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Review Article

Elicitation strategies of *in-vitro* cultures for the sustainable use of medicinal plants

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Abstract: Medicinal plants are highly traded for its promising potential against different types of diseases including cancers. Development of elicitation strategies for increased production of important anticancer compounds from *in vitro* cultures of medicinal plants has proved very productive. For this purpose, different stresses are applied to *in vitro* cultures to produce increased amounts of the compounds. For instance, cell cultures are produced via stem explants in the Murashige & Skoog basal medium supplemented with different concentrations of plant growth regulators (PGRs). The extracts from samples are then subject to flavonoid and phenolic content assessment, antioxidant quantification and Chromatographic analysis. In our experiments, among the many PGRs, Thidiazuron (TDZ) triggered higher quantities of biomass and total flavonoid & phenolic content (191.03 µg quercetin/mg and 202.8 µg gallic acid equivalent/ mg, respectively) through cell cultures of *F. indica*. Similarly, sucrose induced the maximum biomass among the different carbon sources (fructose, glucose, maltose, and sucrose) given in different concentrations to cell cultures of *F. indica* while glucose produced the maximum phenolic content followed by fructose when harvested after 42 days. Manipulation in the supply of light to the cultures with a combined effect from other chemicals, a significant effect was seen on growth and secondary metabolism such that dark-grown cell cultures treated with Methyl Jasmonate (Me-J) gave the highest TPC. High-performance liquid chromatography analysis revealed an increased quantity of secondary metabolites. In conclusion, cell cultures of *F. indica* treated with Thidiazuron and grown in dark in the presence of glucose as a sugar source and Me-J as elicitor gives enhanced quantities of important anticancer secondary metabolites.

Keywords: *F. indica, Callus*, Thidiazuron, *Anticancer, in vitro*

1. INTRODUCTION

Herbal medicine is the primary source of medical treatment in developing countries. Infact, the world health organization (WHO) describes herbal medicine as the major source of medicine (80%) in the world [1]. Asian countries such as Pakistan are rich in diverse medicinal plants [2, 3]. Plants that have been used for anticancer, anti-fever, anti-hyperglycemic, anti-inflammatory, antiseptic, antiviral, hepato-protective, ischemic, immune-stimulating, sedative, and spasmolytic properties [4-7]. Therefore, research has been conducted in-depth on the investigation of compounds in the extracts of different parts from many different medicinal plants. The components of medicinal plants harbor a wide range of medicinal compounds that have promising healing as well as preventive properties. It is, however, asserted that individual plants grown naturally do not contain enough medicinal compounds. These need to be concentrated in extracts which in turn need harvesting a huge amount of plant biomass to extract only ample quantities of medicinal compounds. This causes overharvesting and thus a threat to the existence of important plant species. Further to endangerment, seasonal and geographic dependence, climate dependence, and non-uniform metabolic profile of wild-grown plants calls are some limitations in the use of wild-grown medicinal plants [8]. This calls for the need of *in-*

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vitro cultures to grow important medicinal plants that are independent of season, geography, climate and have a uniform and novel medicinal compounds expressed through manipulations [9]. Apart from these advantages, *in-vitro* cultures offer room for strategies that can enhance the production of these medicinal compounds. This type of manipulation is known as elicitation [10].

An example of such an important plant that we work on in our lab is *Fagonia indica*. *F. indica*, commonly known as "true herb", is famous for its variety of medicinal activities especially anticancer potential. It is a member of the genus *Fagonia* and family *Zygophyllaceae*. The species of the genus *Fagonia*, especially, *F. indica* is famous for its diverse class of medicinally important compounds including terpenoids, flavonoids, and other polyphenolic compounds. The medicinal activities of *F. indica* especially antioxidant and anticancer activities may be attributed to its phenolic compounds. However, isolation only from wild-grown *F. indica* does not guarantees sustainable production of these metabolites. This is because of limitations with wild-grown plants such as over-harvesting, endangerment, seasonal and geographic dependence and variations in metabolic profiles of the plant. *In-vitro* cultures promise to deal with these limitations as they are independent of seasons and geography. Especially, cell cultures promise sustainable, uniform and homogeneous production of secondary metabolites. The current study highlights the various strategies for the enhancement of phenolic compounds through the establishment of feasible cell cultures of medicinal plants.

2. MATERIALS AND METHODS

This is a research review article that mainly focuses on strategies used with different plants for the elicitation of phenolic compounds. For reporting the data as secondary research, a thorough search was performed through scholarly databases including google scholars, PubMed, the web of science and Scopus. The search terms used alone or in combination were "medicinal plants", "herbal medicine", "plant metabolites", "plant *in-vitro* cultures", "elicitation", "and phenolic compounds".

3. RESULTS

3.1. Strategies for Production of Higher Biomass and Secondary Metabolites

Manipulations during *in-vitro* growth of plant tissues and cells bring changes to the accumulation of biomass and secondary metabolites in the cultures. Such manipulations are done at various levels with the aim of increasing biomass accumulation and production of medicinally important secondary metabolites. The many different strategies to manipulate *in-vitro* growth of plant cultures for increased yield can be classified into two broad categories: Manipulations in the medium composition, and environmental conditions.

3.2. Manipulations in the Medium Composition

The composition of the media has a prominent effect on the growth of *in-vitro* cultures and the production of secondary metabolites. The standard medium used for *in-vitro* cultures is MS medium. MS medium is comprised of MS salts (4 g/L of water) mixed with carbohydrate in the form of sucrose (30 g/L) and a gelling agent such as agar (8 g/L) [11]. Any type of manipulations in the basic composition of medium or addition of extra ingredients will affect the growth of cultures and production of secondary metabolites directly. For instance, changing the type and concentration of carbohydrate, mineral salts and introduction of several types and concentrations of PGRs such as auxins and cytokinin will drastically affect the growth parameters and secondary metabolites *in-vitro* [12, 13]. The most important media manipulations can be further classified as follows.

3.2.1 Manipulations of Carbohydrate Type and Concentration

In-vitro cultures require a continuous carbohydrate source in the media. As discussed above, the standard carbohydrate used in MS medium is sucrose at the ratio of 30 grams per liter of the medium. Sucrose is preferred because it is easily transported across the membranes and unlike monosaccharides, it is not rapidly metabolized and thus available for growth for a longer time [14]. Sucrose acts both as a metabolite and signaling molecule in plants and thus altered levels change the quantity of sucrose derived metabolites and sucrose-specific signaling

that in turn affects the plant growth, development, and physiology [15]. Carbohydrates like glucose, fructose, and maltose have been employed in media for *in-vitro* cultures to enhance the yield of secondary metabolites. For instance, callus cultures of *Gossypium hirsutum* L. (cotton) when grown in the presence of different sugars, showed that increasing the sucrose concentration increased the secretion of phenolic compounds. Similarly, increased biomass accumulation was observed in the presence of 3% maltose compared to other types of sugars [16]. Plantlets of *Metroxylon sagu* also showed better results in response 3% sucrose supplementation compared to other types of sugars. Therefore, the alteration in the sugar type and concentration from the generally optimized protocol results in the higher secretion of phenolic compounds and other secondary metabolites.

3.2.2 The Effects of Changes in Mineral Composition

Mineral salts in the media play a pivotal role in the growth and metabolism of the plant *in-vitro* cultures. MS medium is comprised of major and minor salts i.e. macro and micronutrients and they make an indispensable part of the medium [17]. These salts contain different elements notably nitrogen, magnesium, phosphorus, calcium, sodium, zinc and iron. All these are used as salts in the medium at different ratios, some being higher and some in minute quantities [11]. Changes in the ratio of these elements affect the ratio of mineral salts which in turn affect the growth and secondary metabolism of the plants. For instance, changes in nitrogen level directly affect the ratio of nitrate to ammonia which alters the course of secondary metabolism *in-vitro* [18]. Numerous studies have shown that increasing the levels of NO_3^- in proportion to NH_4^+, results in increased metabolite content. Increased levels of NO_3^- has a stimulatory effect on the yield of with anolide A and gymnemic acid in hairy root cultures of *Withania somnifera* [19]. The effect of nitrogen levels on cell and tissue cultures of different plants such as *Capsicum annuum*, *Solanum laciniatum*, *Artemisia annua*, and *Morinda cetrifolia* have been evaluated and optimized long ago [20]. Another essential macronutrient, phosphorus in the form of phosphates is also a vital component of the medium required for plant growth. The concentration of phosphorus affects the growth of plant cultures and influences the production of secondary metabolites. For example, Abdolzadeh, Wang [21] showed that deficiency of phosphorus resulted in the death of leaves in *Lupinus* species while excess or higher concentration inhibited cluster root formation in the plant. Furthermore, altering the levels of phosphates changes the production of secondary metabolites. For example, studies have reported elevated levels of different metabolites such as phenolics and alkaloids in *Gymnema sylvestre* [22], *Catharanthus roseus, Peganum harmala* and *Nicotiana tabacum* [23].

3.2.3 Effects of Plant Growth Regulators

PGRs are signaling molecules, actively involved in the regulation of growth and metabolism of plants. The two main classes of PGRs; auxin or cytokinin, are widely studied for their effects on plant growth, development, and secondary metabolism. Auxins are either natural such as indole-3-acetic acid (IAA), PAA, and indole-3-butyric acid (IBA) or synthetic such as NAA, 2,4-). Auxins play a vital role in cell division, elongation, and differentiation, and substantially influence the structure and function of cells and tissues [24]. Similarly, cytokinin such as BA, and Kinetin (Kn) are also reported to influence plant growth and development greatly [25, 26]. Different studies have demonstrated the role of cytokinin in the regulation of many aspects of plant growth and development including embryogenesis, root, and shoot branching, meristematic activity, phyllotaxis, and vascular development [27]. For example, studies have shown BA as the most suitable PGR for somatic embryogenesis in *Hygrophila spinosa*, *Sapindus mukorossi*, *Albizia lebbeck* [28, 29]. The role of auxins and cytokinin in secondary metabolism has been studied in many different plants [20]. For instance, NAA or IAA has a stimulatory effect on secondary metabolites production [23]. Similarly, Saeed, Ali [30] found that the administration of exogenous PAA to adventitious root cultures enhances the production of important phenolic compounds. PGRs other than auxin and cytokinin, such as Gibberellins, is also known to enhance the production of secondary metabolites [31]. Other studies have reported the combined use of auxin and cytokinin [32, 33]. One important PGR is TDZ, which is classified as cytokinin but believed to play roles both as auxin and cytokinin [34]. This PGR has a significant effect on *in-vitro* morphogenesis with roles in the initiation of callus cultures, shoots, somatic

embryos [34] and regeneration [35]. TDZ has been found very effective in yield-enhancement in secondary metabolism [36]. For instance, TDZ based enhancement of phenolic compounds has been reported in callus cultures of *Artemisia absinthium* [37] and *in-vitro* grown plantlets of *Cucumis anguria* [38]. Similarly, application of TDZ to callus and suspension cultures of *Salvia fruticose* resulted in the enhanced production of rosmarinic acid [39].

3.2.4 Chemical Elicitation of Secondary Metabolism

Secondary metabolism in plants is basically its defense system, producing chemical compounds that cope with damage induced by external and internal stresses. This means that application of any exogenous stress agent will stimulate the secondary metabolism of plants leading to the generation of chemical compounds, called secondary metabolites [40]. Secondary metabolites, because of their antioxidant nature, are important medicinal compounds. Strategies are applied during *in-vitro* cultures to enhance the production of these valuable metabolites. The process of triggering the metabolic pathways to produce metabolites in higher amounts is called as elicitation and the agents used for the process are called elicitors. Elicitors may be biotic or abiotic compounds [41]. Biotic elicitor may come from fungi, bacteria, animals and the same plant when it acts on invading pathogens or other plants while abiotic elicitors may be inorganic chemical compounds, metallic ions and very recently metal nano products [42] and environmental stresses. Elicitation through environmental stresses such as irradiation is discussed in the next section (1.9.2). There are many reports available on the effects of different biotic elicitors such as the plant hormone, jasmonic acid and its derivatives [43]. Jasmonic acid (JA) and Me-J belong to a family of cyclopentanone compounds that show a variety of responses in plant systems. Me-J is involved in elicitation of secondary metabolism to produce a diverse number of different metabolites [44]. For example, it enhanced the production of important phenolic compounds in adventitious roots cultures of *Ajuga bracteosa* [30]. Other studies have shown the stimulatory effect of Me-J on cell suspension [45], adventitious roots [46] and hairy root cultures of *Panax ginseng* [47]. Other types of biotic elicitors include Salicylic acid, bacterial extracts, fungal extracts, chitosan, and plant cell wall derivatives [44].

3.2.5 Environmental Manipulations

Culture environment is a key player in the growth and development of *in-vitro* plant cultures. Culture condition such as air supply, temperature, irradiation, and medium pH, etc. directly affect the accumulation of biomass and secondary metabolites from *in-vitro* cultures [23]. Manipulations in any of these culture conditions can be exploited as a process of elicitation of secondary metabolites. For example, numerous studies have reported the use of different temperature regimes on the accumulation of important secondary metabolites. For example, during *in-vitro* cultures of *Hypericum brasiliense*, temperature regimes either lower (17ºC) or higher (36ºC) than the normal (25ºC) induced higher phenolic compounds [48]. Similarly, Zobayed, Afreen [49] found that increasing the temperature during *in-vitro* growth of St. John's wort reduced the photosynthetic capacity and increased the accumulation of important secondary metabolites such as hypericin, pseudo-hypericin, and hyperforin in shoot tissues. Other conditions such as aeration (exchange of gases like carbon dioxide and oxygen) and pH drastically affect the growth and secondary metabolism of plants *in-vitro* [20]. Other abiotic elicitors include chemicals such as acetic acid, CO_2, ethanol, mercuric chloride ($HgCl_2$), copper sulfate ($CuSO_4$), metal ions and physical factors such as drought, extreme temperature shock, high-pressure inorganic salts, and irradiation [41].

3.2.6 The Effects of Light on Secondary Metabolites Accumulation

Light has been studied extensively for its effects on *in-vitro* growth and secondary metabolites accumulation in plants. Light is an important parameter that affects plant cultures in an array of ways ranging from its effect on growth and development [50] to primary and secondary metabolism [51]. Manipulations in light regimes are considered a very effective way of eliciting secondary metabolites in different *in-vitro* cultures. Light is the most important environmental factor affecting the biosynthesis of phenolic compounds [52]. Khan, Abbasi [53] reported enhanced silymarin content in plantlets of *Silybum marianum* grown under the effect of 2 weeks dark and 2 weeks

light. Similarly, Shohael, Ali [51] reported a high total phenolic and flavonoid content in somatic embryos of *Eleutherococcus senticosus* under the effect of fluorescent light. Manipulations in light regimes have been shown to enhance the production of caffeic acid derivatives in hairy root cultures of *Echinacea purpurea* [54], total phenolic production and total secondary metabolites in cell suspension cultures of *Artemisia absinthium* [55], lignans and neolignans in cell cultures of *Linum usitatissimum* [56] and piperine production in *Piper nigrum* [57]. Besides duration of light, intensity and wavelength have also been found to significantly affect the production of secondary metabolites. For example, changing the wavelength of light enhanced antioxidant secondary metabolites in callus cultures of medicinally important *Prunella vulgaris* [58] and *Artemisia absinthium* [59].

4. CONCLUSIONS

In conclusion, elicitation of *in-vitro* cultures to produce commercially and medicinally important phenolic compounds is an important and promising strategy for enhancement. It has the potential to control the overexploitation of medicinal plants as well as regulating the costs associated with producing effective herbal medicine. Elicitation produces uniform metabolic profile as well as can be geared to produce novel secondary metabolites in *in-vitro* cultures of medicinal plants. Diverse and comprehensive studies are needed for the elicitation of the different plant *in-vitro* cultures to build a profile of different effective elicitors for commercially enhanced production of medicinal compounds from plants.

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