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# 65 years of in vitro culture in Poland

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## Abstract

This paper is a short review of historical development of the tissue culture method in Poland. Similarly to the rest of the world, in vitro technology in Poland has progressed in many directions simultaneously. Its main fields are closely interconnected by natural sequences of biological processes and integrade one into another. The best results, driven by the prospects of practical applications, have been achieved within the areas of meristem culture and micropropagation of valuable genotypes, somaclonal variation, production of haploids and DH plants, somatic embryogenesis, in vitro culture of embryos and ovules, improvement of plant disease resistance, somatic hybridization and transformation of plants. Due to the fact that in vitro domain is a very broad science the authors are aware that this review might not fully cover all the scientists working with tissue culture and their achievements.

**Keywords:** history, plant tissue culture, Poland

## The beginnings

In Poland the earliest manifestation of interest in in vitro culture as a novel experimental method took form of an article by Władysław Becker of Warsaw University published in the quarterly Cosmos. The article contained an extensive review of 105 literature reports on medical aspects of in vitro cultures and included the results of research on stimulation of dedifferentiation and regeneration of mature plant tissues conducted in the laboratories of prominent scientists such as G. Haberlandt and R. J. Gautheret [1]. After WWII, in 1948 Jerzy Czosnowski, then a research assistant with the General Botany Chair at Poznań University, visited Roger Gautheret's Plant Biology Laboratory as a recipient of a Polish Government scholarship. At Sorbon University he learned the methods of culturing plant calli and crown-gall tumors. Upon returning home, he continued experiments on three types of callus, including chemical and bacterial tumors of *Vitis vinifera*. He was the first to note the hormonal self-sufficiency of tumors and this fact became the basis of his doctoral thesis [2]. In following years he studied water and nitrogen relations, and changes in enzyme composition of lupine embryo during early developmental stages [3–5]. The results of his work entered the world literature and were cited in the Bonner and Varner's textbook ("Plant biochemistry", 1965, London) laying foundation for Poznań school of tissue culture. Czosnowski's collaborator, Dr. Marian Zieliński also visited the laboratory of R. J. Gautheret, from whom he brought back the cultures of *Marchantia polymorpha*, an important model species for studying the influence of mineral and organic nutrition on the morphology, anatomy and reproduction of liverworts. *Marchantia* for a long time remained a model plant for MSc dissertations completed at AMU General Botany Laboratory.

Janina Rogozińska also visited the Gautheret's laboratory. The outcome of her visit was a doctorate [6] followed by a three-year post-doc with Folke Skoog at the Botanical Institute of University of Madison (Wisconsin, USA). J. Rogozińska participated in the discovery of cytokinins and sought explanation for their role in plant morphogenesis [7]. According to F. Skoog „…the progress in isolation of active cytokinins fraction was achieved owing to the dedicated effort of Janina Rogozińska…". Results of her postdoctoral work with professor W. G. Whaley at Austin State University (Texas, USA) were published in Phytochemistry [8]. At that time this publication was a major achievement in the career of the future Head of Plant Physiology Laboratory at University of Technology and Life Sciences in Bydgoszcz, who later co-authored many other publications [9,10].

Also Alicja Szweykowska, after completing her training at A. Allsoop's laboratory in Great Britain, added tissue cultures to her research methods [11]. Data on the inductive influence of cytokinins on cell divisions in *Ceratodon* and *Funaria hygrometrica* [12] gathered during her 1964 visit to F. Skoog in Madison (Wisconsin, USA) were, however, more significant. Her research focused on the influence of plant growth regulators on the synthesis of proteins and nucleic acids. The emphasis of studies that she continued in subsequent years was morphogenesis, bringing together many biological subdisciplines such as growth and development, developmental morphology, phenotypic plasticity, heterophylly, cell differentiation in response to growth regulators and induction of functional differentiation in plant tissue systems [13–18].

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Meanwhile, at AMU General Botany Laboratory Maciej Zenkteler initiated and rapidly developed research in the field of experimental embryology. His 1961 visit with A. C. Hildebrandt in 1961 and a year-long visit with the world-famous embryologist P. Maheshwari in Delhi in 1964 led to wide-ranging studies in experimental embryology utilizing tissue culture, including cultures of immature embryos [19], in vitro pollination and fertilization of ovaries and ovules [20–23] and generation of haploid plants [24–26]. From the late 60's M. Zenkteler delivered multiple lectures and conducted practical courses, training researchers from other academic centers in Poland to use the in vitro methods.

Thanks to these personalities and to their experiences gained in the best international laboratories, Adam Mickiewicz University in Poznań (General Botany and Plant Physiology Laboratories) became centers of in vitro culture, influencing other research institutions within the country. Research scientists that began their studies in Poznań include: Z. Tomaszewski (work on lupine embryo and ovule culture), A. Rennert (future assistant of professor Potapczykowa at the University of Łódź), R. Antoszewski from Skierniewice, J. Rybczyński from Polish Academy of Sciences' Institute of Plant Genetics in Poznań, B. Skucińska and W. Miszke from the Agricultural University in Cracow. All members of the General Botany Laboratory used various in vitro techniques in their studies of hormone-induced morphogenesis and in developing the methods of micropropagation of cultivated plants.

## Meristem culture and elimination of viruses

In Poland, as in the rest of the world, in vitro methods have been utilized in many areas of research, each emphasizing the practical aspects of this technique. Freeing vegetatively propagated plants from viruses led to their rejuvenation, ensured better rooting success, increased yield and facilitated mass micropropagation of healthy planting material. In France the first plants to be freed from viruses were potatoes and dahlias. The demonstration that shoot apical meristems were free from plant viruses encouraged further work with meristem cultures of useful species. In Poland, K. Zaklukiewicz from the Institute of Potato Breeding in Bonin developed the methods of identification and control of virus diseases of potato [27]. This work initiated subsequent development of potato micropropagation [28], microtuber production [29] and germplasm preservation scheme and the specialization of the departments in Bonin and Młochów, where the gene banks were created of registered potato cultivars free from the most dangerous viruses (X, S, M, Y and potato leaf roll virus) as well as from quarantine pathogens (potato bulb PSTVd) [30,31].

Another species subjected to elimination of multiple viruses was the widely cultivated greenhouse carnation. At Poznań Agricultural University the Chair of Ornamental Plant Culture an in vitro laboratory was created by Władysław Oszkinis (with the assistance of AMU General Botany Laboratory) with the mission to free carnation plants from viruses [32]. After the premature death of W. Oszkinis, the laboratory was moved to the Owińska Greenhouse Enterprise where in 1968 it became the first commercial in vitro culture laboratory in Poland. Based on the theoretical foundations of viral disease control [33,34], in the period 1968–1983 the laboratory was involved in eradication of carnations through the meristem culture and in micropropagation of chrysanthemum, anthurium and gerbera [35,36]. In this laboratory future Heads of laboratories at leading horticultural institutions in Poland gained their training and experience. Initially, the position of scientific director was held by Krystyna Kukułczanka, the director of Wrocław University Botanical Garden. After her 1967 research visit with a virologist Dr. F. Quak in Wageningen she established in the Garden an active Tissue Culture Laboratory and achieved interesting results with micropropagation of bromeliads, sundews and orchids where protocorm proliferation was obtained with the aid of a rotating apparatus [37–41].

The initial period of plant tissue culture in Poland ended on February 14, 1973 when the 1st National Conference on Tissue Culture took place at the Institute of Plant Breeding and Acclimatization in Radzików. Over 30 participants of this conference, including K. Niemirowicz Szczytt, J. Rybczyński, B. Borkowska, E, Bartkowiak, A. Szweykowska, A. Stolarz, J. Rogozińska and M. and E. Zenkteler founded new Section of In Vitro Cultures affiliated to the Polish Botanical Society.

## Micropropagation

Enthusiastic scientists from the innovative Research Institute of Pomology and Floriculture in Skierniewice applied in vitro method to the propagation of ornamental plants and later also to vegetable and fruit crops. Micropropagation was accomplished not only via the simple division of the initial explants but, especially, by using the cytokinin induced mass proliferation of daughter plants that retained the traits of the parental plant [42–44]. The application of new methods to stimulate growth and development by using appropriate sets of growth regulators [45,46], the proper choice of explant types, introduction of new chemicals [47], testing of antibiotics for control of bacterial diseases [48], all brought about spectacular achievements. The team: M. Hempel, E. Gabryszewska, M. Podwyszyńska and T. Orlikowska made a significant scientific contribution to rapid development of the Research Institute of Pomology in Skierniewice and the horticultural industry in Poland in the 7th and 8th decades of the XX century. The extensive educational effort of the Skierniewice center consisting of multiple thematic conferences, demonstrations, training courses and lectures for horticultural growers encouraged the establishment of numerous private firms specializing in micropropagation of crop plants and rooting of micro-cuttings for which the demand was overwhelming. Towards the end of the 1970's Poland had 120 in vitro laboratories, i.e. more than the entire United States of America.

## Somaclonal variation

Under the influence of high concentrations of growth regulators, plants obtained via adventitious regeneration from somatic cells had permanently altered phenotypic traits such as plant height, stem habit, flowering and fruiting phenology and biochemical traits, such as the presence or absence of particular proteins. B. Borkowska [49] was among those Polish researchers that relatively early took notice of this variability, recognizing, in addition, the transient epigenetic variability determined both physiologically and environmentally, as well as the apparent variability occurring in chimeras [50]. In vitro cultures thus became a new source of variability for plant improvement. Intensively propagated regenerants, microcuttings

obtained from callus tissue and cell suspensions or protoplasts, all show modifications that are caused by gene mutations and by alterations in the number and structure of the chromosomes (inversions, deletions, translocations). Also an altered methylation pattern of nuclear and cytoplasmic DNA may be involved in this variability [50]. The frequency of somaclonal variants is very high in protoplast and somatic embryo cultures. For breeding purposes, this frequency has been further increased by applying chemical and physical mutagenic factors. According to T. Malepszy the use of mutagens in in vitro cultures allows researchers to produce large populations of biochemical mutants, whereas selective factors accelerate the recovery of genetically modified forms. Somaclonal variability, mutagens and techniques of their application and the inheritance patterns of the mutated traits have become important subjects of studies in in vitro cultures [51,52].

## Haploids and double haploid plants

Under experimental conditions haploid formation is induced by androgenesis caused by the action of stress on the whole plant, anthers or isolated microspores. In Poland, the 70's and 80's brought numerous publications on the induction of androgenesis, principally in members of the Solanaceae [24,25,53,54].

In the IHAR (Institute of Plant Breeding and Acclimatization) laboratories, research effort was mainly focused on the generation of haploid embryos and formation of double haploids (DH) in triticale [55,56], barley [57,58], wheat [59], oat [60] and oil rape [61,62]. Teresa Cegielska-Taras and her team developed a method for production of dihaploid rape using an alteration of the developmental program in isolated, colchicine-treated microspores subjected to a 30–35°C temperature treatment. Microspore embryos were obtained directly, without the callus proliferation stage. Conversion of the embryos into diploid plants was achieved by using a low temperature treatment. In vitro cultures of anthers and microspores allowed shortening of the time required for selection of homozygotic lines to one generation in contrast to the conventional selfing procedure that required 5–6 generations [63,64].

Currently, the list of cultivated species that have originated from haploids derived through in vitro androgenesis includes barley [58], wheat [59], rape [62], carrot [65–67], cucumber [68] and cabbage [69]. Production of embryos from female gametophyte cells via apogamy or parthenogenesis ensures stability of DH lines (while retaining a low rate of albinism). Haploid gynogenetic plants have been obtained in the process of breeding of high yielding hybrid cultivars in sugar beet [70,71] and onion [72,73].

In addition to anther and microspore cultures, haploids can be obtained through wide (interspecific or intergeneric) hybridization using the so-called *Hordeum bulbosum* method. In a *H. vulgare* × *H. bulbosum* hybrid, chromosomes of *H. bulbosum* were spontaneously eliminated both from the hybrid embryo and the endosperm, and the haploid embryo reached maturity after being transferred to an artificial medium. Haploid embryo production through elimination of chromosomes of one parent has been reported, among other species, in wheat [74] and barley [75]. During the 50 years that have elapsed since the first successful experiments on androgenesis and gynogenesis, a vast progress has taken place in understanding of this process. Androgenesis in vitro has been studied using advanced cytological, embryological and molecular methods. It has been used in genomics and proteomics, and DH lines have been used in genetic mapping, aiding the search for molecular markers of qualitative and quantitative traits [58].

## Culture of zygotic and hybrid embryos

With the introduction of in vitro methods to Poland, cultures of zygotic embryos started to be commonly used in physiological research and in studying plant responses to biotic and abiotic stresses [76]. At AMU in Poznań, isolated embryonic axes of *Lupinus luteus* have become a favorite model system in the studies of primary aminoacid biosynthesis [77] and aspartate metabolism [78]. Isolated lupine cotyledons have been used in studies of protein profiles during the development of these structures [79]. In subsequent decades, isolated axes were also used in studies of metabolic changes caused by carbohydrate starvation in leguminous plants [80].

Already in the early 60's, to eliminate prezygotic fertilization barriers Polish scientists used the methods of direct pollination of pistils with attached perianth, ovaries placed on artificial media with walls partly or entirely removed and isolated ovules [81]. These methods were applied with good effects to species possessing numerous ovules borne on large placentas, such as representatives of families Caryophyllaceae, Solanaceae, Brassiceae and Liliaceae. The success of in vitro fertilization was lower in species with small ovaries or reduced placentas [82]. In many combinations of wide crosses the only way to obtain hybrid plants was to isolate embryos at an early stage to prevent their malnutrition caused by poor endosperm development. Proembryos and embryos isolated at an early developmental stage ("embryo rescue") required media enriched with aminoacids or endosperm extracts for their further development [83].

Other measures used to rescue embryos included their contact with ovule tissue, osmotic adjustment of the media and lowering the mineral nitrogen level [84–86].

The range of questions associated with in vitro cultures of interspecific and integeneric hybrids is very wide and diverse [87–90]. These questions have been given much attention because of their significant practical implications, such as broadening of the species' genetic variability, creation of new gene combinations and the production of haploids.

The understanding of hybridization biology, fundamental for successful plant breeding, led in the 70's to the production of triticale. New varieties of this grain crop harmoniously combine the advantages of both parental species: wheat and rye. This major achievement was possible because of a long-term cooperation between leading cytogeneticists (Czesław Tarkowski) and plant breeders (Tadeusz Wolski). Thanks to the work of these researchers, the best, high yielding varieties of triticale are of Polish origin. In the 80's, Janusz Zimny and his team obtained new useful qualities in cereals (including triticale) by taking advantage of wide hybridization, androgenesis and somatic embryogenesis [55,90]. Another major success of a long term breeding effort was the production of an intergeneric hybrid *Festulolium* by Z. Zwierzykowski from the Polish Academy of Sciences Institute of Plant Genetics. This highly stress resistant grass crop is much valued by farmers [91,92].

Hybrid embryos and plants were obtained in many combinations of intergeneric pollination between *Salix* and *Populus*. The objective of these attempts was to introgress the genes of poplar, the genus with large biomass increments, into the

genome of the fast-growing short rotation willow, and are therefore important from the point of view of sustainable energy economy [93–97]. Intergeneric hybridization has also been used to improve other species grown for their rapid biomass accumulation, such as *Miscanthus ×giganteus* [98–100]. Regeneration of somatic embryos will facilitate vegetative propagation of *Sida hermaphrodita*, a species with poor seed setting success [101].

## Somatic embryogenesis

Regeneration of embryos in tissue culture through somatic embryogenesis (SE) is a classical example of mass asexual propagation. Jan Rybczyński from Polish Academy of Sciences Botanical Garden pointed out the superiority of somatic embryogenesis over adventitious organogenesis, especially in clonal propagation of genetically stable material [102–104]. There has been a growing number of reports on direct somatic embryogenesis and embryo formation from leafy or cotyledonary explants in wheat [105], rye [102], gentian [106], clover [104–107], cucumber [108] and coniferous trees [109]. This method has not only become widely used in plant improvement procedures but has also been applied to the micropropagation of ornamental bulbs from the Liliaceae family [110], such as *Tulipa*, *Hyacinthus* [111], *Leucojum* and *Galanthus* [112].

When investigating the phytohormonal regulation of somatic embryogenesis, E. Kępczyńska from Szczecin University evaluated the influence of gibberelins [113], ABA and MeJA [114,115], ethylene [116] and auxins on specific phases of SE (i.e. induction, proliferation, differentiation, maturation and embryo regeneration) in two model species: *Medicago sativa* and *M. truncatula*. She found that endogenous jasmonates and ABA (natural growth inhibitors) restrict proliferation and growth of proembryogenic callus but stimulate germination and conversion of alfalfa embryos [116]. She continues her work on increasing the rates of germination and conversion of embryos of cultivated plants, aiming to formulate the optimal conditions for somatic embryo production.

The genetic determinants of reversion of plant developmental program and turning on the direct SE program in *Arabidopsis* were analyzed by M. Gaj and her research team from Silesia University at Katowice. While investigating the molecular mechanisms controlling the plasticity of somatic cells, she identified transcription factors, some of which exhibited unique expression patterns in the embryogenic cells. She demonstrated a differentiating and auxin dependent expression pattern of the gene *bHLH109* during SE, a drastically lower level of its expression in nonembryogenic callus tissue and its low activity in tissue stimulated towards organogenesis [117–119]. Further progress of these studies depends on determining whether the expression of the genes *LEC1* and *FUS3*, important in the zygotic embryogenesis, also influences the development of somatic embryos [120].

Artificial seed technology is an attractive and applied research field that has been developing for more than a decade. It involves the utilization of somatic embryos, axillary buds or growing tips for pelleting in hydrated (hydrogel) or dehydrated (calcium alginate) capsules [121,122]. Growing tips and axillary buds of numerous medicinal plants have been subjected to pelleting procedure to produce planting material [123]. The use of artificial seeds as planting material has been accepted for species exhibiting poor seed set or low germination rates, however this technology still requires improvement if mass production of such seeds is to become economically viable. Another use for pelleted somatic embryos or isolated embryonic axes is the long-term storage of wild, protected or cultivated species in gene banks [124]. The method involves subjecting the embryos to the cryoprotection procedure and storage under the cryopreservation regime (i.e. gradual temperature decrease to −196°C).

## Secondary metabolites

Callus of medicinal plants was produced for the first time by the in vitro method at the end of the 60's. Purification and identification of chemical structure of active compounds present in tissues of medicinal plants and in in vitro derived callus lines was the standard already at this early stage of phytotherapeutical studies [124–126]. Leaders in the field at that time were L. Skrzypczak from Pharmacology Department of the Medical University in Poznań and M. Furmanowa from Pharmacology Department of the Medical University in Warsaw. Progress in the production of metabolites in cultures took place only after the methods were developed for cell proliferation in suspension cultures and the conditions for biosynthesis of therapeutic compounds were optimized [127]. Cell cultures may therefore constitute an alternative to field cultivation of medicinal plants in Poland. In cell cultures the concentrations of bioactive metabolites, such as pigments, volatile oils, antioxidants, polysaccharides, was stimulated by physical or chemical factors to exceed many times the levels present in plant tissues. Overproduction of secondary metabolites in *Lithospermum erythrorhizon* (shikimic acid), *Ruta graveolens* (coumarins), *Catharanthus roseus* (serpentine and ajmalicine), and others was achieved by stimulation of the cell suspensions with elicitors or precursors, or by oxygenation or alteration of the pH of the medium [128,129]. Especially valuable plants are those producing anti-cancer compounds, such as paclitaxel isolated from *Taxus baccata* cultures, harringtonine isolated form tissues of *Cephalotaxus harringtoniana*, and cytostatic compounds such as vinblastine and vincristine [130,131]. Hopes are high for hupercine, a substance obtained from *Huperzia selago*, that may slow down the progress of Alzheimer's disease [132]. The production of biologically active substances based on biotransformation of exogenous compounds has proceeded on a commercial scale owing to the many years of research by A. Chmiel and H. Wysokińska from the Medical University of Łódź [133]. An efficient synthesis of bioproducts in *Agrobacterium rhizogenes* LBA 9402 transformed hairy root cultures has been achieved in a mist bioreactor [134,135]. The controlled aeration ensured a high biomass growth and steady levels of synthesis of biologically active metabolites. In shoots transformed by *Agrobacterium tumefaciens*, Ti plasmid elements introduced into plant cell genome induce the formation of stem teratomas. Extracts from teratomas of *Drosera aliciae* contaning naphtochinones and flavonoids showed a very high antibacterial activity [136].

Currently, in Polish pharmacological research centers studies are being conducted on the second-generation biopharmaceuticals – new drugs isolated from recalcitrant cultures. Pharmaceutical industry is turning back to natural bioproducts, supported by the progress of ethnobotany and ethnopharmacology, and the search for new, more efficient sources of biologically active compounds is very advanced [137].

## Disease resistance

The cultures of tissues, cells and protoplasts were not only useful in the production of pathogen free plants in the 60's and 70's but have been used in the later period in the studies of plant-pathogen interactions.

In physiological studies on raspberry callus tissue, infection by the fungus *Didymella applanata* enhanced the synthesis and accumulation of polyphenols, and caused an increase in peroxidase activity. The fungus also caused changes in the host cell membrane properties. The rate at which *D. applanata* hyphae colonized raspberry calli in the presence of auxins and cytokinins was proportional to the concentration of these growth regulators [138,139].

The susceptibility of cultured lupine embryonic axes to infection by *Fusarium oxysporum* was shown to be connected to low sugar concentration in the medium. The lowering of endogenous concentration of soluble carbohydrates led to a decreased osmoticum in the axes facilitating the growth of the infecting hyphae. In addition, the low intensity of respiration restricted the amount of energy needed for defense (synthesis of the PR proteins decreased) [80].

In the search for sources of resistance in flax, principal genes involved in biosynthesis of antioxidants and in the control of phenylopropanoid pathway responsible for counteracting *Fusarium* infections have been identified [140]. The level of terpenoids in flax tissues increased in response to the infection by *Fusarium* [141].

Another method of building up plant resistance against pathogens was the creation of androgenic plants with an increased disease resistance. One of the first achievements of this approach was the demonstration of an enhanced resistance to parasitic wilt caused by *Fusarium oxysporum* in *Linum usitatissimum* anther culture regenerants. This system was later used to create a transgenic flax [142]. Another interesting study tested *Fusarium* resistant wheat cultivars for their suitability for androgenesis and generation of haploid lines with an elevated pathogen resistance [143]. Double haploids of tobacco also showed an enhanced resistance to PVT [144].

Bacterial or fungal toxins introduced into the medium are commonly used as a selective factor in tissue cultures. In the resistance-breeding program using somaclones of strawberry cv. Filon and cv. Teresa, selection was conducted using homogenized mycelium of *Verticillium dahlii* [145,146]. Filtered, unpurified *Alternaria radicina* toxin filtrate applied to carrot protoplast cultures induced resistance of regenerants to the pathogen. Toxins at various concentrations were the selective factors [147]. Mycelial cultures of macromycetes have also been initiated with the goal to use filtrates of their toxins [148].

When in the 80's of the XX-th century somatic hybridization methods became available, they made possible the transfer of resistance traits from the wild species into cultivated varieties. Hybrids between *Solanum tuberosum* and *S. pinnatisectum* inherited resistance to *Phytophtora infestans* and hybrids between *S. tuberosum* and *S. brevidens* showed resistance to A, X and Y potato viruses and to fungi *Verticillium* and *Alternaria* as well as the bacteria *Erwinia*. The *Brassica oleracea* × *B. napus* hybrids gained resistance to *Erwinia carotovora* [149].

An alternative method to resistance breeding in vitro involved the inoculation of symbiotic micro-organisms: *Pseudomonas* (bacterization) or *Glomus* (mycorrhization) either in vitro or post vitro in order to protect weaned-off plants from soil-borne pathogens after planting in the field [150]. The application of molecular methods (DNA and RNA analysis, hybridization of nucleic acids, GISH, RFLP, RAPD and PCR reaction) made it possible to detect and identify pathogens in stock plants, initial explants and in plant material propagated in vitro, thus complementing the pathogen control methods [151].

Expectations that genetic engineering techniques would offer many new possibilities for viral and fungal disease control have not been fully met. Researchers planned to obtain plants resistant to viruses by introducing fragments of viral DNA into the plant genome. This so called pathogen dependent resistance (PDR) is highly specific towards the virus from which cDNA used for plant transformation originated but also makes it possible to introduce resistance to more than one viral strain. Work is currently ongoing on introducing resistance to Y virus and leaf roll virus into potato using the transgene technology [152].

## Somatic hybridization and transformation of plants

Pioneering work involving the use of cell wall digesting enzymes, the release of protoplasts, and the optimization of conditions for their culture was conducted by Edward Pojnar at Cracow Agricultural University [153,154]. Optimization of protoplast culture of tomato, pea and ornamental asparagus has been continued by A. Pindel and members of his laboratory [155,156]. The team led by W. Orczyk and A. Nadolska-Orczyk, after 40 years of studies of potato protoplasts, has improved the technique of isolation, stimulation of regeneration (from protoplasts through cells and tissues to plants) and the methods of fusion and hybridization of related and unrelated species [157]. The number of interspecific and intergeneric hybrids of species from the families Solanaceae and Brassicaceae obtained via somatic hybridization has exceeded 50 [149]. The fusion of protoplasts in electrical field or by the chemical method (using PEG) resulted in production of tetraploid potato cultivars. The diploid components were united within species or between species, introducing the genome of a wild, disease resistant species into a cultivated variety [158,159]. Fusion of tomato and potato followed by backcrossing yielded tomato plants carrying cytoplasmic male sterility as well as cybrids (called pomato/topato) [149]. In subsequent years, activities of the team turned to vector-assisted (using *A. tumefaciens* [160]) and vectorless protoplast transformation by direct gene transfer in the presence of poliethylene glycol, electroporation, microinjection or DNA delivery by a gene gun [160]. The introduction of DNA into protoplasts demands a high regenerability of the cultures as a prerequisite of production of transformed plants [161]. This has been achieved in cases of *N. tabacum*, *B. rapa*, *Brassica napus*, *Paeonia hybrida*, *Zea mays* and *Lactuca sativa* protoplasts.

The development of an efficient regeneration system in wheat and barley (organogenesis, androgenesis, somatic embryogenesis) was a prelude to the transformation of grain crops through the introduction and integration of foreign DNA with the use of *A. tumefaciens* and combinations of various selective genes and the reporter gene GUS [162]. Experiments were initiated towards genetic transformation in planta by inoculation of wheat and barley spikes with a suspension of *A. tumefaciens*. This work has now been discontinued.

In compliance with EU requirements, the 22 June 2001 Act banned the cultivation of genetically modified plants and

the production of modified food and drugs and required the obligatory registration and monitoring of "confined use of GMO" by appropriate Authorities. The banning of cultivation of genetically modified plants caused researchers to turn their attention to molecular basis of genetic processes, such as detection of DNA sequence polymorphism, acquired stress resistance, creation of gene constructs. The need arose to develop quick identification methods for GMOs present in specific products [163]. The lack of approval for transgenic plants by the Polish society and the threat to crop biodiversity imply that further studies are required on the impact of transgenic plants on the environment and that exhaustive information on the results must be widely distributed.

## Summary

The list of accomplishments of Polish in vitro specialists includes hundreds of methodological protocols for organogenesis induction, production of haploids and dihaploids, somatic embryogenesis, hybridization and transformation of cultivated species. Many phenomena and processes have been explained and new valuable cultivars were produced. The data have been published in thousands of original articles, dozens of reviews, and textbooks: "Plant biotechnology" ("Biotechnologia roślin") [164] (already in its third edition), "Plant cell and tissue culture" ("Hodowla komórek i tkanek roślinnych") [74], "Biotechnology in genetics and plant breeding" ("Biotechnologia w genetyce i hodowli roślin") [52], "Plant cells under stress" ("Komórki roślinne w warunkach stresu") [165] and in laboratory manuals [166]. A forum for wide-ranging information exchange is provided by the National Conferences on Plant Tissue Culture and Biotechnology organized every three years under the auspices of Polish IAPTC section and In Vitro Culture Section of the Polish Botanical Society.

Thirteen such conferences have been held to date and the attendance was high (180–260 participants). They were organized alternately by various prominent research centers representing different specializations. The first of these meetings took place in 1972 and was organized by IHAR Radzików staff and the most recent one – in 2012 in Rogów, and was organized by the Polish Academy of Sciences' Botanical Garden in Warsaw. Additionally, since 1996 F. Dubert from the Institute of Plant Physiology of the Polish Academy of Sciences in Cracow has been organizing a biennial national conference "Application of in vitro cultures in plant physiology". The tremendous progress and acceleration in the field took place after the introduction of biotechnological methods, molecular analyses and transgenesis, as well as digital data recording and computer analysis allowing visualization and modeling of biological processes in order to study their causes and the perspectives for their practical applications. Future development of disciplines related to in vitro culture will occur in association with genetics and biotechnology, providing substrates for pharmaceutical, chemical and fermentation industries and contributing to creation and utilization of renewable energy sources and raw materials.

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## Authors' contributions

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