences were found in the genetic fidelity study of tissue culture-derived plantlets or the later-acclimatized plants of *V. jatamansi* using two distinct markers (RAPD and ISSR) to measure similarity with the mother plant. To meet the rising demands of the pharmaceutical industry, true to type *V. jatamansi* plants can be mass-propagated using this efficient method. The entire process of plant regeneration to planting in the field was devised within 4 months, which is rather quick. A quick and effective callus induction strategy was developed in the current investigation utilizing 1.5 mg/L NAA+15 µM SNP, and shoot induction and rooting of *in vitro V. jatamansi* plants were then accomplished using 10% CW+15 µM SNP. The frequency of callus development and shoot organogenesis in *V. jatamansi* has been shown to be enhanced by SNP's interaction with other plant growth regulators. A successful *in vitro* adventitious root

culture system of *V. jatamansi* Jones was established from *in vitro* produced plants, and the efficiency of adventitious root formation supplemented with different PGRs and elicitors was assessed. The total phenolics, flavanoids and antioxidant activity was high in *in vitro* roots extracted from plants grown in MS+10% CW containing medium and also in the roots grown in MS+NAA (2mg/L) containing medium. The accumulation and existence of valepotriates in the produced biomass were examined. The biomass and production of compounds like valeric anhydride, isovaleric acid, and other valeric acid derivatives that are important for medicine were greatly boosted by treatments with NAA (2.0 mg/L). The biomass and subsequent accumulation of bioactive metabolites showed a wide variation of responses after adventitious root cultures were induced with MeJA and SA. More than SA, MeJA boosted biomass, but it had no impact on the phytoconstituents in the root cultures. The SA treated roots showed a lower level of valepotraite compounds than MeJA treated cultures, where no such compounds were discovered. In order to increase and scale up the synthesis of valepotriates, these preliminary findings can be used to change a variety of additional biotic and abiotic elicitors as well as PGRs in the future. Thus, the adventitious root culture method might be planned as a productive and different source for producing crucial medicinal valepotriates *in vitro*. In the roots of *V. jatamansi in vitro* raised plants, NAA-treated roots, and wild plant, valuable bioactive components such valeric acid were accumulated and detected by GC-MS analysis. Gene expression studies revealed the high expression level of sesquiterpene synthase genes (TPS5, TPS6) in *in vitro* root produced in MS+NAA (2mg/L) as compared to the *in vivo* plant of *V. jatamansi* Jones.

In *T. cordifolia*, *in vitro* mass propagation technique was established using nodal segment in MS+BAP (5mg/L)+10% CW medium. The plants produce through nodal segment culture are true to type plants. Also the genetic fidelity was confirmed using RAPD and ISSR primers. The phytochemical analysis for the total phenolics, flavanoids and antioxidants was carried out which revealed the high phenolics, flavanoids and antioxidant activity in wild stem extracts, followed by *in vitro* plant extracts followed by wild leaf extracts. Subsequently important bioactive compounds and palmitine was detected in both *in vitro* and wild stem extracts which is of high biological importance. Even though the concentration of phenolics, flavonoids, antioxidant and no of important metabolites was found to be higher in wild stem of *T.*

cordifolia as compared to the *in vitro* plant extracts, the metabolites can be increased further by using different strategies.

6.2 Recommendations

The following recommendation are made from the present research:

- Large production of this *V. jatamansi* plant roots using our current established protocol should be done to conserve this germplasm.
- Technology developed from this research findings can be transformed for its commercialization and ex situ conservation which will be helpful to upgrade the economic status of rural people by cultivation of tissue cultured raised *V. jatamansi* and *T. cordifolia* reintroduction from lab to land (nature). This can also be helpful to reduce the dependency of the pharmaceutical industries to collect from the wild.

CHAPTER 7

SUMMARY

A well-known subtropical and temperate traditional medicinal plant of Nepal, *V .jatamansi* Jones, commonly known as Sugandhawal, is a member of the Caprifoliaceae family. This plant is an important source of raw materials for the local economy, the pharmaceutical industry, and the perfume industry. The rhizomes, and essential oil of this plant are widely used in traditional medicine to treat a variety of ailments. This plant is therefore one of the most traded and exploited plant species in Nepal. Similarly *T. cordifolia,* also known as gurjo/giloy, is a climbing shrub common in the tropical and subtropical regions of Nepal. It is a popular medicinal plant in Ayurveda and in ancient Ayurvedic literature and medicine around the world. *T. cordifolia* is indeed one of the most important plants that has tremendous pharmaceutical uses due to its ability to fight many diseases and is also used as an important medicinal plant during Covid 19 pandemic. Due to the increasing demand from the pharmaceutical and aromatic sectors in boththe domestic and international markets, both the volume and value of tradein these two plant species have increased significantly over the years. In this context, the conservation and sustainable use of these species using various biotechnological approaches is very important.

Current research is focused on the conservation and sustainable use of these species using plant tissue culture technology and phytochemical evaluation and characterization of bioactive compounds in both species has also been carried out. Genetic evaluation of the mother and *in vitro* plants was also confirmed by molecular markers. Valepotriate, an important compound in the formation of valerian, a drug for epilepsy was also characterized in the roots and the gene expression of this compound was compared with the roots of the wild and *in vitro* plants. Different biotic elicitor elicitor strategies were also tested on *V. jatamansi* compared to the wild species. Phytochemical evaluation of the total phenols, flavonoids, antioxidants and GC-MS for the characterization of the production of important bioactive metabolites were experimented in both species. Both plants were collected from Dabur Nepal, Banepa, and Kathmandu. For the production of *in vitro* plants of *V. jatamansi*, shoot bud explants were used with MS medium treated with different concentrations of growth

regulators and growth additives. Sodium nitroprusside was also used to establish callus and regenerate whole plants. Genetic clonal fidelity was confirmed in both wild and *in vitro* plants using RAPD and ISSR markers. *In vitro* roots were used to form adventitious roots in different liquid media. The elicitors studied to evaluate adventitious root formation and growth kinetics were methyl jasmonate and salicylic acid. Phytochemical evaluation was performed to compare total phenols, flavanoids and antioxidant activity in both wild and *in vitro* samples. Characterization of valepotriates using GC MS technology was performed for both the wild and *in vitro* samples. Gene expression analysis was also performed for the sesquiterpene synthase gene responsible for the production of valepotriates in the *in vitro* root extracts and the wild plants.

Nodal segments of *T. cordifolia* were used to produce *in vitro* plants using MS media supplemented with various combinations of growth regulators and additives. Genetic clonal fidelity was confirmed in both wild and *in vitro* plants using RAPD and ISSR markers. Phytochemical evaluation was performed to compare total phenolics, flavanoids and antioxidant activity in both wild and *in vitro* samples with the standard curve. Characterisation of important bioactive compounds using GC- MS technology was carried out for the wild and *in vitro* samples.

In *V. jatamansi*, the shoot culture, and the root culture were established in medium containing MS+10% CW. Acclimation of the *in vitro* plants was also done in a mixture of cocopeat and soil in a ratio of 3:1. A rapid and efficient protocol for callus induction was also optimized with 1.5 mg/L NAA+15µM SNP and shooting and rooting of the *in vitro* plants was achieved with 10% CW+15 5µM SNP. *In vitro* adventitious roots were formed in medium containing MS+NAA (2mg/L) medium. During stimulation of root culture with methyl jasmonate and salicylic acid, methyl jasmonate induced higher biomass compared to salicylic acid, but did not contribute to the production of many phytoconstituents, while salicylic acid showed the presence of some valepotriate compounds despite lower biomass. Phytochemical evaluation confirmed the presence of high level of phenolics, flavanoids and antioxidants in the *in vitro* samples compared to the wild samples. High accumulation valepotriates was observed in the *in vitro* samples of roots cultured in medium containing MS+NAA (2mg/L) compared to other in *vitro* cultures and wild roots by GC MS analysis. The gene expression study revealed that the sesquiterpene synthase genes (TPS5, TPS6) are used to produce valepotriates in the *in vitro* root cultures of *V. jatamansi* in contrast to the *in vivo* plant root cultures.

In *T. cordifolia*, the technique of *in vitro* mass propagation technique using nodal segments in MS+BAP (5mg/L) +10% CW medium was introduced. The plants produced by nodal segment culture are true to type. Genetic clonal fidelity was also confirmed with RAPD and ISSR primers. Phytochemical analysis of total phenolics, flavanoids and antioxidants was carried out and revealed that phenolis, flavanoids and antioxidants was carried out and revealed that phenols, flavonoids and antioxidants were highest in the extracts from the wild stem extracts, followed by the extracts from the *in vitro* plants and the extracts from the wild leaves. Subsequently important bioactive compounds were detected in both the *in vitro* and wild stem extracts together with palmitin, which is of great biological importance.

The result of the present investigation was significant. A rapid and reliable protocol for whole plant establishment, including analaysis of genetic fidelity in *V. jatamansi* and *T. cordifolia*, was developed. Callus establishment and plant regeneration were also achieved with sodium nitroprusside in *V. jatamansi*. Adventitious root cultures and triggering strategies for high accumulation of valepotriates using GC MS anlaysis were developed as well as high expression of sesquiterpene synthase gene in *V. jatamansi*. Important bioactive compounds including palmitin, have also been characterised in *T. cordifoilia*. Thus this research will help to conservation these two valuable medicinal plants in nature and promote the production of bioactive compounds *in vitro*.

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APPENDIX

LIST OF PUBLICATIONS

List of scientific publications from Ph.D. thesis

1. **Pandey, S**., Sundararajan, S., Ramalingam, S., & Pant, B. (2020). Effects of sodium nitroprusside and growth regulators on callus, multiple shoot induction and tissue browning in commercially important Valeriana jatamansi Jones. *Plant Cell, Tissue and Organ Culture (PCTOC)*, 1-8. https://doi.org/10.1007/s11240-020-01890-7
2. **Pandey, S**., Sundararajan, S., Ramalingam, S., Baniya, K., & Pant, B. (2020). Rapid clonal propagation and valepotriates accumulation in cultures of Valeriana jatamansi Jones, a high- value medicinal plant. https://doi.org/10.5073/JABFQ.2020.093.022
3. **Pandey, S.,** & Pant, B. (2020). Establishment of *in vitro* cultures of valuable medicinal plant Valeriana jatamansi jones, its conservation and production of bioactive metabolites. http://www.envirobiotechjournals.com/EEC/Vol26OctSuppl20/EEC-6.pdf
4. **Pandey, S.,** Sundararajan, S., Ramalingam, S., & Pant, B. (2022). Elicitation and plant growth hormone-mediated adventitious root cultures for enhanced valepotriates accumulation in commercially important medicinal plant Valeriana jatamansi Jones. Acta Physiologiae Plantarum, 44 (1), 1-13. https://doi.org/10.1007/s11738-021-03319-w
5. **Pandey, S.,** Maharjan, L., & Pant, B. (2023). In vitro Propagation and Assessment of Genetic Homogeneity using RAPD and ISSR Markers in Tinospora cordifolia (Wild.) Hook. F. & Thoms, An Important Medicinal Plant of Nepal. *Journal of Nepal Biotechnology Association, 4)1), 27-36.* https://www.nepjol.info/index.php/jnba/article/view/53443
6. Pokharel, B. R., **Pandey, S**., Manandhar, M. D., & Pant, B. (2023). Comparative study of essential oil in wild and in vitro cultures of Valeriana jatamansi Jones in Nepal. *Plant Biotechnology Reports*, 1-9. https://doi.org/10.1007/s11816-023-00832-x

Plant Cell, Tissue and Organ Culture (PCTOC)
https://doi.org/10.1007/s11240-020-01890-7

RESEARCH NOTE

Effects of sodium nitroprusside and growth regulators on callus, multiple shoot induction and tissue browning in commercially important *Valeriana jatamansi* Jones

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Abstract

The effects of nitric oxide (NO) donor sodium nitroprusside (SNP) and various growth regulators on callus induction and shoot organogenesis in the commercially and pharmaceutically important medicinal plant *Valeriana jatamansi* is presented. Four different growth regulators at different concentrations were tested for their callusing efficiency followed by supplementation of SNP to the best responding growth regulator. Maximum callusing frequency (91.18%) was observed with the supplementation of 1.5 mg/L 1-Naphthaleneacetic acid + 15 µM SNP. 10% Coconut water (CW) was found to be the best growth regulator for the induction of shoots. The multiple shoot formation was also found to be enhanced with the supplementation of SNP. Maximum response for in vitro shoot multiplication was observed when the medium was supplemented with 10% CW + 15 µM SNP (89.32%) as compared to shoots induced in medium supplemented with 1.5 mg/L Benzyl aminopurine + 15 µM SNP (79.46%) respectively. No separate root induction medium was required as CW and CW + SNP supplementation was found to be sufficient enough to produce profuse rooting of the in vitro regenerated plants. Results revealed that SNP enhanced the callus and shoot induction frequencies. Supplementation of SNP also significantly reduced callus browing, allowed the tissues to recover and regenerate. This proposed in vitro culture system will be an effective mass propagation strategy for both commercial utilization and conservation of this highly valued plant species.

Key Message

First report using SNP and growth hormone supplementation for enhanced propagation in *Valeriana jatamansi* presented. Supplementation of SNP enhanced the callus, multiple shoot induction and reduced callus browning in the in vitro cultures of *V. jatamansi*.

Keywords Coconut water · Plant growth regulators · Sodium nitroprusside · *Valeriana jatamansi*

Sushma Pandey and Sathish Sundararajan have equally contributed to the research.

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Introduction

Numerous plant species are exploited for their medicinal properties, which depleted their wild population and made their place in the rare and endangered list (Pant 2014). *Valeriana* is one such genus that is distributed in the Himalayan region (Prakash 1999; Rajkumar et al. 2011). The roots and rhizomes possess medicinal compounds that are used to treat a multitude of diseases and ailments, which are attributed to valerenic acid and valepotriates (Prakash 1999; Singh et al. 2010). The indiscriminate collection from the wild has led to its large-scale depletion in recent years which has necessitated its replenishment, cultivation and has prompted studies on in vitro propagation as an alternative source of the

plant and to produce their therapeutically and commercially important metabolites.

Nitric oxide (NO) is a small ubiquitous gaseous, highly diffusible bioactive molecule. NO plays important roles in several physiological processes including plant growth, development, and physiology such as seed germination, root initiation, plant height, stomatal closure, photosynthesis, fruit ripening, organ senescence, floral regulation and stimulates plant defense mechanism to minimize the damage caused by oxidative stress (Khan et al. 2012, 2017). Recently, NO are exploited in plant tissue culture protocols (Rico-Lemus and RodrÍguez-Garay 2014). Sodium nitroprusside (SNP) is the most common NO donor owing to its relatively low cost, well-documented application, and sustained NO production (Zandonadi et al. 2010).

The positive effects of exogenous supplementation of NO and/or SNP on in vitro propagation has been demonstrated in various plant species including *Arabidopsis*, tobacco, and parsley (Tun et al. 2001), cucumber (Pagnussat et al. 2002), mung bean (Huang and She 2003), tomato (Correa-Aragunde et al. 2004), Chinese crab apple (Han et al. 2009), *Dioscorea opposita* (Xu et al. (2009), cherry rootstocks (Sarropoulou et al. 2015) and *Canscora diffusa* (Subiramani et al. 2019). All these reports indicate that NO and/or SNP plays a role in cell division, as well as in shoot regeneration and multiplication. The present study was undertaken to determine the effects of SNP and other growth hormones on the callus induction and shoot proliferation in the *V. jatamansi*. To the best of our knowledge, this is the first report on the use of SNP in in vitro cultures of *V. Jatamansi.*

Materials and methods

Valeriana jatamansi plants were collected from Banepa, Kavrepalanchok, Nepal (27.6332° N, 85.5277° E), and established in the departmental garden of Central Department of Botany, Tribhuvan University. The whole plants were sterilized by rinsing in running tap water for 15 min followed by treating the plants with Tween 20 (Himedia, India). The plants were disinfected with 0.1% $HgCl_2$ (mercuric chloride) for 5 min followed by rinsing five times with sterile water. The explants were then blot dried using a Whatman filter paper and inoculated in test tubes supplemented with MS medium (Murashige and Skoog 1962) containing 3% sucrose (w/v) and solidified with 0.8% agar. The nodal segments from sterile in vitro plants were then inoculated in MS medium supplemented with 10% Coconut Water (CW) and was periodically subcultured. All the cultures were maintained at 25 ± 2 °C with a 16 h photoperiod. All the chemicals (plant tissue culture grade) used in the study were procured from Himedia, India.

The leaf explants from in vitro grown plants were excised aseptically and inoculated onto MS medium supplemented with various plant growth regulator's (Himedia, India), such as NAA, BAP, 6 Furfuryl aminopurine, Kinetin (KN) and 2,4-Dichlorophenoxyacetic acid (2,4-D) at different concentrations (0.5,1.0,1.5,2.0 and 2.5 mg/L). To optimize the SNP (Himedia, India) concentration, different concentrations of SNP (5, 10, 15, 20, 30 μM) was supplemented in the media in a separate experiment for the best responding growth regulator. Explants inoculated onto medium devoid of growth regulators in MS medium were maintained as control. The cultures were incubated at 25 ± 2 °C under a 16/8 h photoperiod, with a light intensity of 40–50 μmol m^{-2} s^{-1}, and subcultures were carried out at a 7-day interval.

After 20–25 days of culture, the calli were transferred to regeneration medium supplemented with various concentrations of NAA, BAP (0.5,1.0,1.5, and 2.0 mg/L) and 1,5,10,15 and 20% CW. SNP at different concentrations as mentioned above was supplemented to the best responding medium after the growth assessments. The percentage of shoots formed, and the mean number of shoots/explant were recorded. The well-grown shoots after 4 weeks of culture were then transferred to pots containing a mixture of soil and vermicompost (2:1 ratio) and acclimatized in the plant containment facility under continuous illumination at 26 ± 2 °C and 75% relative humidity.

All experiments were repeated thrice, using three replicates each containing fifteen explants. The values are expressed as means ($n = 3$) ± SD. The data were further analyzed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test ($p < 0.05$) with the aid of SPSS (version 17, Chicago, USA) statistical package program. $p < 0.05$ was considered as indicative of significance.

Results and discussion

Due to its tremendous potential for treating a multitude of diseases in humans, it is necessary to conserve, find sustainable and alternative ways to propagate *Valeriana* plant species. It is well known that callus induction can be an efficient and appropriate strategy towards micropropagation of various plant material. Das et al. (2013) reported that the levels of endogenous plant growth regulators vary depending upon the plant species and tissues and therefore the explant choice might have a significant influence on the process of callus induction. Different concentrations of NAA, BAP, KN, and 2,4-D were tested for their callus induction proficiency with the leaf explants of *V. jatamansi*. BAP among different concentrations at 0.5 mg/L induced a callus induction response of 51.21%. Among the different concentrations of KN, maximum response (58.18%) was observed with a concentration of 2.0 mg/L. 2,4-D induced a high frequency of

callus induction (70.18%) with a concentration of 1.5 mg/L. Among the four tested choices of growth regulators, NAA (1.5 mg/L) induced the highest frequency of callus induction with 76.17% explants producing calli (Supp. Table S1).

The leaf explants inoculated onto the callus induction medium swelled up after 4 days and callus formation was observed after 10–12 days of incubation (Fig. 1a). The callus formed were routinely subcultured onto a fresh medium at 7-day intervals. The calli grown in medium supplemented with BAP produced root hair-like structures (Fig. 1b). However, the calli grown in medium supplemented with 2,4-D (Fig. 1c) and NAA (Fig. 1d) did not show any root or hairy structures from the calli. No callus induction was observed with the explants inoculated onto medium devoid of growth regulators (control). Increasing the concentration of growth regulators (2.0 and 2.5 mg/L) resulted in a drastic decrease in the callus induction frequencies among all the tested growth hormones. Previously callus and suspension cultures have been used for plantlet regeneration of *V. wallichii* (Mathur 1991) and *V. edulis* (Castillo et al. 2000). Das et al. (2013) reported optimum callus induction frequency in *V. jatamansi* Jones while using rhizome explants on media supplemented with 2,4-D and highest regeneration frequencies were obtained by supplementing MS medium with thidiazuron and NAA. Chen et al. (2014) suggested that 2,4-D and NAA produced embryogenic calli which differentiated into adventitious shoots and somatic embryos in *V. officinalis*. However, in the present study NAA alone was sufficient

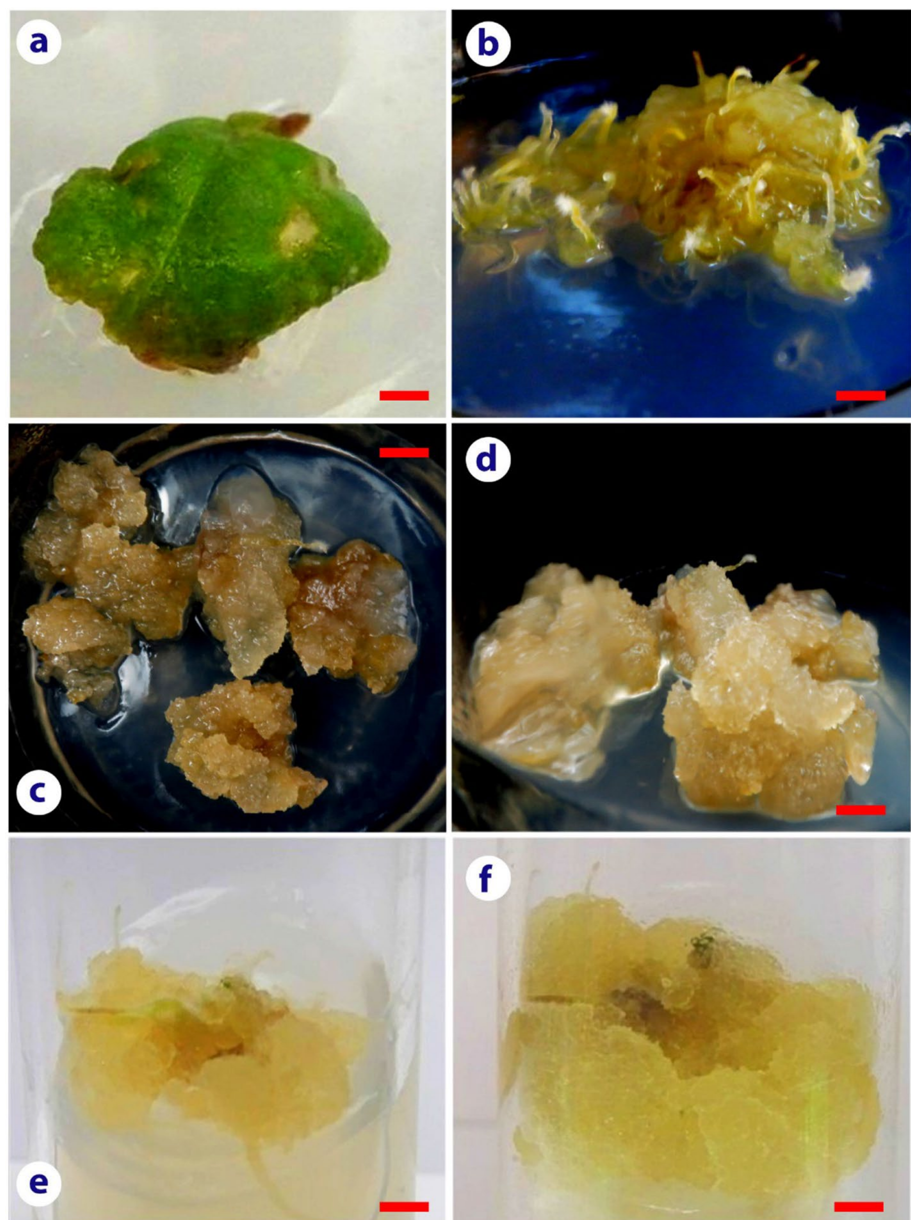

Fig. 1 Callus induction of *Valeriana jatamansi* leaf explants with different plant growth regulators and sodium nitroprusside combinations. **a** Leaf explant 7 days post inoculation onto callus induction medium, **b** callus induced from leaf explants supplemented with 0.5 mg/L BAP, **c** callus induction from leaf explants with 1.0 mg/L 2,4-D, **d** callus induction with 1.5 mg/L NAA, **e** callus induced from leaf explants after 14 days of inoculation onto medium supplemented with 1.5 mg/L NAA + 10 µM SNP, **f** callus induced from leaf explants after 14 days of inoculation onto medium supplemented with 1.5 mg/L NAA + 15 µM SNP. Scale bar = 1 cm

enough to induce maximum callusing response and this could be interpreted to the fact the callus induction percentages vary depending on explant choice and plant species.

SNP derived NO has recently been considered a new phytohormone (Leterrier et al. 2012) and has been used for developing various plant tissue culture protocols (Rico-Lemus and RodrÍguez-Garay 2014). Ötvös et al. (2005) reported that in *Medicago sativa,* SNP in combination with auxins promoted activation of cell division and formation of the embryogenic cell and further suggested that 10 µM SNP as a sole entity in the medium did not influence the cell division, however, supplementation of SNP along with 2,4-D significantly increased the cell division frequency. A significant increase in the callus fresh weight was reported in *Hyoscyamus niger* when the medium was supplemented with SNP (50 µM) with other plant growth regulators suggesting the synergism existing between SNP and growth regulators on the callus induction frequencies (Samsampour et al. 2018). To optimize the dosage of SNP required to enhance the callus induction potential, the best responding concentration in all the four tested growth regulators was chosen and SNP at four different concentrations was supplemented. The exogenous supplementation of SNP significantly enhanced the callus induction frequency. The calli induced in medium supplemented with SNP showed friable and compact nature (Fig. 1e, f) compared to the calli grown in medium with growth regulators and devoid of SNP. The results of SNP supplementation on callus induction with four different growth regulators are depicted in Supp. Fig. S1. Maximum callus induction (91.18%) was achieved with the combination of NAA (1.5 mg/L) + 15 µM SNP and invariably the combination of NAA (1.5 mg/L) with all tested SNP treatments produced a better callusing response (Supp. Fig. S1a). The next best response (86.55%) was achieved with the medium supplemented with 2,4-D (1.5 mg/L) + 15 µM SNP (Supp. Fig. S1b). The medium supplemented with 0.5 mg/L (BAP) + 10 µM SNP induced 83.50% callus induction (Supp. Fig. S1c). Compared to the other tested growth regulators along with SNP supplementation, medium supplemented with KN responded with lower callusing percentages (Supp. Fig. S1d). The results demonstrated that the addition of SNP in different growth regulators greatly enhanced the callus induction frequencies of *V. jatamasni* leaf explants. When the medium was supplemented with BAP, the concentration of 1.5 mg/L induced maximum shoot induction response (66.10%) with 26.32 shoots per explant. NAA, on the other hand, failed to induce higher frequencies of shoot induction with only 1.0 mg/L treatment showed a frequency of 27.32% shoot formation with 12.17 shoots per explant. The treatments with three other concentrations also failed to produced proficient shoot induction. Similar to our results, previously a 100% callus induction percentage in cherry rootstocks (Sarropoulou et al. 2015) and 87% callus induction in *D. opposite* Thunb tuber (Xu et al. 2009) was reported with the exogenous supplementation of SNP. Similar findings using SNP was reported by Kalra and Babbar (2012), where supplementation of SNP stimulated callogenesis in *Albizzia lebbeck*.

Coconut water is a natural substance containing high levels of zeatin and related forms, kinetin, and N6-(D2-isopentenyl)- adenine (iP)-like precursors (Tan et al. 2011). The positive effects of CW supplementation alone or in combination with growth regulators for the in vitro growth and development of various plant species have been reported previously (Maharjan et al. 2019). Among the tested growth regulators, CW remained the best regulator in inducing shoots from the in vitro calluses of the *V. jatamansi* (Supp. Table S2). The highest shoot induction frequency was obtained with 10% CW which produced 81.22% shoot induction with maximum shoots per explants (34.31). Interestingly, the 20% CW also enhanced the shoot induction percentage, however it reduced the mean shoot numbers compared to the lower concentrations of CW. The results suggest that 10% CW is the optimum concentration of growth regulator required to induce efficient multiple shoot induction in callus explants of *V. jatamansi.*

The effects of SNP on the multiple shoot induction efficiencies were tested by supplementing different concentrations of SNP to medium containing 1.5 mg/L BAP and to the medium supplemented with 10% CW. The calli transferred to BAP + SNP medium started producing green shoot buds (Fig. 2a) along with prominent hairy root-like structures (Fig. 2b) and the shoot buds turned to distinct shoots after 10 days. However, in the calli inoculated onto the medium containing CW and SNP, green-colored buds started appearing early on day three of the inoculation (Fig. 2c, d) and the shoots elongated within 6–7 days' post-inoculation. The shoot formation and elongation were early in the CW + SNP combination compared to that of BAP + SNP treatments (Fig. 2e, f). CW + 15 µM SNP induced maximum response (89.32%) with 35.42 mean shoots compared to the response obtained with the medium supplemented with BAP + SNP (Supp. Fig. S2a, b). In both cases of BAP and CW, the addition of increased concentration of SNP (30 µM) reduced the shoot induction percentages, and a drastic reduction in shoot numbers was also observed. Similar to our results, Kalra and Babbar (2010) reported that by increasing the number of meristems, nitric oxide could enhance the regeneration response and suggested that NO might play a vital role in regulating the gene expression related to the meristem differentiation. Similar results were observed by Karthik et al. (2019) in *Glycine max* where the exogenous supplementation of SNP enhanced the in vitro responses. The individual shoots obtained from calli inoculated onto BAP + SNP (Fig. 3a–c) and CW + SNP treatments were excised and allowed to proliferate and the shoots from CW + SNP

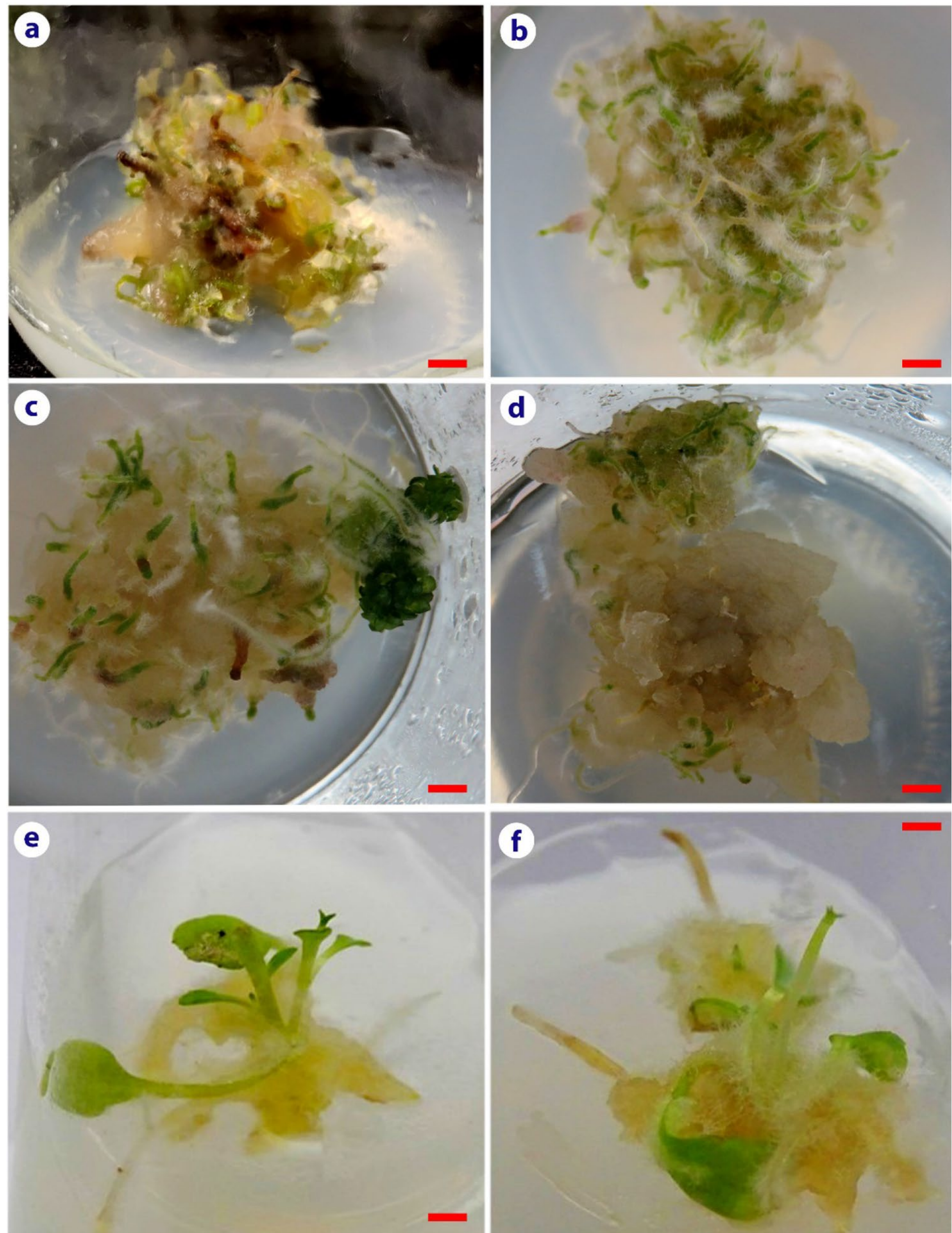

Fig. 2 Effect of growth regulators and sodium nitroprusside on shoot induction of callus from *Valeriana jatamansi*. Shoot formation from callus supplemented with **a** 1.5 mg/L BAP + SNP 10 μM, **b** 1.5 mg/L BAP + SNP 15 μM, **c** 10% CW + 15 μM SNP, **d** 10% CW + 10 μM SNP, **e**, **f** rapid shoot initiation and formation of distinct individual shoots from two individual callus explants supplemented with 10% CW + 15 μM SNP. Scale bar = 1 cm

supplementation showed rapid proliferation (Fig. 3d, e) and the leaves of the plants were relatively broader and greener (Fig. 3f) compared to BAP treatments. Zebarjadi et al. (2011) used an additional rooting medium for rooting of in vitro derived *V. officianlis* plantlets, however, during the rooting experiments, there was no need for an extra medium for root induction since the supplementation of coconut water itself induced roots and CW + SNP treatments further enhanced the formation of roots in the medium (Fig. 3g).

Callus browning results in plant growth inhibition and reduced cell proliferation. Yoruk and Marshall (2003) suggested that a multitude of factors cause callus browning in in vitro tissue culture systems and excessive accumulation of reactive oxygen species (ROS) along with the organelles disorganization, results in limiting plant growth and eventually cell death (Sharma et al. 2012). A higher concentration of growth regulators resulted in callus browning and hyperhydricity (Fig. 4a). The callus browning symptoms were evident in all the tested growth regulators at higher dosages especially NAA and BAP (Fig. 4b). The calli browning resulted in stunted shoots, shoot necrosis, and eventual death of the plant when left in the medium devoid of SNP (Fig. 4c, d). However, when the calluses were transferred to medium containing SNP, callus browning was reduced and later allowed plants to regenerate (Fig. 4e, f). Some of the calli derived from SNP supplemented medium showed hyperhyrdicity symptoms especially

Fig. 3 Influence of sodium nitroprusside on shoot and root induction of *Valeriana jatamansi.* **a** Shoot proliferation from callus explants supplemented with 1.5 mg/L BAP + 15 µM SNP, **b**, **c** individual shoots excised and allowed to proliferate in 1.5 mg/L BAP + 15 µM SNP fresh medium, **d** shoot proliferation from callus explants supplemented with 10% CW + 15 µM SNP, **e**, **f** individual shoots excised and allowed to proliferate in 10% CW + 15 µM SNP fresh medium showing rapid proliferation and broader leaf structures upon transfer to fresh medium, **g** root formation with 10% CW and 10% CW + 15 µM SNP. Scale bar = 2 cm

with the SNP concentration of 20 and 30 µM. Similar to that of our observations, Subiramani et al. (2019) reported hyperhydricity in *C. diffusa* in vitro propagation mediated by SNP supplementation and reduced callus browning of *D. opposita* tuber explants (Xu et al. 2009) and *Ficus religiosa* (Hesami et al. 2018).

Conclusion

Valeriana plant species are being overexploited for commercial/pharmaceutical utilization owing to its high demand for the roots and rhizomes that contain

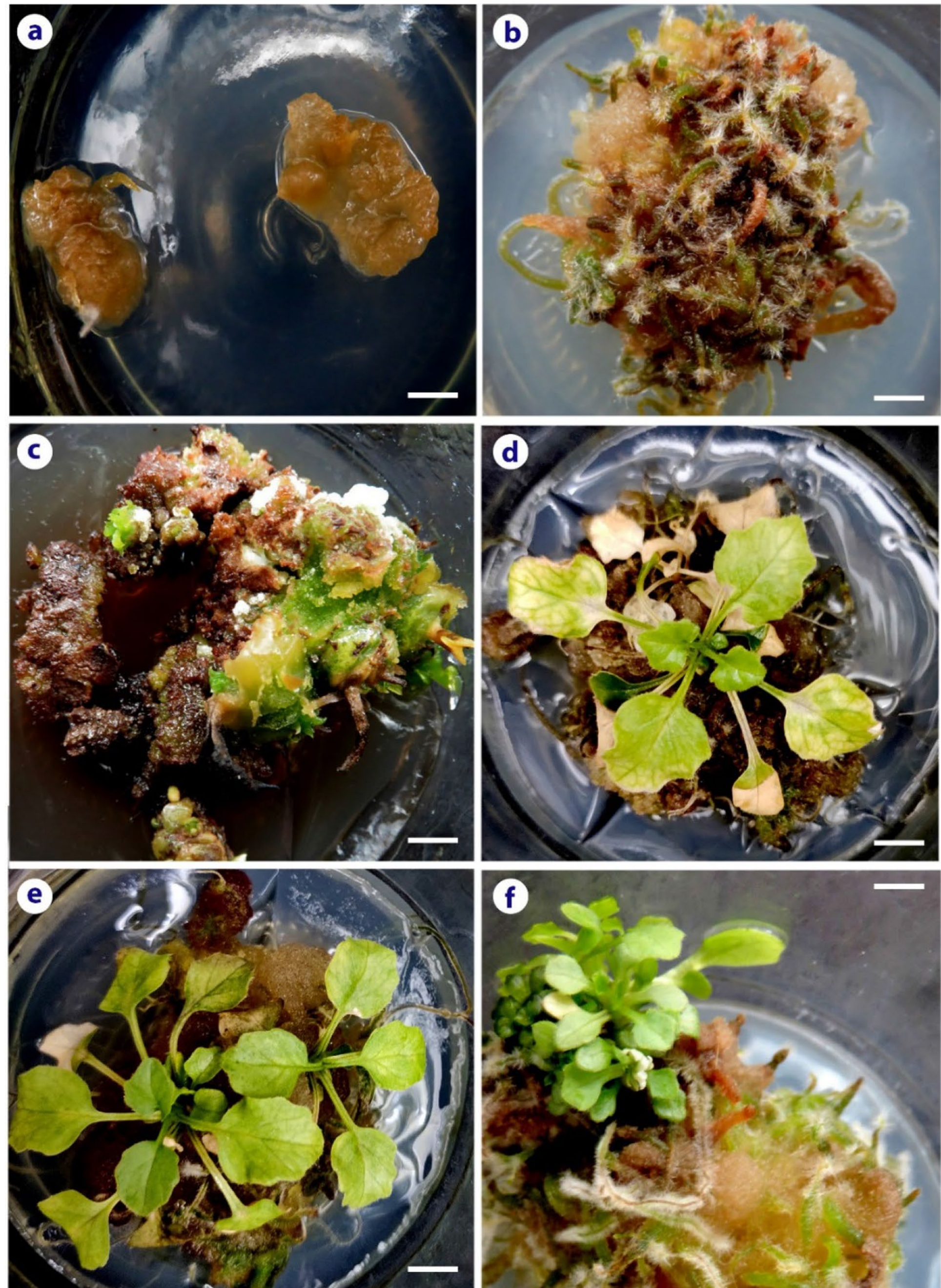

Fig. 4 Effects of Sodium nitroprusside on in vitro callus browning and shoot regeneration of *Valeriana jatamansi*. **a** Callus browning and hyperhydricity symptoms with medium supplemented with BAP 2.5 mg/L, **b** calli turning brown after 7 days of culture on shoot induction medium supplemented with 2.0 mg/L NAA, **c** callus browning and shoot necrosis during culture of explants in medium supplemented with NAA 2.0 mg/L, **d** proliferating shoot withering after 12 days of incubation in medium supplemented with 2.0 mg/L BAP, **e** calli initially cultured in 2.0 mg/L BAP transferred to BAP mg/L + 15 µM SNP showing recovery and shoot proliferation after 12 days of incubation, **f** calli initially cultured in 2.0 mg/L NAA transferred to NAA 2.0 mg/L + 10 µM SNP showing recovery with initiation of multiple shoots after 6 days of incubation. Scale bars = 1 cm

valepotriates. It is imperative to optimize an efficient protocol for in vitro propagation of *V. jatamansi* to meet the high commercial demand as well as for its conservation. In the present study, we demonstrated that exogenous supplementation of SNP has a significant effect on in vitro callus induction and shoot organogenesis of *V. jatamansi*. A rapid and efficient protocol for callus induction was optimized using 1.5 mg/L NAA + 15 µM SNP and subsequently shoot induction and rooting of in vitro *V. jatamansi* plants was achieved using 10% CW + 15 µM SNP. It has also been shown that SNP interacts with other plant growth regulators to improve the frequencies of the callus formation and shoot organogenesis in *V. jatamansi.* Thus, we suggest that this optimized tissue culture system using SNP in the present study will be a viable strategy in the in vitro propagation of this medicinally important and endangered plant to meet its demands for pharmaceutical industries and aid in the conservation of the plant species.

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Author contributions SP executed the experiments and carried out the experimental analysis. SS prepared the manuscript. RS edited and critically evaluated the manuscript. BP conceptualized the research design and edited the manuscript.

Compliance with ethical standards

Conflict of interest All authors read, approved the manuscript, and declare that there is no conflict of interest.

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

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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-

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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.

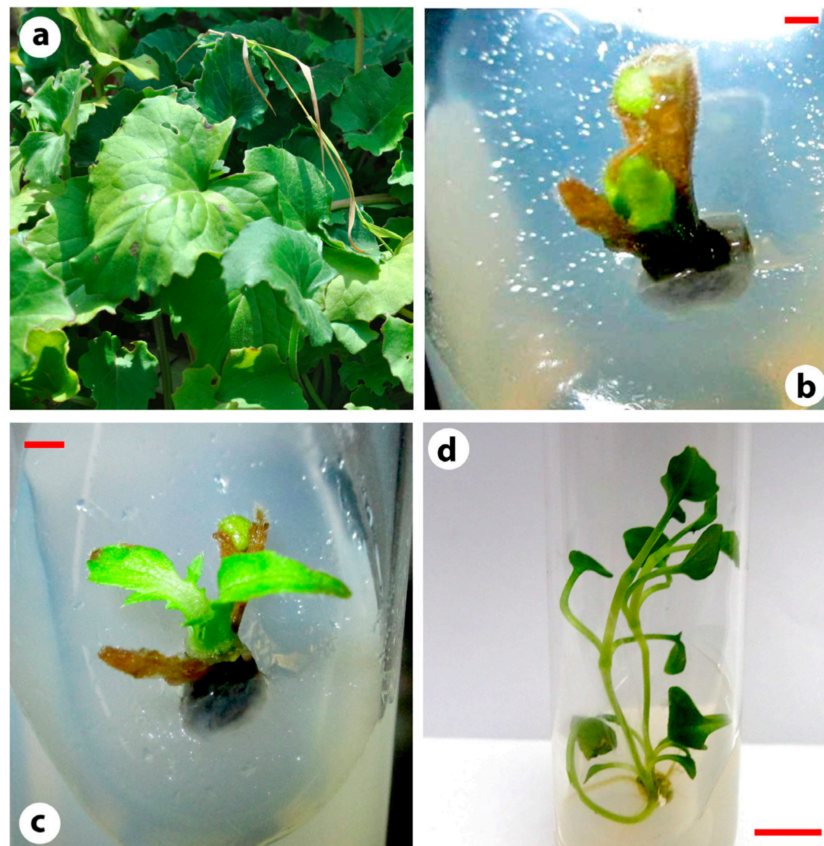

Fig. 1: *In vitro* regeneration of *V. jatamansi* shoot bud explants on MS+BAP (2 mg/L) (**a**) Mother plant of *V. jatamansi*; (**b**) shoot bud explants post 7 days incubation; (**c**) shoot bud initiation after 12 days of culture; (**d**) shoot elongation after 30 days of culture. Scale bar = 1cm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

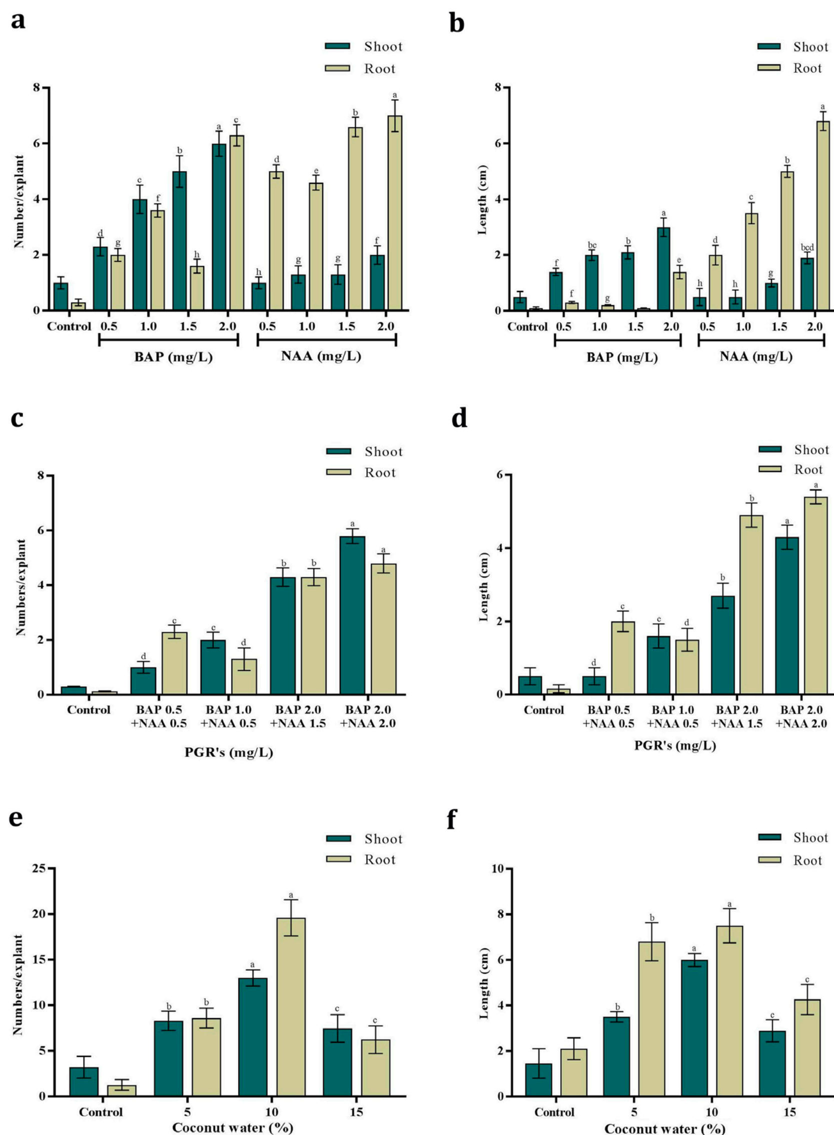

Fig. 2: Effect of PGR'S on shoot and root induction from nodal explants of *V. jatamansi* (**a**) mean number of shoots and roots per explant with different concentrations of BAP and NAA; (**b**) mean shoot and root lengths with different concentration of BAP and NAA. (**c**) mean number of shoots and 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

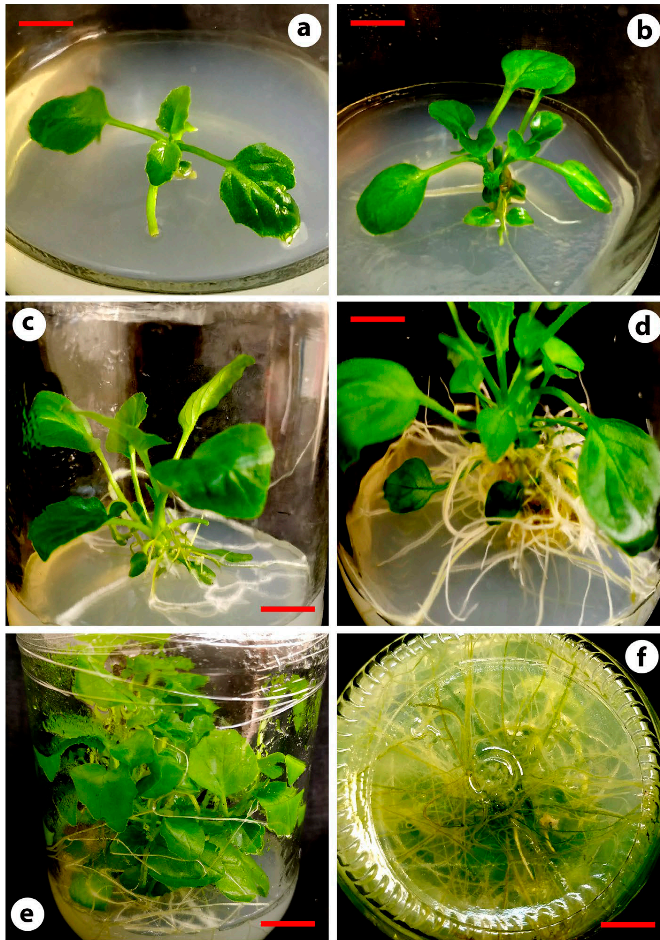

Fig. 3: *In vitro* regeneration of *V. jatamansi* nodal segments in MS+ 10% Coconut water (**a**) Nodal segment of *V. jatamansi*, after 5 days of culture; (**b**) multiplications of shoot after 8 days; (**c**) shoot multiplication after 15 days of culture; (**d**) adventitious root formation after 25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

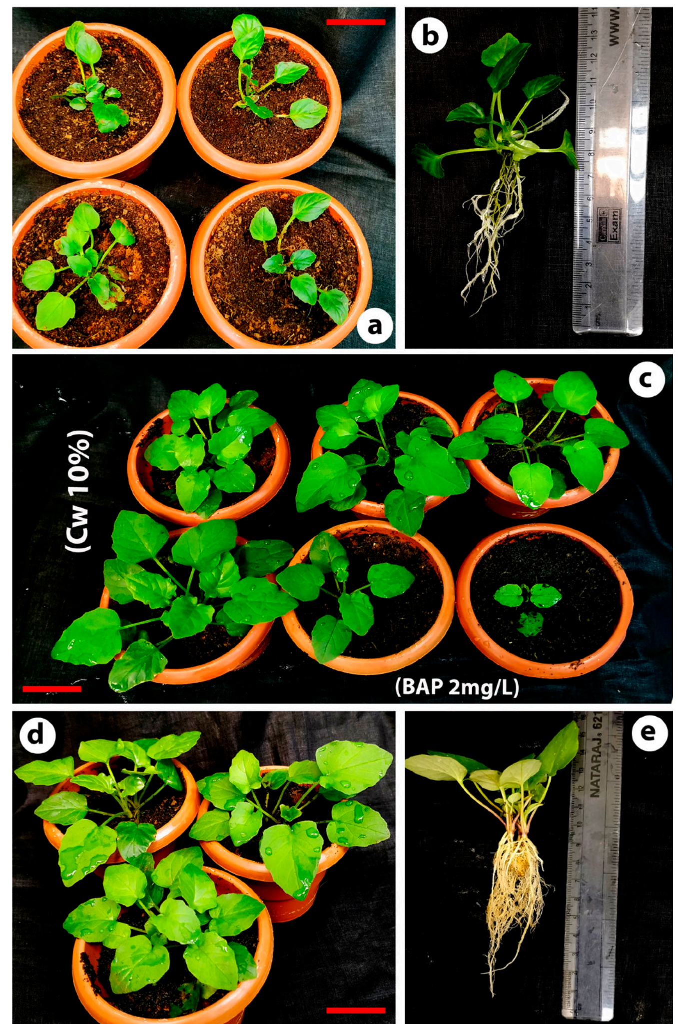

Fig. 4: Acclimatization of in vitro regenerated plantlets of *V. jatamansi* (**a**) Plants of *in vitro V. jatamansi* hardened on coco peat: soil (3:1); (**b**) roots formed prior to acclimatization; (**c**, **d**) acclimatized *in vitro* plantlets from BAP (2 mg/L) and coconut water (10%) treatment 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

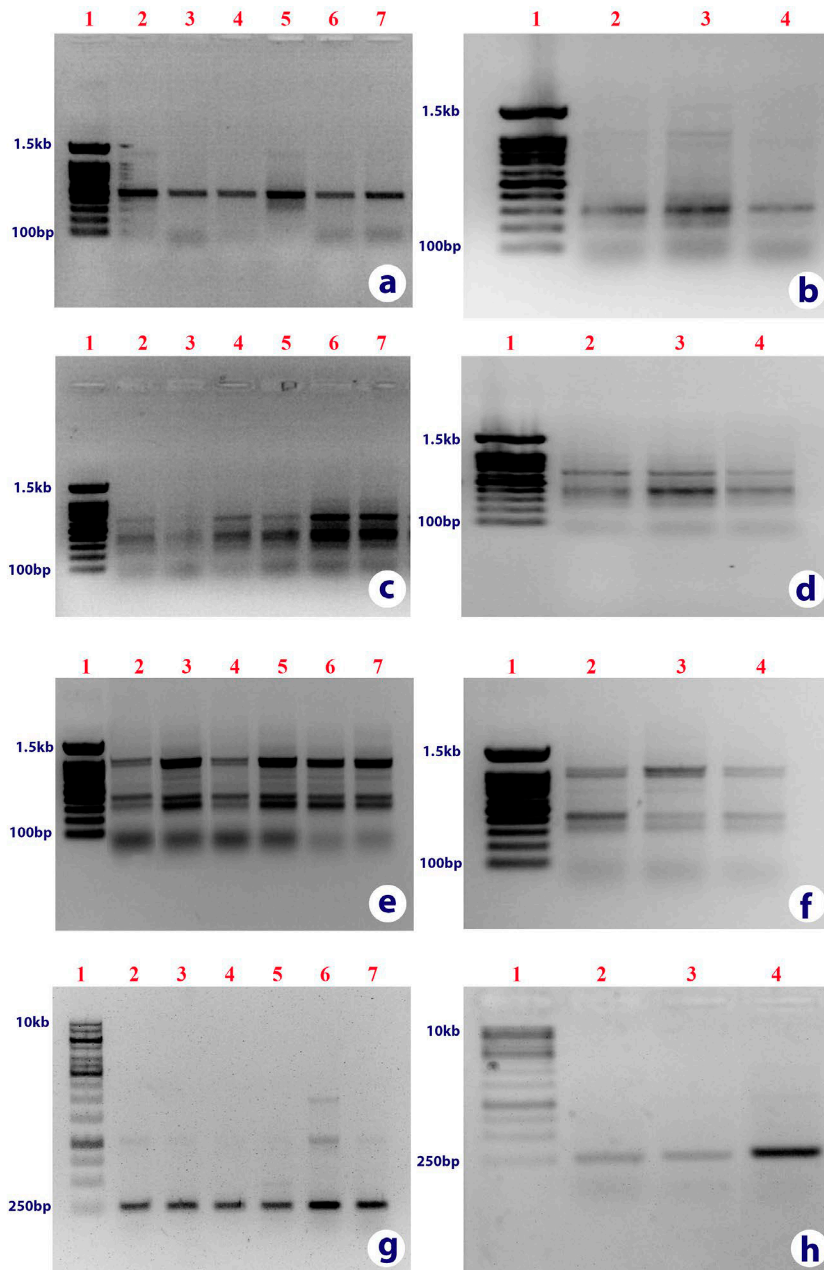

Fig. 5: Representative image depicting the genetic fidelity analysis in in vitro, mother and acclimatized plants of *V. jatamansi* using RAPD and ISSR primers (**a**) OPB 07 Primer **1**-marker, **2** to **6** *in vitro* plants; **7** mother plant; (**b**) OPA 07 Primer **1**-marker, **2** mother plant; **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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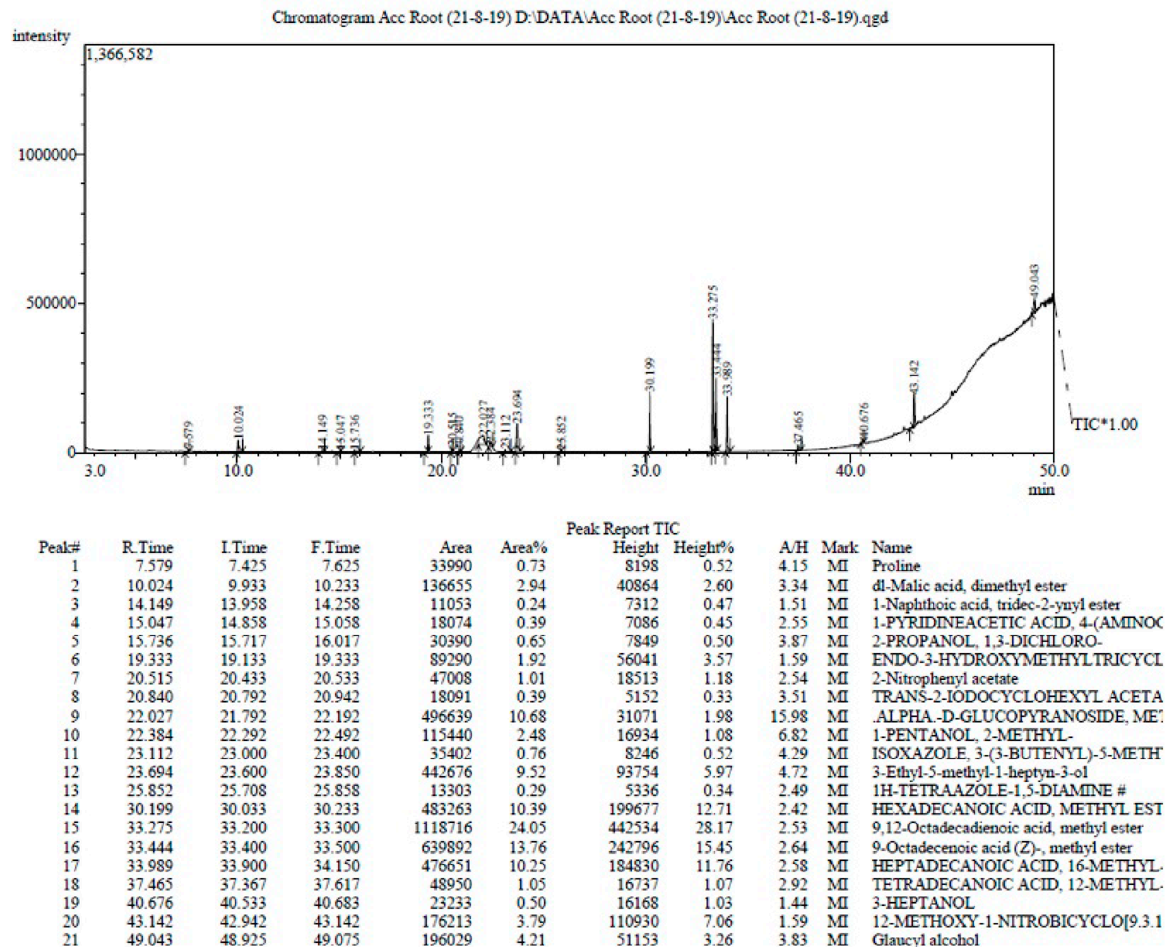
Conflict of interest

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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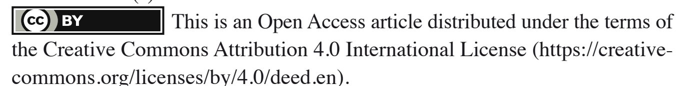
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Establishment of *in vitro* cultures of valuable medicinal plant *Valeriana jatamansi* jones, its conservation and production of bioactive metabolites

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ABSTRACT

Valeriana jatamansi Jones is a perennial herb, belonging to the family Valerianaceae. The pharamaceutical significance of this plant is mainly because of the rhizomes and roots. The species has become threatened in its natural habitats due to exploitation of its rhizomes for drug preparation in pharmaceutical industries. *In vitro* plant cell and tissue culture would not only surmount the limitations of vegetative propagation but also can hasten the production of clonal material for field planting and production of unlimited amount of secondary metabolites. In the present study, MS medium supplemented with 2 mg/L of Benzyl amino purine (BAP) produced maximum shoot bud regeneration with shoot numbers (6), shoot lengths (3 cm), root numbers (6.3), and root length (1.4 cm). Methanolic *in vitro* extracts of *V. jatamansi* resulted in the highest phenolic content (55± 1.00 mg GAE/g) compared to the wild sample (49±1.00 GAE/g) and the highest flavanoid content (219±1.00 mg QE/g) as compared to the wild (124±1.00 mg QE)/g). DPPH activity was also highest in *in vitro* extracts (84.33±0.577%) as compared to the wild (78.33±0.569%). GC-MS profiling of the wild plant extracts revealed the presence of important bioactive compounds including valeric anhydride, valerenol. A reproducible protocol was established using shoot bud explants which will be benefitted towards utilization of natural resources for the production of pharmaceutically important compounds at a large scale from *V.jatamansi.*

Key words: *Secondary metabolites, Plant tissue culture, Phytochemical analysis, GC-MS*

Introduction

Valeriana jatamansi, a valuable medicinal plant is an herb, belonging to the family valerianaceae, also known as Sugandhawal have multiple uses in traditional and modern systems of medicine and also a native plant species of Himalaya, distributed widely in the tropical and subtropical regions of the world (Prakash *et al*., 1999). The essential oil and extracts from the roots and rhizomes is used worldwide (Singh *et al*., 2010), and is highly demanded in drug industry (Das *et al*., 2013). In Nepal, this species has been banned for export due to its high medicinal properties and also been prioritized for research and development (Chaudhary *et al*., 2016). The presence of valeopotriates, a group of monoterpenoids having epoxy group and beta-acetoxy isovaleric acids has been a source of high medicinal importance in this plant (Kaur *et al*., 1999). The pharmaceutical

Abbreviations

MS: Murashige and Skoog, 1962; mg/L: milligram per liter; GAE/g: Gallic acid equivalent per gram; QE/g: Quercetin equivalent per gram; DPPH: 2,2-diphenyl-1-picrylhydrazyl; GC-MS: Gass chromatography mass spectrometry; BAP: Benzyl amino purine.

benefits of valeopotriates is due to its tranquilizing properties which play a major role in its its effectivity in leprosy (Singh *et al*., 2010). Roots and rhizomes of the species has been reported to cure obesity, skin diseases, epilepsy, insanity and skin poisoning (Prakash *et al*., 1999). In India this species has been listed in high trade consumption between 100 and 200 metric tons as the plants have been continuously used in industries thus depleting the natural resources (Jugran *et al*., 2015). Thus conservation of this species is very important to meet the needs of the pharmaceutical industries. The roots and rhizomes are continuously being harvested from large quantities from the wild to meet the demand of pharmaceutical industries because of valeopotriates and valtrates which contributes to its multipurpose medicinal value, thus leading to the conservation and sustainable utilization (Kumar *et al*., 2012). Naturally, the herb is propagated through seeds for the propagation, but still has limitation for propagation due to dormant nature of the seeds (Kaur *et al*., 1999). Therefore, plant tissue culture can be a alternative for *in vitro* propagation in a large scale within a small period of time as this method has been reliable and useful for conservation of important medicinal plants and production of important phytochemicals (Pant *et al*., 2014). Previously, the bioactive constituents in north-east India analyzed by GC-MS revealed the presence of twenty-one compounds (Das *et al*., 2011). Though there are reports on propagation of *V. jatamansi* Jones (Das *et al* 2013., Kaur., 1999, Jugran *et al*., 2015), this paper focuses on using shoot bud explants for *in vitro* propagation of *V. jatamansi*. Phytochemical analysis for the total phenolics, flavanoids and antioxidant activity of the wild roots and also the *in vitro* roots was determined and compared followed by GC-MS of the wild plants to characterize the valuable secondary metabolites.

Materials and Methods

Plant material and surface sterilization

The fresh plants of *V. jatamansi* were collected from Dabur Nepal, Kathmandu and planted in the departmental garden of Tribhuvan University. Shoot buds explants were excised and rinsed in running tap water followed by sterilization with few drops of Tween 20 (Himedia, India) and rinsing in sterile water for 30 min. The explants were then treated with 0.1% $HgCl_2$ (mercuric chloride) for 5 min followed by rinsing five times with sterile water to remove traces of $HgCl_2$. The explants were dried using a Whatman filter paper and inoculated onto Murashige and Skoog (MS) (Murashige and Skoog 1962) medium containing 3% sucrose (w/v), 0.1% myoinositol supplemented with different combination of BAP, prior adjusting the pH of the medium to 5.8 prior to autoclaving (121 ºC, 20 min), solidified with 0.8% plant tissue culture tested agar-agar (w/v) (Himedia, India) The cultures were maintained at 25 °C ± 2 °C and 1000 flux lux under 16/8 h photoperiod with 75% relative humidity (RH).

Inoculation in culture medium and Shoot Proliferation, Elongation and root formation

Explants (shoot buds) were cultured on MS basal medium supplemented with different concentrations BAP (0.5 to 2 mg/L). All cultures were incubated under 16h photoperiod with a light intensity of 55 $\mu molm^{-2}s^{-2}$ provided by cool white fluorescent lamps (Phillips, India) at 25 ± 2 °C. Subcultures were carried out after 4-weeks and number of shoots were calculated.

Acclimatization and Hardening

After 5-6 weeks, the *in vitro* raised plantlets were taken out from the culture medium, rinsed with distilled water. The plantlets were subsequently transplanted into plastic pots containing a mixture of coco peat: soil (3:1), and placed in green house.

Phytochemical analysis

Plant material and extract preparation

The fresh roots of wild plant as well as *in vitro* plants were washed under running tap water, dried with filter paper and shade dried at room temperature. 100 mg of dried tissue was homogenized in 100 mL of methanol and extractions were carried on an orbital shaker (REMI, India) with constant stirring at 180 rpm for 24 h followed by centrifuging at 10,000 rpm for 15 min and the supernatant ltered through lter paper. Measurements for biochemical parameters were taken in Lamda-35 double beam spectrophotometer (Perkin-Elmer, USA).

Determination of total polyphenols, total flavanoid

Quantitative estimation of total phenolic content (TPC), and total flavanoid content (TFC), using the extracts were carried out according to the methods

described by Singleton and Rossi (1965) and Chang *et al.*, (2002). The results of TPC, TFC, were expressed in mg standard gallic acid equivalent (GAE) and quercetin equivalent (QE) per gram of dry tissue respectively.

Antioxidant activity assay

DPPH radical scavenging assay

The DPPH radical activity was estimated using the method of Liyana-Pathiranan and Shahidi, (2005). A solution of 0.135 mM DPPH in methanol was prepared, 1.0 mL of this was mixed with 1.0 mL of different concentrations (0.02 – 0.1 mg) of the extract prepared in methanol, following the reaction mixture vortexed in dark at room temperature 27 ºC for 30 min The absorbance was measured spectrophotometrically at 517 nm. DPPH radical scavenging activity was calculated as follows

DPPH radical scavenging activity (%) = [(Abs control – Abs sample)]/(Abs control)] x 100 where Abs control is the absorbance of DPPH radical + methanol; Abs sample is the absorbance of DPPH radical + sample extract /standard.

GC MS analysis

The extracts of the wild roots were analyzed using gas chromatography and mass spectrometry GC-MS 4000 (Varian, USA)system with a HP-5MS agilent column (30m × 0.25 mm, 0.25 5 film thickness). Injector temperature was 28 C. Oven temperature programme used was holding at 50 ºC for 5min, heating to 28 ºC at 3 ºC/min, and keeping the temperature constant at 28 ºC for 7min. Helium was used as a carrier gas at a constant flow of 1.0 mL/min and an injection volume of 0.20 5L was employed. The important bioactive compounds were studied according to previous literatures and their biological properties.

Data Analysis

All the samples were analyzed in triplicates. The values were expressed as means of triplicate analysis of the samples (n = 3) ± SD. All the data were further analyzed using a one-way analysis of variance (ANOVA) followed by Duncan's multiple range test ($p< 0.05$) with the aid of SPSS (17 version) 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.

Results and Discussion

Establishment of *in vitro* cultures from shoot buds

Das *et al.*, (2013) reported efficient callus-mediated shoot regeneration system for the large-scale production of *V. jatamansi* Jones. The effect of composition and contents of growth regulators in culture media on the accumulation of secondary metabolites in *in vitro* cultures is well known Maurmann *et al*., 2006. In our study, the shoot bud, explants inoculated on MS media supplemented with various concentration of BAP (Table 1), for shoot initiation and proliferation. Shoot initiation was observed in the medium supplemented with BAP (Fig 1B), particularly among the various concentrations of BAP tested 2 mg/L induced maximum shoot initiation (6.0 shoots per explant) after 7 days of culture (Fig 1C, D). The average shoot length (3.0 cm), average root numbers (6.3) and average root length (1.4 cm) were also highest compared to other tested concentration of BAP (Table 1). High multiplication and rooting rate was achieved using combination of BAP (1.5µM) along with either NAA or IAA (0.5 µM), and GA_3 by Purohit *et al*., 2015. This combination of BAP and NAA has been used for high-frequency shoot multiplication for several species including *V. jatamansi.* Kaur *et al*., (1999) also reported

Table 1. Statistical analysis of all the treatments using ANOVA. Data were taken after 5 weeks of culture.Data presented as mean±SD

Plant Growth Regulators BAP (mg/L)	Average shoot number	Average shoot length (cm)	Average root number	Average root length (cm)
0	1.0±0.00	0.5±0.00	0.3±0.33	0.16±0.16
0.5	2.3±0.33	1.4±0.06	2.0±0.57	0.3±0.33
1	4.0±0.57	2.0±0.00	3.667±0.33	0.2±0.03
1.5	5.0±0.00	2.1±0.05	1.667±0.33	0.1±0.057
2	6.00±0.00	3.0±0.10	6.33±0.33	1.4±0.17

Table 2. Contents of total phenolic and flavonoid of *in vitro* and wild extract. Values are expressed as mean ± SD (n=3).

Sample	Extract	Total phenol (mg gallic acid g^{-1})	Total flavonoids (mg quercetin g^{-1})
In vitro	Methanol	55± 1.00	219±1.00
Wild	Methanol	49±1.00	124±1.00

that shoot buds cultured on medium fortified with BA and IAA or NAA induced shoots, and subsequently produced roots on the same medium. During our experiments using BAP, root formation was also observed (Fig 1C, D). Previous literatures reported that cytokinins BA, TDZ, zeatin or Kinetin had no effect on callus induction, shoot organogenesis Das *et al*., 2013. But in our experiment BAP produced plantlet regeneration. The production of roots in *Valeriana* species is very important because the valuable bioactive metabolites is accumulated in roots and roots are harvested to meet the demands of industries. The acclimatized plantlets were established showed and 80 % survival rate (Fig 1E,F). Being one of the medicinally rich plant, the knowledge of the biochemical constitution of *V. jatamansi* is essential. The methanolic *in vitro* root extracts of *V. jatamansi* possessed the highest phenolic, flavanoid content and antioxidant activity when compared with wild extracts (Table 2, 3). The phenolic content of all the extracts was considerably high, which could be a major contributing factor to the antioxidant activity of this plant extracts. According to Asekun *et al*., (2007) high phenolic content of plant extracts could be responsible for their enhanced antioxidant activity. Flavonoids, a groups of natural compounds also classified as natural phenolics by Agrawal *et al*., (1989). Both valepotriates and valerenic acids were detected in root tissues of wild plants when profiled using GC-MS (Table 4). Thus this protocol can be useful for isolation and characterization of medicinally important metabolites valepotriates. The proposed tissue culture system will be able to maximize the production of these plants.

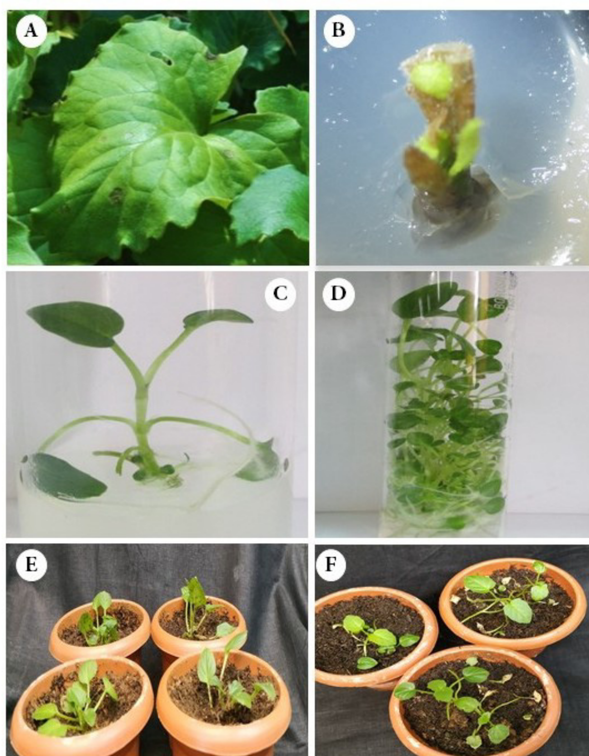

Fig. 1. *In vitro* regeneration of *V. jatamansi* in MS+ BAP (2mg/L) (**A**) Mother plant of *V. jatamansi*; (**B**) shoot bud explants post 3 days incubation; (**C**) Shoot initiation after 8 days of culture (**D**) Shoot elongation after 30 days of culture. (**E,F**) Plants of *V.jatamansi*, hardened on Coco peat : soil (3:1).

Table 3. Total antioxidant capacity of the extracts. Values are expressed as mean ± SD (n=3).

Sample	Extract	DPPH(%)
In vitro	Methanol	84.33±0.577
Wild	Methanol	78.33±0.569

Conclusion

In the present research, an efficient protocol for *in vitro* propagation of *V. jatamansi* using shoot bud as explants was established. This protocol will be useful for the conservationof this valuable medicinal plant. This paper presents the phytochemical potential of *in vitro* propagated plants to produce high amounts of phenolics, flavanoids and antioxidants. Important bioactives compounds including Valerenol and Valeric anhydride, a class of valepotriates was also detected from the wild roots of *V.jatamansi*, which is a pharmaceutically important compounds and simultaneously the *in vitro* cultures can also be used for the production of the same without disturbing the natural habitat.

Table 4. List of major compounds detected in GC-MS analysis of in wild roots of *V. jatamansi*with biological importance.

Name of compound	Retention Time (min)	Molecular Formula	MW (g/mol)	Biological importance	References
Valeric anhydride	22.727	$C_{10}H_{18}O_3$	186	Neuroprotective	Raina *et al.*,2015
Valerenol	27.276	$C_{15}H_{24}O$	220	Essential oil	Raina *et al.*,2015
Hexadecanoic acid	34.360	$C_{17}H_{34}O_2$	270	Antimicrobial activity, anti cancer activity	Chowdhary *et al.*, 2015
DL-Proline	20.939	$C_6H_9NO_3$	143	Drought-stress tolerant	Hayat *et al.*, 2012
Octadecanoic acid	38.158	$C_{19}H_{36}O_2$	296	Anti-inflammatory	Senguttuvan *et al.*, 2014

Recommendation

From the present research, since the pharmaceutical industries harvest all the wild resources, the present protocol can be useful to produce the plant irrespective of season, which can be highly benefitted to the farmers to produce the plants throughout the year. Supplementation of BAP as a growth hormone can aid in producing more plants in short duration, which will be a great advantage for the pharmaceutical industries as well as the farmers.

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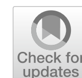
ORIGINAL ARTICLE



Elicitation and plant growth hormone-mediated adventitious root cultures for enhanced valepotriates accumulation in commercially important medicinal plant *Valeriana jatamansi* Jones

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Abstract

Adventitious root cultures of medicinally important *Valeriana jatamansi* Jones were induced in vitro and the growth kinetics of the root cultures were assessed upon supplementation of indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), 1-naphthaleneacetic acid (NAA) and elicitors methyl jasmonate (MeJA) and salicylic acid (SA). NAA (2.0 mg/L) induced the highest biomass among the tested plant growth regulators (PGRs) and among the elicitor treatments, MeJA induced higher biomass of the root cultures compared to SA. Further, the root cultures obtained from each treatment were analyzed for their phytoconstituents and antioxidant potential. Total phenolic content (TPC), total flavonoid content (TFC), and antioxidant potential were found to be highest with the extracts of roots grown in liquid medium supplemented with NAA (2.0 mg/L). The elicitor MeJA induced higher biomass, but lesser phytoconstituents compared to NAA. SA, on the other hand, did not produce significant biomass. The GC–MS analysis revealed that NAA-treated roots accumulated 50 bioactive compounds similar to that of wild roots in which 48 compounds were detected including important valepotriates such as valeric anhydride, valeric acid, and derivatives. The SA elicitation resulted in the detection of 23 compounds including some valeric acid derivatives, and MeJA-treated cultures accumulated 21 compounds but did not aid in the production of valepotriates. Our results demonstrate that *V. jatamansi* root cultures can be effectively used for the large production of valepotriates by supplementing NAA.

Keywords Adventitious roots · Elicitation · GC–MS · Valepotriates · *Valeriana jatamansi*

Abbreviations

A/H	Area/height
CW	Coconut water
DPPH	2,2Diphenyl-1-picrylhydrazyl
DW/L	Dry weight per liter
FW	Fresh weight
GC–MS	Gas chromatography–mass spectrometry
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
MeJA	Methyl jasmonate
NAA	1-Naphthaleneacetic acid
PGRs	Plant growth regulators
SA	Salicylic acid
TPC	Total phenolic content
TFC	Total flavonoid content

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Sushma Pandey and Sathish Sundararajan equally contributed to the research.

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Introduction

The genus Valeriana consists of more than 200 species of which three important species, *Valeriana jatamansi*, *Valeriana officinalis,* and *Valeriana edulis* are most widely used in various medicinal systems owing to their pharmaceutical properties especially aromatic oils (Bos et al. 1998; Chen et al. 2014). The plant's rhizome and roots contain

essential oil, which is a valuable aromatic oil used extensively worldwide (Mathela et al. 2005). *V. jatamansi* finds its use in a multitude of medicinal systems including Ayurvedic, Unani, and modern systems (Das et al. 2013). Among various compounds, the essential oils derived from the roots and rhizomes contain patchouli alcohol and bornyl isovalerate (Bos et al. 1998). Various pharmacological studies carried out with the essential oil components of the *Valeriana* species revealed that they possess antifungal, antibacterial, anxiolytic, and sedative properties (Wasowski et al. 2002). The presence of valepotriates which are nonglycosidic iridoid esters is known to be the reason behind the sedative properties exhibited by the plant. Valtrate, acevaltrate, and didrovaltrate, the major valepotriates present in *V. jatamansi,* are responsible for the plants' various medicinal properties (Gupta et al. 1986).

Due to the overexploitation by the drug industries for the valuable metabolites, *V. jatamansi* is documented to be one of the highly exploited plant species of the Himalayan range. The indiscriminate collection of this plant in the recent decade has led to its exhaustion in the wild and thus necessitates its replenishment and cultivation. To date, plants collected from the wild contribute to all the demands of various industries (Gupta et al. 2006). In the recent past, the demand for in vitro culture techniques has grown by leaps and bounds, as it not only offers a practicable tool for rapid propagation, but also aids in the conservation of vulnerable medicinal plants at the brink of extinction (Abraham et al. 2010). The cell culture system can be a providential and reliable strategy to generate substantial quantities of high-value secondary metabolites (Fazal et al. 2014). PGRs have been widely used in the biosynthesis of various secondary metabolites, including therapeutic compounds (Vanisree et al. 2004).

Adventitious root cultures are one of the most significant systems for biomass increase and generating commercially valuable metabolites (Baque et al. 2012; Wang et al. 2013). Both hairy and adventitious root cultures have been most widely used for this purpose due to the high efficacy and the easy upscaling of root cultures to bioreactors (Cui et al. 2013). Sivanandhan et al. (2012) suggested that the growth conditions of adventitious root cultures can be easily controlled compared to other culture systems. The adventitious roots are relatively more stable and large quantities of secondary metabolites can be accumulated. Further, using in vitro cultures, it is feasible to produce relatively high biomass in a limited time compared to field-grown plants (Murthy et al. 2008; 2016). It is reported that auxin promotes the formation of adventitious roots and that auxin signaling and transport regulate the plant's root length, number, root hair as well as the direction of root growth (Yan et al. 2014). NAA, a synthetic auxin is widely used at relatively lower doses to promote auxin-type responses in various plant growth developmental stages including cell growth and division, and rooting (Sun and Hong 2010).

Manipulation of biotic and abiotic elicitors can stimulate the accumulation of medicinally important metabolites in plant culture systems (Sivanandhan et al. 2012; Sivakumar et al. 2019). Radman et al. (2003) suggested that elicitation is an effective tool for improving the biosynthesis of secondary metabolites. Depending on the nature and form of the elicitors, they can be predominantly categorized into biotic and abiotic compounds. Saini et al. (2013) reported successful enhancement of isoflavones using abiotic elicitors SA and MeJA in *Glycine max*. Wang et al. (2015) successfully used MeJA in liquid *Hypericum perforatum* cultures for improving flavonoid production. Biotic elicitors have been successfully used in suspension cultures of *Psoralea corylifolia* for the accumulation of high psoralen content (Ahmed and Baig 2014). It is suggested that the appropriate type and concentration of elicitors vary with plant species and the intended metabolite (Ho et al. 2018). Consequently, it is imperative to examine the role and impact of such compounds on the growth and accumulation of metabolites in economically significant medicinal plants. With this background, an attempt was made to investigate the effects of PGRs and elicitors MeJA and SA on the adventitious root cultures and subsequent accumulation of high-value valepotriates.

Materials and methods

In vitro propagation of *V. jatamansi* Jones

Valeriana jatamansi plants collected from Dabur Nepal, Kavre, Banepa were grown at the Central Department of Botany, Tribhuvan University. Well-established plants with prominent leaves and nodes were rinsed off with running tap water to remove all soil and debris, followed by sterilization using a few drops of liquid detergent Tween 20 (Himedia, India) and rinsing in sterile water for 30 min followed by treatment with 0.1% $HgCl_2$ (mercuric chloride) for 5 min. The explants were then rinsed with sterile water to remove traces of $HgCl_2$. The shoot tips were excised from the disinfected plants, blot dried using a sterile Whatman filter paper, and placed on sterile Petri plates. The shoot tips were then inoculated in test tubes containing MS (Murashige and Skoog 1962) medium fortified with 3% sucrose (w/v) and 0.1% myoinositol, and solidified with 0.8% agar until prominent shoot induction. The in vitro plants were then multiplied in MS medium supplemented with 10% coconut water (CW) and subcultured at regular intervals. All the cultures were maintained at 25 ± 2 °C with a 16 h photoperiod with a light intensity of 40–50 μmol/m^2 s supplied by cool white fluorescent lamps (Philips, India).

In vitro root multiplication

In vitro root multiplication was performed by excising sterile roots (1.0 g) from the well-developed plants and inoculated into culture flasks containing MS liquid media supplemented with different PGRs at various concentrations, i.e., NAA [0.5, 1.0, 1.5 and 2.0 mg/L], IBA [0.5, 1.0, 1.5 and 2.0 mg/L], and IAA [0.5,1.0,1.5 and 2.0 mg/L]. Liquid MS media devoid of PGRs was used as a control. All the cultures were grown in an orbital shaker set at 80 rpm at 25 ± 2 °C under 16 h photoperiod.

Growth kinetics of adventitious root cultures

The assessment of the adventitious root culture's growth was carried out periodically using an initial inoculum concentration of 0.1 g dry weight per liter (DW/L) in 100 mL of MS medium supplemented with different hormones under the same growth conditions as mentioned above. Fresh weight (FW) of biomass was recorded every 10 days till day 40. Further, the biomass was taken out, rinsed with sterile distilled water thrice and shade dried to record the dry weight (DW) of the samples.

Elicitor treatment

For elicitation experiments, MeJA and SA were prepared in an aqueous solution of 50% ethanol (v/v) and filter-sterilized through a 0.22 µm Millipore filter (Sartorius, Germany). The initial inoculum and culture conditions were maintained as described above. MeJA at 0, 1, 3, 5, and 10 µM; and SA at 0, 60, 100, and 300 µM concentrations were used. Different concentrations of elicitors were added to the cultures and the response of the treatments was assessed periodically up to 40 days, followed by harvesting the root cultures which were used for the biochemical and GC–MS analysis.

Sample extraction for biochemical and analytical analysis

The in vitro root cultures from all the growth hormonal combinations after the initial growth assessments and wild samples were extracted with methanol for 48 h with constant shaking and centrifuged at 4000*g* for 15 min. The supernatant was pooled and stored at 4 °C which was later used for biochemical assays to assess the total phenolic, flavonoid contents and antioxidant activity.

Total phenolic content (TPC)

The TPC of the extracts was determined using the Folin–Ciocalteu method with gallic acid as standard (Qiu et al. 2010). Extracts (2 mL) were mixed with 2.5 mL of the Folin–Ciocalteu reagent at 10% (v/v). After 2 min, 7.5% (p/v) Na_2CO_3 (2 mL) was added, followed by incubation for 10 min at 50ºC. The absorbance was read at 755 nm and the results are expressed as gallic acid equivalent per gram of dry biomass weight (mg GAE/g DW).

Total flavonoid content (TFC)

The TFC was determined by the flavonoid–aluminum ($AlCl_3$) complexation method (Ghasemi et al. 2009) using quercetin as a standard. Briefly, the extract (1 mL) was mixed with 5% (p/v) $NaNO_2$ (0.3 mL), followed by incubation for 5 min at room temperature (RT). Then, 0.5 mL of 2% (p/v) $AlCl_3$ was added and the sample was gently shaken and neutralized after 6 min with 1 N NaOH (0.5 mL). The absorbance was read at 425 nm and samples without $AlCl_3$ were used as blank. TFC was calculated using the standard calibration of quercetin alcoholic solution and expressed as milligrams of quercetin equivalent per gram of dry biomass weight (mg QE/g DW).

DPPH radical scavenging assay

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical activity was estimated according to Liyana-Pathiranan and Shahidi (2006). 1.0 mL of DPPH in methanol (0.135 mM) was mixed with the methanolic extract (1.0 mL). The reaction mixture was thoroughly vortexed in dark at RT for 30 min and the absorbance was measured at 517 nm. All the samples were analyzed in triplicate. The radical scavenging activity was calculated as follows:

DPPH radical scavenging activity (%) = [(Abs control−Abs sample)]/(Abs control)] × 100, where Abs control is the absorbance of DPPH radical + methanol; Abs sample is the absorbance of DPPH radical + sample extract/standard.

GC–MS analysis

The best responding PGR and elicitor treatment among the tested concentrations based on root suspension growth kinetics and phytochemical analysis were analyzed using gas chromatography coupled with a mass spectrometry (GC–MS 4000, Varian, USA) system (Pandey et al. 2020). 0.20 µL volume of sample was injected into HP-5MS Agilent column (30 m × 0.25 mm, 0.25 µ film thickness). The oven temperature was programmed from 70 °C at the rate of 5 °C/min to 280 °C with the hold time of 4 min and 8 min, respectively. Helium was used as a carrier gas at a constant flow of 1.0 mL/min and a split ratio of 10:1. The temperatures of the injector were maintained at 280 °C. For mass spectrometry, ion source temperature was maintained at 200 °C and ionization energy was set at 70 eV.

Statistical analysis

All the experiments were completely randomized and repeated thrice. All data were statistically analyzed using analysis of variance (ANOVA) and the presented as means ± SD. The data were further analyzed by Duncan's multiple range test ($p < 0.05$) with the aid of SPSS (version 17, Chicago, USA). $p < 0.05$ was considered as indicative of significance.

Results

Growth kinetics of adventitious root cultures

The nodal explants of *V. jatamansi* plants were inoculated onto the MS medium and allowed to proliferate (Fig. 1a). The well-proliferated in vitro plants (Fig. 1b, c) were used as a source to harvest roots and the roots were inoculated in the liquid MS medium supplemented with different PGRs at different concentrations. The growth progression of the adventitious root cultures from the initial inoculum was assessed based on the FW and DW analysis. The control cultures (basal media devoid of PGRs) showed a slower growth response (Fig. 1d), whereas the cultures grown on a medium supplemented with IAA (Fig. 1e), IBA (Fig. 1f)

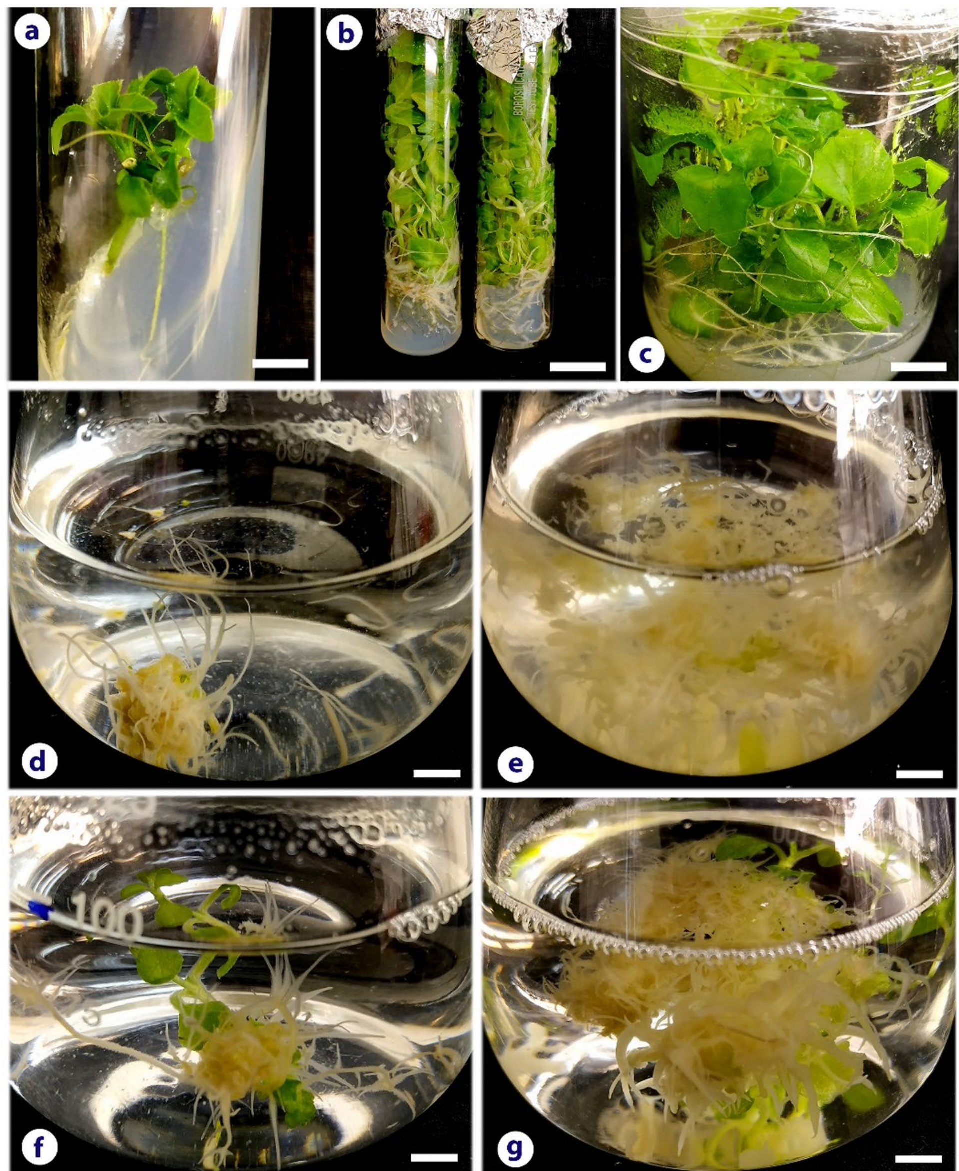

Fig. 1 Establishment of in vitro *V. jatamansi* plants and adventitious root cultures **a** shoot proliferation from nodal segment explant of *V. jatamansi*; **b**, **c** establishment of in vitro plants during routine subculture; **d** adventitious root formation in MS basal media after 25 days; **e** adventitious root formation in medium supplemented with IAA (2.0 mg/L) after 25 days; **f** adventitious root and prominent shoot formation in medium supplemented with IBA (1.5 mg/L) after 25 days; **g** adventitious root cultures established in medium supplemented with NAA (2.0 mg/L) after 25 days. Scale bar = 2 cm

showed better proliferation of roots. However, NAA induced profuse rooting and large clumps of root formation were observed with NAA (2.0 mg/L) (Fig. 1g).

Among the tested PGRs at different concentrations, the addition of NAA (2.0 mg/L) resulted in the highest biomass accumulation with an FW of 7.43 g and a DW of 3.06 g respectively (Fig. 2a, b) compared to the control (2.78 g of FW and 1.0 g DW). In general, NAA supplemented liquid cultures showed a relatively better root biomass accumulation. The growth progression of the adventitious roots treated with different choices and concentrations of PGRs was evident till day 30 and the proliferation was reduced

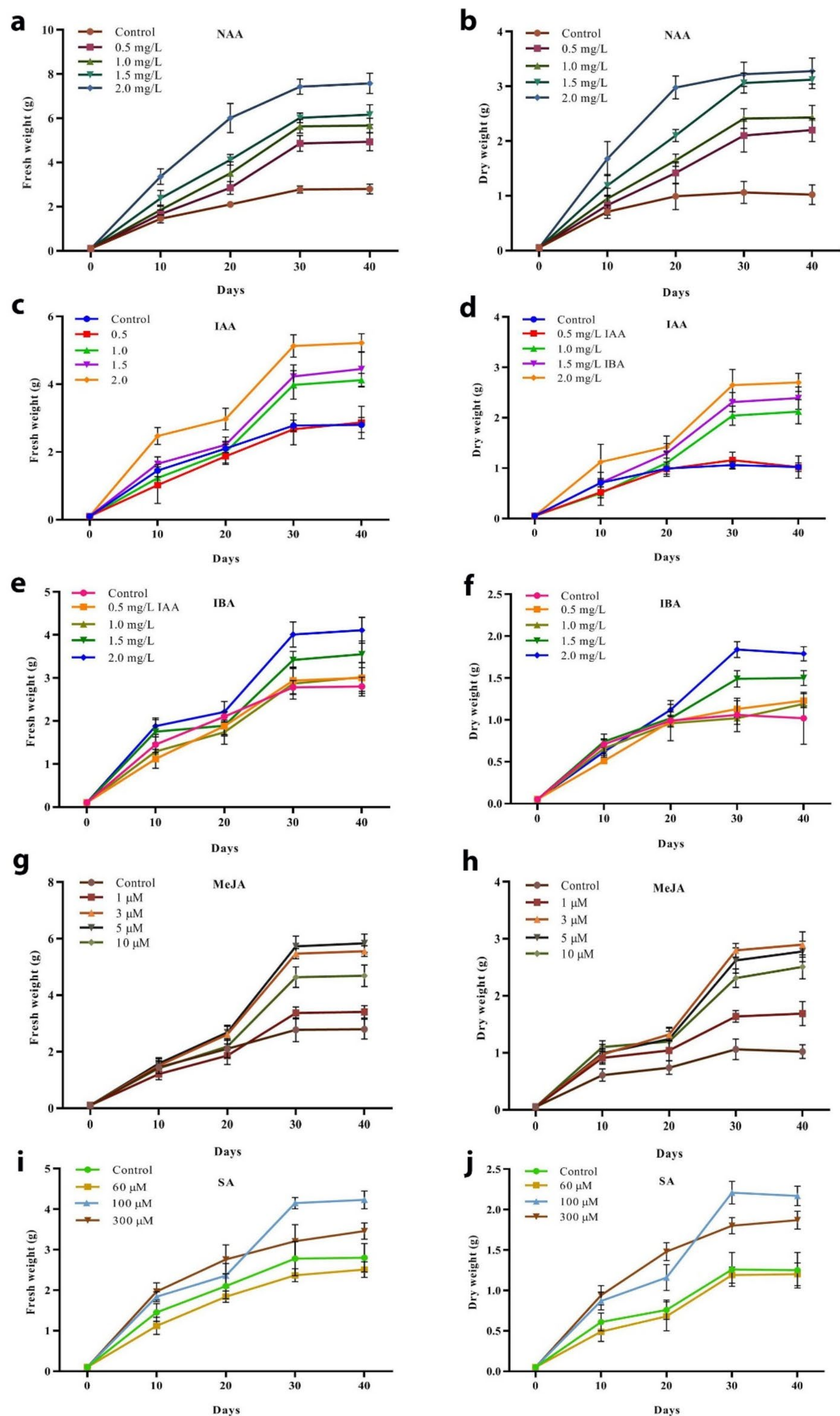

Fig. 2 Fresh and dry weight analysis of adventitious root cultures grown in medium supplemented with different growth hormones and elicitors at various concentrations. **a** FW, **b** DW analysis of root cultures grown in medium containing NAA; **c** FW, **d** DW analysis of root cultures grown in medium containing IAA; **e** FW, **f** DW analysis of root cultures grown in medium containing IBA; **g** FW, **h** DW analysis of root cultures grown in medium containing MeJA; **i** FW, **j** DW analysis of root cultures grown in medium containing SA. Data collected till 40 days of incubation and the values are presented as mean ± SD of three replications

after day 30 (Supp. Table 1). On the other hand, IAA induced 5.13 g of biomass with 2.0 mg/L (Fig. 2c, d) and 4.23 g when the medium was supplemented with 1.5 mg/L concentrations respectively. Comparatively among the three tested PGRs, the IBA-treated cultures responded poorly with a maximum FW of 4.01 g and 4.11 g with 2.0 mg/L on days 30 and 40, respectively (Fig. 2e, f). However, both IAA and IBA failed to increase the biomass of roots as compared to NAA. It was also observed that the initiation of shoot portions resulted in all the PGRs tested in the liquid cultures. However, the most apparent growth of shoots was observed with the cultures treated with IBA (Fig. 1f) which could well be a reason for less root growth in IBA supplemented media which in turn reduced the final biomass levels.

The elicitor-treated root cultures were also assessed periodically for the FW and DW and it was found that the maximum biomass accumulation was observed with the MeJA-treated cultures at the end of 30 days (Supp. Table 2). Compared to MeJA at 1 µM (Fig. 3a), MeJA at 3 µM (Fig. 3b), 5 µM (Fig. 3c) and 10 µM (Fig. 3d) showed enhanced root proliferation. MeJA at 3 µM and 5 µM showed FW of 5.47 g and 5.73 g respectively (Fig. 2g, h). SA, on the other hand, did not show significant biomass accumulation compared to MeJA as well as the growth hormonal treatments (Fig. 3e–g). The FW ranged between 2.37 g (60 µM) and 4.15 (300 µM) among the different concentrations tested at the 30-day mark (Fig. 2i, j). Comparatively, between the elicitation and PGR's experiments, it was observed that the PGR NAA (2.0 mg/L) was the best choice to induce adventitious roots in the liquid cultures of *V. jatamansi.*

Phytoconstituent and antioxidant potential of adventitious root cultures

The root cultures treated with PGRs and elicitors after growth kinetic analysis were tested for their total phenolic, flavonoid contents, and their antioxidant potential was determined using DPPH assay. Along with the roots harvested from plants grown in the wild, the best responding cultures with PGR treatments (2.0 mg/L NAA, 1.5 mg/L IBA and 1.0 mg/L IAA) based on FW and DW were taken for the analysis (Fig. 3h). The maximum phenolic content (58.69 mg GAE/g) was observed with the roots cultured in NAA (2.0 mg/L), which was significantly higher than that of the other PGRs-induced roots and that of the wild roots (42.0 mg GAE/g) (Fig. 4a). The IBA (1.5 mg/L) and IAA (1.0 mg/L)-induced root cultures showed 46.78 and 46.00 mg GAE/g respectively and the least amount of phenolics (28.66 mg GAE/g) was observed with that of roots grown in the basal medium devoid of PGRs. Interesting observations were made during the experiments with the elicitor-treated roots cultures with the TPC. The FW and biomass accumulation were found to be higher with MeJA supplemented roots as stated before. However, the TPC was found to be higher with the SA-treated root cultures, as 100 µM SA resulted in the TPC of 50.75 mg GAE/g. Contrastingly, the MeJA-treated roots showed TPC in the range of 41.00 and 48.56 mg GAE/g, which was comparatively lesser than that of SA-treated root culture extracts (Fig. 4b). The flavonoid contents of the adventitious root cultures also followed the same pattern as that of phenolic contents. The PGRs-treated root cultures showed higher flavonoid content, especially the cultures induced with 2.0 mg/L NAA showed 248.33 mg QE/g (Fig. 4c). The TFC of IBA-treated roots was 103.33 mg QE/g and that of IAA-treated roots was 95.0 mg QE/g. The least TFC was recorded with that of the roots grown in the basal medium (73.00 mg QE/g) and the wild samples exhibited TFC of 125.09 mg QE/g. Similar to the results obtained for TPC, among the elicitor-treated roots, SA treatment enhanced the TFC, i.e., SA (100 µM)-treated roots showed a TFC of 99.31 mg QE/g and SA (300 µM)-treated roots showed 120.00 mg QE/g TFC, which was the highest among the elicitor treatments (Fig. 4d). On the other hand, there was no significant increase in the TFC of the root cultures that were grown in medium containing MeJA except for MeJA (5 µM) which showed a TFC of 117.66 mg QE/g. The antioxidant potential of the hormonal and elicitor-treated root cultures revealed that the highest of antioxidant potential was observed in the wild roots (79.04%) and the cultures grown in NAA (2.0 mg/L) showed DPPH radical scavenging percentage on par (78.51%) with that of the wild roots (Fig. 4e). The SA-treated roots showed an antioxidant potential of 73.88 and 71.11% with 100 µM and 300 µM respectively (Fig. 4f).

GC–MS analysis

The GC–MS analysis of the methanolic extracts of wild samples along with roots cultured in NAA (2.0 mg/L), MeJA (5 µM), and SA (100 µM) was carried out and the wild *V. Jatamansi* revealed the presence of 48 compounds which included 6 major bioactive compounds (Fig. 5a, Supp. Table 3). The GC–MS characterization of the root extracts from NAA (2.0 mg/L) treatment revealed the presence of 50 compounds which included major bioactive compounds such as valeric anhydride, propanoic acid, hexadecanoic acid, octadecanoic acid, butanoic acid, proline, isovaleric acid and valeric acid (Fig. 5b, Supp. Table 4). Interestingly the elicitor-treated root fractions showed relatively fewer compounds compared to that of the wild and NAA-treated samples. The extracts of SA (100 µM) revealed the presence of 23 compounds including major bioactive compounds divalerate, butanoic acid, pentanoic acid, valeric acid, furaldehhyde, and palmitic acid (Fig. 5c, Supp. Table 5). The root extracts of MeJA revealed the presence of 21 compounds; however, the valepotriates were not detected, instead only

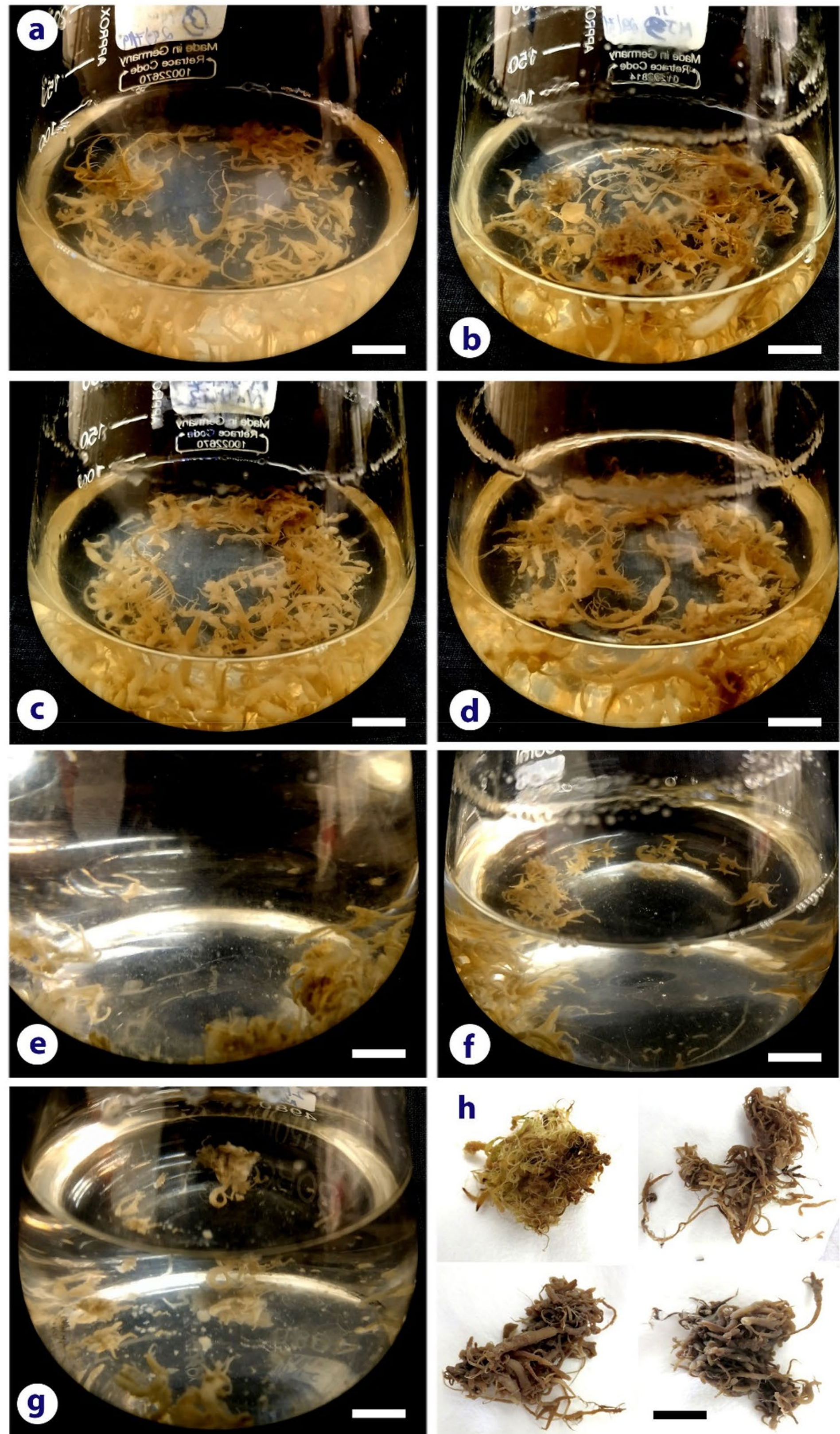

Fig. 3 Elicitation experiments with *V. jatamansi* adventitious root cultures. Root cultures after 8 days of elicitation with MeJA and SA **a** MeJA 1 µM; **b** MeJA 3 µM; **c** MeJA 5 µM; **d** MeJA 10 µM; **e** SA 60 µM; **f** SA 100 µM; **g** SA 300 µM; **h** representative images of part of adventitious roots grown in NAA 2 mg/L (top left), MeJA 3 µM (top right), MeJA 5 µM (bottom left) and SA 100 µM (bottom right) harvested for extract preparation. Scale bar = 1 cm

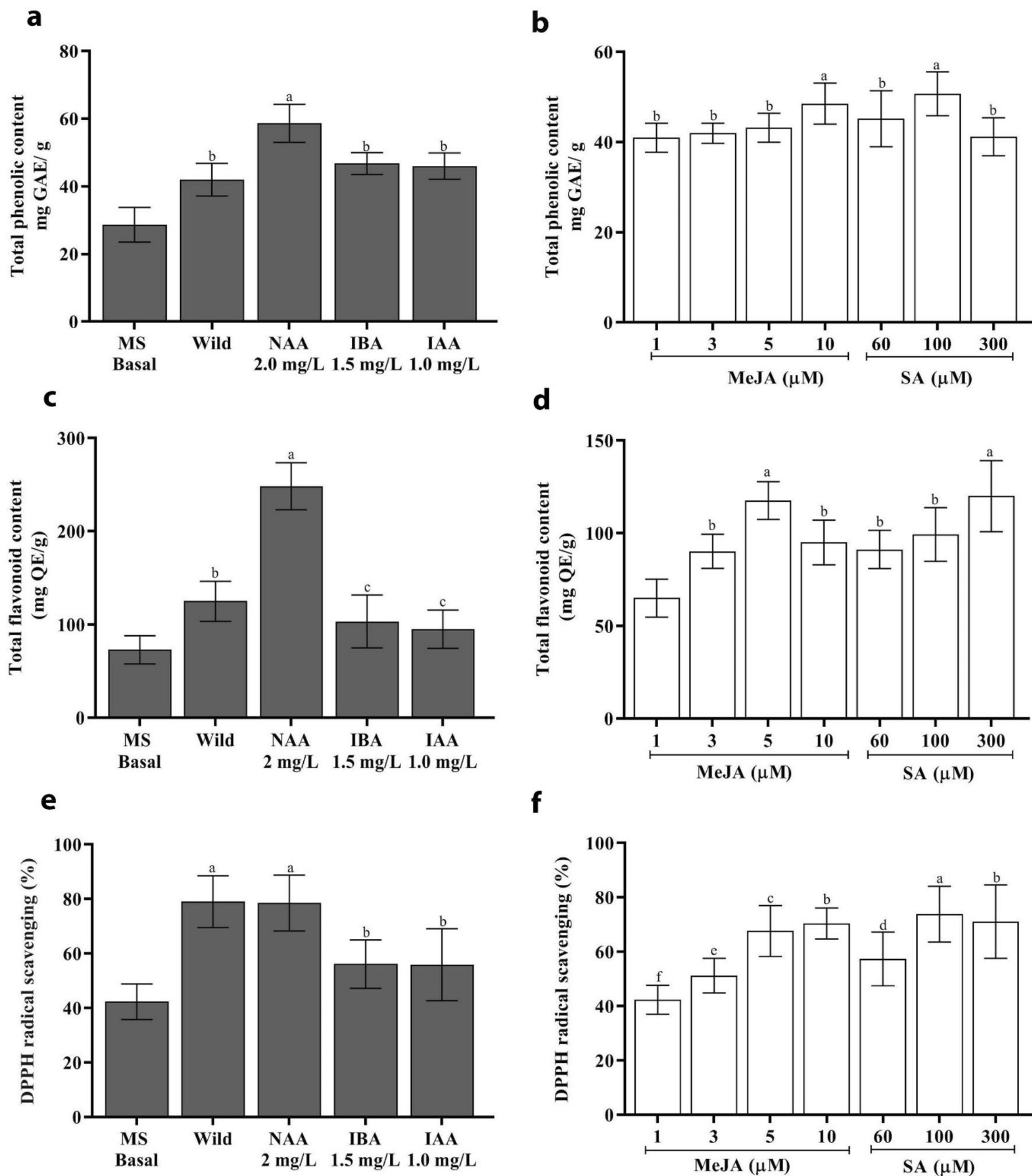

Fig. 4 Biochemical analysis of the adventitious root cultures under different treatments. **a** TPC of adventitious root extracts from cultures grown supplementing different PGRs; **b** TPC of adventitious root extracts from cultures grown supplementing different elicitors; **c** TFC of adventitious root extracts from cultures grown supplemented with different growth hormones; **d** TFC of adventitious root extracts from cultures grown supplemented with different elicitors; **e** antioxidant potential of root extracts from cultures grown in different growth hormones; **f** antioxidant potential of root extracts from cultures grown in different elicitors; values represents the mean ± SD. Means following the same alphabets are not significantly different, according to Duncan's multiple range test ($p < 0.05$)

different classes of fatty acids, alcohols, and esters were detected (Fig. 5d, Supp. Table 6).

Discussion

In vitro plant cell cultures represent one of the viable alternatives to conventional methods for increasing bioactive compounds to meet the ever-increasing industrial demands (Khan et al. 2017). Among various plant cell cultures

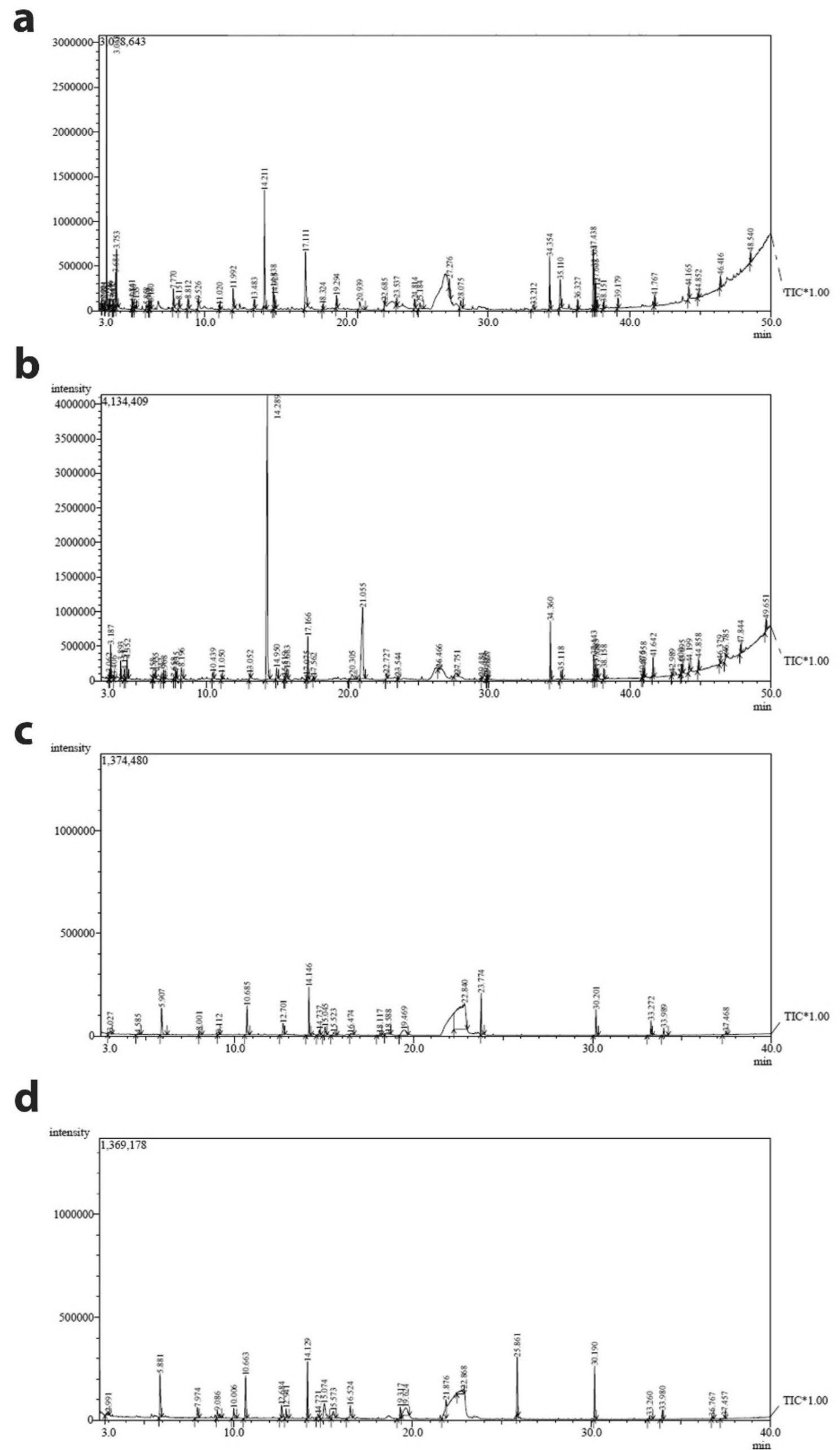

Fig. 5 GC–MS analysis of extracts derived from adventitious root cultures grown in different supplements. Chromatograms of extracts from **a** wild roots, **b** NAA (2 mg/L), **c** MeJA 5 μM, **d** SA 100 μM

utilized for the production of high biomass and in turn important bioactive compounds in limited time and space, adventitious root cultures are most widely preferred (Saeed et al. 2017). Rani et al. (2017) suggested that numerous strategies including elicitation, temperature stress, and PGR manipulation can be employed toward the production of commercially and pharmaceutically important metabolites via adventitious roots. Fazal et al. (2014) reported maximum adventitious root biomass accumulation from *Prunella vulgaris* L. callus immersed in a liquid medium containing 1.0 mg/L of NAA. NAA, in general, showed better biomass accumulation compared to that of IAA and IBA-treated cultures. Similar to our results, the addition of NAA accelerated adventitious root in terms of induction rate, the number of roots per explant, and root length in *Aloe vera* (Lee et al. 2011) and *A. Paniculata* (Sharma et al. 2013). In experiments with various plant species, a rapid increase in the adventitious root formation was observed at lower concentrations of NAA (1.0 and 2.0 mg/L), and higher concentrations of NAA subsequently reduced the root numbers (Raju and Prasad 2010; Sun and Hong 2010). Peeters et al. (1991) reported that in tobacco, NAA was absorbed sixfold faster than IAA and suggested that the ability of NAA as the most potent inducer of adventitious roots could be because the absorption and utilization of NAA is more rapid than other growth hormones under in vitro conditions. From the results of our study, it is concluded that 2.0 mg/L NAA is the best PGR to ensure high-frequency rooting in *V. jatamansi* liquid cultures. In many studies involving adventitious root suspension cultures, NAA has been reported to be the most effective growth regulator for enhanced biomass accumulation (Yan et al. 2014; Khan et al. 2015; Saeed et al. 2017). Khan et al. (2015) suggested that compared to the solid medium, liquid media fortified with NAA resulted in efficient adventitious root formation. Further, the liquid cultures are more plausible for plant cell growth and development owing to the easy availability of nutrients, PGRs, and water. Our results and observations are consistent with the report of Saeed et al. (2017) where enhanced root biomass accumulation with NAA was obtained in *Ajuga bracteosa* Wall ex Benth and the possible reason suggested is due to NAA's longer half-life compared to other PGRs in the plant cells.

Polyphenols are the largest group of phytochemicals that exhibit higher antioxidant activity and these polyphenols are most effective in quenching free radicals, immune system stimulation, gene expression regulation, and other cellular processes (Ahmad et al. 2013). Manipulating the plant tissue culture conditions including the choice and concentration of PGRs is a sustainable strategy to obtain enhanced production of economically valuable metabolites (Palacio et al. 2012). In contrast to the results obtained in our study, Fazal et al. (2014) reported the lowest TPC in dry biomass of adventitious roots of *P. vulgaris* L. when the medium was fortified with NAA (2.0 mg/L) and enhanced TPC was observed with cultures treated with NAA at lower concentrations. In their study, they observed maximum TPC, TFC, and DPPH radical scavenging activities with root cultures grown in a medium supplemented with 0.5 and 1.5 mg/L NAA. Khan et al. (2017) reported the positive effects of NAA compared to other hormones like IAA and IBA in adventitious root cultures of *Fagonia indica* and suggested that the proficiency of NAA toward adventitious root formation compared to other hormones can be attributed to the irreversible conjugation and oxidation of those PGRs in the plant cell. Similarly, the positive effects of NAA at 2.0 mg/L in biomass production and subsequent higher TPC, TFC, and antioxidant potential of the in vitro root extracts were observed.

Elicitation has been most widely followed and is also considered an effective approach for the production and high-level accumulation of medicinally important metabolites (Yue et al. 2016). MeJA triggers a series of stress signaling pathways to produce multiple classes of defense-related compounds such as phenolic acids, flavonoids, and alkaloids (Saeed et al. 2017). In the cultures grown with elicitors MeJA and SA, the biomass accumulation was low in the case of SA, but the TPC, TFC, and antioxidant potential were found to be on par with that of the cultures grown with PGRs, but could not exceed the TPC, TFC contents of root cultures grown in NAA (2 mg/L). The cultures grown with the supplementation of MeJA showed a higher biomass production, but lacked in TPC and TFC. Numerous reports suggest that exogenous supplementation of MeJA enhances the production of useful medicinal compounds in plant cell cultures of various plant species (Faizah et al. 2018). Previous reports in various plant species suggest that flavonoid and anthraquinone accumulation in adventitious and hairy root cultures was improved upon elicitation with MeJA (Lee et al. 2015; Perassolo et al. 2017; Han et al. 2019). In contrast to the results obtained in our study where the addition of MeJA enhanced the biomass but reduced the phytoconstituent accumulation, MeJA was found to inhibit the root growth and favored the metabolite production with cell and adventitious root cultures of *Oplopanax elatus and Talinum paniculatum* (Jiang et al. 2017; Faizal and Sari 2019). These results suggest that the TPC, TFC, and antioxidant potential of elicitor-treated roots are biomass independent. This type of biomass- dependent and PGRs-independent, and biomass-independent and PGRs-dependent DPPH-based antioxidant activities have been previously reported in various plant species (Ali and Abbasi 2013; Tariq et al. 2014). However, the same biomass and PGRs dependence /independence could not be applied to the root cultures grown in different PGRs, since the phytoconstituent composition and antioxidant activity were dependant on the biomass accumulation with different PGRs and their concentrations in the present investigation.

The GC–MS analysis also revealed similar findings to that of the phytoconstituent analysis when the root cultures were grown in NAA (2.0 mg/L) and accumulated 50 compounds similar to that of the wild roots where the presence of 48 compounds was identified. However, the presence of bioactive compounds was found lesser in elicitor-treated root cultures. The presence of commercially and pharmaceutically important valepotriates including divalerate, and valeric acid were detected in root cultures grown in NAA and SA. The valeric acid functions as an anticonvulsant agent, behenic alcohol finds its use in the cosmetic industry, ascorbic acid functions as a cofactor in photosynthesis, and palmatine is an alkaloid that takes part in plant metabolism. Interestingly, there was no detection of such compounds in MeJA-treated cultures. Das et al. (2013) reported higher valepotriates content when NAA was supplemented in the in vitro callus cultures of *V. jatamansi*. Apart from this report, the essential oils from the wild plants alone of *V. jatamansi* have been analyzed for their antioxidant and phytochemical compositions by various researchers to date (Thusoo et al. 2014). On the other hand, *V. officianalis* and *V. walichhi* have been extensively studied for their medicinal importance and their in vitro adventitious and hairy root cultures have been assessed for their valepotriates contents (Nikolova et al. 2017; Nandhini et al. 2018). However, to the best of our knowledge, this is the first report on the accumulation of valepotriates in in vitro root cultures of *V. Jatamansi* mediated by PGRs and elicitation.

Conclusion

An efficient in vitro adventitious root culture system of *V. jatamansi* Jones was established from in vitro raised plants and the proficient adventitious root growth supplemented with different PGRs and elicitors was assessed. The generated biomass was investigated for the presence and accumulation of valepotriates. Treatments with NAA (2.0 mg/L) considerably enhanced the biomass and production of medicinally important compounds including valeric anhydride, isovaleric acid, and other valeric acid derivatives. Elicitation of adventitious root cultures with MeJA and SA showed variable responses on the biomass and subsequent accumulation of bioactive metabolites. MeJA induced higher biomass compared to SA, but did not enhance the phytoconstituents of the root cultures. However, the SA-treated roots showed the presence of few valepotraite compounds compared to MeJA-treated cultures where no such compounds were detected. These preliminary results presented here can be used further to manipulate various other biotic and abiotic elicitors, as well as PGRs for enhancing and large-scale valepotriates production in the future. Hence, this adventitious root culture system can be strategized as an efficient and alternative source for generating pharmacologically important valepotriates in vitro.

Author contribution statement SP executed the experiments. SS did the experimental analysis. SP and SS prepared the manuscript. SR contributed to the research design, supervised, and evaluated the manuscript. BP conceptualized the research, evaluated and edited the manuscript.

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Declarations

Conflict of interest All authors read, approved the manuscript, and declare that there is no conflict of interest.

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Research Article

In vitro Propagation and Assessment of Genetic Homogeneity using RAPD and ISSR Markers in *Tinospora cordifolia* (Wild.) Hook. F. & Thoms, An Important Medicinal Plant of Nepal

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Abstract

The Menispermaceae family includes the widely glabrous, succulent, climbing shrub *Tinospora cordifolia* (Gurjo), which has been found to have a variety of pharmacological and ethnomedicinal characteristics. *T. cordifolia* is also one of the most commercially exploited plants in pharmaceuticals. The nodal segment explants were initially cultured on Murashige and Skoog (MS) medium supplemented with various concentrations of 6-benzyl amino purine (BAP), and/or Kinetin (KIN) among which MS +BAP (2.0 mg/l) induced shoot initiation after 7 days of post-inoculation. The nodal segments were then excised and treated with various concentrations of BAP, BAP with KIN, and coconut water for the proliferation among which 5 mg/l induced significant nodal segment proliferation (9.0 nodal segments/per explant) and shoot length (8.0 cm). The efficacy of coconut water in increasing the nodal segment proliferation of *T. cordifolia* was tested and the shoot proliferation increased significantly at 5% and 10% of coconut water, however, the maximum response of shoot number (23.0), shoot length (12 cm) was in the MS medium supplementation with BAP (5mg/l) and 10% coconut water. The genetic fidelity of these plants was also confirmed by random amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) markers in wild and in vitro cultures. This protocol is an efficient way for the in vitro mass propagation of true-to-type plantlets of *T. cordifolia* which provides a basis for germplasm conservation and sustainable utilization.

Keywords: Coconut water, Genetic fidelity, ISSR, Plant tissue culture, RAPD

Introduction

Tinospora cordifolia (Willd.) Hook. f. & Thoms is an extensively spreading, glabrous, succulent, woody climbing shrub belonging to the family Menispermaceae. It is distributed throughout the tropical region of Nepal, India, Sri Lanka and China ascending to an altitude of 1,200 m asl. It thrives in the tropical region in forests and other habitats (DPR, 2007). The genus *Tinospora* has around thirty-two different species distributed in tropical Africa, Madagascar, Asia to Australia, and the Pacific Island (Forman, 1981; Mabberley, 2005). In Nepal, only two species of *Tinospora* have been

reported are *Tinospora cordifolia* (Willd.) Hook. f. & Thoms. and *Tinospora sinensis* (Lour.) Merr. (Checklist of flowering plants of Nepal, 2000). It is known as Gurjo in Nepali. *T. cordifolia* is a rather large, widely disseminating, glabrous, dioecious, perennial deciduous climber that may be found growing on a variety of hedges and trees up to an altitude of 1000 m. It is often found growing in dry deciduous woods in tropical and subtropical countries. The plant has been demonstrated to possess multiple ethnomedicinal, pharmacological, and medicinal activities but systematically updated information is lacking on the therapeutic effectiveness of *T. cordifolia*. *T. cordifolia* is one of the most commercially exploited plants in pharmaceuticals. In the Indian system of medicine (ISM), there is an estimated 10,000 tonnes of annual demand for this species, which is used to prepare crude herbal medications (Singh, 2004). This plant has medicinal use primarily because it contains a variety of bioactive substances, including glucosides and alkaloids like berberine (Singh et al., 2003). Especially in Ayurveda, stems, roots, leaves, and starch derived from the stems and roots are used for therapeutic purposes (Singh et al., 2003). The root is a powerful emetic and is used for visceral obstructions. Its water extract is used in leprosy (Nayampalli et al., 1982). The root also exhibits an antidiabetic effect (Gupta et al., 1967). The extracts of stems, leaves, barks and roots show strong antioxidant activities (Stanley et al., 1999). Decoction of the leaves is used to treat gout, while the bitter substance in the stem is used to treat debility, dyspepsia, fever, and urinary illness (Singh et al., 2003). The leaves, bark, and roots of this plant are primarily responsible for its pharmaceutical significance because they contain a variety of bioactive substances, including alkaloids, glycosides, lactones, saponins, tannins, steroids, polysaccharides, and aliphatic compounds with various medicinal uses. Intense interest in the plant has been sparked by the identification of its active ingredients and their biological significance in the prevention of disease (DPR, 2007). A wide number of chemical compounds like aporphine, alkaloids, diterpenes, berberine, palmatine, tembertarine, magniflorine, choline and tinosporin have been isolated from this plant (Forman et al., 1981). It has been demonstrated that an extract from the stems of *T. cordifolia* increases the immunological protection response to a commercially available vaccine for infectious bursal illness in chicks (Sachan et al., 2019). The surface glycoprotein, receptor binding domain, RNA-dependent RNA polymerase, and major protease of the SARS-CoV-2 virus had strong binding affinities for berberine, isocolumbin, magnoflorine, and tinocordiside in in vitro investigations (Sagar & Kumar, 2020).

There is always a chance of variation happening within the in vitro-raised plants for their genetic homogeneity whenever a plant tissue culture technology is utilized for plant multiplication. Observations of somaclonal differences in in vitro-raised plants are frequent. Because of their excellent reproducibility, reliability, simplicity, and cost-effectiveness, RAPD and ISSR markers have the most widespread uses among the many markers and are more frequently utilized by researchers (Sultana et al., 2022). Molecular markers are frequently employed to examine the variation within the germplasm to determine the genetic similarity between the in vitro-grown plantlets and the mother plant (Mittal & Sharma, 2017). Simple sequence repeats (SSR), inter simple sequence repeats (ISSR), and random amplified polymorphic DNA (RAPD) have historically been utilized extensively in the assessment of clonal fidelity in a variety of plant species (Bhattacharya et al., 2015; Oliya et al., 2021; Pandey et al., 2020). Also, it is important to use many molecular markers to assess the clonal fidelity of plants grown in tissue culture (Palombi & Damiano, 2002). Because *T. cordifolia* contains a lot of phenolics, the advancement of plant tissue culture technology holds tremendous promise for the quick reproduction of plant germplasm. For the short- to medium-term conservation of significant plant species, it is a potent instrument. The return of plants into their original habitats and large-scale plant propagation are made possible through tissue culture technologies (Leander & Rosen, 1988; Lernda & Svensson, 2000). The present investigation was carried out to produce a reproducible protocol for in vitro propagation and assessment of genetic homogeneity in *T. cordifolia*.

Materials and Methods

Plant material and surface sterilization

Fresh *T. cordifolia* plants were procured from Dabur Nepal, Kavre, and Banepa and planted in the garden of the Tribhuvan University, Central Department of Botany. Excised nodal segment explants were

sterilized with a few drops of liquid detergent Tween 20 (Himedia, India) and then washed in sterile water for 30 minutes to eliminate all soil and debris. To get rid of any remaining $HgCl_2$ residues, the explants were treated with 0.1% $HgCl_2$ for five minutes. They were then rinsed five times with sterile water.

Inoculation of explant, shoot proliferation, elongation and root formation

Nodal segments explants were inoculated on MS basal medium supplemented with different concentrations and combinations of PGRs, which were chosen based on the primary response of the cultures which were BAP (0.5-2 mg/l), Kinetin (KIN) (0.5-2 mg/l), and combination of BAP (0.5 mg/l) + KIN (0.5 mg/l), BAP (1.0 mg/l) + KIN (0.5 mg/l), BAP (2 mg/l) + KIN (1.5 mg/l), BAP (2 mg/l) + KIN (2 mg/l), MS+ BAP (5 mg/l) and MS+BAP (5 mg/l)+ coconut water (5, 10%). After the proliferation of nodal segments, the shoots were inoculated in MS+IBA (0.5-2 mg/l) for rooting. All cultures were incubated under a 16/8-hour photoperiod using white fluorescent tubes (Phillips, India) at 25 ± 2°C. After 4 weeks of inoculation, the frequency and number of shoots developed were counted. Visual observations were used to record morphological modifications.

Genetic stability of in vitro-developed plants compared with mother plants

Selection of samples for DNA isolation: Young and healthy leaves harvested from the wild (mother plant collected from natural habitat) and five explants, each set derived from in vitro culture nodal segments grown on MS+BAP (5 mg/l) + 10% coconut water were used as explants for DNA extraction.

Extraction of DNA by CTAB method: Genomic DNA of in vitro plant leaves and wild leaves was extracted by using the modified CTAB method (Doyle, 1990). Approximately 0.2 g of leaf samples were taken and ground to fine powder by using a motor and pestle in liquid nitrogen. About 750 µl of CTAB buffer (2% CTAB, 0.5M EDTA, 5M NaCl, 1M Tris-HCl, pH 8.0, 0.2% β-mercaptoethanol) was added to make the fine paste and transferred into a clean sterilized microcentrifuge tube. Samples were incubated at 65 °C for 45 minutes in a recirculating water bath. After incubation, samples were centrifuged at 10,000 rpm for 5 minutes to spin down cell debris. A clean, sterilized microcentrifuge tube was then used to transfer the supernatant, and an equal volume of chloroform and isoamyl alcohol (24:1) was added. This mixture was then gently inverted several times (5-8 minutes). The upper aqueous phase was transferred to the microcentrifuge tube after it was centrifuged once more for 10 minutes at 12,000 rpm. An approximately equal volume of ice-cold propanol was added to each sample. To precipitate the DNA, the tube was slowly inverted many times. The tube was kept at -20 °C for one hour. The pellet was then created by spinning DNA at 12,000 rpm for five minutes. After discarding the supernatant, 400 µl of ice-cold 70% ethanol were used to wash the particle. To remove the salt, it was centrifuged once more for 5 minutes at 10,000 rpm. The ethanol was then thrown away, and the pellet was allowed to dry for 30 minutes, during which ethanol was evaporated. It was then re-dissolved in TE buffer (40µl) and kept at 4°C. Using a UV-spectrophotometer and agarose gel electrophoresis at a concentration of 1%, the quality and amount of the isolated DNA were evaluated. At the end, DNA samples were stored at -20°C after the concentration of the extracted DNA was adjusted to 10–20 ng.

DNA amplification and RAPD, ISSR analysis

The chosen explant DNA was amplified using five 14-decamer ISSR primers and a set of ten 10-decamer RAPD primers. The RAPD and ISSR assays were performed using a polymerase chain reaction (PCR) reaction in a thermal cycler using a 15 µl reaction volume, nuclease-free water, 1µ l Taq polymerase (0.5 U µl), 0.5 l dNTPs (0.2 mM), 1 l primer, and template DNA (50 ng) (Biorad, USA). Cycling condition was also optimized for identifying the best cycling conditions. The amplification cycle for RAPD primers was initial denaturation at 92 ºC for 5 min, 92 ºC for 1 min, 45 cycles of 35-60 ºC for 1 min, 72 ºC for 1 min, 72 ºC for 5 min. The amplification cycle for ISSR markers was initial denaturation at 95 ºC, 94 ºC for 1 min, 35 cycles of 42-60 ºC, 72 ºC for 2 min and 72 ºC for 7 min.

Data analysis

Triplicates of each sample were used for analysis. The samples' triplicate analysis results were expressed as means (n = 3) with SD. With the use of the SPSS, all the data were then further examined

using a one-way analysis of variance (ANOVA) and Duncan's multiple range test ($p < 0.05$). $p < 0.05$ was regarded as indicative of significance, and the DMRT analysis was used to rate the response percentage. Manual scoring was done on the banding pattern in the genetic fidelity analysis using RAPD and ISSR markers.

Results and Discussion

Establishment of in vitro cultures from nodal segment explant

In the present study, the nodal segment explants were initially cultured on an MS medium supplemented with various concentrations of BAP, and/or KIN for axillary shoot initiation and proliferation (Table 1). This treatment was chosen based on the positive response of the nodal segment in different concentrations of plant growth hormones. Nodal segment explants cultivated on medium enriched with BAP (2.0 mg/l) (Figure 1A) produced shoot from the nodal explants after 7 days of inoculation, among the tested concentrations of BAP (Figure 1B).

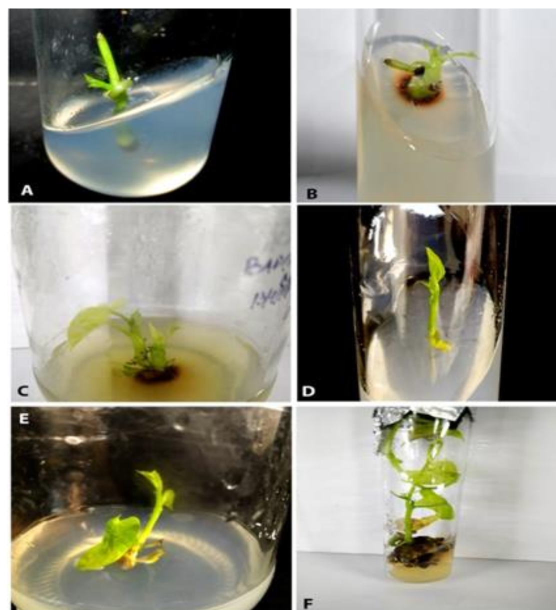

Figure 1: In vitro regeneration of *T. cordifolia* nodal explant. (A) inoculation in MS+BAP (2 mg/l); (B) after 7 days of inoculation in BAP (2mg/l); (C) after 25 days of inoculation in BAP (2 mg/l); (D) inoculation in MS+KIN (2 mg/l); (E) after 7 days of inoculation in MS+KIN (2 mg/l); (F) after 25 days of inoculation in MS+KIN (2 mg/l).

Nevertheless, it was found that when transferred to MS+KIN (2 mg/l), the proliferation rate was modest (Figure 1C), and only two shoots per explant were formed at the end of 30 days (Figure 1D). After 10 days, the proliferation rate significantly decreased, and even two subcultures onto a new medium spaced two weeks apart had little effect on the proliferation rate (i.e., number of shoots and roots). However, compared to different concentrations of BAP, MS+KIN (2 mg/l) was effective in producing shoots with more nodal segments (7.33±0.57) and (3.06±0.057 cm shoot length) (Figure 1F, Table 1).

Table 1: Effect of different concentrations and combinations of PGRs on the proliferation of nodal explant of *T. cordifolia*.

PGRs			Average shoot number	Average shoot length (cm)
BAP (mg/l)	Kinetin (mg/l)	Coconut water (%)		
0	0	0	0.66±0.57	0.23±0.057
1.5	-	-	2.66±0.57	0.6±0.10
2	-	-	1.66±0.57	1.16±0.15
0	1.5	-	4.33±0.57	2.06±0.15
0	2	-	7.33±0.57	3.06±0.057
0.5	0.5	-	2.3±0.57	2.13±0.15
1	0.5	-	3.66±0.57	3.23±0.15
2	1.5	-	2.33±1.15	2.10±0.17
2	2	-	5.66±1.15	3.040.057
5	0	-	9.33±0.57	8.63±0.55
5	-	5	13.33±1.52	9.43±0.38
5	-	10	20.33±0.57	12.10±0.10

Mass multiplication and rooting of the nodal segment

The nodal explants from in vitro generated shoots cultured in a medium containing BAP (2 mg/l) were inoculated into MS + BAP (5 mg/l) alone and supplemented with coconut water (5, 10%) in order to investigate the effectiveness of coconut water in boosting the shoot proliferation of *T. cordifolia*. The shoot proliferation increased significantly at 5 and 10% (Figure 2D), however, the maximum response was attained with the supplementation of 10% coconut water-containing medium producing more shoots (20.33 ±0.57) with 12.10± 0.10 cm (Figure 2H) as compared other concentration of coconut water. All the experiment was performed using control MS medium without plant growth regulators. Coconut water (10%) supplementation resulted in a maximum response about shoot numbers (20.0) and shoot length (12.0 cm). The

results indicated that medium supplemented with coconut water significantly increased the shoot induction of nodal explants (Figure 2H) as compared to the control MS basal medium (Table 1, Figure 2E, Figure 3). Similarly, rooting was also observed when the shoots grew in MS+BAP (5 mg/l)+10% CW containing medium was inoculated in MS+IBA (0.5 mg/l) (Figures 2F and 2G).

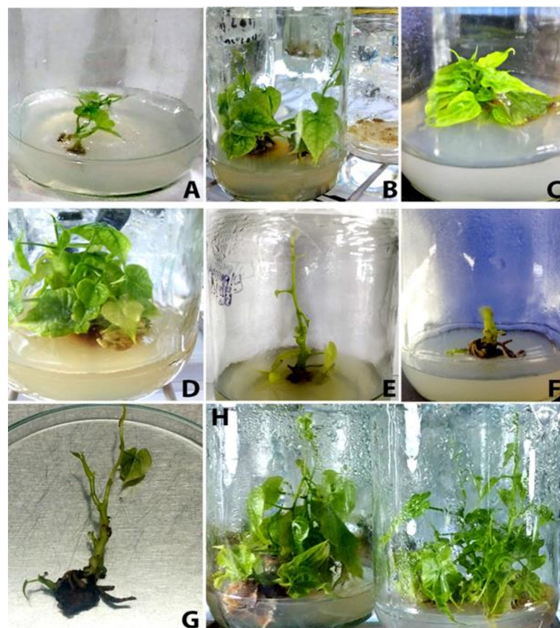

Figure 2: Mass multiplication of *T. cordifolia* nodal explant. (A) inoculated in MS+BAP (5 mg/l); (B) after 10 days of inoculation in BAP (5 mg/l); (C) after 25 days of inoculation in BAP (5 mg/l); (D) inoculated in MS+BAP (5 mg/l)+ 5% CW; (E) after 15 days of inoculation in MS basal medium; (F, G) rooting in MS+BAP (0.5 mg/l); (H) after 25 days of inoculation in MS+BAP (5mg/l)+ 10% CW.

Different protocols have been reported for the in vitro propagation of *T. cordifolia* (Sultana et al., 2013) reported the development of multiple shoots in high frequency was achieved in the nodal explant culture. They used MS basal medium throughout this investigation with different combinations and concentrations of BAP, Kinetin and Thidiazuron (TDZ). Within 30 days of inoculation, MS media with BAP (2 mg/l), Kinetin (4 mg/l), and TDZ (0.20 mg/l) generated a maximum of 10.29 shoots per explant, while half-strength MS medium supplemented with IBA (2 mg/l) produced roots. Earlier, a micropropagation protocol was developed using *T. cordifolia* collected from Northeast India (Handique et al., 2009). MS basal medium was used throughout this experiment with different combinations and concentrations of BAP, Kinetin and IAA. In this experiment, the nodal segments produced fewer shoots in MS+ BAP (2 mg/l) and KIN (2 mg/l) (Figure 1). Increasing the concentration of coconut water in MS+BAP (5 mg/l) containing medium supported the growth of the plant resulting in a higher number (20.33±0.57) of nodal segments from shoots with high shoot length (12.10± 0.10) (Table 1, Figure 2H). This is because the enhanced growth activity of coconut water can be attributed to its growth regulator content specifically cytokinin (Chugh et al., 2009), and this has been reported in *V. jatamansi* (Pandey et al., 2020). Similarly, high concentrations of IBA (1, 1.5 and 2 mg/l) did not produce many roots, as compared to IBA (0.5 mg/L) which produces prominent roots in *T. cordifolia* (Figure 2G). This is the first report of in vitro propagation of this species with the supplementation of coconut water in this species in Nepal.

RAPD and ISSR analysis

In the present study, ten RAPD 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 (Table 2). The number of bands varied from 1 to 4 with a total number of 22 bands for RAPD primer with band size between 100-1200 bp (Figure 4). Similarly, out of the 10 primer sets of ISSR markers, 5 primers (HB8, HB9, HB10, HB11 and 17898B) gave reproducible bands patterns between the in vitro raised plantlets and the mother plant (Table 3). The number of bands varied from 1 to 3 with a total number of bands for ISSR primer with band size between 100-400 bp (Figure 5).

In the case of plants that are significant commercially, determining the genetic homogeneity between the mother plant and in vitro regenerated plants is crucial. There are numerous variables, including dietary content, stress, hormonal balance, and culture time, which are significant contributors to in vitro variability (Khan et al., 2011). It is crucial to determine if the in vitro regenerants are genetically identical to the mother plant or not, particularly if the research's goal is to commercialize the specific plant species for its medicinal value. ISSR and RAPD primers have been successfully used to demonstrate clonal stability and to detect possible genetic variations within the in vitro-

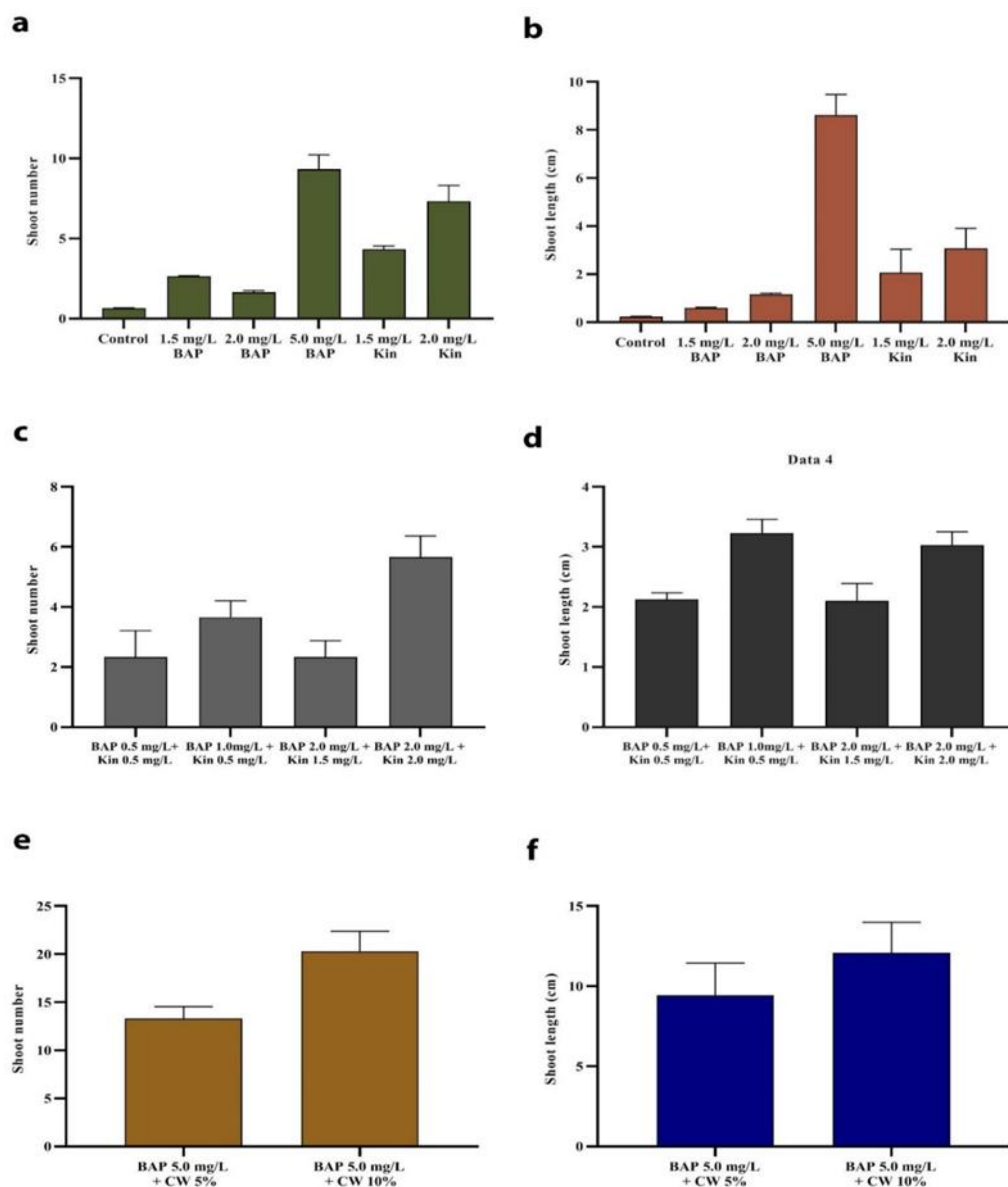

Figure 3: Effect of PGRs on shoot induction from nodal explants of *T. cordifolia*. (**a**) number of shoots per explant with different concentrations of BAP and Kin; (**b**) shoot length with different concentrations of BAP and Kin; (**c**) number of shoots per explant with different concentrations of BAP and Kin; (**d**) shoot length with different concentrations of BAP and Kin; (**e**) number of shoots per explant with different concentrations of BAP and coconut water; (**f**) shoot length with different concentrations of BAP and coconut water.

regenerated plantlets in several in vitro propagated medicinal and commercially important plant species like *Withania sominifera* (Nayak et al., 2012), *Alhagi maurorum* (Agarwal et al., 2015) and *Withania coagulans* Dunal (Tripathi et al., 2018). It is known that micropropagated tissues are easily exposed to somaclonal variations, especially during long-term cultures (Larkin & Scowcroft, 1981). In this regard, RAPD and ISSR markers are the favoured method used to determine the genetic stability of regenerated plants and wild plants of *T. cordifolia* (Mittal et al., 2017). The RAPD analysis revealed that the in vitro-derived plants of *T.*

cordifolia exhibited the same banding patterns as that of mother plants which produced 22 bands of approximately 100-1400 bp confirming that no genetic variation occurred in the DNA of in vitro regenerated plantlets (Figure 4, 5).

Table 2: RAPD primers with total number of bands amplified.

Primer	Sequence (5'-3')	Length (bp)	Annealing temp.	DNA band produced
OPA 08	GTGACGTAGG	10	44.2	2
OPA 10	GTGATCGCAG	10	44.2	1
OPA 18	AGGTGACCGT	10	44.2	2
OPE 08	TCACCACGGT	10	44.2	2
OPC 11	AAAGCTGCGG	10	44.2	2
UBC 292	TGCCGAGCTG	10	44.2	1
OPB 07	GGTGACGCAG	10	45.0	3
OPD 04	TCTGGTGAGG	10	46.0	4
UBC 210	CCGGGGTTTT	10	46.0	2
UBC 465	AGCTGAAGAG	10	44.2	3

Because of genetic variation, the resultant plant could not have the same qualities as the parent plant. Physiological/biochemical, morphological, and molecular methods can all be used to identify these somaclonal variants (Bairu et al., 2011). Morphological and biochemical approaches are inferior to molecular techniques among them. random amplified polymorphic DNA markers are frequently used in genetic variation studies in tissue-culture-derived plants because they require less DNA, are simpler to use, less expensive, more reliable, take less time, and don't require prior knowledge of the nucleotide sequence of the organism being studied (Devarumath et al., 2002). Various RAPD-based studies in *T. cordifolia* have been reported which were specific to wild populations only, however, the results of such studies could not be so reliable due to low reproducibility and low level of polymorphism of RAPD marker.

Table 3: ISSR primers with total number of bands amplified.

Primer	Sequence (5'-3')	Length (bp)	Annealing temp.	DNA band produced
HB 08	GAGAGAGAGAGAGG	14	52.3	1
HB 09	GTGTGTGTGTGTGG	14	52.3	3
HB 10	GAGAGAGAGAGACC	14	52.3	2
HB 11	GTGTGTGTGTGTCC	14	52.3	1
17898 B	CACACACACACAGT	14	52.3	2

Contrarily, the ISSR markers revealed higher heterozygosity and thus can be attributed to the detection of high polymorphism which makes them the most preferred DNA marker (Lade et al., 2020). This study reports confirmation of genetic homogeneity with RAPD and ISSR markers of the in vitro and mother plant.

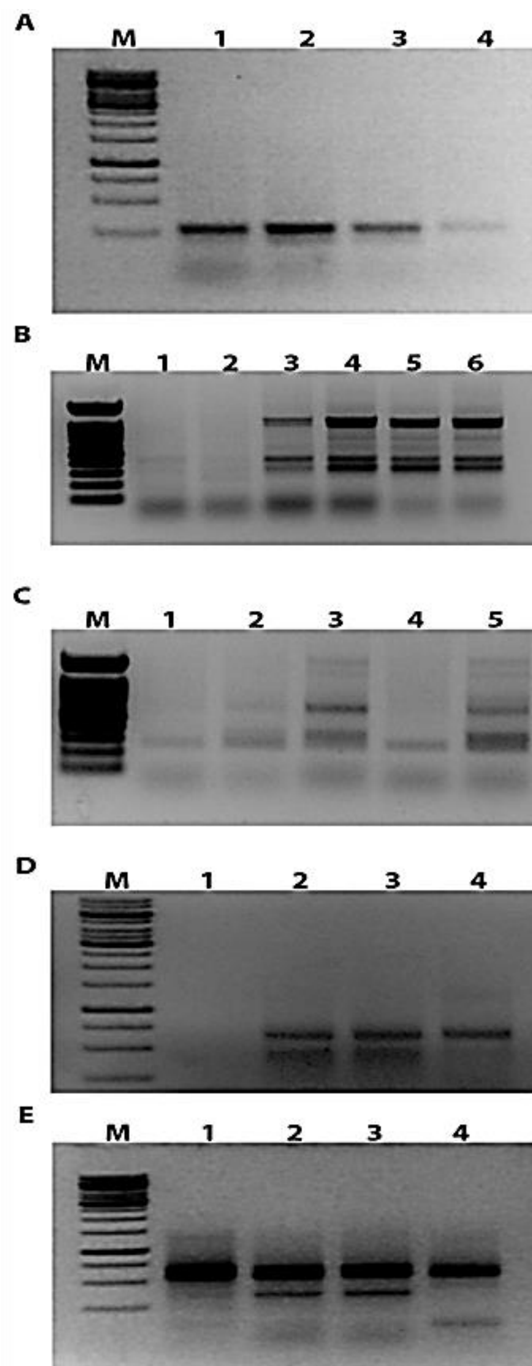

Figure 4: Representative images depicting the genetic fidelity analysis of in vitro and mother plant of *T. cordifolia* using RAPD markers depicting the molecular ladder of 100 bp -1.5 kb. (A) OPA 10 primer, 1 mother plant, 2 to 4 in vitro plants; (B) OPB 07 primer, 3 mother plant, 4 to 6 in vitro plants; (C) OPD 04 primer, 1 to 4 in vitro plants, 5 mother plant; (D) OPE 08 primer, 2 mother plant, 3 and 4 in vitro plants; (E) OPA 18 primer, 1 mother plant, 2 to 4 in vitro plants.

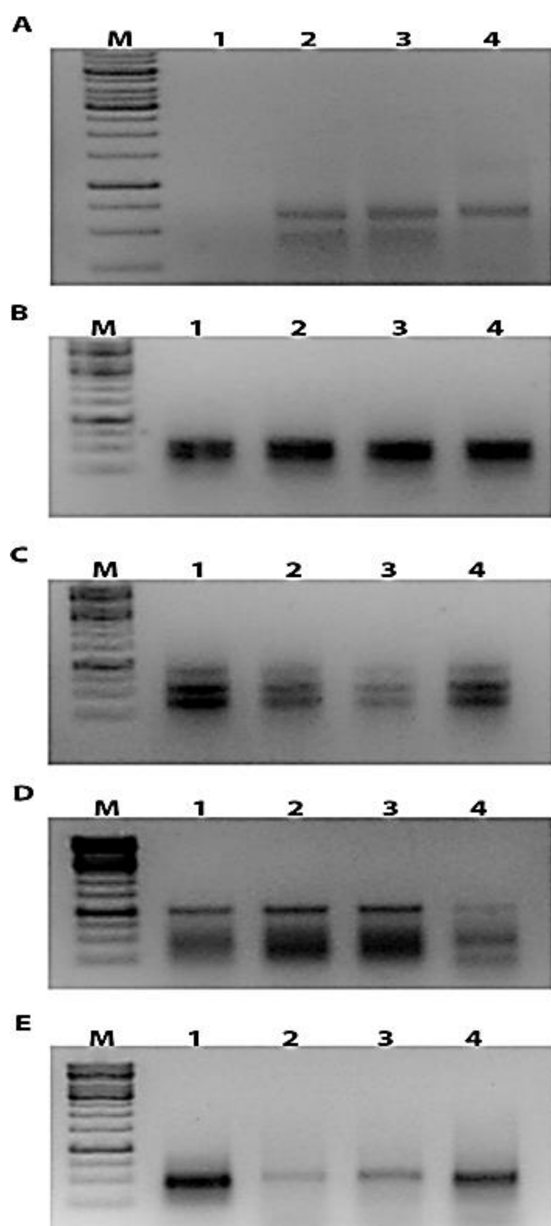

Figure 5: Representative images depicting the genetic fidelity analysis of in vitro and mother plant of *T. cordifolia* using ISSR marker depicting the molecular ladder of 100 bp -1.5 kb. (A) 17898 B primer, 1 to 3 in vitro plants, 4 in mother plant; (B) HB 08 primer, 1 to 3 in vitro plants, 4 in mother plant; (C) HB 09 primer, 1 to 3 in vitro plants, 4 mother plant; (D) HB 10 primer, 1-mother plant, 2-4 in vitro plants; (E) HB 11 primer, 1-mother plant, 2-4 in vitro plants.

Conclusion

An efficient *in vitro* propagation technique was developed in *T. cordifolia,* using nodal segment by using coconut water in the highly effective culture medium. The plants produce through nodal segment culture generated true-to-type plants. Also, the genetic fidelity was confirmed using RAPD and ISSR primers. The current strategy can be an alternative way for germplasm conservation and producing *T. cordifolia* plantlets in large quantities which is highly useful in pharmaceutical industries.

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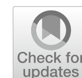

Comparative study of essential oil in wild and in vitro cultures of *Valeriana jatamansi* Jones in Nepal

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Abstract

Comparative analysis was carried out to determine the variation in the composition of essential oils of hairy roots produced in vitro and in vivo rhizomes of *Valeriana jatamansi* Jones collected from three different districts in Nepal i.e., Pyuthan (1200 m), Gulmi (3000 m), Godawari (1400 m) was carried out. In vitro adventitious root proliferation was established in MS media supplemented with Naphthalene acetic acid (NAA). GC–MS analysis were performed on the essential oils extracted from the sample. The findings showed that the yield of the essential oil obtained from the hydrodistillation of in vitro adventitious roots and dried rhizomes ranged between 0.52 and 0.80%. The highest percentage of essential oil (0.80%) were produced in the in vitro adventitious roots and minimum in the in vivo rhizomes collected from Pyuthan district, i.e., (0.52%). GC–MS analysis revealed a total of 17 volatile compounds in the essential oil from the dried rhizomes of *V. jatamansi* Jones from different locations and in vitro adventitious roots. It was found that the compound carotol was present only in sample from Pyuthan district, but absent in other samples. The compound matricarin was found in samples from Gulmi and Godawari district but absent in samples from Pyuthan district and in vitro adventitious roots. Similarly, the compound jatamansone was found in samples from Gulmi, Godawari, and adventitious roots but absent in sample from Pyuthan district. The compounds patchoulinone, 1, 2-butyl octyl ester of benzene dicarboxylic acid, and bis (2-ethyl hexyl) phthalate, one of the major compounds were present only in sample from adventitious roots The findings of the present study showed that the essential oil composition in both in vitro and in vivo roots, at different altitudes significantly affect the essential oil content of *V. jatamansi* Jones.

Keywords Essential oil · Adventitious root · GC–MS · *Valeriana jatamansi*

Introduction

Valeriana jatamansi Jones, a perennial herb locally known as nakalli jatamansi also commonly known as Indian Valerian grows in moist and shady places with creeping rhizome and belongs to Family Menispermeaceae. The plant can reach a height of 45 cm tall with thick, horizontal, nodular, and aromatic rootstock. *V. jatamansi* Jones is one of the important medicinal plants of Nepal and is found mostly at an altitude of 1200–3000 m from the sea level mainly distributed from north-western Himalayas to southwest China including Afghanistan, India, Nepal, and Bhutan (Jugran et al. 2019; Pandey and Pant 2020; Pant 2014; Press et al. 2000). Being highly medicinal, this species have been highly exploited in Nepal, and its rhizomes, roots and obtained oil has been traded since many years in Nepal (Pyakurel et al. 2017; Charmakar et al. 2021). Due to increasing demand from pharmaceutical and aromatic industries in national and international markets, volume and value of trade have been expanded over decade, which resulted in unregulated harvestings from the wild (Charmakar et al. 2021). The aromatic oil of three Himalayan species of genus *Valeriana* namely, *V. jatamansi*, *V. edulis* and *V. officinalis* are distributed which shows high pharmaceutical properties (Bos et al. 1998; Chen et al. 2014). The high medicinal value of this plant is mainly due to the presence of valerenic acid and valepotriates obtained from the roots and rhizomes which are highly aromatic. Therefore, this roots and rhizome has been used to treat numerous diseases and ailments. The

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root oil is used in perfumery and tobacco flavoring industry (Prakash 1999; Singh et al. 2006). The extracts is also widely used for drug preparation in Indian, British and Chinese pharmacopeia (Bhatt et al. 2012).

Essential oil isolated from this plant is widely used for commercial purpose in pharmaceutical industries. More than 290 compounds mainly monoterpenes and sesquiterpenes are the main constituents of the oil from roots and rhizomes (Jugran et al. 2019; Pandey et al. 2020). Apart from these, several other compounds like valerenic acid, isovaleric acid, valtrate, didrovaltrate, bornyl isovalerate, α, β and γ-patchoulene, patchouli alcohol, and 8-acetoxy patchouli alcohol have also been identified from the volatile oil and patchouli alcohol was the major constituent mainly from the roots and rhizomes from Nepal (Bos et al. 1998; Jugran et al. 2019; Pandey et al. 2020). The oil obtained from the leaf and root of *V. jatamansi* consisted of maaliol, methyl valeric acid and maaliol from root (Sati et al. 2005). Bhatt et al. (2012) performed GC and GC–MS analysis of the essential oil extracted from wild and planted material of *V. jatamansi.* The findings revealed the presence of 20 essential compounds. Patchouli alcohol was present in highest amount in *V. jatamansi* samples of planted source than wild. Other major compounds detected in both the samples (wild and planted) were α-guaiene, α-humulene, and, seychellene, whereas δ-guaiene was isolated only from wild samples. The roots and rhizomes of *V.jatamansi* Jones are stimulant, carminative, and antispasmodic, useful in hysteria, epilepsy, and neurosis. The plant is used in hypochondriacs, nervous unrest, and similar emotional state. It has remarkable influence on the cerebrospinal system (Sharma 2003). Oil of Valeriana is used as cholera drops to be safe from cholera and also is used in soap and perfumery. The energentene made from the juice of fresh roots has been recommended as a narcotic in insomnia. Moreover, it also has a slight influence over blood circulation, increasing heart force, and treatment of cardiac palpitations (Das et al. 2021). Fabio Colonna for the first time in 1592 noticed valerian as a specific drug for epilepsy (Eadie 2004). In India, the dried rhizomes are used in perfumes (Annonymus 2000). The variation in the quantity of oil is partly due to the influence of locality. The root of plant growing in a dry, stony soil yields higher oil than one found in moist and fertile land (Fierster et al.1984). Similarly, essential oil and extract of the *V. jatamansi* species are used as flavoring agent. Approximately, 30 products are commercially available that contain Valerian oil in cosmetic and other pharmaceutical industries (Raina and Negi 2015).

Valerian oil consists of valerinic acid, formic acids, and acetic acids. Valerian oil possess an unpleasant odor due to the presence of isovalerinic acid. Isovalerinic acid is liberated during the process of drying, and the decomposition of the chief constituent bornyl isovalerate due to fermentation (Guenther 1960). The cultivation of this plant for a commercial purpose is less practiced. However, the available literatures reveal that this plant is usually propagated by the division of old plants. Vegetative propagation is best compared to propagation by seeds. The micropropagation of the plant has been practiced by following different protocols for substitute of conventional methods of propagation (Pandey et al. 2020). Biotechnological approaches especially, plant tissue culture aid as a supplement to the mass scale production of the bioactive plant metabolites (Rao and Ravishankar 2002). This plant species has been under severe threat due to habitat loss, high medicinal value and overexploitation. Due to its overexploitation, plant tissue culture can be considered as a best alternative to reduce its loss from habitat. In the present investigation, in vitro root culture of *V. jatamansi* was established for mass production of adventitious roots and a comparative study of biochemical constituents in in vitro produced adventitious roots of *V. jatamansi* Jones and the rhizomes collected from natural habitat was performed. Previously we had reported high accumulation of valepotriates in in vitro root cultures of *Valeriana jatamansi* Jones (Pandey et al. 2022).

The present study was thus focused on the study of essential oil obtained from in vitro grown adventitious roots and the in vivo rhizomes collected from different localities of Nepal and analyzing their major chemical constituents.

Materials and methods

Production of in vitro adventitious roots

The in vitro plants that were generated with axillary shoot bud explants on BAP supplemented media and MS medium supplemented with coconut water were used as explants for the formation of the complete plantlet. An excising sterile roots (1.0 g) of well-developed plants which were collected from Nepal at an altitude of 1500 m were used to perform i*n vitro* root multiplication previously established in the laboratory (Pandey et al. 2020). The in vitro plant collected for the production of adventitious roots was one-year-old plant which was maintained in the laboratory by doing regular subculture. The roots of the plants were inoculated on the MS solid media containing different auxins at different concentrations, i.e. NAA (0.5, 1.0, 1.5 and 2.0 mg/L), Indole-3-butyric acid (IBA) (0.5, 1.0, 1.5 and 2.0 mg/L) and Indole-3-acetic acid (IAA) (0.5, 1.0, 1.5 and 2.0 mg/L). As control, solid MS media devoid of plant growth regulators was used. All the cultures were maintained at 25 ± 2 °C with a 16 h photoperiod grown in a culture room maintained at 25 ± 2 °C.

Collection of ex vitro plant materials

The *ex vitro* plant materials used for the present research are the rhizomes which were collected from one-year-old plant of *V. jatamansi* Jones. The rhizomes of in vivo plants were collected from three districts of Nepal Pyuthan, Gulmi, and Lalitpur, at an altitude of 1200, 3000 and 1400 m. The materials were collected during September and October 2017.The collected plant materials were air-dried at room temperature (± 25 °C) for a week and powdered for the extraction of essential oil.

Extraction of essential oil

The essential oil was extracted by hydrodistillation in Clevenger's apparatus. Different samples from the different localities (100 g fresh weight) were hydro-distilled separately in a Clevenger's apparatus using distilled water (500 mL) for 12 h and the essential oil was distilled, collected, and extracted with diethyl ether. The ether extract was dehydrated over anhydrous sodium sulfate, filtered, and was distilled off to obtain the essential oil. To extract the essential oil from *in vitro* hairy roots (100 g), powdered material was used in separate small-sized Clevenger's apparatus. The extraction of oil was carried out following (British Pharmacopoeia Commission 1998). The volume of the essential oil was measured directly in the extractor. The essential oil was collected, dehydrated over anhydrous sodium sulfate, and stored in sealed, labeled glass vials at 4 °C until further analysis. Total yield percentage was calculated as volume (ml) essential oil per 100 g of plant dry matter (AOAC 1990).

GC–MS analysis

Analysis of the oil using gas chromatography and mass spectrometry was carried out in the Institute of Engineering and Chemical Science, Singapore. GC–MS 4000 (Varian, USA) system with a HP-5MS agilent column (30 m × 0.25 mm i.d., 0.25 μ film thickness) was used for analysis. Injector temperature was 280^0C. Oven temperature program used was holding at 50 °C for 5 min, heating to 280 °C at 3 °C/min, and keeping the temperature constant at 280 °C 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 identification of components of the essential oil was based on comparison of their mass spectra with those stored in NIST05 library or with mass spectra from literature.

Statistical analysis

All experiments were repeated three times, using three replicates, each containing fifteen explants. The values are expressed as means ($n = 3$) ± SD. Further, one-way analysis of variance (ANOVA) was performed followed by Duncan's multiple range test ($p < 0.05$). The data were analyzed using SPSS (version 17, Chicago, USA) statistical package program. $p < 0.05$ was considered as indicative of significance.

Results and discussion

Growth kinetics of adventitious root cultures

The well proliferated in vitro plants were used as a source to harvest roots and the roots were inoculated in the MS medium supplemented with different growth regulators at different concentrations. The assessment of growth progression of the adventitious root cultures from the initial inoculum were done based on fresh and dry weight analysis (Fig. 1a–g, Table 1). The control cultures (basal media without growth hormones) recorded a fresh weight of 2.56 g which was relatively similar to IAA and IBA treated cultures (Table 1). IAA induced 4.52 g of biomass with 2.0 mg/L and 3.91 g when the medium was supplemented with 1.5 mg/L, respectively. Between IAA and IBA treated cultures, IBA was the least responsive growth hormone with a maximum of fresh weight (3.51 g) with a concentration of 2 mg/L (Table 1). Comparatively, NAA showed better increase in the biomass of root than both the growth hormones (IAA and IBA) (Table 1). Among the tested growth hormones at different concentrations, the highest biomass accumulation was resulted in the NAA (2.0 mg/L) supplemented medium, with a fresh weight and dry weight of 6.41 g and 2.91 g, respectively (Table 1, Fig. 1d–g). Overall, NAA supplemented cultures showed a relatively better adventitious root biomass accumulation (Table 1, Fig. 1d–g). Disappearing of the natural population is continuous and to minimize the loss, in vitro cultures provide an important aspect for large scale propagation of desired species and isolation of important metabolite and essential oils (Zhao and Tang 2020). Adventitious root culture can be an important strategy for isolation of essential oil and high valued medicinal compounds using different plant growth regulators (Saeed et al. 2017; Rani et al. 2017). Zhao and Tang (2020) also reported enhanced production of valtrate using methyl jasmonate. Different auxin treatment was used for adventitious root

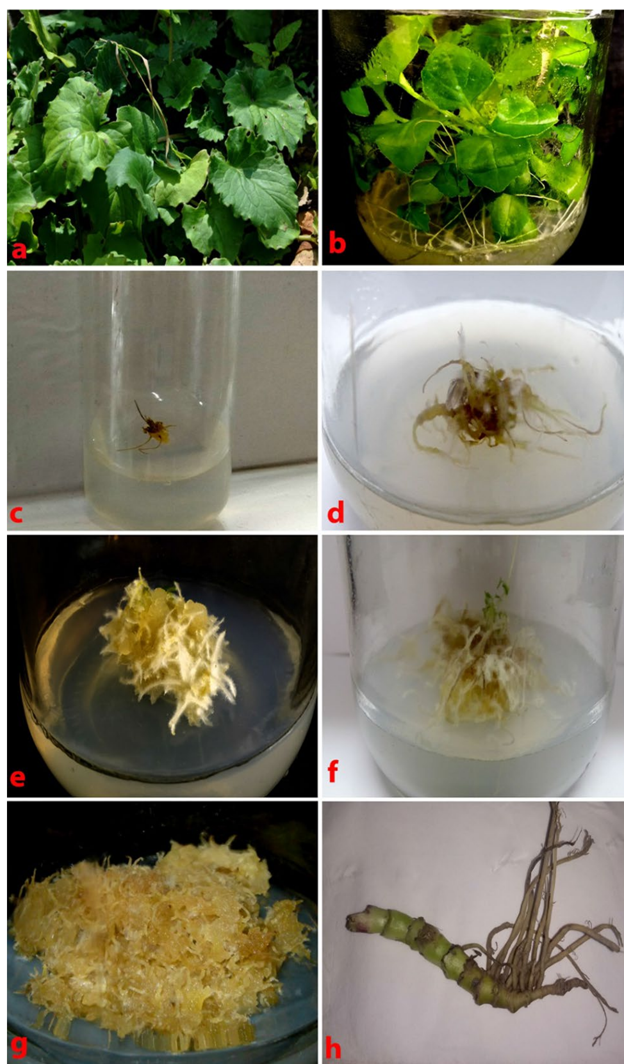

Fig. 1 Establishment of in vitro *V. jatamansi* adventitious root cultures (**a**) Wild Plant of *V. jatamansi*; (**b**) shoot proliferation from nodal segment explant of *V. jatamansi* (**c**) root inoculation in MS media supplemented with NAA (2.0 mg/L) post 2 days (**d**, **e**, **f**, **g**) adventitious root formation in MS media supplemented with NAA (2.0 mg/L) at 7, 10, 15, 30 days (**h**) representative image of rhizome collected from different altitudes in Nepal

culture in *Andrographics paniculata*, only NAA was able to induce adventitious roots showing high growth (Sharma et al. 2013). At lower concentrations of NAA, an increase in the adventitious root formation was observed (1.0 mg/L and 2.0 mg/L) in different species, however, higher concentrations of NAA reduced the root numbers (Raju and Prasad 2010; Sun and Hong 2010). In tobacco, the uptake of NAA was six times higher than IAA absorption, moreover, an uptake of NAA is more rapid than other growth hormones under in vitro conditions (Peeters et al. 1991). In case of *Passiflora pohlii* Mast, the stem segments showed higher rate of adventitious roots on MS medium supplemented with

Table 1 Fresh and dry weight analysis of adventitious root cultures of *Valeriana jatamansi* Jones grown in medium supplemented with different growth hormones at different concentrations

Treatments	Day 0		Day 30	
	FW (g)	DW (g)	FW (g)	DW (g)
Control MS basal	0.1	0.05	2.56 ± 0.015	0.89 ± 0.010
NAA (mg/L)				
0.5	0.1	0.05	3.99 ± 0.015	1.96 ± 0.015
1.0	0.1	0.05	5.06 ± 0.015	2.1067 ± 0.011
1.5	0.1	0.05	5.84 ± 0.010	2.31 ± 0.011
2.0	0.1	0.05	6.41 ± 0.015	2.916 ± 0.015
IAA (mg/L)				
0.5	0.1	0.05	1.99 ± 0.015	0.95 ± 0.010
1.0	0.1	0.05	3.10 ± 0.015	1.526 ± 0.015
1.5	0.1	0.05	3.91 ± 0.030	1.196 ± 0.0152
2.0	0.1	0.05	4.52 ± 0.026	1.346 ± 0.015
IBA (mg/L)				
0.5	0.1	0.05	2.116 ± 0.015	0.986 ± 0.005
1.0	0.1	0.05	1.933 ± 0.015	0.85 ± 0.010
1.5	0.1	0.05	2.913 ± 0.0152	1.130 ± 0.020
2.0	0.1	0.05	3.513 ± 0.0152	1.230 ± 0.020

Data presented as mean ± SD of three replications. The data was analyzed using one-way analysis of variance (ANOVA)

NAA as compared to IBA and IAA (Simao et al. 2016). Our results are in accordance to these previous reports, where best concentration for higher root biomass was found in NAA (2 mg/L) solid medium.

Essential oil yield

The yield of essential oil from the rhizomes of *V. jatamansi* ranged between 0.52 and 0.80% from the samples collected from different altitudes and habitats as well as in vitro obtained hairy roots (Table 2). The result showed the increase in essential oil yield with increased in the

Table 2 Essential oil yield from in vivo rhizomes at different altitude and adventitious root cultures of *Valeriana jatamansi* Jones

S.N	Locality	Altitude	Amount of essential oil (%)	Means ± SD
1.	Pyuthan	1200 m	0.52	0.5200 ± 0.01000%
2.	Gulmi	3000 m	0.69	0.6800V0.01000%
3.	Godawari	1400 m	0.57	0.5800 ± 0.01000%
4.	In vitro hairy roots		0.80	0.8333 ± 0.01528%

Data presented as mean ± SD of three replications. The data was analyzed using one-way analysis of variance (ANOVA)

altitude between 1600 and 3000 m, while decreased thereafter. Similarly, the yield of essential oil in in vitro adventitious roots was higher than wild rhizomes. The lowest yield was 0.52% found in wild rhizomes at 1000–1200 m altitude in Pyuthan, followed by 0.57% at 1400 m in Godavari, while the highest yield was 0.69% from wild rhizomes in gulmi at 3000 m altitude (Table 2). The essential oil yield was comparatively highest in in vitro hairy roots with 0.80% (Table 2). The essential oil yield from different parts of *V. jatamansi* has been previously reported (Verma et al. 2011; Bhatt et al. 2012; Jugran et al. 2020). But no reports have been found in comparative analysis of wild rhizomes and in vitro produced adventitious roots. It has been reported that the oil yield depends on several factors such as local habitat and its environmental conditions, season, time of collection, genetic differences etc. (Verma et al. 2011).

Essential oil composition

GC–MS analysis of the essential oil of *V. jatamansi* rhizomes identified a total of 17 compounds. This includes the samples collected from different populations and habitats along with the in vitro adventitious roots. All the compounds (excluding the trace components) are listed in (Table 3, Fig. 2, Fig. 3 Supp). The major components of the oil were found to be 3-methyl butanoic acid, 3-methyl pentanoic acid, borneol acetate, β-patchoulene, α-guaene, α-panasinsen, α-patchoulene, and patchouli alcohol in all the four samples analyzed (Table 3, Fig. 2b, c, i, g, d, e, f, m). The compound jatamansone (Table 3, Fig. 2k, Fig. 3l Supp) was found in samples from Gulmi, Godawari, and in vitro adventitious hairy roots but it was absent in sample from Pyuthan. The compound carotol (Table 3, Fig. 2j, Fig. 3k Supp) was found only in the sample from Pyuthan but it was absent in other samples. Similarly, matricarin (Table 3, Fig. 2l, Fig. 3j Supp)

Table 3 GC–MS analysis of the essential oil composition of *Valeriana Jatamansi* Jones rhizomes from different altitude, habitats and in vitro adventitious root culture

S.No	Name of the Compound	Retention Time	Different Habitats			
			Pyuthan	Godavari	Gulmi	In vitro hairy roots
1	3- Methyl butanoic acid	4-5	✓	✓	✓	✓
2	3- Methyl pentanoic acid	5.4-5.43	✓	✓	✓	✓
3	Borneol acetate	8.20	✓	✓	✓	✓
4	β- Patchoulene	9.01	✓	✓	✓	✓
5	α- Guaene	9.29	✓	✓	✓	✓
6	α- Gurjunene	9.37	✓	✓	✓	✓
7	α- Panasinsen	9.49	✓	✓	✓	✓
8	α- Patchoulene	9.54	✓	✓	✓	✓
10	Patchouli alcohol	10.89	✓	✓	✓	✓
11	Carotol	10.10	✓	-	-	-
12	Matricarin	11	-	✓	✓	-
13	Jatamonsone	12.74	-	✓	✓	✓
14	Patchoulinone	11.26	-	-	-	✓
15	1,2–butyl octyl ester of benzene dicarboxylic acid	11.72				✓
16	Bis (2-ethyl hexyl) pythalate	11.72	-	-	-	✓

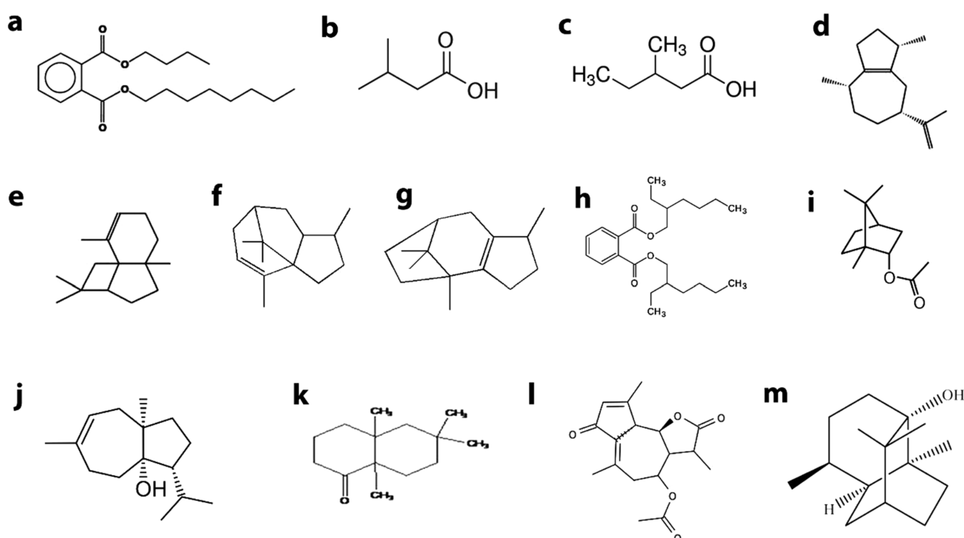

Fig. 2 Structures of the major components identified from essential oils from rhizome and in vitro adventitious roots of *Valeriana jatamansi Jones* **a** 1, 2-butyl octyl ester of benzene dicarboxylic acid **b** 3- methyl butanoic acid **c** 3- methyl pentanoic acid **d** α- guaene **e** α- panasinsen **f** α- patchoulene **g** β- patchoulene **h** Bis 2 pthalate **i** borneal acetate **j** carotol **k** Jatamansone **l** matricarin **m** patchouli alcohol

was present in samples from Godawari and Gulmi but absent in samples from Pyuthan and in vitro adventitious roots. The compounds like patchoulinone and 1, 2-butyl octyl ester of benzene dicarboxylic acid and bis (2-ethyl hexyl) phthalate were present only in in vitro adventitious roots (Table 3, Fig. 2a, h) Fig. 3m, n, Supp). It was found that twenty-six peaks were observed in the chromatogram out of which twelve compounds could be identified in samples from Pyuthan (Table 3). The number of peaks observed in the chromatogram of samples from Gulmi was 26 out of which 13 compounds could be identified (Table 3). Similarly, out of 29 peaks from samples of Godawari. 13 compounds could be identified (Table 3) and out of 24 peaks of adventitious roots 15 compounds (Table 3) could be identified. The compound jatamansone was found in samples collected at an altitude of 1400 m and 3000 m, as well as in in vitro adventitious roots, but it was absent in samples collected at an altitude of 1200 m. The compound carotol was found only in the sample collected at an altitude of 1200 m but absent at other altitudes as well as in in vitro roots. Similarly, matricarin was present in samples collected at an altitude of 1400 and 3000 m but absent in lower altitude as well as in in vitro adventitious roots. Patchoulinone, 1,2–butyl octyl ester of benzene dicarboxylic acid and Bis (2-ethyl hexyl) phthalate was only found in oil isolated from in vitro adventitious roots and were absent in other samples. The variation in the presence and absence of these compounds among the different samples collected from different locations could be attributed to plant's origin, geographical locations, different environmental factors, climate, genetic variation, and harvesting seasons (Verma et al. 2011). Among all studied populations, the essential oil yields from three diverse locations of the western Himalayan region were found to be higher from roots (0.35–0.43%) as compared to rhizomes (0.05–0.08%). However, we have reported that the wild rhizomes at different altitudes were comparatively higher with a maximum of 0.67% and the essential oil in in vitro roots was0.80% which was higher than the wild rhizomes (Verma et al. 2013).On the contrary, (Singh et al. 2013) reported higher oil content in rhizomes (1.66%) compared to roots (0.60%). It has been reported that herbs from diverse locations in the temperate Himalayan region may have influence the essential oil yield and its chemical composition due to varied morphological and genetic features (Dhiman et al. 2020). Effect of altitude may be one of the factor responsible for the varied production of essential oil isolated from rhizomes of three different areas with different altitudes as suggested by (Mahdavi et al. 2013) in their study where they reported that the essential oil was higher at highest altitude i.e.; 3200 m whereas it was comparatively lower at the lowest altitude i.e.; 1600 m in *Tanacetum polyphatum*, Iran. Similarly (Khalil et al. 2020) also reported that *S. thymbra* plants growing at higher altitude gave better yield carvacerol, an anticancer compound in essential oil as compared to lower altitude. Packter. (1981) reported that soil was one of the physiochemical factor affecting the quality and quantity of essential oils of medicinal plants. Phuyal et al. (2019) reported that in *Xanthoxyllum armatum*, the essential oil yield from the samples collected from wild population at 1400 m was lowest (0.16%) as compared to (2.478%) at 1990 m. In our study we have not performed the experiment on soil parameters and the rhizomes were collected from the plants of *V. jatamansi* from three different locations which was morphologically similar in size.

Mathela et al. (2005) identified three important terpenoids (maaliol, patchouli alcohol, and 8-acetoxypatchouli alcohol) among which, patchouli alcohol (13.4–66.7%) had the highest percentage in its essential oil (Verma et al. 2011). Thusoo et al. (2014) identified 7 major sesquiterpenes in the essential oil extracted from *V.jatamansi* roots, β-vatirenene,

β-patchoulene, dehydroaromadendrene, β-gurjunene, patchouli alcohol, β-guaiene, and α-muurolene. Similarly to this report, in our study also α- Patchoulene and β- Patchoulene were identified with a high retention time of 9.54 and 9.01 min (Table 3, Fig. 2f, g, Fig. 3d, h Supp). In another report, 20 compounds were identified in root oil where the maximum percentage of compounds was Patchouli alcohol (52.8%) (Rawat et al. 2017) which is in agreement with our study where Patchouli alcohol was identified with the retention time of 10.89 min (Table 3, Fig. 2m, Fig. 3i Supp). Various chemical constituents like patchouli alcohol, alpha-patchoulene, and beta-patchoulene are considered important for perfume industry (Dhiman et al. 2020). Apart from this, we have reported the presence of a new compound Patchoulinone, 1,2–butyl octyl ester of benzene dicarboxylic acid and Bis (2-ethyl hexyl) phthalate in in vitro adventitious roots (Table 3, Fig. 2a, h, Fig. m, n, o Supp). Similar to our results, the essential oil extracted from the root bark of *Uvaria narum* was found to contain 15% of bornyl acetate, 8% of patchoulinone, and a tricyclic sesquiterpene ketone (Wiart 2006). GC–MS analysis of methanolic extracts prepared from the leaves of the mother plant of *V. wallichii* revealed the presence of 37 peaks where bis (2-ethyl hexyl) phthalate was also detected (Sharma et al. 2020). Patchoulinone has been reported to have a calming influence, stops flatulence and counteracts putrefaction whereas, Phthalates are the esters of phthalic acid and are mainly used as plasticizers (Manay et al. 2014). Current day, pharmaceuticals are dependent on plant-derived metabolites, with new products being discovered constantly. Nevertheless, the constant and uniform supply of plant pharmaceuticals has often been compromised. One alternative for the production of important plant active compounds can be achieved through in vitro plant tissue culture, as it assures independence from geographical conditions by eliminating the need to rely on wild plants (Adhikari et al. 2013; Pant et al. 2021) and may also enable us to discover new compounds that may not be present in the wild plants (Espinosa Leal et al. 2018, Pant 2014, Pandey et al. 2020). Thus the essential oil from the in vitro roots can be commercialized for the public demand which ultimately will help in conserving the endangered or red listed plants species and also ensure the stability of the wild populations.

Conclusion

The in vitro generated biomass along with wild populations were investigated for presence of medicinally important metabolites in the essential oils. The variation in the essential oil yield and the chemical composition of *V. jatamansi* rhizomes and in vitro adventitious roots could be account to the genetic, geographical and environmental factors. The highest recorded yield was found in vitro adventitious roots (0.80%), followed by 0.69% at an altitude of 1600–3000 m, 0.57% at an altitude of 1400 m, and the least recorded yield was 0.52% at an altitude of 1200 m. A few major components like α-patchoulene, β-patchoulene, Patchouli alcohol, Jatamansone were identified from both the wild rhizome and in vitro adventitious roots, However, Patchoulinone, 1, 2–butyl octyl ester of benzene dicarboxylic acid was identified only in in vitro adventitious roots which has not been reported previously in this plant. The yield percentage was also comparatively higher in in vitro adventitious root culture than the wild-collected rhizomes. This is the first report elucidating the comparative study of essential oils and their components with respect to samples collected from different altitudes in Nepal and establishment of an in vitro adventitious root culture system. Therefore, in vitro culture technique can be a promising technology for the production of commercially important and desirable secondary metabolites at large scale which will ultimately minimize the collection pressure from their natural habitat which may lead to the depletion as well as the loss of the plant species completely.

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Author contributions BRP executed the experiments. SP did the experimental analysis. BRP and SP prepared the manuscript. MDM contributed to the research design, and evaluated the manuscript. BP conceptualized the research, evaluated and edited the manuscript.

Data availability All the data are available in the manuscript except Fig. 3. is provided as an additional supplementary material.

Declarations

Conflict of interest All authors read, approved the manuscript and declare that there is no conflict of interest.

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CONFERENCES

1. International Conference (January 10-12, 2017) on "Biodiversity, Climate Change Assessment and Impacts on Livelihood" organized by the Central Department of Botany, Tribhuvan University, Nepal; Agriculture and Forestry University, Nepal; City University of New York, USA and Institute for Global Agriculture and TechNology Transfer, USA (Poster Presentation)

   Title of the presentation: Plant Tissue Culture: An appraisal for Biodiversity Conservation
2. International Conference on Medicinal, Aromatic and Nutraceutical plants from Mountaineous areas organized by Department of Life sciences and Department of Biotechnology, Graphic Era (Deemed to be University) and American Council of Medicinally active Plants (ACMAP), USA on February 14-16, 2019 in Dehradhun, India (Oral Presentation)

   Title of the presentation: Establishment of in vitro culture of valuable medicinal plant Valeriana jatamansi Jones and modification strategies for conservation and production of bioactive metabolites
3. International Youth Conference on Science, Technology and Innovation organized by Nepal Academy of Science and Technology(NAST) on 21-23rd October 2019, Kathmandu, Nepal.(Oral Presentation)

   Title of the presentation: Enhanced Valepotriate accumulation in *in vitro* cultures of *Valeriana jatamansi* Jones, a pharmecutically important plant followed by GCMS profiling and Gene expression analysis
4. 9th International Plant Tissue Culture and Biotechnology Conference organized by University of Dhaka and Bangladesh association of Plant Tissue Culture, Dhaka, Bangladesh on8-10 February, 2020. (Oral Presentation)

   Title of the presentation: Rapid propagation, GC-MS analysis for bioactive compounds and assessment of genetic fidelity using RAPD and ISSR markers in *Valeriana jatamansi* Jones, a high-value medicinal plant
5. International Symposium on "Advances in Plant Biotechnology and Genome Editing", 42nd Meeting of Plant Tissue Culture Association, India, Organized by ICAR- Indian Agricultural Research Institute, Ranchi, India on April 8-10 2021. (Oral Presentation)

Title of the presentation: Adventitious root cultures for enhanced valepotriates accumulation in commercially important medicinal plant *Valeriana jatamansi* Jones.

6. International Conference on Biodiversity and Bioprospecting organized by Department of Plant Resources, Thapathali, Kathmandu, Nepal on 22-24 June, 2022 (**Oral Presentation**).

   Title of the presentation: Rapid propagation, GC-MS analysis for bioactive compounds and assessment of genetic fidelity using RAPD and ISSR markers in *Tinospora cordifolia* (Wild.) Hook. f. & Thoms, a high-value medicinal plant.

7. International Conference on Biotechnology "Academia to Industry" organized by Nepal Biotechnology Association, Kathmandu, Nepal on March 17-19, 2023. (Oral Presentation)

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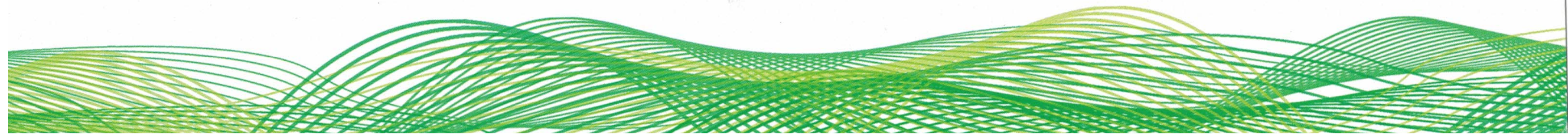

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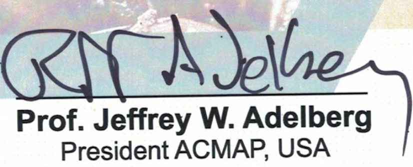

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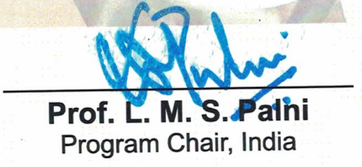

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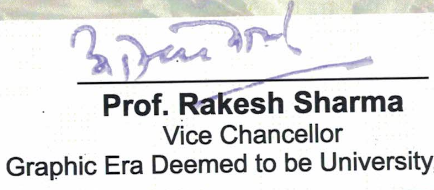

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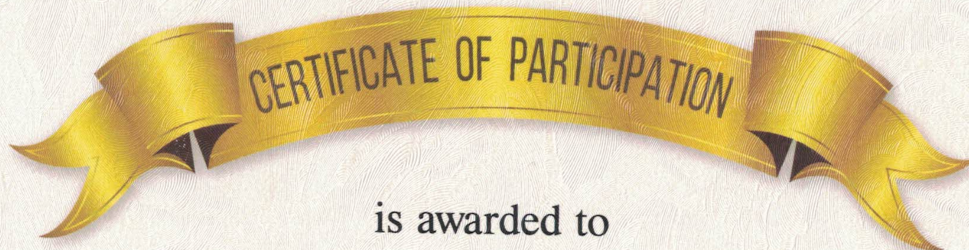

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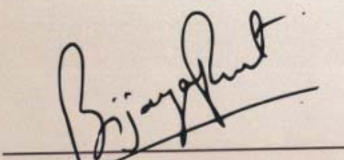

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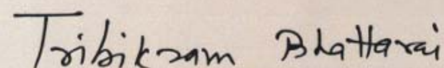

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