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Polyamines, Gelling Agents in Tissue Culture, Micropropagation of Medicinal Plants and Bioreactors

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1. Introduction

Currently, tissue cultures of species of agricultural importance have wide applicability in industrial production processes. Tissue culture is a name given to a set of techniques that allow the regeneration of cells, tissues and organs of plants, from segments of plant organs or tissues, using nutrient solutions in aseptic and controlled environment. This regeneration is based on the totipotency of plant cells. Totipotency is a capability indicating that plant cells, in different times, may express the potential to form a new multicellular individual. Tissue culture appears to be a good alternative to conventional propagation, requiring less physical space, with high multiplication rate, without incidence of pests and diseases during cultivation, and enabling higher control of the variables involved. Thus, in the *in vitro* environment, with the required stimuli and appropriate conditions, different cell types express different behaviors, possibly leading to cell multiplication and differentiation into a specific tissue, characterized by a form and a function, which may lead to the regeneration of a new individual.

The discovery of this feature in plant cells is indistinguishable from the first studies on tissue culture in the early twentieth century by Heberlandt in 1902, which were followed by the first practical results reported by White in 1934 [1].

Over the years, various tissue culture techniques were developed, being micropropagation, meristem culture and somatic embryogenesis, the most used. The degree of success of any technology employing cultured cells, plant tissues or organs, is mainly dependent on the choice of the nutritional components and growth regulators which control, in a large extent, the developmental *in vitro* pattern. Therefore, it is crucial to evaluate the nutritional and

metabolic needs of cells and tissues of each species to be cultivated. In general, the choice of the medium is carried out taking into account, in addition to these needs, the purpose of the *in vitro* cultivation, maximizing plant development.

In general, the culture medium is composed of inorganic salts, reduced nitrogen compounds, a carbon source, vitamins and amino acids. Other compounds may be added for specific purposes, such as plant growth regulators, gelling agents, organic nitrogen compounds, organic acids and plant extracts.

Throughout the history of tissue culture, various kinds of culture media have been developed. However, the MS (Murashige & Skoog) medium [2] is the most widely used for the regeneration of dicots, and therefore it has a great importance in the applications of tissue culture in agriculture.

2. The use of polyamines as growth regulators

Many plant growth regulators have been used in vitro. Generally, literature reports on the use of auxins, cytokinins and gibberellins and different balances of auxins and cytokinins to encourage the development of specific organs. An auxin/cytokinin ratio of 10 induces the rapid growth of undifferentiated callus, a ratio of 100 leads to root development and a ratio of 4 favors development of shoots [3].

However, some studies report on the use of polyamines, as growth regulators. Polyamines (PAs) are low molecular weight aliphatic amines, implicated in various physiological and developmental processes in plants [4], such as growth regulation, cell division and differentiation, and also in the plant response to various sources of stresses. The exogenous application of PAs has been used by many researchers to provide or enhance growth and cell division [4]. The most common types of PAs are spermidine (SPD), spermine (SPM), and their diamine precursor, putrescine (PUT).

In plants, the mechanisms regulating both the biosynthesis and degradation of polyamines are less studied than in other organisms. Some papers proposed the use of exogenous polyamines during the processes leading to *in vitro* plant and callus formation [5,6] and endogenous PAs concentration has been related to several organo-genetic processes[7].

Beneficial effects of polyamines on *in vitro* regeneration and somatic embryogenesis were documented in several crops. Promoter effect of PAs in the conversion of somatic embryos or shoot regeneration has been evidenced in several plant species. The exogenous addition of PAs, mainly SPD or PUT at 2.0 mM concentration, not only elevated the endogenous levels of PAs but also enhanced the frequency of conversion of protocorm-like-bodied (PLBs) to shoots in *Dendrobium huoshanense* [8]. On the other hand, spermidine showed no effect on *Dendrobium* "Sonia". Among various tested polyamines, the maximum number of PLBs was produced with 0.4 mM putrescine treatment. The increase (1.0 mM) or decrease (0.2 mM) of SPD concentration caused a decrease in the production of PLBs. All treatments with spermidine and spermine resulted in the production of less number of PLBs than control. Thus, different responses to the application of different polyamines may occur [9].

High levels of free putrescine were correlated with the ease of cultures to reach stabilization, as observed with juvenile tissues. Adult tissues, containing low levels of putrescine, were difficult to stabilize in culture [10]. Furthermore, it has been reported [11] that the application of putrescine may reduce the production of unwanted ethylene and can enhance morphogenesis.

The exogenous application of polyamines has shown positive effect in the micropropagation of several species, for example, in buds from newly developed shoots, obtained from forced outgrowth of mature field-grown hybrids of hazelnut trees (*Corylus avellana* L.), cultured *in vitro* on MS medium [3] and on a modified Driver and Kuniyuki medium [12] [DKW] DKW/Juglans containing 6.7 μM, 11.1 μM or 15.5 μM N-6-benzyladenine (BA), supplemented with or without a combination of polyamines (0.2 mM putrescine + 0.2 mM spermidine + 0.05 mM spermine). The effects of culture medium and BA were found to be insignificant on explant response. Polyamines were found to have a strong effect on both shoot elongation and on the number of buds per shoot. Polyamines increased both the mean shoot elongation by 83% and the number of buds per shoot by 41%, compared to controls. In the presence of polyamines, shoot elongation continued up to 4.0 cm, while in the absence, the shoot elongation reached only 2.0 cm. Moreover, the results indicate that polyamines added to culture medium can improve the stabilization of cultures and enhance the morphogenic capacity of mature explants [13].

The effects of exogenous polyamines on somatic embryo formation in carrot (*Daucus carota* L.) cells were investigated [14]. The results showed an enhancement of somatic embryo formation following the addition of spermine. However, this effect was not always reproducible. Spm addition increased the DNA content in cells, regardless the development and the lag time of somatic embryo formation, and suppressed the protein secretion from cells.

Many studies have shown the beneficial effects of applying various polyamines on the rooting process. Polyamines have been shown to be key factors, in conjunction with auxins, in the process of adventitious rooting production [15,16]. The *in vitro* rooting of wild-type tobacco (*Nicotiana tabacum* cv. Xanthi) shoots was promoted by polyamines in the absence of any other growth regulator and was inhibited by two inhibitors of polyamine metabolism [17]. According to authors, some arguments causally implicated Put in the inductive rooting phase of poplar: i) the transient increase of Put did not occur in the non-rooting cuttings, ii) it was observed only in the basal rooting zone, iii) inhibitors of Put biosynthesis, such as DFMO and DFMA (α-α-difluoromethylornithine and difluoromethylarginine, respectively) applied prior to, or at the beginning of, the inductive phase, inhibited rooting [18], iv) an inhibitor of Put conversion into Spd and Spm, CHA (cyclohexylamine, an inhibitor of Spd synthase), promoted the accumulation of endogenous Put and favored rooting in the absence of auxin, v) the administration of Put, prior to, or at the beginning of, the inductive phase, stimulated rooting [19]. Some studies correlated this effect with PAs antioxidant properties, such as during the morphogenesis of *Hemerocallis sp*. It was verified that exogenous Put, alone (10 μM Put), with Spd (10 μM Spd + 10 μM Put) or with Spm (10 μM Spm + 10 μM Put), induced the best results, regarding both the number and the height of

differentiated shoots. The combination of the three polyamines in culture medium (10 μM SPD + 10 μM SPM + 10 μM PUT) induced the highest percentage of microplant formation, whereas the treatment with SPD and SPM (10 μM SPD + 10 μM SPM) led to the highest amount of necrotic tissue (65%). Spermine alone (10 μM Spm) was effective in controlling oxidative processes. In fact, oxidative stress during *in vitro* cultivation of plants occurs with high frequency [8].

However, in some species, exogenous polyamines do not play a significant role on *in vitro* growth, such as in *Hancornia speciosa*. In this specie, the application of exogenous polyamines did not improve the growth of callus [20]. Conversely, in most studies it has been reported that the use of specific polyamines or polyamine combinations can enhance the performances of tissue culture in some plant species, especially in those with low rooting potential, browning and hyperhydricity.

3. Alternative gelling agents

The *in vitro* cultivation of plant tissues is generally carried out in a solid or semi-solid nutrient medium, using gelling agents. Traditionally, agar is used, which is a polysaccharide extracted from seaweeds. This hydrocolloid is composed of agarobiose (3-β-D-galactopyranosyl-(1,4)-3,6-anhydro-α-L-galactose) [21]. The main differences among different agar-products are due to the impurities, their level and composition, which can vary according to manufacturers. Agar has been widely used since it has convenient gelling properties and stability during tissue culture. In all media used for *in vitro* culture of plants, agar is the major source of unknown variations [22], besides it's a high costs.

Gums, such as gelan, produced by bacteria and commercialized under the name of Gel-Gro®, Gerlite® (Kelko, Merck) and Phyta-gel® (Sigma), are polysaccharides that do not contain contaminating materials. Moreover, these products are used in lesser amount per liter than agar, to obtain the same consistency. They are added to the medium at approximately one fourth the concentration of agar. Furthermore, they appear more transparent. Despite emerging as alternatives to agar, the high cost of these products still limits their use in commercial cultures. These polysaccharides are imported from North America and Europe and therefore this leads to increasing costs for further micropropagation applications.

An alternative for cost reduction is the partial replacement of some of these gelling agents with other polysaccharides. Starch is an inexpensive alternative among studied gelling agents, and its use may reduce the costs of tissue culture. Nevertheless, starch is hydrolyzed by plant amylolytic enzymes during the in vitro culture. To circumvent this occurrence, the increase of air exchange in the bottle and consequently the increase of evaporation of excess water, may reduce this drawback [23].

Tropical countries possess a lot of starchy, little studied, native species, whose characteristics could serve still unfilled market niches. It should be considered that, among five of the raw products used in world for starch production, four are of tropical origin:

potato, cassava, maize and rice. Thus, the possibility to find native starch with specific properties is quite high in tropical regions.

In early experiments, using maize starch as gelling agent, the growth and differentiation of cultured plant cells from tobacco and carrot have been increased. In a medium solidified with starch, cell dry weight increased more than three times with respect to cells grown in a medium gelled with agar [24].

In India, several tissue culture studies were developed using gums and starches derived from tropical species, such as true sago palm (*Metroxylon sagu*) and "isubgol" (*Plantago ovata Forsk.*). Results revealed that these gelling agents were satisfactory in the micropropagation of chrysanthemum (*Dendrathema grandiflora* Tzvelev). Moreover, the cost of cultures was less than 5% of that of commercial agar, resulting in an alternative low-cost gelling agent for industrial scale micropropagation [25].

Isubgol was also successfully used as gelling agent for culture media for *in vitro* germination, formation of aerial parts and roots of *Syzygium cuminii* and cultures of anthers in *Datura innoxia*. Cultures showed similar responses to those observed in medium solidified with agar [26].

Katira gum, derived from the bark of *Cochlospermum religiosum*, was used as gelling agent in the culture medium for micropropagation of *Syzygium cuminii* and for somatic embryogenesis of *Albizzia lebbeck*. Various combinations of agar (0.2-0.6%) and starch (1-3%) were added to increase the firmness of the medium [27].

Guar gum, derived from the endosperm of *Cyamopsis tetragonoloda* and locust bean gum from carob (*Ceratonia siliqua*) (composed of mannose:galactose ratios of 1.6:1 and 3.3:1, respectively) is commercially produced in great amounts. Others, such as tare and fenugreek gums, have also been used [28]. These gums were used as gelling agents for *in vitro* multiplication and regeneration as well for germination of *Linum usitatissimum* and *Brassica juncea*, for the multiplication of aerial parts of *Crateavea nurvala*, and their subsequent rooting, for the *in vitro* androgenesis of anthers on *Nicotiana tabacum*, and for somatic embryogenesis of callus cultures on *Calliandra twedii*. The media were gelled with 2, 3 or 4% gum, as compared to agar (0.9%), and for all species the morphogenetic responses were improved in the medium gelled with guar gum [29].

Xanthan gum has minimal change in viscosity over a wide temperature range, and presents good gelling ability in a broad pH range (3.2-9.8). Xanthan gum, a microbial desiccation-resistant polysaccharide, is commercially produced by aerobic submerged fermentation of *Xanthomonas campestris*. It has a β-(1,4)-D-glucopyranose glucan backbone with side chains of (3,1)-α-linked D-mannopyranose-(2,1)-β-D-glucuronic acid-(4,1)-β-D-mannopyranose, on alternating residues. This gum, cream-colored, odorless, free flowing powder, hydrates rapidly in cold and hot water to give a reliable viscosity, even at low concentrations. It is highly resistant to enzymatic degradation, extremely stable over a wide pH range, and forms highly pseudoplastic aqueous solutions [30].

A gelling agent developed in Brazil (patent PI9003880-0 FAPESP/UNESP) was tested as an alternative to agar in the micropropagation of sweet potato (*Ipomoea batata*). The product consists of a mixture of starch from seeds of pigeonpea (*Cajanus cajan*) and cassava starch (*Manihot esculenta*) at a ratio of 2:3, being used as a gelling agent in the MS culture medium at a concentration of 7%. It was observed that this starch mixture increased the fresh weight of cultures when compared to microplants grown on agar. Thus, this starch mixture represents a good alternative for agar replacement in the micropropagation of sweet potato. Moreover, this substitution reduced by over 94% the final cost of the culture medium, demonstrating a high economic validity [25].

Another successful Brazilian experience deals with xyloglucans, extracted from the seeds of jatoba (*Hymenaea courbaril*) and mixed with agar to prepare a solid medium for the micropropagation of apples. The performance of this new mixture, composed of 0.4% agar and 0.2% xyloglucan (w/v), was verified on *Malus prunifolia Borkh* and cv. *Jonagored* (*Malus domestica*), and compared to the medium solidified with agar (0.6 %, w/v). The growth and the multiplication of shoots were higher in the modified medium. Furthermore, a lower incidence of hyperhydricity and a higher percentage of shoot rooting in the absence of auxin, were observed. When 0.25 mM indole-3-butyric acid (IBA) was added to both the media, the modified medium gave better results in terms of rooting percentage and root quality than the traditional agar medium [31].

The efficacy of the partial substitution of agar by galactomannans (GMs), obtained from seeds of *Cassia fastuosa* (cassia) and *Cyamopsis tetragonolobus* (guargum - a commercial GM), was tested in the micropropagation of strawberry (*Fragaria x ananassa* Duchesne cv. Pelican). GMs were mixed with agar in the proportion of 0.3/0.3 % (w/v) in MS medium, and the performances were compared with the behavior of the medium containing only agar (0.6% w/v). Strawberry shoots, grown in the modified medium, showed both an enhanced cell proliferation and a higher length of roots with respect to controls. These results showed that agar could be partially replaced by GMs, since experimental data confirmed a favorable interaction between the two polysaccharides.

Another study, involving guar gum extracted from the seeds of *Cassia fastuosa* or *Cyamopsis tetragonolobus*, mixed in equal proportions with agar to a final concentration of 0.3% (w/v) for each type of gelling agent, was carried out on the micropropagation of "Durondeau" pear (*Pyrus communis* L. cv. Durondeau). The production of multiple shoots and the formation of roots from shoots were compared with a control media, solidified with agar alone at a concentration of 0.6 % (w/v). In the medium solidified with the mixtures of agar/guar and agar/cassia GM, an increase in the number of regenerated shoots of 32 and 17%, respectively, was obtained. The modified media promoted both a higher number of roots and increased the rooting percentage. A maximum of 91% rooting was obtained in the medium solidified with the agar/cassia GM, containing 9.8 mM indole-3-butyric acid. With this medium, less callus formation at the base of the shoot was also observed [23].

In a study on the micropropagation of african violet, the cultivation of shoots in liquid medium, with cotton wad and different combinations of starch, semolina, potato powder as an alternative to agar, was tested. The highest frequency of regeneration was found in media containing agar (0.8%) or with a combination of starch, semolina, potato powder (2:1:1) and starch (6%) plus agar (0.4%). The maximum numbers shoots was produced in media containing agar (0.8%), the combination of starch (6%), plus agar (0.4%) and in liquid medium with cotton wad substrate. The best shoot proliferation took place in liquid medium with cotton substrate. The results showed that the combination of starch, semolina, potato powder (2:1:1) and 6% starch, plus 0.4% agar, can be suitable alternatives for agar alone in shoot regeneration step, but shoot number will result lower than in agar alone. These options are cheaper than agar [32].

The type of medium (liquid or semi-solid) can directly affect the rooting process. Although the liquid medium positively influences the availability of water, nutrients, hormones and oxygen levels, the vast majority of protocols for micropropagation were established in semi-solid media [33].

Some other agar substitutes have been tested, such as corn starch mixtures (Gelrite®), used for shoot proliferation of apples, pears and red raspberries [34]. However, their use may induce some problems during micropropagation. Gelrite®, for example, causes hyperhydricity and, in some cases, vitrification on regenerated shoots [35]. Hyperhydric shoots are characterized by a translucent aspect due to a chlorophyll deficiency, a not very developed cell wall and high water content. The losses of up to 60% of cultured shoots or plantlets, due to hyperhydricity, have been reported in commercial plant micropropagation [36], reflecting the importance of this problem. The hyperhydricity of shoots was induced in *Prunus avium*, after four weeks multiplication cycles in Gelrite® [37]. This performance has been confirmed by other authors [38]. The vitrification symptoms (thick and translucent stems and leaves, wrinkled and curled leaves) were found on 100 % shoots, after 21 days culture.

Besides the properties of these described substances, it is not always possible to use these agents. In studies with *Eucalyptus grandis* x *E. urophylla* in liquid medium containing aluminum, an acrylic blanket was used to support the shoots, because the addition of $AlCl_3.6H_2O$ decreased medium pH (to around pH 3.5), affecting medium ionic balance, not allowing the gelation of agar [39].

4. Micropropagation of medicinal species

Medicinal and aromatic plants are of great importance for the pharmaceutical industry and traditional medicine in several countries. Just to emphasize the importance of medicinal plants in their various aspects for human health, data from the World Health Organization (WHO) indicated that 80% of the population uses them as basic drugs, in traditional medicine, in the form of plant extracts or their bioactive compounds.

Micropropagation of some medicinal plants has been achieved through the rapid proliferation of shoot-tips and ancillary buds in culture. The success of micropropagation of medicinal species opens perspectives for the production of seedlings, followed by selection of superior genotypes, their clonal multiplication for obtaining genotypic uniformity within the plants to use in plantations at high productivity. Numerous limiting factors have been reported to influence the success of *in vitro* propagation of medicinal plants and, therefore, it is unwise to define a particular protocol for the micropropagation of these species [40].

In fact, a large-scale application depends mainly on the development of an efficient protocol for proliferation, rooting and acclimatization of explants in *ex vitro* conditions.

Jaborandi plant (*Pilocarpus microphyllus*) is a tree, extensively used in the pharmaceutical industry for the manufacture of drugs, used for the treatment of glaucoma. The micropropagation of this specie was conducted on apical explants grown in MS medium, supplemented with 6.66 mM benzyl-aminopurine (BAP) [41]. *Echinodorus scaber* is an herbaceous water plant with cleansing, anti-ophidic, diuretic, anti-rheumatic and anti-inflammatory effects, used in traditional medicine to normalize uric acid, treating gout and osteoarthritis, and also used for the manufacturing of soft drinks. The protocol for this specie consists in the inoculation of nodal explants in MS medium, supplemented with 1 mgL^{-1} BAP [42].

For the micropropagation of *Tournefortia paniculata*, a shrub whose leaves are used as decoction for diuretic and urinary infections, the shoots were grown on WPM (Lloyd & McCown) medium, supplemented with 1 mg L^{-1} BAP, and shoot rooting was induced in the same medium, without growth regulators. For acclimatization, microplants were placed in a commercial substrate, and cultured in a greenhouse, and then transplanted in the field [43].

Lipia gracilis plant is rich in essential oils, containing thymol and canvacrol, as main chemical compounds, responsible for the proven antimicrobial activity. A protocol for its micropropagation has been defined, using MS medium supplemented with 1 mg L^{-1} BAP [44].

The proliferation success, expressed as number of new produced shoots, is the most important parameter that should be optimized *in vitro*, since often the proliferation process is slow and, as consequence, the number of produced plants is limited. This feature can also be combined to obtain secondary metabolites. Micropropagation ensures a uniform production, which cannot be guaranteed by cultivation of seeds of allogamous plants, due to the high genetic variability of offsprings, which cannot guarantees the uniformity on types and levels of metabolites. Several protocols for medicinal plants have been described [42], but many species still require further studies, in particular, to increase or maintain the production of desirable metabolites.

The micropropagation of medicinal plants is particularly important if the plant produces few seeds, seeds with low germination potential and needs special care to reach the mature

stages. As an example, *Jatropha elliptica*, an herbaceous native species in Brazilian cerrado with purgative properties, used in the treatment of severe itching, syphilis and treatment of snake bites, presents low seed germination potential, about only 6%. The developed micropropagation protocol consists of MS medium, supplemented with 0.5 μmol L^{-1} indolacetic acid (IAA) and 1 μmol L^{-1} BAP for the multiplication step, and 5 μmol L^{-1} naphthalene acetic acid (NAA) for shoot rooting [45].

An efficient micropropagation protocol has been proposed for *Stevia rebaudiana*, an important antidiabetic medicinal plant. The highest frequency (94.5%) of multiple shoot regeneration and maximum number of shoots (15.7 shoots per explant) were obtained on MS medium, supplemented with 1.0 mg L^{-1} BAP. Furthermore, the *in vitro* derived nodal explants, grown in the same nutritional conditions, produced a total of 123 shoots per explant after only three subcultures. The highest frequency of rooting (96%) was obtained on half-strength MS medium, in the presence of 0.4 mg L^{-1} NAA. The rooted plantlets were successfully transferred into plastic cups, containing sand and soil, in the ratio of 1:2, and subsequently transferred in the greenhouse [46].

Some medicinal plants are "recalcitrant" to vegetative propagation, and in these cases, the technique of *in vitro* cultivation, and the use of different plant growth regulators, can synergistically help plant production. *Byrsonima intermedia* A. Juss is a shrub found in Brazilian cerrado, whose husk presents medicinal activities in diarrheas and dysenteries. The *Byrsonima* genus presents low germination rate and slow plantlet emergency, which makes sexual propagation difficult. The effect of different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D), thidiazuron (TDZ) and BAP in callus formation, was evaluated. The results demonstrated that there was no formation of callus in leaf explants maintained in the absence of 2,4-D, and that the addition of TDZ and BAP had no influence in the callogenetic process. For callus induction and proliferation, the results suggested the use of MS medium, supplemented with 1.0 mgL^{-1} 2,4-D, maintaining the explants in the dark [47].

Another species, presenting difficulties in spreading, is the Brazilian ginseng (*Pfaffia tuberosa*), whose roots are used to produce drugs and dietary supplements. A protocol for its micropropagation was developed using nodal explants cultivation in MS medium supplemented with 1 mmolL^{-1} thidiazuron (TDZ), followed by shoot subculture on the same medium, lacking of growth regulators. The methodology was proven to be valid, due to the high rate of multiplication, good development of shoots, roots and great adaptation to *ex vitro* conditions [48].

The use of tissue culture techniques is also of great importance in the development of new plant products, by preserving germplasms. Micropropagation is a useful technique to preserve the gene pool, especially in cases where small amounts of viable seeds are produced. As an example, canopy (*Canopy macrospyphonia*), a medicinal species, considered highly vulnerable and widely used by traditional communities inhabiting Brazilian cerrado.

For this species, micropropagation would be an alternative to avoid genetic erosion. Thus, a protocol for its *in vitro* micropropagation and conservation in germplasm bank was developed. It was found that half diluted MS medium (MS/2), without growth regulators, promoted the proliferation of shoots (4 per bud), shoots elongation (5.2 cm), number of buds (6-8) and reduced vitrification (4%). Seedlings were grown for three months in MS/2 culture medium, supplemented with 2% sucrose, 4% mannitol, 2 mgL^{-1} calcium pantothenate, under germplasm Bank conditions, showing 40% survival. Regarding substrates, seedlings grew better in Plantmax®, where 40% of the seedlings survived and the majority showed root formation [49].

Other plants threatened with extinction can be grown *in vitro*, to maintain a germplasm bank, and to guarantee the possibility of increasing their population, as is the case of *Rhinacanthus nasutus* (L.) Kurz., used as a potent ethno-medicinal plant for various diseases, including cancer. Since the plant is collected throughout the year for roots and leaves, it increasingly disappeared from its natural habitat, and, as a consequence, its distribution in nature is reduced to an alarming rate. Natural resurgence of this plant is through seeds and stem cuttings. Low seed germination and viability is another factor that hampers the natural propagation of this plant. The possibility to regenerate whole plants from leaf derived callus was reported. High numbers of shoots (36.5) were obtained through an intervening callus phase. The composition of the media had a significant effect on both percentage of callus formation and subsequent plantlet regeneration. Optimum callus induction (98.8%) was obtained in MS medium, supplemented with kinetin (Kn) (4 mgL^{-1}) and indolbutiric acid (IBA) (0.5 mgL^{-1}). However, the highest callus regeneration (96%) was observed in MS medium, supplemented with 3 mgL^{-1} Kn and 0.5 mgL^{-1} NAA. An important finding in this study was that the ISSR (Inter Simple Sequence Repeat) analysis of *R. nasutus* results in shoots with low degree of variation and statistically not significant differences [50].

Cytotoxic activities of plant extracts against human tumor cell lines can be studied utilizing micropropagation. The stabilization of shoots and roots of *Cistus creticus* subsp. *creticus* L. in *in vitro* culture in solid or liquid MS medium, without addition of plant growth regulators, was studied [51]. Authors verified the presence of labdane diterpenes in shoot extracts, but they were absent in root extracts. The cytotoxic activity of shoot extracts, by sulforhodamine B (SRB) assay on five human cancer cell lines, was verified. In particular, active extracts showed cytotoxic activity on HeLa (cervix), MDA-MB-453 (breast) and FemX (melanoma) cancer cells, with IC$_{50}$ reaching 80.83 mg mL^{-1} on HeLa, 76.18 mg mL^{-1} on MDA-MB-453, and 87.52 mg mL^{-1} on FemX cells, respectively.

A wide application of micropropagation techniques on medicinal plants for clonal propagation and production of virus free plants, if characterized by low immunity to infections, was reported. This is the case of *Aloe vera*, a species traditionally propagated by seedling production, where there is a high probability of disease occurrence in planting material, depending on the injury done to mother plants, seedlings and lateral shoots, at the time of harvest. Thus, micropropagation presents the advantage of a system with high genetic quality and health, and high income [52].

5. Biochemical responses of plants cultivated in vitro

The *in vitro* culture can be used to study the biochemical mechanisms of plant survival, as a function of medium changes, which may produce different biochemical and physiological responses. The *in vitro* cultivation of plants can induce different responses according to the medium used, such as the presence of regulators, gelling agents, minerals, and other components. The concentration of many plant metabolites responds to these changes more or less quickly, but all endogenous molecules may show variations, mainly due to some kind of stress. Stress intensity (pressure to change exerted by a stressor) is not easily quantified. Stress could occur at a low level, creating conditions that are marginally non-optimal, with little expected effect. However, if this mild stress continues for long time, becoming a chronic stress, the physiology of plants is likely to be altered [53].

Tissue culture has been used as a model to study biochemical responses to different stress types on medicinal plants, as reported for oregano (*Origanum vulgare L.*) by using shoot buds as potential model system for studying carbon skeleton diversion from growth to secondary metabolism, as adaptive response to nutrient deficiency. Nutritional stress caused a moderate increase of constitutive free proline, and exogenous proline affected growth and phenolic antioxidant content of oregano shoots, compared to control. The role of proline, and its association to redox cycles, can be considered as a form of metabolic signaling, based on the transfer of redox potential amongst interacting cell pathways, which in turn elicits phenolic metabolism, stimulating carbon flux through pentose phosphate oxidative pathway [54]. Other studies also showed changes in response to mineral stresses, as the study carried out using micropropagation on *Eucalyptus grandis x E. urophylla*, grown with the addition of aluminum (6.75, 13.5 and 27 mgL^{-1} of $AlCl_3.6H_2O$). It was observed that the metal affected the ionic equilibrium of the culture medium, the morphology of the shoots, and that the reduced medium pH induced an increase in polyamines content and a higher acid phosphatase activity [41].

As mentioned above, hyperhydricity is another problem that can occur using Gelrite®. This was observed in *P. avium* shoots, causing a production of higher amounts of ethylene, polyamines, and proline, which are substances considered as stress markers. The higher activity of glutathione peroxidase (GPX, EC 1.11.1.9), involved in organic hydroperoxide elimination, suggested an increased production of these last compounds in hyperhydric state [39].

Plant cell culture is a methodology, which can be used to study or to produce some active metabolites, such as polyphenols. *In vitro* culture can be used to explore new industrial, pharmaceutical and medical potentialities, such as the production of secondary metabolites, like flavonoids. This technique was applied to the investigation of *Coriandrum*, and a detailed analysis of individual polyphenols on *in vivo* and *in vitro* grown samples, was performed. *In vitro* samples also gave a high diversity of polyphenols, being C-glycosylated apigenin (2983 mg kg^{-1} d.w.) the main compound. Anthocyanins were found only in clone

A, certainly related to purple pigmentation, and peonidin-3-O-feruloylglucoside-5-O-glucoside was the major anthocyanin found (1.70 mg kg^{-1} d.w.) [55].

These studies show the potential of tissue cultures in plant tissue technology aimed to the production of antioxidants compounds. Therefore, even with the increase of these compounds, plants may present reduced growth, or other problems, such as hyperhydricity. On the other hand, studies involving bioreactors can be an alternative for the production of secondary metabolites.

6. Bioreactors

The use of bioreactors in laboratories and biofactories is already a reality and the trend of its increasing application is indisputable. While conventional micropropagation uses small flasks, with a small number of plants per flask and requires intense manipulation of the cultures, then, involving a large amount of skilled work, the bioreactor uses large bottles containing liquid medium with large amounts of plants, which reduces significantly the demand of skilled operators. Bottles used in conventional micropropagation typically contain less than 0.5 L of culture medium, while bioreactors, on the other hand, may contain amounts ranging from 1.5 to 20 L [56].

Pioneering studies, published in the 90's, showed the superiority of bioreactors on plant multiplication rates, when compared to conventional systems with semi-solid or liquid medium. In the propagation of pineapple (*Ananas comosus*), the multiplication rate in bioreactors was four times higher than that obtained in conventional systems [57]. In banana (*Musa acuminata*) this multiplication rate was five times higher [58] and in sugarcane (*Saccharum edule* Hassk.), it was six [59].

Bioreactors are used in the micropropagation of several crops, including ornamental and medicinal plants, vegetables and fruits. Studies showed that more than 40 plant species are commercially propagated in bioreactors [60]. By cultivation in bioreactors, different plant parts can be obtained, such as buds, somatic embryos, bulbs, shoots, calluses, protocorm and others. Bioreactors are currently being used for commercial micropropagation in U.S., Japan, Taiwan, Korea, Cuba, Costa Rica, Netherlands, Spain, Belgium, France [61], and Brazil (http: biofrabricasdemudas.blogspot.com).

The design of the first bioreactors, used for propagation of plants, was derived from fermenters used for cultivation of bacteria and fungi cultivation for industrial purposes. Currently, there are several types of bioreactors developed specifically for *in vitro* plant cultivation [62]. Among the different types of plant micropropagation bioreactors, the Temporary Immersion Bioreactor (TIB), developed in the mid 90's, stands out [61]. This type of bioreactor consists of two glass or plastic vessels, one of which contains the plant material in culture (plant culture vessel), while the other stores the culture medium. The transfer of the medium to the flask containing the plant material occurs through silicone hoses, driven by positive pressure of an air pump. Generally, explants are immersed in the culture

medium temporarily. The soaking process occurs at regular intervals of predetermined time. When the time of immersion is finished, the air pump starts and a solenoid valve drive the air flow to the opposite direction. The circulating air in the system is sterilized by passing through filters with pores of 0.22 to 0.44 μm, coupled to silicone hoses (Figure 1).
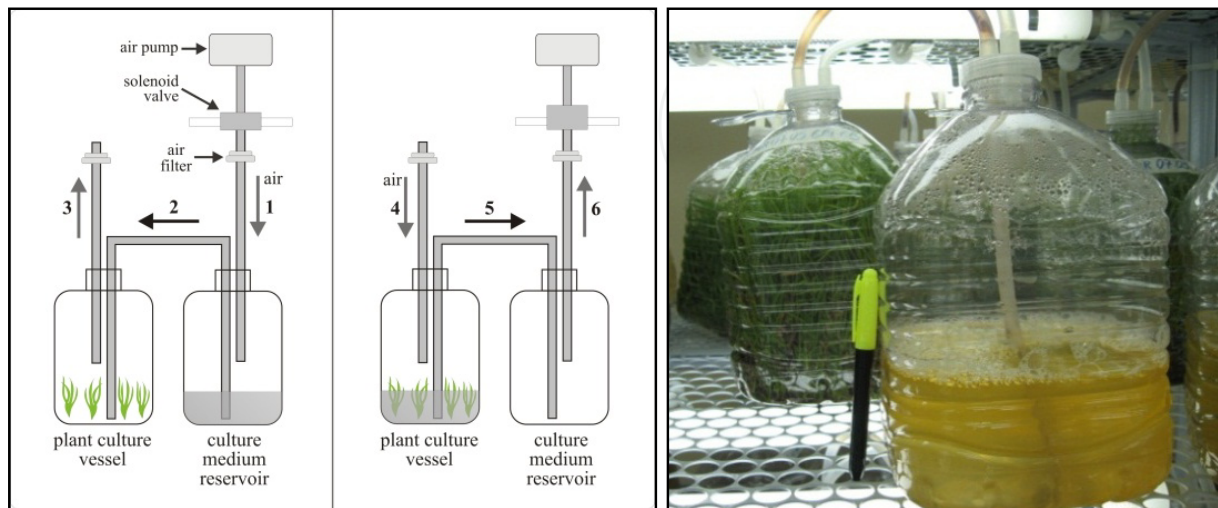

Figure 1. Temporary immersion bioreactor (TIB): The transfer of the medium to the plant culture vessel occurs by a positive pressure of the air pump and the explants are temporarily immersed, after this, the air pump starts and the medium returns to the culture medium reservoir (A) scheme, (B) picture.

The main advantages of temporary immersion bioreactors in plant propagation process include: (1) the liquid medium is in contact with the entire surface of the explants (leaves, roots; etc.), increasing the absorption surface of nutrients and growth regulators; (2) the forced aeration provides excellent oxygen supply and prevents the buildup of harmful gases, resulting in a better crop growth; (3) the movement of explants inside the bioreactor results in reduced apical dominance expression, favoring the proliferation of axillary buds; (4) significant reduction in manpower due to the lower handling of vessels, labeling, etc.; (5) the production of a large number of seedlings, favoring large scale production [59, 63, 64]. The high rates of multiplication, the rapid growth of crops, and the reduction of the need for labor and cost for the culture medium, by avoiding gelling agents, make the technology of micropropagation in temporary immersion bioreactors a conventional static medium in liquid or semi-solid systems.

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