Chapter 1

General introduction: a personal reflection on the use of liquid media for *in vitro* culture

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1. Early attempts: Gottfried Haberlandt and his successors

When studying biology in the 1960s I started *in vitro* culture of pea roots at a students' course. Before that, I had never heard about Haberlandt's experiments published in 1902, and about his successors like Robbins (1922) or Kotte (1922) who grew pea root tips *in vitro* for the first time. Since students' literature searches were carried out manually in the 1960s by evaluating the relevant literature and by going back to cited previous publications, important information was often overlooked or found only by chance.

Today, electronic databases ensure a comprehensive literature overview. Publications older than thirty years which are not stored as electronic data, however, are in danger of being forgotten. This can result in reports on "new" experiments supplied for publishing "for the first time". Since reviewers and publishers sometimes do not recognize the repetitions of publications from the pre-electronic period, recent articles may present old stories being declared as new.

The translation of Haberlandt's pioneering publication from German to English by Krikorian and Berquam in 1969 led to the international acceptance of Haberlandt's role in initiating cell and tissue culture research as a new field of Plant Physiology and Applied Botany. Today, students working with plant cultures *in vitro* cannot but come across Haberlandt's name.

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When Haberlandt (1902) started his experiments he used liquid medium for culturing isolated cells from bracts of *Lamium purpureum* in Knop's solution supplemented with 1 or 5 % (w/v) sucrose. The cells survived for more than one month. However, cell division was never observed. Similar results were achieved by Bobilioff-Preisser (1917) who cultivated mesophyll cells of *Viola lutea* and *Thunbergia alata*. The cells were kept alive on agar-gelled medium for 2-4 months. They died earlier when grown in liquid medium. No cell divisions were achieved under either of the culture conditions.

Literature on *in vitro* culture using liquid or agar-gelled media was reviewed by Fiedler (1938/1939), who summarized the unsuccessful efforts from 1902 until 1938. The review also includes reports on fruitless attempts to cultivate small tissue pieces. The recommendation based on the experiences of that period was, preferably, to use agar-gelled media. The culture conditions on agar media seemed to mimic best the natural environment, especially due to optimal oxygen supply when culturing the cells, tissues and organs on the medium surface. An exception among the numerous disappointing experiments was the successful cultivation of excised root tips of pea and corn on both agar-gelled or liquid media (Robbins 1922 a, b; Kotte 1922 a, b). A continuous growth of tomato roots tips in liquid medium was achieved by White (1934) when excising and subculturing newly-formed root meristems. This enabled more precise investigation into media compositions and other environmental factors. A considerable success in tissue culture was achieved when Nobecourt (1939), White (1939) and Gautheret (1939) reported, almost simultaneously, the indefinite growth of *Nicotiana* callus on both auxin-containing medium or on auxin-free medium when using *Nicotiana* tumour tissues which do not require growth substances.

Finally, nearly 60 years after Haberlandt's first experiments, differentiated mesophyll cells of *Macleaya* directly isolated from leaves developed in liquid media into cell clusters and calli forming organs and somatic embryos (Kohlenbach 1959, 1965, 1966, 2003). Thus the totipotency of differentiated cells predicted by Haberlandt in 1902 had been confirmed. For reviews on the early history of plant cell, tissue and organ culture see (*e.g.*). Street (1973), Gautheret (1982, 1985), Härtel (2003), Höxtermann (2003).

2. Cell suspensions for secondary metabolite production

Cell suspensions are defined as single cells or small cell aggregates in agitated liquid media. Suspensions of cultured cells capable of long-term subculturing were described first for *Nicotiana* and *Tagetes* (Muir, 1953; Muir et al., 1958) and *Daucus* (Steward and Shantz, 1956). Nickell (1956) demonstrated the feasibility of growing cell suspensions like 'micro-organisms' by cultivating highly dispersed suspensions of cells derived from hypocotyls of *Phaseolus vulgaris*. A new technique for isolating and cloning single cells by plating of filtrated suspensions on an agar layer (Bergmann, 1959, 1960) ensured the selection of somaclonal variants or mutants, to establish variant clones. Detailed overviews on culture techniques and growth patterns in cell suspension cultures were given (*e.g.*) by Rajasekhar et al. (1971), Wilson et al. (1971) and King and Street (1973). A few years later, secondary metabolite production using cell suspensions was one of the main topics of the First International Congress on Medical Plant Research "Plant Tissue Culture and its Biotechnological Application" in Munich 1976. The preface of the congress proceedings emphasized optimistically: "*The possibility of growing plant cells like microorganisms offers a basis for the development of new technologies. The production of the natural products of higher plants by means of plant cell suspension cultures seems to be possible. Consequently one can conceive the future industrial production of primary and secondary plant products by fermentation of plant cells. Prior to the industrial application of plant cell cultures, however, several premises need to be investigated. Cell culture strains have to be selected which produce the desired natural product in the highest possible yields and which simultaneously show high growth rates. Suitable fermentation technologies together with growth and production media for mass cultivation of plant cells have to be developed*" (Barz et al., 1977).
Alfermann et al. (2003) summarized the strategy outlined by Zenk et al. in 1977 as follows:

"1. *Screen plants for high accumulation of the desired natural compound(s)*
2. *Initiate callus cultures from selected high producing parent plants*
3. *Analyze these cultures for the desired product(s)*
4. *Establish cell suspension cultures from producing callus strains*
5. *Analyze the suspension cultures*
6. *Select high producing cell lines via single cell cloning using random selection based on somaclonal variations or mutagenic treatment*
7. *Ultimate objective: Selection of stable high producing cell lines*
8. *Further improvement of product yields by optimization of the culture process."*

At that time numerous secondary products were already detected in plant tissue cultures of various plant species (Butcher, 1977). Today we have to admit that only four plant cell culture systems are or were used for large scale commercial production: Shikonin from *Lithospermum erythrorhizon,* ginsenosides from *Panax ginseng*, purpurin from *Rubia akane* and paclitaxel from *Taxus* sp. (Alfermann et al., 2003).

Two reasons may explain the relatively poor success of the theoretically convincing strategy for biotechnological production of secondary compounds. 1. Cell culture systems have to produce the metabolites more cheaply than do the conventional systems. This cannot be achieved in most cases by cell cultures. 2. Dedifferentiated cells in liquid media usually do not accumulate the desired compounds in quantities sufficient for commercialisation.

Based on the negative experience of the last decades new strategies are now being developed, like the identification and incorporation of genes for special biosynthetic steps which may alter the pattern of natural product accumulation (Alfermann et al., 2003) or the culture of highly differentiated organs, shoots or plants instead of suspended cells. Temporary immersion systems as described in this volume may solve some of the problems in accumulating the desired secondary products in future.

3. Suspension culture for plant propagation

3.1 Propagation via adventitious or axillary shoots

Enthusiastic discussions on the use of suspension culture for rapid plant propagation started in the early 1970s. Ben-Jaacov and Langhans (1972) estimated that one shoot tip of *Chrysanthemum* can produce 100,000 plantlets in less than one year when subculturing suspended organogenic callus in rotating vessels and afterwards placing the cultures on stationary medium for plantlet development. Two years later Earle and Langhans (1974 a, b) described a method using liquid Murashige and Skoog medium (1962). According to the authors that system "*could produce up to 9 x* 10^{14} *plantlets or 90 billion 15 cm plants within a year, a great increase over the number possible via conventional propagation*". There is no doubt that for *Chrysanthemum* such high numbers of plants can be achieved from suspension culture. Nevertheless, the described techniques are not used in Europe for vegetative propagation of *Chrysanthemum*, one of the most commercially-significant cut flower and ornamental pot plant. The reason for this unexpected situation is that conventional propagation of *Chrysanthemum* by cuttings produced in the open in southern countries and

rooted in greenhouses in Northern Europe, result in cheaper plants than can be obtained from *in vitro* culture. Additionally, the risk of undesired somaclonal variants arising from proliferating cell aggregates can be avoided. However, suspension cultures were successfully used for mutagenic treatments and mutant selection in *Chrysanthemum* (Huitema et al., 1989, 1991; Preil et al., 1991).

Similarly, high numbers of plants accessible from liquid cultures of *Chrysanthemum* were also reported for other species. For *Aechmea,* Zimmer and Pieper (1975) determined propagation rates of 8 to 11-fold after four weeks subculture and calculated a theoretical production of 70 billion plants per year. In other bromeliads, when using single leaves which were separated from *in vitro*-grown plants, it was assumed to be theoretically possible to obtain more than 500 plants in a year, starting with one mother plant (Hosoki and Asahira, 1980). For carnations, Earle and Langhans (1975) reported that after four month of axillary shoot proliferation in liquid medium (assuming 60-fold increase every six weeks) 100,000 flowering plants may arise per year from one initial shoot tip. Although using a chimeral carnation cultivar, no alteration from the original type was observed, indicating that the chimeral arrangement of the petal tissue had not been disturbed by the culture procedure, i.e. only axillary shoots were initiated resulting in true-to-type clonal progeny. Propagation of chimeral cultivars *via* adventitious shoots in every case would result in rearrangements of the apical constitution and loss of the original phenotype.

Higher multiplication rates in liquid media compared to cultures on agar-gelled media were reported for many species. For example, in some *Rhododendron* cultivars, shoot production in liquid medium was 10-fold higher than with agar-gelled medium (Douglas, 1984). According to Bergervoet et al. (1989) in *Cucumis sativus* a 50 ml suspension culture might yield about fifteen hundred plants arising from adventitious buds. The growth of *Rosa chinensis* shoots cultured in liquid medium was superior to those cultured on two-phase (solid-liquid) medium or solid medium alone (Chu et al., 1993).

Despite many promising results, the use of liquid culture systems has not yet become a routine technique in the propagation of the economically most significant species. The risk of hyperhydricity (vitrification) is one of the main reasons. Further, handling of cultures on agar-gelled media is easier, in most cases for commercial laboratories. The orchid micropropagation industry is an exception to this rule. The propagators use liquid media routinely either in the initial phase of the propagation process or for proliferation of protocorm-like bodies (PLB's). Pieper and Zimmer (1976) reported that within 98 days more than 1.1 kg protocorms of *Cymbidium* were produced in liquid medium after inoculating aerated stationary vessels

with 15 g PLB's. The authors calculated that 280,000 single protocorms could be obtained from one kg of protocorm biomass. A comprehensive description of micropropagation techniques including the use of liquid media was compiled for almost all orchid species of economic interest by Arditti and Ernst (1993).

3.2 Propagation via somatic embryos

Liquid media were used from the beginning of research on developmental pathways of cells leading to somatic embryos (Steward et al., 1958; Reinert, 1958). Plantlet regeneration was achieved when agar-gelled medium was inoculated with freely suspended cells or cell aggregates from *Daucus* suspension cultures (Steward et al., 1958). The similarities of cell suspension derived regenerants with zygotic embryos were soon stated (Steward, 1958). Thus the totipotency of cells and its significance for morphology and embryology had been definitely confirmed (Steward, 1968; Steward et al., 1970) as predicted by Haberlandt (1902), who summarized: " *… finally, I think that I am not making too bold a prophecy if I point to the possibility that one could probably succeed in regenerating artificial embryos from vegetative cells.*"

Tisserat et al. listed 32 families of angiosperms, 81 genera, and 132 species that have been described as producing somatic embryos as early as in 1979. Bajaj (1995) estimated that somatic embryos had been induced in more than 300 plant species belonging to a wide range of families. Examples for somatic embryogenesis achieved in 180 species of herbaceous dicots were given by Brown et al. (1995), whereas KrishnaRaj and Vasil (1995) listed 120 species of herbaceous monocots. Additionally, somatic embryogenesis has been reported for approximately 150 woody species and related hybrids (Dunstan et al. 1995).

Embryogenesis offers several advantages over other developmental pathways due to possibilities of automation of various process stages (Cervelli and Senaratna, 1995; Ibaraki und Kurata, 2001). Although substantial progress in understanding the biology of somatic embryogenesis has been achieved in the past (Merkle et al., 1995; Yeung, 1995; Nomura and Komamine, 1995; Dudits et al., 1995), insufficiencies still exist from the practical point of view, e.g. large differences in embryogenic response to cultural conditions, even in closely related genotypes, and the variation in hormonal requirement at different stages of embryo development.

Considerable progress towards commercialisation of somatic embryo production for mass propagation was achieved in conifer species (Gupta and Timmis, this volume). In *Cyclamen* a yield of some 90,000 plantlets from one litre of embryogenic suspension culture seems to be possible

(Winkelmann et al., 1998). Another study in *Cyclamen* described that from one litre of suspension culture 52,000 embryos may germinate (29 % germination rate) resulting in 33,800 plantlets *in vitro* (65 % conversion rate). In total of *27*,000 young plants (80 % acclimatisation rate) could be obtained after 38 weeks culture period (Hohe et al., 2001).

Difficulties in the application of somatic embryogenesis to plant propagation of insufficiently characterised genotypes may arise from their unknown need for auxin and the duration of auxin application. In many cases, only relatively few cells from heterogeneous embryogenic cultures are able to develop into normal embryos, whereas the major part of the cell population differentiated either into callused or aberrant embryos or non-embryogenic vacuolated cell clusters. Malformed embryos regenerate sometimes in high numbers, e.g. embryos lacking cotyledons or plumules, multiheaded embryos or those with excessive root growth. Such variability of embryogenic cultures prevails, even when suspensions were cultured according to standard protocols.

Lack of reproducibility of propagation protocols is reported for many species. Variation in achieved embryo numbers is evidently often caused by loss of embryogenic competence of cultures during subcultivation. Since uniformity of embryos and reproducibility of the propagation process is one of the prerequisites of any industrial production, synchronisation of embryo development remains one of the most important problems. Whether embryogenesis becomes economically feasible for commercial mass-propagation of a wide range of cultivars, will depend on essential improvements for the embryo regeneration pathway.

Some studies have shown that capacity for embryogenesis is heritable. Two dominant genes with complementary interaction are assumed to control somatic embryogenesis in *Medicago sativa* (Wan et al., 1988; Yu and Pauls, 1993), *Cucumis sativus* (Nadolska-Orczyk and Malepszy, 1989), *Dactylis glomerata* (Tar'an and Bowley, 1997) and *Trifolium pratense* (McLean and Nowak, 1998). In *Cyclamen* a genetic control of the regeneration ability *via* somatic embryogenesis by two dominant major genes with epistatic interaction was postulated (Püschel et al., 2003). Further literature is listed by Henry et al. (1994). In future breeding programmes, selection of genotypes with high embryogenic competence might result in cultivars specifically designed for propagation *via* somatic embryos.

4. Bioreactors for plant propagation

The term "bioreactor" is not precisely defined. It is in use for large-scale vessels for plant biomass production. Bioreactors are normally connected to units controlling temperature, pH, aeration, stirring and various other devices. However, in some cases *in vitro* culture vessels that deviate from Erlenmeyer flasks, Petri dishes or culture boxes are called "bioreactors", too, when innovations and improvements of vessel design were introduced.

Bioreactors have been primarily developed for culturing microorganisms and later for plant cell suspensions to accumulate cell biomass for secondary metabolite production. For industrial cultivation of plant cells, the largest European bioreactor cascade consisting of connected vessels of 75 litres, 750 litres, 7.5 m^3, 15 m^3 and 75 m^3 volumes was established and used for suspension cultures of *Echinacea purpurea, Rauwolfia serpentina* and some other species by the Diversa Company in Ahrensburg, Germany (Rittershaus et al., 1989; Westphal, 1990). Needless to say, for plant propagation purposes bioreactors of a few litres are sufficiently large because high numbers of propagules can be obtained from small vessels.

The aim of bioreactor application is to provide optimum growth conditions by regulating chemical or physical parameters to achieve either maximum yield and high quality of the propagules, or to keep the production costs as low as possible by integration of automated facilities and simple low-cost devices. Since the mid of 1980s various publications stressed the advantages of bioreactors and discussed diverse designs or application strategies (e.g. Ammirato and Styer, 1985; Styer, 1985; Preil, 1991; Takayama, 1991; Denchev et al., 1992; Takayama and Akita, 1994, 1998; Heyerdahl et al., 1995; Ziv, 2000; Ziv, this volume).

4.1 Organogenic cultures

Plant species suitable for propagation through organogenic pathways in various types of bioreactors have been listed by Takayama (1991), Takayama and Akita (1994), Ziv (2000) and Paek et al. (this volume): for example *Anoectochilus, Anthurium,* apple, banana, *Begonia,* Boston fern, *Chrysanthemum, Colocasia, Dieffenbachia, Dioscorea,* garlic, *Gladiolus,* grape, *Hippeastrum, Lilium, Narcissus, Nerine,* Ornithogalum, *Phalaenopsis,* pineapple, *Pinellia*, poplar, potato, *Saintpaulia, Sinningia (Gloxinia), Spathiphyllum, Stevia* and strawberry. Many other species are potential candidates for scaling-up organogenic cultures. In general monocotyledonous plants, including orchids, are easier to handle in liquid systems than dicotyledonous species which tend to become more hyperhydric (vitrified) and etiolated when high plant densities accumulate in

the bioreactor. An impressive example for mass propagation of shoots of *Stevia rebaudiana* using a bioreactor of 500 litre volume was given by Akita et al. (1994) and Takayama and Akita (1994).

4.2 Embryogenic cultures

In 1985 Ammirato and Styer outlined strategies for large-scale manipulation of somatic embryos in suspension culture using bioreactors. In the years following this, right up to the present day, numerous authors have repeatedly restated the general principles of bioreactor application, stressing the advantages of embryogenic pathways for plant propagation, including the production of artificial seeds (for literature on problems of artificial seeds see Redenbaugh, 1993).

No integration of bioreactors for the industrial production of somatic embryos has been achieved in any economically-important crop plant until now. Some of the difficulties in regulation of embryogenic processes have been already discussed in section 3.2. Nevertheless, in various species, bioreactor cultures can be used as a part of the propagation process, especially in scaling-up of pro-embryogenic cell masses and globular or heart-shaped embryos for further development on agar-gelled media or growth in temporary immersion systems (TIS).

Reports on embryo production in bioreactors includes a wide range of taxonomically diverse species, e.g. alfalfa (Stuart et al., 1987), sandalwood (Bapat et al., 1990), sweet potato (Bieniek et al., 1995) and *Picea sitchensis* (Ingram and Mavituna, 2000), chosen at random. Investigations on the effects of oxygen supply, carbon dioxide accumulation or medium pH in the bioreactors are of extraordinary importance for the more precise regulation of embryo development. In *Euphorbia pulcherrima* dissolved oxygen tension (pO_2) of 60 % and in *Clematis tangutica* pO_2 of 15 % resulted in high embryo yield (Preil et al., 1988; Luttmann et al., 1994). In *Cyclamen,* a significantly higher number of germinating embryos was obtained from cultures grown at 40 % pO_2 than from those grown in Erlenmeyer flasks or in bioreactors aerated with 5 %, 10 % and 20 % pO_2 (Hohe et al., 1999a). Embryo production in *Daucus* decreased significantly at 10 % dissolved oxygen (600 and 170 somatic embryos per ml at 100 % and 10 % pO_2, respectively) (Jay et al., 1992). In contrast, Shimazu and Kurata (1999) reported that the total number of carrot somatic embryos was unaffected by increasing oxygen concentrations in the range of 4-40 % pO_2. However, oxygen enrichment enhanced the number of torpedo and cotyledonary-stage embryos. These contradictory results may be due to the application of varying culture parameters, or to differently responding *Daucus* genotypes.

Carbon dioxide accumulation in bioreactor culture of *Cyclamen* was severely growth-inhibiting in comparison to CO_2-concentrations determined in Erlenmeyer flasks. By removing CO_2 from the aeration gas of the bioreactor, cell growth was as high as achieved in Erlenmeyer flasks. However, the regeneration ability of cells after being cultured in bioreactors with CO_2 accumulation, was better than those from Erlenmeyer flasks or bioreactors without CO_2-accumulation (Hohe et al., 1999b). A considerable increase of embryogenic biomass in bioreactor culture of *Clematis tangutica* was achieved when 2.5 % or 5 % (v/v) CO_2 was supplemented to the aeration gas. Supplement of 10 % (v/v) CO_2 reduced embryo yield severely (Barbon-Rodriguez, 2001). CO_2 seems to be much more involved in embryogenic processes than was expected in the past. Not surprisingly, pH of the medium also influences somatic embryogenesis. In *Daucus,* embryo production was highest when the pH of the hormone-free medium was maintained at 4.3. However, most of the embryos did not develop into plantlets. Cultures grown at pH 5.8 produced less embryos which, in contrast to pH 4.3, were able to continue development into plantlets (Jay et al., 1994). These observations indicate that control of environmental factors in bioreactors is essential to overcome difficulties in somatic embryo production and conversion of embryos into plants suitable for commerce.

5. Temporary immersion systems

A periodic immersion technique in which the plant tissue spends periods immersed in the liquid medium alternating with periods in the air was first described by Steward et al. (1952) and modified by Steward and Shantz (1956). In the so-called “Steward apparatus” or “Auxophyton”, tubes are fastened with their long axes parallel to the radius of discs which are rotated at 1 rpm by a shaft held at an angle of 10-12°. The liquid medium runs from one end of the tube to the other leaving the tissue in the gas phase and *vice versa*. By similar means, Harris and Mason (1983) achieved alternate exposure and submergence of explants when tilting a flat-bottomed vessel in one direction to expose the tissue to air and in the opposite direction to submerge them in the medium. The first automated device operating according to the principle of ebb and flood was designed by Tisserat and Vandercook (1985). This soon initiated discussions on growing *in vitro* cultures which are temporarily immersed. A breakthrough was achieved when Alvard et al. (1993) introduced the RITA® vessel and Teisson and Alvard (1995) stressed temporary immersion as a new concept of plant *in vitro* cultivation using liquid media. These ideas fertilized worldwide activities in the application and testing of temporary immersion systems

(TIS), resulting in variations in vessel design, equipment and treatments such as immersion time and frequency, depending on the requirements of different plant species and cultivars.

An overview on TIS was given by Etienne and Berthouly (2002) and Berthouly and Etienne (this volume). One can only speculate why this culture technique was adopted so late as a simple, low-cost and effective propagation method, which is applicable to almost all commercially-interesting species including those listed in section 4.1. The principles of the RITA® system and of the twin-flask system of Escalona et al. (1999) represent the most convincing technical solutions combining low-cost devices and easy handling of the cultures. The practical advantages of TIS became evident when culturing e.g. somatic embryos of *Musa* spp. (Escalant et al., 1994), *Hevea brasiliensis* (Etienne et al., 1997), *Citrus* (Cabasson et al., 1997) and *Coffea arabica* (Etienne-Barry et al., 1999) as well as shoots of sugarcane (Lorenzo et al., 1998) and pineapple (Escalona et al., 1999) or potato microtubers (Jimenez et al., 1999). For details see Berthouly and Etienne (this volume).

The physiologically most important advantage of TIS is the efficient gaseous exchange between plant tissue and gas phase inside the vessel. Multiple daily air replacement by pneumatic transfer of the medium ventilates accumulated gasses like ethylene or CO_2. Additionally, uptake of nutrients and hormones over the whole explant surface ensures maximum growth. There is no doubt that TIS will play an outstanding role in future mass propagation of plants *in vitro*.

6. Concluding remarks

Liquid culture systems have significant effects on the multiplication rates and morphology of shoots, somatic embryos, microtubers or bulblets produced *in vitro*. Liquid media favour tissue organisation similar to that of aquatic plants. This, however, can complicate the acclimatisation of the propagules at the end of the propagation process. Therefore, steps have to be taken to avoid hyperhydricity (vitrification), the most serious disorder in liquid culture systems. Optimisation of aeration in bioreactors and TIS in many cases leads to propagules of excellent quality which can be acclimatized easily. Nevertheless, it must be noted that a chance contamination will quickly develop and disperse in liquid medium and is likely to lead to total loss of the culture.

Compared to agar-based systems, liquid systems are more adaptable to automation and are, therefore, suitable for the reduction of labour and costs, as media can be changed easily during scaling-up, and the cleaning of

culture vessels is simplified. Some figures on reduction of production costs in TIS are discussed by Berthouly and Etienne (this volume).

In any case, the use of liquid media is only one part of the propagation scheme for various plant species, because establishing *in vitro* cultures usually starts on agar-gelled medium. After the scaling-up and multiplication phase in liquid media, gelled and solid media are used again in most cases for rooting of shoots or conversion of embryos. During this 'pre-acclimatisation' phase, tissues previously adapted to the liquid environment become 're-organised' and conditioned for the *in vivo* environment.

Temporary immersion systems will play a dominant role in future micropropagation of plants. The principles of temporary immersion of tissue cultures were already in discussion fifty years ago; they are easily understood by tissue culturists. Nevertheless, suitable equipment such as RITA® had to be invented before ideas of TIS application in mass propagation took root. The development of useable protocols and guidelines are a prerequisite for the integration of new elements in traditional *in vitro* culture systems design. The organizers of the First International Symposium on "Liquid Systems for *In Vitro* Mass Propagation of Plants" in 2002 set out to establish such guidelines.

References

Akita M, Shigeoka T, Koizumi Y & Kawamura M (1994) Mass propagation of shoots of *Stevia rebaudiana* using a large scale bioreactor. Plant Cell Rep. 13: 180-183

Alfermann AW, Petersen M & Fuss E (2003) Production of natural products by plant cell biotechnology: results, problems and perspectives. In: Laimer M & Rücker W (ed) Plant Tissue Culture 100 years since Gottlieb Haberlandt (pp. 153-166). Springer, Wien New York

Alvard D, Cote F & Teisson C (1993) Comparison of methods of liquid medium culture for banana micropropagation. Effects of temporary immersion on explants. Plant Cell, Tiss. Org. Cult. 32: 55-60

Ammirato PV & Styer DJ (1985) Strategies for large scale manipulation of somatic embryos in suspension culture. In: Zaitlin M, Day P & Hollaender A (eds) Biotechnology in Plant Science: Relevance to Agriculture in the Eigthies (pp. 161-178). Academic Press, New York

Arditti J & Ernst R (1993) Micropropagation of Orchids. John Wiley & Sons, Inc. New York

Bajaj YPS (1995) Somatic embryogenesis and its applications for crop improvement. In: Bajaj YPS (ed) Biotechnology in Agriculture and Forestry 30 – Somatic embryogenesis and synthetic seed Vol I (pp. 114-125). Springer, Berlin

Bapat VA, Fulzele DP, Heble MR & Rao PS (1990) Production of sandalwood somatic embryos in bioreactors. Current Science 59: 746-748

Barbon-Rodriguez R (2001) Efecto del dioxido de carbono sobre la embriogenesis somatica de *Coffea arabica* L. cv. Caturra rojo y *Clematis tangutica* K. Tesis, Universidad Central de las Villas, Cuba

Barz W, Reinhard E & Zenk MH (1977) Preface. In: Barz W, Reinhard E & Zenk MH (eds) Plant Tissue and Its Bio-technical Application (pp. V-VI). Springer, Berlin

Ben-Jaacov J & Langhans RW (1972) Rapid multiplication of *Chrysanthemum* plants by stem-tip proliferation. HortScience 7: 289-290

Bergervoet JHW, van der Mark F & Custers JBM (1989) Organogenesis versus embryogenesis from long-term suspension cultures of cucumber (*Cucumis sativus* L.). Plant Cell Rep. 8: 116-119

Bergmann L (1959) A new technique for isolating and cloning cells of higher plants. Nature 184: 648-649

Bergmann L (1960) Growth and division of single cells of higher plants *in vitro*. J. General Physiol. 43: 841-851

Berthouly M & Etienne H (2004) Temporary immersion system: a new concept for use liquid medium in mass propagation. (This volume pp. 161-190)

Bieniek ME, Harrell RC & Cantliffe DJ (1995) Enhancement of somatic embryogenesis of *Ipomoea batatas* in solid cultures and production of mature somatic embryos in liquid cultures for application to a bioreactor production system. Plant Cell, Tiss. Org. Cult. 41: 1-8

Bobilioff-Preisser W (1917) Beobachtungen an isolierten Palisaden- und Schwamm parenchymzellen. Beihefte Bot. Centralbl. 33: 248-274

Brown DCW, Finstad KI & Watson EM (1995) Somatic embryogenesis in herbaceous dicots. In: Thorpe TA (ed) *In vitro* embryogenesis in plants (pp. 345-416). Kluwer Academic Publishers, Dordrecht

Butcher ND (1977) Secondary products in tissue culture. In: Reinert J & Bajaj YPS (eds) Plant Cell, Tissue and Organ Culture (pp. 667-693). Springer, Berlin

Cabasson C, Alvard D, Dambier D, Ollitrault P & Teisson C (1997) Improvement of *Citrus* somatic embryo development by temporary immersion. Plant Cell. Tiss. Org. Cult. 50: 33-37

Cervelli R & Senaratna T (1995) Economic aspects of somatic embryogenesis. In: Aitken-Christie J, Kozai T & Smith MAL (eds) Automation and Environmental Control in Plant Tissue Cultures (pp. 29-64). Kluwer Academic Publishers, Dordrecht

Chu CY, Knight SL & Smith MAL (1993) Effect of liquid culture on the growth and development of miniature rose (*Rosa chinensis* Jacq. 'Minima'). Plant Cell, Tiss. Org. Cult. 32: 329-334

Denchev PD, Kuklin AI, & Scragg AH (1992) Somatic embryo production in bioreactors. J. Biotechnology 26: 99-109

Douglas GC (1984) Propagation of eight cultivars of *Rhododendron in vitro* using agar-solidified and liquid media and direct rooting of shoots *in vivo*. Scientia Horticulturae 24: 337-347

Dudits D, Györgyey J, Bögre L & Bako L (1995) Molecular biology of somatic embryogenesis. In: Thorpe TA (ed) *In vitro* Embryogenesis in Plants (pp. 267-308). Kluwer Academic Publishers, Dordrecht

Dunstan DI, Tautorus TE & Thorpe TA (1995) Somatic embryogenesis in woody plants. In: Thorpe TA (ed) *In vitro* Embryogenesis in Plants (pp. 471-538). Kluwer Academic Publishers, Dordrecht

Earle ED & Langhans RW (1974a) Propagation of *Chrysanthemum in vitro*: I. Multiple plantlets from shoot tips and the establishment of tissue cultures. J. Amer. Soc. Hort. Sci. 99: 128-131

Earle ED & Langhans RW (1974b) Propagation of *Chrysanthemum in vitro*: II. Production, growth, and flowering of plantlets from tissue culture. J. Amer. Soc. Hort.Sci. 99: 352-358

Earle ED & Langhans RW (1975) Carnation propagation from shoot tips cultured in liquid medium. HortScience 10: 608-610

Escalant JV, Teisson C & Cote F (1994) Amplified somatic embryogenesis from male flowers of triploid banana and plantain cultivars (*Musa* spp.). *In Vitro* Cell. Dev. Biol. 30: 181-186

Escalona M, Lorenzo JC, Gonzalez B, Daquinta M, Gonzalez JL, Desjardins Y & Borroto CG (1999) Pineapple (*Ananas comosus* L. Merr.) micropropagation in temporary immersion systems. Plant Cell Rep. 18: 743-748

Etienne H, Lartaud M, Michaux-Ferriere N, Carron MP, Berthouly M & Teisson C (1997) Improvement of somatic embryogenesis in *Hevea brasiliensis* (Müll. Arg.) using the temporary immersion technique. *In Vitro* Cell. Dev. Biol. 33: 81-87

Etienne H & Berthouly M (2002) Temporary immersion systems in plant micropropagation. Plant Cell, Tiss. Org. Cult. 69: 215-231

Etienne-Barry D, Bertrand B, Vasquez N & Etienne H (1999) Direct sowing of *Coffea arabica* somatic embryos mass-produced in a bioreactor and regeneration of plants. Plant Cell Rep. 19: 111-117

Fiedler H (1938/39) Die pflanzliche Gewebe- und Organkultur. Sammelreferat. Z. Bot. 33: 369-416

Gautheret R (1939) Sur la possibilité de realiser la culture indéfinie des tissus de tubercules de carotte. C.R. Acad. Sci. Paris 208: 118-120.

Gautheret RJ (1982) Plant tissue culture: the history. In: Fujiwara A (ed) Plant Tissue Culture (pp. 7-12). Proc. 5th Intl. Cong. Plant Tissue & Cell Culture, Tokyo 1982

Gautheret RJ (1985) History of plant tissue and cell culture: A personal account. In: Vasil IK (ed) Cell Culture and Somatic Cell Genetics, Vol. 2 Cell Growth, Nutrition, Cytodifferentiation and Cryopreservation (pp. 1-59). Acad. Press, Inc. Orlando, San Diego, New York

Gupta PK & Timmis R (2004) Mass propagation of conifer trees in liquid cultures. Progress towards commercialization. This volume (pp. 379-392)

Haberlandt G (1902) Culturversuche mit isolierten Pflanzenzellen. Sitzungsber. Akad. Wiss. Wien, Math.-Naturwiss. Kl. 3: 69-92; translated by Krikorian AD & Berquam DL 1969: Experiments on the culture of isolated plant cells. Bot. Rev. 35: 68-88

Härtel O (2003) Gottlieb Haberlandt (1854-1945): a portrait. In: Laimer M & Rücker W (ed) Plant Tissue Culture 100 years since Gottlieb Haberlandt (pp. 55-66). Springer, Wien New York

Harris RE & Mason EBB (1983) Two machines for *in vitro* propagation of plants in liquid media. Can. J. Plant Sci. 63: 311-316

Henry Y, Vain P & de Buyser J (1994) Genetic analysis of *in vitro* plant tissue culture responses and regeneration capacities. Euphytica 79: 45-58

Heyerdahl PH, Olsen OAS & Hvoslef-Eide AK (1995) Engineering aspects of plant propagation in bioreactors. In: Aitken-Christie J, Kozai T & Smith L (eds) Automation and Environmental Control in Plant Tissue Culture (pp. 87-123). Kluwer Academic Publishers, Dordrecht

Höxtermann E (2003) Cellular 'elementary organisms' *in vitro*: The early vision of Gottlieb Haberlandt and its realization. In: Laimer M & Rücker W (ed) Plant Tissue Culture 100 years since Gottlieb Haberlandt (pp. 67-91). Springer, Wien New York

Hohe A, Winkelmann T & Schwenkel HG (1999a) The effect of oxygen partial pressure in bioreactors on cell proliferation and subsequent differentiation of somatic embryos of *Cyclamen persicum*. Plant Cell Tiss. Org. Cult. 59: 39-45

Hohe A, Winkelmann T & Schwenkel HG (1999b) CO_2 accumulation in bioreactor suspension cultures of *Cyclamen persicum* Mill. and its effect on cell growth and regeneration of somatic embryos. Plant Cell Rep. 18: 863-867

Hohe A, Winkelmann T & Schwenkel HG (2001) Development of somatic embryos of *Cyclamen persicum* Mill. in liquid culture. Gartenbauwissenschaft 66: 219-224

Hosoki T & Asahira T (1980) *In vitro* propagation of bromeliads in liquid culture. HortScience 15: 603-604

Huitema JBM, Preil W, Gussenhoven GC & Schneidereit M (1989) Methods for the selection of low-temperature tolerant mutants of *Chrysanthemum morifolium* Ramat. by using irradiation cell suspension cultures. I. Selection of regenerants *in vivo* under suboptimal temperature conditions. Plant Breeding 102: 140-147

Huitema JBM, Preil W & de Jong J (1991) Methods for the selection of low-temperature tolerant mutants of *Chrysanthemum morifolium* Ramat. by using irradiation cell suspension cultures. III. Comparison of mutants selected with or without preselection *in vitro* at low-temperature. Plant Breeding 107: 135-140

Ibaraki Y & Kurata K (2001) Automation of somatic embryo production. Plant Cell Tiss. Org. Cult. 59: 179-199

Ingram B & Mavituna F (2000) Effect of bioreactor configuration on the growth and maturation of *Picea sitchensis* somatic embryo cultures. Plant Cell, Tiss. Org. Cult. 61: 87-96

Jay V, Genestier S & Courduroux JC (1992) Bioreactor studies on the effect of dissolved oxygen concentrations on growth and differentiation of carrot (*Daucus carota* L.) cell cultures. Plant Cell Rep. 11: 605-608

Jay V, Genestier S & Courduroux JC (1994) Bioreactor studies of the effect of medium pH on carrot (*Daucus carota* L.) somatic embryogenesis. Plant Cell, Tiss. Org. Cult. 36: 205-209

Jimenez E, Perez N, de Feria M, Barbon R, Capote A, Chavez M, Quiala E & Perez JC (1999) Improved production of potato microtubers using a temporary immersion system. Plant Cell, Tiss. Org. Cult. 59: 19-23

King PJ & Street HE (1973) Growth pattern in cell cultures. In: Street HE (ed) Plant Tissue and Cell Culture (pp. 269-337). Blackwell Sci. Publ., Oxford

Kohlenbach HW (1959) Streckungs- und Teilungswachstum isolierter Mesophyllzellen von *Macleaya cordata*. Naturwiss. 46: 116-117

Kohlenbach HW (1965) Über organisierte Bildungen aus *Macleaya cordata* Kallus. Planta 64: 37-40

Kohlenbach HW (1966) Die Entwicklungspotenzen explantierter und isolierter Dauerzellen. I. Das Streckungs- und Teilungswachstum isolierter Mesophyllzellen von *Macleaya cordata*. Z. Pflanzenphysiol. 55: 142-157

Kohlenbach HW (2003) The developmental potentials of isolated mesophyll cells and protoplasts. In: Laimer M & Rücker W (ed) Plant Tissue Culture 100 years since Gottlieb Haberlandt (pp. 99-103). Springer, Wien New York

Kotte W (1922a) Wurzelmeristem in Gewebekultur. Ber. Dtsch. Bot. Ges. 40: 269-272

Kotte W (1922b) Kulturversuche mit isolierten Wurzelspitzen. Beitr. Allg. Bot. 2: 413-434

Krikorian AD & Berquam DL (1969) Plant cell and tissue cultures: The role of Haberlandt. Bot. Rev. 35: 59-88

KrishnaRaj S & Vasil IK (1995) Somatic embryogenesis in herbaceous monocots. In: Thorpe TA (ed) *In Vitro* Embryogenesis in Plants (pp. 155-204). Kluwer Academic Publishers, Dordrecht

Lorenzo JC, Gonzalez BL, Escalona M, Teisson C, Espinosa P & Borroto C (1998) Sugarcane shoot formation in an improved temporary immersion system. Plant Cell Tiss. Org. Cult. 54: 197-200

Luttmann R, Florek P & Preil W (1994) Silicone-tubing aerated bioreactors for somatic embryo production. Plant Cell, Tiss. Org. Cult. 39: 157-170

McLean NL & Nowak J (1998) Inheritance of somatic embryogenesis in read clover (*Trifolium pratense* L.). Theoretical and Applied Genetics 97: 557-562

Merkle SA, Parrott WA & Flinn BS (1995) Morphogenic aspects of somatic embryogenesis. In: Thorpe TA (ed) *In Vitro* Embryogenesis in Plants (pp. 155-204). Kluwer Academic Publishers, Dordrecht

Muir WH (1953) Cultural conditions favouring the isolation and growth of single cells from higher plants *in vitro*. Ph.D. Thesis. Univ. Wisconsins, U.S.A.

Muir WH, Hildebrandt AC & Riker AJ (1958) The preparation, isolation and growth in culture of single cells from higher plants. Am. J. Bot. 45: 589-597

Murashige T & Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. 15: 473-497

Nadolska-Orczyk A & Malepszy S (1989) In vitro culture of *Cucumis sativus* L. 7. Genes controlling plant regeneration. Theoretical and Applied Genetics 78: 836-840

Nickell LG (1956) The continuous submerged cultivation of plant tissues as single cells. Proc. Natn. Acad. Sci. U.S.A. 42: 848-850

Nobécourt P (1939) Sur la pérennité et l'augmentation de volume des cultures de tissues végétaux. C.R. Soc. Biol. 130: 1270-1271

Nomura K & Komamine A (1995) Physiological and biochemical aspects of somatic embryogenesis. In: Thorpe TA (ed) *In Vitro* Embryogenesis in Plants (pp. 249-266). Kluwer Academic Publishers, Dordrecht

Paek KY, Chakrabarty D & Hahn EJ (2004)Application of bioreactor systems for large scale production of horticultural and medicinal plants. This volume (pp. 93-114)

Pieper W & Zimmer K (1976) A simple, inexpensive apparatus for *in vitro* propagation of tissues. Gartenbauwissenschaft 41: 221-224

Preil W, Florek P, Wix U & Beck A (1988) Towards mass propagation by use of bioreactors. Acta Horticulturae 226: 99-106

Preil W (1991) Application of bioreactors in plant propagation. In: Debergh PC & Zimmerman RH (eds). Micropropagation (pp. 425-445). Kluwer Academic Publishers, Dordrecht

Preil W, Huitema JBM & de Jong J (1991) Methods for the selection of low-temperature tolerant mutants of *Chrysanthemum morifolium* Ramat. by using irradiation cell suspension cultures. II. Preselection *in vitro* under low-temperature stress. Plant Breeding 107: 131-134

Püschel AK, Schwenkel HG & Winkelmann T (2003) Inheritance of the ability for regeneration via somatic embryogenesis in *Cyclamen persicum*. Plant Cell, Tiss. Org. Cult. 72: 43-51

Rajasekhar EW, Edwards M, Wilson SB & Street HE (1971) Studies on the growth in culture of plant cells. XI. The influence of shaking rate on the growth of suspension cultures. J. Exp. Bot. 22: 107-117

Redenbaugh K (1993) Synseeds. Applications of synthetic seeds to crop improvement. CRC Press Boca Raton, Ann Arbor, London, Tokyo

Reinert J (1958) Morphogenese und ihre Kontrolle an Gewebekulturen aus Carotten. Naturwissenschaften 45: 344-345

Rittershaus E, Ulrich J, Weiss A & Westphal K (1989) Large scale industrial fermentation of plant cells: Experiences in cultivation of plant cells in a fermentation cascade up to a volume of 75000 litres. BioEngineering 5: 28-34

Robbins WJ (1922a) Cultivation of excised root tips and stem tips under sterile conditions. Bot. Gaz. 73: 376-390

Robbins WJ (1922b) Effect of autolyzed yeast and peptone on growth of excised corn root tips in the dark. Bot. Gaz. 74: 59-79

Shimazu T & Kurata K (1999) Relationship between production of carrot somatic embryos and dissolved oxygen concentration in liquid culture. Plant Cell, Tiss. Org. Cult. 57: 29-38

Steward FC, Caplin S & Millar FK (1952) Investigations on growth and metabolism of plant cells. I. New techniques for the investigation of metabolism, nutrition and growth in undifferentiated cells. Ann. Bot. 16: 57-77

Steward FC & Shantz EM (1956) The chemical induction of growth in plant tissue cultures. In: Wain RL & Wightman F (eds) The Chemistry and Mode of Action of Plant Growth Substances (ed) (pp. 165-186). Butterworths Ltd., London

Steward FC, Mapes MO & Mears K (1958) Growth and organised development of cultured cells. II. Organisation in cultures grown from freely suspended cells. Amer. J. Bot. 45: 705-708

Steward FC (1958) Growth and development of cultivated cells. III. Interpretations of the growth from free cell to carrot plant. Am. J. Bot. 45: 709-713

Steward FC (1968) Totipotency of angiosperm cells. Its significance for morphology and embryology. Phytomorphology 17: 499-507

Steward FC, Ammirato PV & Mapes MO (1970) Growth and development of totipotency cells. Some problems, procedures and perspectives. Ann. Bot. 34: 761-787

Street HE (1973) Introduction. In: Street HE (ed) Plant Tissue and Cell Culture (pp. 1-10). Blackwell Sci. Publ., Oxford

Stuart DA, Strickland SG & Walker KA (1987) Bioreactor production of alfalfa somatic embryos. HortScience 22: 800-803

Styer DJ (1985) Bioreactor technology for plant propagation. In: Henke RR, Hughes KW, Constantin MJ & Hollaender A (eds) Tissue Culture in Forestry and Agriculture (pp. 117-130) Plenum Press, New York, London

Takayama S (1991) Mass propagation of plants through shake- and bioreactor-culture techniques In: Bajaj YPS (ed) Biotechnology in Agriculture and Forestry Vol 17 (pp. 495-515). Springer Verlag, Berlin

Takayama S & Akita M (1994) The types of bioreactors used for shoots and embryos. Plant Cell, Tiss. Org. Cult. 39: 147-156

Takayama S & Akita M (1998) Bioreactor techniques for large-scale culture of plant propagules. Adv. Hort. Sci. 12: 93-100

Tar'an B & Bowley SR (1997) Inheritance of somatic embryogenesis in orchardgrass. Crop Science 37: 1497-1502

Teisson C & Alvard D (1995) A new concept of plant *in vitro* cultivation liquid medium: Temporary immersion. In: Terzi M, Cella R, Falavigna A(eds) Current Issues in Plant Molecular and Cellular Biology (pp. 105-110). Kluwer Academic Publishers, Dordrecht

Tisserat B, Esan EB & Murashige T (1979) Somatic embryogenesis in angiosperms. Hortic. Rev. 1: 1-78

Tisserat B & Vandercook CE (1985) Development of an automated plant culture system. Plant Cell, Tiss. Org. Cult. 5: 107-117

Wan Y, Sorensen EL & Liang GH (1988) Genetic control of *in vitro* regeneration in alfalfa (*Medicago sativa* L.). Euphytica 39: 3-9

Westphal K (1990) Large-scale production of new biologically active compounds in plant-cell culture. In: Nijkamp HJJ, van der Plas LHW & van Aartijk J (eds), Progress in Plant Cellular and Molecular Biology (pp. 601-608). Kluwer Academic Publishers, Dordrecht

White PR (1934) Potentially unlimited growth of excised tomato root tips in a liquid medium. Plant Physiol. 9: 585-600

White PR (1939) Potentially unlimited growth of excised plant callus in an artificial medium. Am. J. Bot. 26: 59-64

Wilson SB, King PJ & Street HE (1971) Studies on the growth in culture of plant cells. XII. A versatile system for the large scale batch or continuous culture of plant cell suspensions. J. Exp. Bot. 21: 177-207

Winkelmann T, Hohe A & Schwenkel HG (1998) Establishing embryogenic suspension cultures in *Cyclamen persicum* ‚Purple Flamed'. Adv. Hort. Sci. 12: 25-30

Yeung EC (1995) Structural and development patterns in somatic embryogenesis. In: Thorpe TA (ed) *In Vitro* Embryogenesis in Plants (pp. 205-248). Kluwer Academic Publishers, Dordrecht

Yu KF & Pauls KP (1993) Identification of a RAPD marker associated with somatic embryogenesis in alfalfa. Plant Mol. Biol. 22: 269-277

Zenk MH, El-Shagi H, Arens H, Stöckigt J, Weiler EW & Deus B (1977) Formation of indole alkaloids serpentine and ajmalicine in cell suspension cultures of *Catharanthus roseus*. In: Barz W, Reinhard E & Zenk MH (eds) Plant Tissue Culture and Its Bio-technical Application (pp. 27-43). Springer, Berlin

Zimmer K & Pieper W (1975) Weitere Untersuchungen zur Kultur *in vitro* von *Aechmea*. Gartenbauwissenschaft 3: 129-132

Ziv M (2000) Bioreactor technology for plant micropropagation. Horticultural Reviews 24: 1-30

Ziv M (2004) Simple bioreactors for mass propagation of plants. This volume (pp.77-91)

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