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Secondary metabolite production and low cost *In-vitro* propagation of *Podophyllum hexandrum*. (An endangered medicinal plant). A review

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Abstract
The western Himalaya is a rich repository of unique plants that are valued for their medicinal properties. Many of these plants are extensively utilized in pharmaceutical industries, and there is a huge global demand for them. Since most of these plants have become rare, threatened, or endangered, there is an urgent need to conserve them. *Podophyllum hexandrum* is among one of them which is an endangered medicinal plant. Hence for the regeneration and conservation of the *Podophyllum hexandrum* micro propagation is one of the major tools. The primary application of micro propagation has been to produce high quality planting material. However, micro propagation technology, being a capital-intensive industry is expensive. Low-cost tissue culture technology is the adoption of practices and use of equipment to reduce the unit cost of micro propagule and plant production. Low cost options should lower the cost of production without compromising the quality of the micro propagules and plants. In low cost technology cost reduction is achieved by improving process efficiency and better utilization of resources. Plants cell culture technologies were introduced at the end of 1960‘s as a possible tool for both studying and producing plant secondary metabolites. Plants secondary metabolites are unique sources for pharmaceuticals food additives, flavors and others industrial values. Commercial importance of these secondary metabolites has resulted in a great interest in its production and in exploring possibilities of enhancing its production by means of tissue culture technology.

Keywords: *Podophyllum hexandrum*, secondary metabolite, low cost, *In-vitro* propagation

Introduction

Plant kingdom, comprising about 250,000 species, is a repository of probably hundreds of thousands of low-molecular-weight structurally complex chemical compounds known as secondary metabolites (Narayani *et.al* 2017) [15]. These high-value metabolites are biosynthesized through phenylpropanoid, mevalonate, 2-C-methyl-d-erythritol-4-phosphate, amino acid, glucose, acetate-malonate pathway, or combined pathways. Secondary metabolites have animportant role in the interaction between plants and their environment (e.g., defense against herbivores and pathogens, protection against ultraviolet light, etc.) and, thus, are vital for their existence and subsistence. They are accumulated in specific tissues and structures (e.g., vacuoles, specialized glands, trichomes, etc.), and their production is affected by several factors, like genotype, plant physiology, climate, environmental conditions, and pathogens; in some cases, they are only produced during certain developmental stages (Shitan N 2016. Isah *et.al* 2018) [18, 6].

Secondary metabolites are not essential as primary metabolites as these are not directly involved in growth, development and reproduction of organisms. They are organic compounds which are not directly involved in survival of plants but they produce some products which aid them in their normal growth and development. Secondary metabolites are compounds biosynthetically derived from primary metabolites but more limited in distribution in the plant kingdom, being restricted to a particular taxonomic group (species, genus, family, or closely related group of families). Secondary compounds have no apparent function in a plant's primary metabolism but often have an ecological role; they are pollinator attractants, represent chemical adaptations to environmental stresses, or serve as chemical defenses against microorganisms, insects and higher predators, and even other plants (allelochemics). Secondary metabolites are frequently accumulated by plants in smaller quantities than are primary metabolites. As a result, secondary metabolites that are used commercially as biologically active compounds (pharmaceuticals, flavors, fragrances, and pesticides) are generally higher value-lower volume products than the primary contrast to primary metabolites, tend to be synthesized in specialized cell types and at distinct developmental stages, making their extraction and purification metabolites. Thus, compared to primary metabolites (bulk chemicals), many secondary metabolites can be considered as specialty materials or fine chemicals. Secondary metabolites are often large organic molecules that require a large number of specific enzymatic steps for production

- Synthesis of tetracycline requires at least 72 separate enzymatic steps for production.
- Starting materials arise from major biosynthetic pathways.

Examples of commercially useful plant secondary metabolites are nicotine, the pyrethrins, and rotenone, which are used in limited quantities as pesticides, and certain steroids and alkaloids, which are used in drug manufacturing by the pharmaceutical industry.

The production of secondary metabolites by in-vitro cultures usually occurs in a two-step process, biomass accumulation and secondary metabolites synthesis, in which both steps need to be optimized independently (Isah T *et al* 2009, Murthy NH *et al* 2014) [13]. Production could be accomplished by using undifferentiated calli, cell suspension cultures, or organized structures like shoots, roots, or somatic embryos. In some cases, a certain degree

of differentiation may be needed for the biosynthesis to occur (Karuppusamy S 2009) [8]. The use of differentiated organ cultures is required, for instance, when the target metabolite is only produced in specialized plant tissues or glands as is the case of essential oils (Karuppusamy S 2009, Rao SR *et.al* 2002) [8].

Among differentiated tissues, hairy root culture offers new opportunities for the in-vitro production of plant-valuable compounds (Chandra S *et.al* 2011) [5]. Hairy roots are induced by the infection of plants with Agro bacterium rhizogenes, a Gram-negative soil bacterium. During the infection, a DNA segment (T-DNA) from the large root-inducing (RI) plasmid of the bacterium is transferred into the genome of the infected plant. The higher level of cellular differentiation, rapid growth, genetic and biochemical stability, and maintenance facility are some of the advantages of hairy roots (Chandra S *et.al* 2011) [5]. Also, they can accumulate metabolites in the aerial parts of the plant. However, the difficulties in cultivating hairy roots in an industrial system limit their commercial use to produce valuable plant secondary metabolites.

Although there are many studies reporting the production of secondary metabolites using callus cultures and differentiated tissues (Isah T *et.al* 2018, Murthy NH *et.al* 2014, Ali M *et.al* 2016) [13, 6], in most cases, undifferentiated cells are the preferred culture system (Yue W *et.al* 2016). Cell suspension culture is a simple and cost-effective method that has been extensively used to overcome the problems of large-scale production. Plant cell is biosynthetically totipotent, which means that under suitable conditions, each cell has theoretically the capacity to produce compounds identical to those present in the parent plant (Yue W *et.al* 2016) [20]. Plant cell cultures have more immediate potential for commercial application than tissue or organ cultures (Rao SR *et.al* 2002, Xuj *et.al* 2011) [8, 23].

They are considered as a stable system for the continuous production of secondary metabolites of uniform quality and yield. Another great advantage of plant cell cultures is the possibility to synthesize novel products not usually produced by the native plant (Zhang X *et.al* 2011, De Padua RM *et.al* 2012) [25]. This is the preferable biotechnological platform to produce high-value secondary metabolites, as taxol (Patal RA *et.al* 2014, Sharma K *et.al* 2016) [1], resveratrol (Cai Z *et.al* 2011), artemisinin (Baldi A *et.al* 2008) [2], ginsenosides (Jeong C *et.al* 2008), and ajmalicine (Ten Hoopen HJC *et.al* 2002).

Classification of secondary metabolites

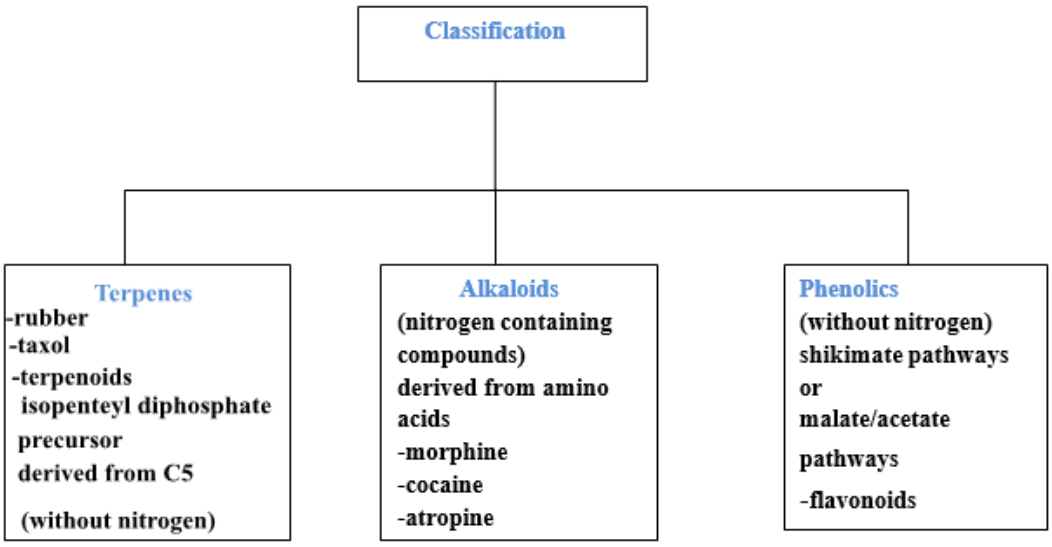

Fig 1

Production of secondary metabolites in callus culture

Callus culture is the culture of dedifferentiated plant cells induced on media usually containing relatively high auxin concentrations or a combination of auxin and cytokinin in *in vitro* conditions (Namdeo, 2007). Callus cultures can be embryogenic or non-embryogenic. Embryogenic calli contain differentiated embryogenically competent cells that can regenerate complete plants through the process called somatic embryogenesis (Ptak, 2013). Madhavi *et al*. (1998) studied the isolation of bioactive constituents from Vaccinium myrtillus fruits and cell cultures. Fruits and casus cultures were extracted and fractionated. Major fractions contained flavonoids, such as cyanidin-3-galactoside, cyanidin-3-glucoside, cyanidin-3-arabinoside and proanthocyanidins. Anthocyanin accumulation in callus was lower (0.08 mg-1 dry cell weight; DCW) than in the fruit (27.3 mg-1 DCW). Callus cultures accumulated both oligomeric (178 mg-1 DCW) and polymeric (436 mg-1 DCW) proanthocyanidins; proanthocyanidins were Similarly present in fruit extracts (oligo- and polymeric, 202 and 1613 mg-1DCW, respectively). Stable and optimized callus cultures are a logical step in the first phase of the cell culture production of plant secondary metabolites, i.e. preparing the inoculums for liquid suspension cultures. Production of secondary metabolites in cell suspension cultures have been widely published and it was proposed as a technology to overcome problems of variable product quantity and quality from whole plants due to the effects of different environmental factors, such as climate, diseases and pests (Yamamoto *et al*., 2002)

Strategies to improve the production of secondary metabolites

In the commercial exploitation of plant cell cultures for the production of high-value secondary metabolites, it is fundamental to achieve high yields and consistent productions.

The production of secondary metabolites in plants is genotype-dependent; thus, the first step to initiate cell or organ cultures is the choice of the parent plant containing higher contents of the secondary product of interest for callus or organ induction, and the selection of high-producing cell/organ lines (Murthy NH *et.al* 2014) [13]. The selection is made by analyzing cell/organ growth and then by quantifying the desired product by chromatographic and spectroscopic techniques (Murthy NH *et.al* 2014) [13]. Nevertheless, even selecting a highly productive line, the production yields are not always adequate, and after long periods of cultivation they lose their production efficiency. Thus, many alternative strategies can be used to stimulate the production of secondary metabolites and obtain efficient yields including traditional and metabolic engineering strategies (Isah T *et.al* 2018. Khani S *et al* 2012) [6].

***In vitro* propagation/ Micro propagation**

Micro propagation is a plant tissue culture technique used for producing plantlets and implies the culture of aseptic small sections of tissues and organs in vessels with defined culture medium and under controlled environmental conditions and has become an increasingly important tool for both science and commercial applications in recent years. It is the foundation on which all biotechnological research rests, because almost all uses of plant biotechnology ultimately require the successful culture of plants cells, tissues or organs. This technique has many advantages over conventional vegetative propagation, as e.g. the propagation of a great number of pathogen-free plants in a short time with high uniformity. The success of micro propagation involves several factors, as the com-position of the culture medium, culture environment, and genotype. The development of procedures for rapid *in vitro* clonal micro propagation of any plants may be a great commercial value to the industry. This review disc uses the different micro propagation techniques and various factors affecting the micro propagation of plants.

Plant micro propagation is an integrated process in which cells, tissues or organs of selected plants are isolated, surface sterilized, and incubated in a growth-promoting aseptic environment to produce many clone plantlets (Alt-man, 2000). The technique of cloning isolated single cells *in vitro* demonstrated the fact that somatic cells, under appropriated conditions, can differentiate to a whole plant. This potential of a cell to grow and develop a multi cellular organism is termed cellular totipotency. This potential of cells or tissues to form all cell types and regenerate a plant is the basic principle of tissue culture. In-vitro culture is one of the key tools of plant biotechnology that exploits the totipotency nature of plant cells, a concept proposed by Haberlandt (1902) and unequivocally demonstrated, for the first time, by Steward *et al*. (1958). Tissue culture is alternatively called cell, tissue and organ culture through *in vitro* condition (Debergh and Read, 1991). It can be employed for large-scale propagation of disease

free clones and gene pool conservation. Now a day's industry has applied immensely *in vitro* propagation approach for large-scale plant multiplication of elite superior varieties. As a result, hundreds of plant tissue culture laboratories have come up worldwide, especially in the developing countries due to cheap labour costs. However, micro propagation technology is more costly than conventional propagation methods, and unit cost per plant becomes unaffordable compelling to adopt strategies to cut down the production cost for lowering the cost per plant. The micro propagation process can be divided in five different stages.

Phase 0: growing mother plants under hygienic conditions. It involves the production of stock plants in green house.

Phase I: initiation of culture. The purpose of this stage is to initiate axenic cultures. It involves the selection of explants, disinfestations and the cultivation under aseptic conditions.

Phase II: rapid regeneration and multiplication of numerous propagules (multiplication phase). Masses of tissues are repeatedly sub cultured under aseptic conditions onto new culturing media that encourage propagule proliferation. The culture can supply shoots for the subsequent propagation phases as well as material that is required to maintain the stock.

Phase III: elongation and root induction or development (rooting phase). This phase is designed to induce the establishment of fully developed plantlets. It is the last period *in vitro* before transferring the plantlets to ex vitro conditions.

Phase IV: transfer to ex vitro condition (acclimatization). Acclimatization is defined as the climatic or environmental adaptation of an organism, especially a plant that has been moved to a new environment (Kozai and Zobayed, 2000).

Successful in-vitro propagation of plants is now being used for commercialization. Many commercial laboratories and national institutes worldwide use *in vitro* culture system for rapid plant multiplication, germplasm conservation, elimination of pathogens, genetic manipulations, and for secondary metabolite production. Annually, millions of plants are routinely produced in- vitro. The great potential of micro propagation for large-scale plant multiplication can be tapped by cutting down the cost of production per plant by applying low-cost tissue culture, which is to adopt practices and proper use of equipment and resources to reduce the unit cost of micropropagule and plant production without compromising the quality. Somatic embryogenesis facilitates cryopreservation, seed development, mutations, and genetic transformation. Recent progress in genetic manipulation of plant cells has opened new possibilities for improvement of plants which is totally depends on tissue culture.

Factors effecting in-vitro propagation

1. Media.
2. Type of explants.
3. Genotype.
4. Source of explants.
5. Orientation of explants.
6. Mineral nutrition.
7. Growth regulators.
8. Carbon source.
9. Gelling agents.

Low cost in-vitro propagation

Tissue culture refers to a set of techniques that permit the regeneration of cells, tissues and organs using nutrient solution in aseptic and controlled environment (Lima *et al*., 2012). Micro propagation or tissue culture method of propagation is one of the techniques with the potential of producing bulk of healthy planting materials without season limitation throughout the year. The recent studies done have shown that a single shoot tip has the potential of producing more than 6000 transformable plantlets per year (Amoo *et*

al., 2011). Tissue culture based plant propagation is carried out in highly sophisticated facilities which are expensive and often are not available in the developing countries. For example the cost media, chemicals, equipment and instruments used in micro propagation such as autoclaves for sterilization of media and instruments are often very expensive. Hence, options to expensive inputs and infrastructure are described and can be sought and developed to reduce the cost in micro propagation (Gitonga, *et al*., 2010). This necessitates the need to source for alternative low cost equipment and chemical facility studies have addressed the problem by decreasing the unit cost of production like low technology tissue culture materials for initiation and multiplication (Gitonga *et al*., 2010).

In low cost technology is achieved by improving process efficiency and better utilization of resources. Low-cost tissue-culture technology will stay a high priority in agriculture, horticulture, forestry, floriculture and medicinal plant of many developing countries for the production of suitably priced high quality planting material. In many developed countries, conventional tissue culture-based plant propagation is carried out in highly sophisticated facilities that may incorporate stainless steel surfaces, sterile airflow rooms, and expensive autoclaves for sterilization of media and instruments, and equally expensive glasshouses with automated control of humidity, temperature and day-length to harden and grow plants.

International scenario

Micro propagation has been identified as a suitable technology in the development projects of UNESCO in Africa and the Caribbean; however, the cost of production must be reduced (Brink *et al*., 1998). The private industry is the most important group that requires cost-effective technology. Many international organizations also agree that tissue culture technology is veryrelevant to agriculture, provided the problem of high cost of production is satisfactorily solved (FAO, 1993). The FAO Committee on Agriculture has perceived plant tissue culture as a main technology for the developing countries for the production of disease-free, high-quality planting material. In the frame of global efforts to halt the loss of by 2010, the Convention on Biological Diversity (CBD 1992; Glowka *et al*. 1994), and then the Global and European Strategies for. Economic and cultural aspects are the encroacher taken into account while establishing the evolved horticulture and farming methods (Khan *et al*. 2021). Plant Conservation (GSPC, ESPC, available at www.plan-taeuropa.org) have prioritized the in situ conservation of rare and threatened plant species and their back up by ex situ conservation in botanic gardens (Sharrock and Jones 2009).

Indian scenario

Low-cost tissue culture technology is the adoption of practices and use of equipment to reduce the unit cost of micro propagule and plant production. Many such facilities established at a high cost are high-energy users, and are run like a super-clean hospital. The requirements to establish and operate such tissue culture facilities are expensive. In India only very low few tissue culture companies are produce tissue culture raised plantlets compare to the developed countries. If the low cost tissue culture technology developed its very useful to the farmers, nursery owners and self-help groups.

Natural propagation/ vegetative propagation

Vegetative propagation or vegetative reproduction is the growth and development of a plan by asexual means. This development occurs through the fragmentation and regeneration of specialized vegetative plant parts. Many plants that reproduce asexually are also capable of sexual propagation.

Vegetative reproduction involves vegetative or non-sexual plant structures, whereas sexual propagation is accomplished through gamete production and subsequent fertilization. In non vascular plants such as mosses and liverworts, vegetative reproductive structures include gemmae and spores. In vascular plants, vegetative reproductive structures include roots, stems, and leaves.

Vegetative propagation is made possible by meristem tissue, commonly found within stems and leaves as well as the tips of roots that contain undifferentiated cells. These cells actively divide by mitosis to allow widespread and rapid primary plant growth. Specialized, permanent plant tissue systems also originate from meristem tissue. It is the ability of meristem tissue to continually divide that allows for plant regeneration required by vegetative propagation.

Podophyllum hexandrum

Classification

Kingdom	Plantae
Division	Mangnoliophyta
Class	Magnoliopsida
Order	Ranunculales
Family	Berberidaceae
Genus	Podophyllum
Species	Podophyllum hexandrum

The Himalayan region is home of numerous highly valued medicinal plants including Podophyllum hexandrum Royle which is a herbaceous, rhizomatous species of great medicinal importance, now endangered in India (Alam *et al*. 2009). Podophyllum hexandrum Royle belonging to family Berberidaceae, is a valuable medicinal herb commonly known as the Himalayan May Apple, grows in the Himalayan alpine and subalpine zones. Underground part i.e. rhizome and roots of the plant yield podophyllotoxin, an active ingredient used as a starting compound for the chemical synthesis of etoposide and teniposide, compound that are effective in treatment of lung cancer, a variety of leukemia's, and other solid tumors (Sharma *et al*. 2000). Though podophyllotoxin is present in different plant, but in sufficient amounts it is present only in some species of genus Podophyllum. Podophyllum comprises about 22 species (Airi *et al*., 1997) and out of the different species screened for podophyllotoxin and related lignans, Indian Podophyllum (Podophyllum hexandrum syn. P. emodi) and American Podophyllum (Podophyllum peltatum) have been found promising with reference to podophyllotoxin contents. Podophyllum hexandrum of Indian origin contains three times more podophyllotoxin than its American counterpart Podophyllum peltatum (Alam *et al*. 2009). There has been massive extraction of its rootstock (official part) over the last several decades leading to destructive harvesting. This has led to severe reduction in its population

density and the species is now listed in endangered plant species category. A species without enough genetic diversity is thought to be unable to cope with changing environments or evolving competitors and parasites. Considering the importance, threat perception and need for sustainable supply of its rootstock, there is need of not only multiplying its stock (by organized cultivation) but also assessing the relative active content concentration in its populations in different agro climatic regions and also in different morphotype. Such studies shall hopefully identify the best stocks/morphotype, which can be further multiplied (through cultivation). Further, studies on population genetic diversity and the structure of population within a species may not only illustrate the evolutionary process and mechanism but also information useful for biological conservation of Podophyllum hexandrum.

Distribution

In India, Podophyllum hexandrum has been reported to be distributed in all the Himalayan states like Jammu & Kashmir, Himachal Pradesh, Uttrakhand and Sikkim. In Jammu & Kashmir it is reported to occur at, Daitwas forest; Gilgit Gulmarg (2,700-3,000 m); Jagran river bank between Kundi & Shikar (3,000- 3,600 m) Kishenganga valley; Kanasar, Jhelum basin (2,400-2,700 m); Khelanmarg (2,700-3,000 m); Lid- was; Muzafarabad range forest (2,400 m); Sind Valley; Tanmarg forest (2,200-2,600 m); Zaskar (3,500m); Mechigaon, Zozila pass (3,500 m). Trumba, Dagoum, Chandanwadi, Seshnag, Kargil, Pissughile, Pahalgam.

Chemical constituents of Podophyllum

Lignans are an important group of natural products occurring in different plants species. Podophyllotoxin is the most important lignan due to its therapeutic value. Plants belonging to family Berberidaceae containing podophyllotoxin. The Indian species Podophyllum hexandrum contains three times more podophyllotoxin than its American counterpart Podophyllum peltatum, which contains other lignans *viz* α and β Peltatin (Bhattacharyya *et al*., 2012). The Indian Podophyllum yields 7-15 % resin as compared to American Podophyllum which yields only 4-8 % resins (Thakur *et al*., 2010 and Qazi *et al*., 2011). The different lignans isolated from roots and rhizomes of P. hexandrum are: 1) Podophyllotoxin; 2) 4'-Demethylpodophyllotoxin 3) Desoxypodophyllotoxin; 4) 4' Demethyldesoxy podophyllotoxin; 5) □-Peltatin; 6) □-Peltatin; 7) Podophyllotoxone; 8) 4'-Demethylpodophyllotoxone; 9) Isopicropodophyllone and 10) 4'-Demethylisopicropodophyllone (Anonymous, 1969; Dewick and Jackson, 1981; Husain, 1993).

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