Retraction Notice

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Editor guiding this retraction: Prof. Sukumar Saha (EIC of AJPS)

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Review on Medicinal Plants Propagation: A Comprehensive Study on Role of Natural Organic Extracts in Tissue Culture Medium

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Abstract

Plant cell, tissue and organ culture is a set of techniques designed for the growth and multiplication of cells and tissues using organic nutrients in an aseptic and controlled physical environment. The exact conditions required to initiate and sustain plant cells in culture, or to regenerate intact plants from cultured cells, are different for each plant species. Tissue culture practices have shown that three factors namely explant choice, medium composition and control of the physical environment, are important in successful cultures. When completely defined plant culture media did not give the desired results, employing natural organic substances have beneficial effects on *in vitro* plant cell and tissue cultures. The composition of different culture mediums and the effects of natural compounds, including the supernatant and freeze dried biomass of well growing algal strains, are presented in the present review.

Keywords

***In Vitro*, Plant Tissue Culture, Medicinal Plants, Culture Medium, Natural Organic Extracts**

1. Introduction

All living cells of a plant are capable of differentiating and dedifferentiating into whole plants. This inherent property of the cells called “cellular totipotency” has led to the concept of tissue culture studies. Plant tissue culture was originally developed as a research tool in order to study the biochemistry and physiology of plants. Plant tissue culture has advanced the knowledge of fundamental botany, especially in the field of agriculture, horticulture, plant breeding, forestry, somatic cell hybridization, phytopathology and industrial production of

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plant secondary metabolites etc. Plant tissue culture has turned into a standard procedure for modern biotechnology and has become one of the cornerstones of present day agriculture.

1.1. *In Vitro* Plant Tissue Culture Aspects/Methods

The term "plant cell and tissue culture" has become the generalised statement for a very broad subject. It covers all aspects of the cultivation and maintenance of any plant material ranging from a single cell to specified organ *in vitro*.

The process of tissue culturing, from explants to mature stage plants involves four basic steps:

- Explant initiation/Culture establishment;
- Shoot multiplication;
- Rooting;
- Acclimatization and Hardening.

In terms of practical aspects majorly five areas can be distinguished: Organ or Meristem cultures, Protoplast cultures, Anther and Microspore cultures, Callus cultures and Cell Suspension cultures ([1] & **Figure 1**).

1.2. Plant Propagation through Organogenesis, Meristem Culture and Somatic Embryogenesis

Mostly isolated primary or secondary shoot meristems (shoot apex, axillary buds) are induced to shoot under aseptic conditions. Generally, this occurs without an interfering callus phase, and after rooting, the plantlets can be isolated and transplanted into soil. Highly valuable single plants can be propagated by using this procedure. This technique has received a broad interest in horticulture for mass propagation of clones for the commercial market, and the production of virus-free plants.

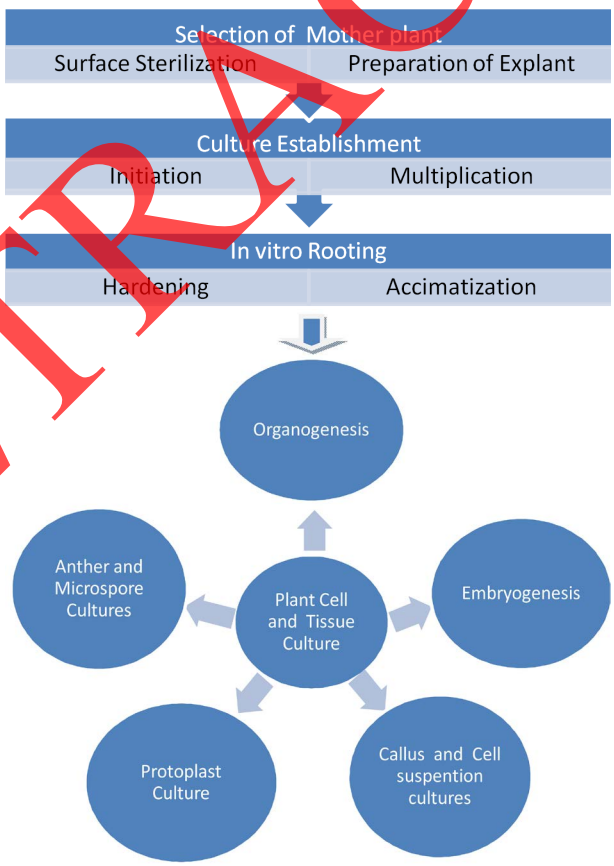

Figure 1. An overview on regular plant cell and tissue culture protocol & practical aspects.

1.3. Callus Cultures

Isolated pieces of a selected tissue (explants) are obtained aseptically from a plant organ and cultured on, or in a suitable nutrient medium. Primary callus culture derived from tissues with high contents of parenchyma or meristematic cells. In such explants, mostly only a limited number of cell types occur, so a higher histological homogeneity exists than that in the entire organ. However, growth of the explant in the nutrient medium usually results in an unorganized mass or clump of cells—a callus consisting largely of cells different from those in the original explant.

1.4. Cell Suspensions

Ideally all cells are isolated in a cell suspension. There are usually a high percentage of cells occurring as multicellular aggregates under practical conditions in these cell populations. A supplement of enzymes is able to break down the middle lamella connecting the cells in such clumps. Often, cell suspensions are produced by mechanical shearing of callus material in a stirred liquid medium. These cell suspensions generally consist of a great variety of cell types, and are less homogenous than callus cultures.

1.5. Protoplast Cultures

Initially the cell wall of isolated cells is enzymatically removed, and the explant is transformed into a single cell culture. This method has been used to study processes related to the regeneration of the cell wall, and to better understand its structure. The main aim in using this approach in the past however has been interspecies hybridizations. Nowadays, protoplasts are still essential in many protocols of gene technology. Plants can be regenerated through somatic embryogenesis to be used in breeding programs.

1.6. Anther or Microspore Cultures

Culturing anthers, or isolated microspores from anthers under suitable conditions, haploid plants can be obtained through somatic embryogenesis or organogenesis. It is possible to produce dihaploids, and within one year a fertile homozygous dihaploid plant can be produced from a heterozygous mother plant. This method is advantageous for hybrid breeding, by substantially reducing the time required to establish inbred lines. During culturing microspores initially a callus is produced, with separate formation of roots and shoots that subsequently join, and in due time haploid plants can be isolated. Another aim in using anther or microspore cultures is to provoke the expression of recessive genes in haploids to be selected for plant breeding.

2. Basic Requirements of Plant Cell and Tissues *in Vitro*

Growth and morphogenesis of plant cells and tissues *in vitro* are largely depends on composition of culture medium. The composition of culture medium, preferable to a certain plant species, is nearly the main task for the establishment of a successful plant cell and tissue culture technique. The underlying principles involved in plant tissue culture are simple. First, it is necessary to isolate a plant part. Second, it is necessary to provide the plant part with an appropriate environment in which it can express its intrinsic or induced potential. Third and most important is the above mentioned procedure must be carried out aseptically [2]. The requirements of cultured plant cells and tissues grown *in vitro* are similar in general to those of intact plants growing in nature. Although the basic needs of cultured plant tissues are similar to those of natural growing plants, in practice, nutritional components promoting optimal growth of tissues under *in vitro* conditions may vary with respect to cultural needs and plant species.

The earliest attempts to culture plant tissues and organs *in vitro* utilized the simple inorganic dilutions of Knop [3], Hoagland and Snyder [4]. They were used widely for the growth of whole plants. There is a small number of standard culture media that are widely used with or without additional organic and in organic supplements such as White's medium [5], Murashige and Skoog medium [6], Gamborg's medium [7], Nitsch and Nitsch medium [8] with lower inorganic compounds, are widely used for obtaining haploid tissues or embryos from cultured anthers. There is an almost unending list of plant tissue culture media with or without modifications to the basic formulation have been reported to be appropriate for *in vitro* cultivation of variety of plant species. The difficulties encountered in obtaining sustained growth of plant tissues *in vitro* can be attributed to

inadequate nutrition and poor choice of plant material. Plants synthesize most of the essential components and they are used in various metabolic processes. Cultured plant cells, tissues and organs lack the capacity to synthesize carbohydrates, most of the vitamins, and plant growth regulators. Plant-culture media generally consist of essential and optional components [9]. The essential nutrients consist of inorganic salts, a carbon and energy source, vitamins, and plant growth regulators. In addition to these standard components, in case of specific needs of particular species or tissues, other components, including organic nitrogen compounds, organic acids, and a wide variety of complex natural extracts, can be important but are optional. When the completely defined media did not give the desired results, enriching the culture medium with natural organic extracts like coconut water (milk), casein hydrolysate, yeast extract, malt extract, tomato and orange juice have beneficial effects on *in vitro* plant cell and tissue cultures.

3. *In Vitro* Studies on Medicinal Plants

Nature has provided a rich source of herbal medicines to cure various ailments of mankind. Plants are one of the most important sources of drugs, since every species in nature is of potential value to human beings. Medicinal plants are of great interest as pharmaceutical industries depend in part on plants for the production of secondary compounds [10]. A large number of medicinal plants are exploited from the natural flora for the commercial production of drugs. During the last few decades as a result of technology advancement and increasing demand of phytomedicines, medicinal plants used in various traditions were investigated chemically and phmacologically for the active chemical constituents and to elaborate new stand recipes for more effective treatments for specific ailments. In view of growing world populations, increasing anthropogenic activities, rapidly eroding natural ecosystems etc., the natural habitat for a great number of herbs and trees are dwindling and many of them are facing extinction [11] [12]. To cope up with this alarming situation, the recent exciting developments in biotechnology have come as a boon, now plant tissue culture become an integral part of biotechnology, which can be of immense help to overcome the problem of depletion of natural herbal wealth through rapid micropropagation methods. There are number of constraints for the propagation and conservation of many taxa through conventional methods like vegetative and seed propagation, which includes slow rate of multiplication, edaphic and climatic factors, dormancy, low percentage of seed set and germination. Sometimes, in spite of a high rate of germination, clonal uniformity cannot be maintained through seeds. Under these circumstances, *in vitro* techniques have been successfully applied to solve many constraints related to conventional clonal propagation in a large number of medicinal plants.

Major Applications of Plant Cell Tissue Culture in Medicinal Crops

- Large scale propagation of elite plant materials.
- Generation of genetically modified fertile individuals, as a model system for fundamental plant cell physiology aspects.
- Conservation of rare, endangered and threatened species.
- Conservation of endemic species.
- Up regulation/higher yields of secondary compounds through cell suspension cultures.
- Plant microbe interactions.

In view of above consideration an overview on medicinal plants propagation is presented. Special emphasis has been given to basic requirements of plant cells and tissues *in vitro*, with special reference to natural organic supplements as growth additives were discussed in detail.

4. Natural Organic Supplements on *in Vitro* Regeneration of Medicinal Plants

Growth and morphogenesis of plant tissue cultures can be improved by small amounts of some organic nutrients, which include vitamins, amino acids and certain undefined supplements. Sometimes it requires supplementation of additional substances in the medium for optimization of growth, the amount of these substances required for successful cultures varies with the species and genotype. Many undefined supplements were employed in early tissue culture media. Many of these amendments can be a source of amino acids, peptides, fatty acids, carbohydrates, vitamins and plant growth substances in different concentrations. Optimization of regeneration protocol supporting the action of growth additives as a supplement of growth regulators will be useful in the establish-

ment of reliable regeneration protocols for various medicinal herbs of economic importance. Application of growth additives is adapted to the cultural needs [13] *i.e.* objectives of the experimental studies like micro propagation, regeneration, cytodifferentiation, androgenesis, biosynthesis of secondary metabolites and biotransformation of cells as well as the particular plant species taken. The first successful cultures of plant tissue involved the use of yeast extract [14]. In the present review, extensive use of natural organic extracts as media supplement in a number of medicinal plants regeneration have been discussed in detailed and represented in a tabulated form **Table 1**.

Table 1. Effect of natural organic extracts as growth additives on *in vitro* regeneration of medicinal plants.

Name of the medicinal plant	Family	PGR concentration (mg/L)	Name of the growth additive	Nature of *in vitro* study	Reference No.
Gymnema sylvestre	Asclepiadaceae	BAP (1.0 mg/L) + KIN (0.5 mg/L)	Yeast extract Malt extract Coconut milk Casein hydrolysate	Regeneration	[61]
Orthosiphon aristatus	Lamiaceae	BAP (1.0 mg/L) + KIN (0.5 mg/L)	Coconut water Watermelon juice Asparagine Glutamine Peptone	Regeneration	[49]
Orthosiphon aristatus	Lamiaceae	BAP (1.0 mg/L) + KIN (0.5 mg/L)	CW (10%) + Glutamine (mg/L) CW (10%) + Asparagine (mg/L)	Regeneration	
Oroxylum indicum L. Vent	Bignoniaceae	BAP (1.0 mg/L)	Coconut milk Casein hydrolysate	Regeneration	[50]
Stereospermum suaveolens (DC.)	Bignoniaceae	BAP (0.1 mg/L)	Casein hydrolysate	Regeneration	[59]
Anogeissus pendula and *A. latifolia*	Combretacea	BA 4.4 mM + IAA 5.7 mM	1) Malt extract 2) Coconut water 3) Casein hydrolysate	Regeneration	[56]
Centella asiatica	Apiaceae	1) 2,4-D (4.52 µM/L) 2) 2,4-D (2.26 µM/L + IAA 2.85 µM/L)	Casein hydrolysate + Glutamine Casein hydrolysate	Somatic embryogenesis	[63]
Plantago ovata	Plantaginaceae—Plantain family	BA 4.4 mM + NAA 2.7 mM BA 4.4 mM + NAA 2.7 mM	Coco nut water Casein hydrolysate	Somatic embryogenesis	[62]
Cucumis sativus	Cucurbitacae	Benzyl adenine (0.044 mM)	L-glutamine	Regeneration	[69]
Syzygium cuminii	Myrtacae	Benzyl adenine (1.0 mg/L) Benzyl adenine (1.0 mg/L)	Casein hydrolysate L-glutamine	Regeneration	[60]
Eclipta alba	Asteracae	BA (4.4 µM) + KIN (4.6 µM)	Coconut water	Regeneration	[43]
Lavandula latifolia	Labiate	BA 8.8 µM + IAA 2.7 µM BA 4.4 mM + IAA 5.7 mM	Coconut water	Regeneration	[65]
Stevia rebaudiana	Asteracae	BAP (2.0 mg/L) + KIN (0.5 mg/L) + NAA	Yeast extract Malt extract Coconut milk Casein hydrolysate	Regeneration	[47]
Asclepias curassavica	Asclepiadaceae	KN (3 mg/L) + NAA (0.5 mg/L)	Coconut water	Regeneration	[51]
Eryngium foetidum	Apiaceae	1) BAP (2 mg/L) and NAA (1 mg/L) 2) BAP (2 mg/L) and NAA (1 mg/L)	Coconut milk Tomato juice Banana Yeast extract Beef extract Peptone Tryptone	Regeneration	[44]

Continued

Hedychium coronarium	Zingiberaceae	BAP (1.0 mg/L)	Casein hydrolysate Coconut milk	Regeneration	[52]
Psorolea carylifolia	Fabacae	BA (5.0 mM) + NAA (0.5 mM)	Casein hydrolysate	Regeneration	[57]
Acacia sinuata	Fabacae	BAP (6.66 µM) + Kn (4.65 µM)	Coconut water	Organogenesis	[45]
Acacia nilotica	Fabacae	2.5 µM − 10 µM 2,4-D + 5 µM − 25 µM BAP	L-glutamine	Somatic embryogenesis	[72]
Acacia sinuata	Fabacae	2,4-D (4.52 µM)	L-glutamine	Somatic embryogenesis	[71]
Acacia catechu	Fabacae	KN (13.9 µM) + NAA (2.7 µM)	L-proline	Somatic embryogenesis	[73]
Acacia auriculiformis	Fabacae	NAA (0.5 mg/L) + BAP (1.0 mg/L)	L-glutamine and L-arginine	Regeneration	[67]
Phyla nodiflora	Verbanaceae	2,4-D (0.6 mg/L) + BAP (1.0 mg/L)	Picloram, coconut water, citric acid	Somatic embryogenesis	[53]
Rauwolfia serpentina	Apocyanaceae	Basal medium	Coconut water	Regeneration	[46]
Arnebia hispidissimia	Boraginaceae	BAP (0.5 mg/L) + Kn (0.1 mg/L)	L-arginine	Regeneration	[65]
Murraya koenigi	Rutacae	2,4-D (2.5 mg/L) + BAP (0.5 mg/L)	Coconut water	Somatic embryogenesis	[56]
Stevia rebaudiana	Asteracae	2,4-D (1.0 mg/L) + BA (0.5 mg/L)	Tryptophan and L-glutamine	Embryogenic callus	[69]

Many undefined additions made to plant tissue culture medium are

- Fluids which nourish immature zygotic embryos;
- Juices, pulps and extracts from various fruits;
- Meat, malt extracts and fibrin digest;
- Extracts of seedlings or plant leaves;
- The extract of boiled potatoes and corn steep liquor;
- Protein hydrolysates;
- Plant sap and the extract of roots or rhizomes.

4.1. Fluids Which Nourish the Embryos (Coconut Water/Coconut Milk)

The most readily obtained fluid with this kind of activity is coconut milk (water). The coconut (*Cocos nucifera* L.) is an important fruit tree in the tropical regions. The edible part of the coconut fruit is the endosperm tissue. Endosperm tissues undergo one of three main modes of development, which are the nuclear, cellular and helobial modes [15] and the development of coconut endosperm belongs to the nuclear mode. Initially, the endosperm is a liquid containing free nuclei generated by a process, in which the primary endosperm nucleus undergoes several cycles of division without cytokinesis (the process in which the cytoplasm of a single eukaryotic cell is divided to form two daughter cells). Cytokinesis then occurs, progressing from the periphery towards the center, thus forming the cellular endosperm layer. At first, the cellular endosperm is translucent and jelly-like, but it later hardens at maturity to become white flesh (coconut meat). Unlike the endosperms of other plants (e.g., wheat and corn), the cellularization process in a coconut fruit does not fill up the entire embryo sac cavity, but instead leaves the cavity solution-filled. This solution is commonly known as coconut water and it is of cytoplasmic origin [16]. Nutrients from coconut water are obtained from the seed apoplasm (surrounding cell wall) and are transported symplasmically through plasmodemata, which is the connection between cytoplasms of adjacent cells into the endosperm [17].

The aqueous part of the coconut endosperm is termed coconut water, whereas coconut milk also refers to the liquid products obtained by grating the solid endosperm, with or without addition of water. The main components of coconut milk are water (ca. 50%), fat and protein [18], whereas coconut water contains mainly water.

Unlike coconut water, coconut milk, which is the source of coconut oil, is generally not used in plant tissue culture medium formulations [19]. Mauney *et al*. purified a growth factor from the aqueous extract of coconut meat which was found to be very potent in promoting growth of tissue cultured plants [20]. Another group, Shaw and Srivastava demonstrated the presence of purine-like substances in coconut meat extract [21]. The extensive use of coconut water as a growth-promoting component in tissue culture medium formulation can be traced back to more than half a century ago, when Overbeek *et al*. first introduced coconut water as a new component of the nutrient medium for callus cultures in 1941 [22]. Besides its nutritional role, coconut water also appears to have growth regulatory properties, e.g., cytokinin-type activity [19]. Some of the most significant and useful components in coconut water are cytokinins, which are a class of phytohormones [23]. Other components found in coconut water include sugars, sugar alcohols, lipids, amino acids, nitrogenous compounds, organic acids and enzymes [24].

4.2. Chemical Composition of Coconut Water

4.2.1. Active Ingredients

The remarkable growth stimulating property of coconut milk has led to attempts to isolate and identify the active principles.

4.2.2. Auxin Activity

Coconut water contains indole-3-acetic acid (IAA), the primary auxin in plants [25]. IAA is a weak acid (pKa = 4.75) that is synthesized in the meristematic regions located at the shoot apex and subsequently transported to the root tip in plants [26]. Auxin is implicated in many regulatory processes in plants especially those relating to plant growth and development [27].

4.2.3. Cytokinin Activity

Coconut water is an important additive in the tissue culture media of several plants, including orchids and traditional Chinese medicinal herbs. The cytokinins found in coconut water support cell division, and thus promote rapid growth. Cytokinins are a class of phytohormones that exert various roles in the different aspects of plant growth and development, e.g., cell division, formation and activity of shoot meristems, induction of photosynthesis gene expression, leaf senescence, nutrient mobilization, seed germination, root growth and stress response [28]. One advantage of coconut water is that it results in considerable plant cell proliferation without increasing the number of undesirable mutations [29]. However, it should be noted that cytokinins cannot completely substitute coconut water's effects. This is due to the presence of other phytohormones (such as auxin and gibberellins [25] [30] or even undefined chemical components which may exert synergistic effects with cytokinins).

Coconut water contains various cytokinins such as kinetin, trans-zeatin and their derivatives were discussed in detail.

4.2.4. N6-Furfuryladenine (Kinetin)

The first cytokinin, kinetin was discovered by Miller *et al*. in Wisconsin. It was a degradation product of herring sperm DNA and was found to be able to promote cell division in plants [31] [32]. Kinetin was previously assumed to be an unnatural and synthetic compound, until in 1996 Barciszewski *et al*. detected it in freshly extracted cellular DNA from human cells and in plant cell extracts [33]. And recently, Ge *et al*. identified kinetin and kinetin riboside from coconut water [34]. Being one of the cytokinins, kinetin has the effects on the plant developmental processes that could be influenced by cytokinins, such as leaf expansion and seed germination. Most importantly, kinetin is well known for its ability to retard senescence in plants [35]. Recent research evidence revealed that oxidative DNA damage plays an important role in cancer development and that dietary antioxidants can provide effective protection against oxidative damage [36]. Kinetin inhibited the formation of 8-oxo-2'deoxyguanosine, which is a common marker of oxidative damage in DNA and also it is known to inhibit oxidative and glycoxidative protein damage *in vitro*. Kinetin riboside exhibited a cytotoxic effect on plant crown-gall cells [37] [38].

4.2.5. Trans-Zeatin

Trans-zeatin was the first naturally-occurring cytokinin identified from a plant source (*Zea maze*) by Letham

[39]. In 1974, Letham identified trans-zeatin in coconut water [40], and a year later, Van Stadens and Drewes verified the presence of both trans-zeatin and trans-zeatin riboside in coconut water [41]. Based on experimental data, trans-zeatin plays a key role in the G2-M transition of tobacco cells. It was found to override the blockade of mitosis caused by lovastatin which inhibits cytokinin biosynthesis and controls cellular entry in mitosis [42]. Trans-zeatin riboside is the most abundant type of cytokinin found in coconut water, trans-zeatin is normally used to induce plantlet regeneration from callus in plant tissue culture.

4.3. Effect of Coconut Water (CW) on Regeneration

Coconut water supplementation to the plant tissue culture medium enhances the *in vitro* plant regeneration of many medicinal crops. An efficient rapid and large scale *in vitro* shoot multiplication of valuable medicinal herb *Eclipta alba* [43] has been standardized from catyledonory explants on proliferative medium supplemented with BA + Kn + 2ip + GA3 + 5% CW. Good regeneration frequencies and multiplication was achieved. In another report it was observed that 10% CM (v/v) showed best result as growth adjuvant in regeneration medium of *Eryngium foetidum* [44]. Similar progressive research findings have been well reported with *Acacia sinuata* [45], in which CW at 10% good regeneration frequencies and shoot multiplication was successfully achieved. In *Rawolfia serpentina* [46] a simple regeneration protocol has been developed using cut ovarian peices cultured on MS medium supplemented with CW at 10% (v/v). In a recent report on *Stevia* [47] CW supplementation as growth additive highly influenced the shoot bud initiation and multiplication, in which two fold increase in shoot multiplication was achieved compared to without CW supplemented cultures. *In vitro* regeneration studies on *Cyamopsis tetragonolobust* [48], it was observed that both 2,4-D and coconut water had a synergistic effect, either of them was not able to produce the same result when used separately. MS medium containing 20% CW (v/v) with 2 mg/L of 2,4-D resulted the maximum increase in regeneration. It was also observed that cultures grown on without CW turned brown after four weeks and need to transfer to fresh medium, whereas addition of coconut water to the medium had made the cultures survival for 8 weeks. In *Orthosiphon aristatus* [49], supplementation of CW reduces the time required for bud break and shoot initiation.

In contrary to the above promissory research findings supplementation of CW as growth additive in tissue culture medium resulted with negative influences on *in vitro* plant system of *Oroxyllum indicum* [50], explants remained totally unresponsive when cultured on MS medium fortified with CM. Significant reduction in frequency of shoot initiation, shoot number and length was observed. Increment in basal callus at cut ends was also observed after 6 - 8 days of inoculation. Callus was light brown in colour and non regenerative in nature. Similar results were reported with another medicinal herb *Asclepias currassavica* [51], in which CW at elevated levels leads to basal callus, as a result shoots remained stunted with robust leaves. This is due to in proper adjustment of cultured cells to excessive organic nutrients in tissue culture medium. Similar trend was followed in an another report on regeneration of *Hedychium coronarium* [52], explants when cultured on MS medium fortified with CM significant reduction in frequency of shoot number and shoot length was observed. Shoots developed were remaining stunted and yellowish in colour.

4.4. Effect of Coconut Water (CW) on Somatic Embryogenesis

The medium type, plant growth regulators and complex organic extracts such as coconut milk in suspension culture, markedly influenced the plant regeneration via somatic embryogenesis in *Phyla nodiflora* [53]. Optimized callus was transferred into suspension culture, which showed globular and heart shaped embryos in MS with 2,4-D, BA and CM (10 ml/L) on 6th sub culture. Further developmental stages such as torpedo and cotyledonary stage embryos showed maturation in 8th and 10th subculture. In an another report callus was developed on MS medium supplemented with auxins, 2,4-D and IBA at different concentrations in combination with 20% CW (v/v), which further leads to somatic embryo initiation from leaf explants of *Murraya koenigi* [54] has been well reported. In contrary to the above research findings, *in vitro* plant regeneration of *Plantago ovata* was attempted through somatic embryogenesis [55]. In which optimum concentration of CW was useful for promoting growth of embryogenic cultures, where as super optimal dose of CW induced polyphenol synthesis, browning of callus leads to eventual death of cells.

4.5. Casein Hydrolysate (CH)

Although protein hydrolysates are a convenient source of substances which may promote plant growth, they are

by nature relatively undefined supplements. The proportion of individual amino acids in different hydrolysates depends on the nature of the source protein and the method by which the product has been prepared. Casein hydrolysates (CH) can be a source of calcium, phosphate, several microelements, vitamins and most importantly, a mixture of up to 18 amino acids. Several casein hydrolysates are available commercially but their value for plant tissue culture can vary considerably. Acid hydrolysis can denature some amino acids and so products prepared by enzymatic hydrolysis are to be preferred. The best can be excellent sources of reduced nitrogen, as they can contain a relatively large amount of glutamine. Several investigators have concluded that casein hydrolysate itself is more effective for plant culture than the addition of the major amino acids which it provides. Although protein hydrolysates are a convenient source of substances which may promote plant growth, they are by nature relatively undefined supplements.

4.6. Effect of Casein Hydrolysate (CH) on Regeneration

Synergistic effect of complex extracts was studied after determining the optimum cytokinin and auxin levels for shoot sprouting to improve the quality of shoots was extensively studied earlier in a number of medicinal plants regeneration. In a recent report on stevia [47] regeneration fortification of CH resulted with good regeneration responses and multiplication of shoots was increased two fold. Similar research findings were reported on *Oroxyllum indicum* [50] regeneration using CH as growth additive. CH enhances the direct shoot regeneration and the number of shoots found to multiply 9.34 fold over control treatments at 20 mg/L CH is well documented. CH has also been found useful in *Anogeissus pendula* and *A. latifolia* [56] *in vitro* studies. The synergistic effect of PGR's and additives has been demonstrated in several medicinal plants. In accordance with these reports, the present investigation also exemplifies the positive modification of shoot induction efficiency by additive CH in combination with PGR's. In corporation of growth additive CH (50 - 200 mg/L) to the medium containing BA (5 µM) + NAA (0.5 µM) facilitated the growth of shoots, but did not affect the regeneration potential of explants in *Psorolea carylifolia* [57]. Growth of regenerated shoots was better at 100 mg/L CH. Induction of healthy shoot formation has been reported in *Crataeva nurvala* [58] using CH. In a recent report on shoot multiplication of *Stereospermum suaveolens* [59], nodal explants cultured on MS medium supplemented with 0.44 µM BAP and 25.0 mg/L CH resulted with 1.2 no. of shoots, 12.6 cm shoot length, and 2.0 nodes per micro shoot were obtained. Similar findings were also reported in another medicinal tree *Syzyzium cumini* [60] *in vitro* study. Nodal explants cultured on MS medium supplemented with different concentrations of BA and KIN. Among these BA (0.5/1.0 mg/L) induced greening and opening of incipient shoot buds, however which do not elongate. Elongation of shoot buds was facilitated on MS with 1.0 mg/L BA supplemented with CH (1.5 mg/L). In *Hedychium coronarium* [52] regeneration, *in vitro* raised plants in basal medium were served as explants cultured on MS medium supplemented with BA (1.0 mg/L) and CH (50 - 200 mg/L). Fortification of selected medium with CH supports the shoot multiplication and elongation. New shoots were obtained after four weeks maximum shoot length (5.5 cm) was obtained within 9 weeks on higher concentrations of CH. Where lower concentrations of CH was unsuitable.

In contrast with above research findings in *Asclepias curassavica* [51] supplementation of CH to the culture medium at 200 - 300 mg/L leads to basal callus at cut ends there by which inhibits the growth, further resulted with stunted shoots. These results are in agreement with *Gymnema* [61] *in vitro* studies, CH did not significantly improve the shoot sprouting frequency, shoot number and shoot length. Increase in concentration of CH resulted with basal callusing at cut ends and suppression of shoot bud proliferation. This is due to non adjustment of cultured plant cells to excessive organic nitrogen in the culture medium.

4.7. Effect of Casein Hydrolysate (CH) on Somatic Embryogenesis

The use of additives such as CH promotes large scale production of *Plantago ovata* [55] through *in vitro* somatic embryogenesis. Addition of CH along with 4.4 µM BA and 2.7 µM NAA in MS media led to increase in callus growth. Media containing 2 gm/L CH were more effective for the growth of embryonic cultures resulting in the formation of mass of nodular and compact callus with more than 65 embryos in 1 gm of callus. Optimum concentration of CH was useful for promoting growth of embryogenic cultures. However a super optimal dose of CH induced polyphenol synthesis and caused browning of callus and also eventual death of embryos. In an another report on *Centella asiatica* [62] somatic embryogenesis MS medium supplemented with 200 mg/L glu-

tamine and 100 mg/L CH in combination with different PGR's exhibited better survival response of explants. The results showed that SE was induced from stolon tips and leaf explants. Among which 2,4-D alone at 4.52 µM con. induced highest somatic embryo initiation. In addition to this 2,4-D (2.26 µM) in combination with IAA (2.85 µM) in leaf explants, moderate amount of somatic embryos were induced.

4.8. Malt Extract (ME)

Malt extract, mainly a source of carbohydrates, was shown to initiate embryogenesis in nucellar explants. Some plant hormones, such as auxins and gibberellins have been identified in malt extract. Although no longer commonly used, malt extract seems to play a specific role in cultures of citrus. Malt extract, mainly a source of carbohydrates, was shown to initiate embryogenesis in nucellar explants.

4.9. Effect of Malt Extract (ME) on Regeneration

In the present review, reports on malt extract supplementation to the culture medium for regeneration is very limited compare to other additives such as coconut water, casein hydrolysate and yeast extract. Though the usage of malt extract was limited, but in some medicinal crops such as *Gymnema sylvestre* [61], *Anogeissus pendula*, *A. latifolia* [56] and *Stevia rebaudiana* [47] enhanced regeneration frequency and shoot multiplication were recorded on malt extract supplementation. ME at a concentration of 100 mg/L enhanced the shoot number, prevented yellowing of leaves and reduced the callus formation at the cut end of the axillary node explants. Similar research findings were reported in a recent report on stevia regeneration using ME. Thus ME was found to be necessary for speedy proliferation of axillary buds and to improve the quality and quantity of shoots in *G. sylvestre* and stevia.

4.10. Yeast Extract (YE)

Culture medium is often supplemented with variety of natural organic extracts which have constituents of organic nature. Yeast extract (YE) is used less as an ingredient of plant media nowadays than in former times, when it was added as a source of amino acids and vitamins, especially inositol and thiamine (Vitamin B1). In a medium consisting only of macro and micro nutrients, the provision of yeast extract was often found to be essential for tissue growth. Yeast extract has been typically added to the media in the range of 0.1 - 1 g/L; occasionally 5, 10 and even 20 g/L respectively. It normally only enhances growth in media containing relatively low concentrations of nitrogen, or where vitamins are lacking.

4.11. Effect of Yeast Extract (YE) on Regeneration

Supplementation of yeast extract as growth adjuvant in plant tissue culture medium is a regular practice and it has been well established in many medicinal plants *in vitro* studies, such as *Eryngium foetidum* [44], *Stevia rebaudiana* [47], *Gymnema sylvestre* [61], *Asclepias curassavica* [51], *Lavandula latifolia* [63]. Majority of medicinal plants shows good regeneration response on supplementing YE to the culture medium at different concentrations. In a recent report, supplementation of YE at a moderate concentration enhances the shoot multiplication rate two folds in *Stevia rebaudiana* [47]. Among the different concentrations of yeast extract used, at 0.05% YE supplemented with 2.0 mg/L BAP + 0.5 mg/L Kin + 0.1 mg/L NAA maximum of 10 shoots with shoot length of 5.8 cm was obtained. The promotive effect of YE on shoot proliferation was reported earlier in *Lavandula latifolia* [64] *in vitro* studies.

In contrast with the above promising research report, in *Asclepias curassavica* [51] supplementation of YE to the culture medium resulted with basal callus growth and decrease in shoot multiplication rate. These results are in agreement with *Gymnema sylvastre* [61] *in vitro* studies. YE did not significantly improve the shoot sprouting frequency, shoot number and shoot length. At higher doses of YE browning of tissues and basal callus growth at cut ends resulted with the suppression of shoot proliferation.

4.12. Banana Homogenate

Homogenised banana fruit is sometimes added to media for the culture of orchids and is often reported to promote growth. The reason for its stimulatory effect has not been explained. One suggestion mentioned earlier is that it might help to stabilise the pH of the medium. Pierik *et al.* found that it was slightly inhibitory to the ger-

mination of *Paphiopedilum ciliolare* [64] seedlings but promoted the growth of seedlings once germination had taken place. In another recent report, good regeneration response was obtained with supplementation of banana homogenate to culture medium of *Erynigium foetidum* [44] belongs to apiaceae family.

4.13. Amino Acids as Reduced Nitrogen

Adding extra reduced nitrogen to the culture medium, in the form of amino acids is beneficial for shoot development.

4.14. Effect of Amino Acids on Regeneration

Amino acids glutamine and asparagine significantly improved shoot multiplication in *Orthosiphon arisstatus* [49] is well documented. In *Syzyzium cumini* [60] *in vitro* studies lower levels of L-glutamine (50 & 100 mg/L) had no significant effect on axillary shoot development. However higher levels (150 & 200 mg/L) effectively supported the elongation of multiple shoots, while highest level (250 mg/L) was totally inhibitory. An improved regeneration protocols were developed for *Arnebia hispidissimia* [65] and *Embelia ribes* [66] *in vitro* using L-glutamine. In an another report on *Acacia auriculoformis* [67] regeneration, hypocotyl explants were excised from 10 day old seedlings cultured on modified MS salts with Kin, BAP, 2,4-D and NAA either singly or in combination. BA (1.0 mg/L) and BA (1.0 mg/L) + NAA (0.5 mg/L) showed highest morphogenetic response. Glutamine enhanced the frequency of calli showing shoots and number of shoots per explant over the control explants. Similar results were also reported in other plant species such as *Cuccumis sativus* [68], where shoot tip explants were cultured *in vitro* on MS medium with L-glutamine as nitrogen source and an optimum concentration of BA (0.044 mM) to study their effect on *in vitro* morphogenesis. The explants grown with 0.068 mM of L-glutamine displayed the highest culture response (74.6%) and greatest shoot number (13.6) per explants at the end of two subcultures. The reduction of nitrate to ammonia is a limiting factor in the cells. Thus, the enhancement of growth rate in the presence of glutamine could be explained on the basis that it provides a readily available source of nitrogen to the plant cells. Also, glutamine is relatively non-toxic, hence it helps in enabling the cells to maintain high growth rate for a longer period.

4.15. Effect of Amino Acids on Somatic Embryogenesis

Influence of amino acids as growth additives on embryogenic callus induction has been well established in *Stevia rebaudiana* [69], where amino acids tryptophan and glutamine has showed pronounced effect on embryogenic callus initiation. Similar research findings were recorded in somatic embryogenic studies of *Acacia* species [70]. Various amino acids namely glutamine, alanine and proline were added individually to MS liquid suspension medium containing 4.52 µM 2,4-D, 10% coconut water prior to autoclaving to determine their effect on enhancing the frequency of somatic embryo production in *Acacia sinuate* [71]. MS liquid medium with 4.52 µM 2,4-D and 87.64 mM sucrose was used as control for this study. Glutamine at 342.46 µM enhanced somatic embryo production along with 4.52 µM 2,4-D, 10% coconut water and 87.64 mM sucrose. In *Acacia nilotica* [72] somatic embryos converted only in the presence of glutamine. All stages of embryos developed at optimal concentration of 342.46 µM glutamine. However higher concentrations of glutamine (>342.46 µM) in the medium significantly decreased the frequency of somatic embryogenesis compared to the control. When compared to glutamine, proline and alanine had little effect in enhancing the frequency of somatic embryo induction. Medium supplemented with proline was found to be beneficial for somatic embryo production in *Acacia catechu* [73]. These findings suggest that amino acids may play an important role in enhancing somatic embryogenesis *in vitro*.

5. Algal Biomass on *in Vitro* Regeneration of Medicinal Plants

Synthetic growth regulators enhance and accelerate the production of *in vitro* plants with good agronomical traits. The approach of using cyanobacterial cultures would overcome many barrier of micropropagation where costly synthetic chemicals are involved. Cyanobacteria or blue green algae are prokaryotic photosynthetic microorganism that produces a wide array of substances, including plant growth regulators. These include antibiotics, vitamins and plant growth regulators [74]. Among the growth regulators, gibberellins, auxin, cytokinin, ethylene, abscisic acid and jasmonic acid have been detected in cyanobacteria [75]. The reason for choosing

standard cyanobacterial culture is that it is known to produce the growth regulators auxin, cytokinin, gibberellin and other bioactive chemicals which are documented. These bioactive components produce vitamins, minerals, polyunsaturated fatty acid, carotenes, and other pigments that have an antioxidant activity to receiving attention.

The dilution of freeze-dried biomass from some micro algae and cyanobacteria could be useful for the improvement of *in vitro* culture media of economically important medicinal crops. Recent report on using cyanobacterial culture medium for *in vitro* callusing of *Stevia rebaudiana* [76] has been witnessed, in which MS salts and cyanobacterial extract as liquid medium were used for initiation and multiplication of adventitious shoot buds to ensure high yields of biomass. The combined effect of cyanobacterial media and MS (70 ml + 30 ml) was found optimal for maximum shoot proliferation showing 20 shoots per explant with 9.5 cm shoot length. Similar results were also obtained with another medicinal herb *Becopa monneri* [77], micropropagated *in vitro* using cyanobacterial medium. Best response was obtained on cyanobacterial medium supplemented with 2.0 mg/L Kn with 80% regeneration frequency with a maximum of 5.0 shoots per explant. In another report using *cyanobacterial* culture medium for standardizing regeneration protocol of *Becopa monneri* [78] [79], shoot lets were regenerated from nodal explants of stem through auxiliary shoot proliferation. Earlier many reports clearly stated the usage of either cyanobacterial extracts or extracellular products (filtrate) as media supplement. But in a recent published work, first time reported the use of cyanobacteria in regenerating somatic embryogenic callus and as a substitute to MS media [80]. In which cyanobacteria*i* medium showed significant promotive effect on somatic embryo initiation and induction of shoot primordial. This is a novel approach on stevia regeneration using cyanobacteria in the culture medium.

Recently published research works, reports the use of cyanobacterial extracts/filtrate and extracellular products. Further research are needed in this direction for using cyanobacterial cultures or extracellular products as media supplements for regular plant tissue culture practices. Since, these promissory photosynthetic microorganisms are known to excrete a large number of vital substances which have definite roles in plant *in vitro* development.

6. Conclusion

Efficient plant regeneration is the primary objective of many studies in plant tissue culture. Micropropagation system provides a method for rapid regeneration of various medicinal crops of high economic value. The improved *in vitro* plant culture system has the potential for commercial production of medicinal crops on large scale. During the past decade remarkable progress resulted in plant biotechnology has been witnessed with a constant flow of improved transformation regeneration protocols for many medicinal crops. A good regeneration protocol is always needed for genetic transformation studies for up-regulation of secondary compounds. In such instances usage of natural organic extracts in culture medium rejuvenates the *in vitro* plant system resulted with good regeneration frequencies and enhanced shoot multiplication, however these findings have been reported and well discussed in this review. Supplementation of natural organic extracts as additives for standardizing regeneration protocols of commercially important medicinal crops has been increased significantly. These promissory organic extracts described in this review would certainly be of increasing importance in near future in the field of medicinal plants research, such as genetic transformation studies and scale-up of secondary compounds through cell suspension cultures in bioreactors.

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Abbreviations

BAP—6 Benzyl Amino Purine
Kin—Kinetin
NAA—Naphthalene Acetic Acid
IAA—Indole Acetic Acid
MS medium—Murashige and Skoog medium
CW—Coconut Water
CH—Casein Hydrolysate
ME—Malt Extract
YE—Yeast Extract

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