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REVIEW in POLISH BOTANY CENTENNIAL

Application and Improvement of In Vitro Culture Systems for Commercial Production of Ornamental, Fruit, and Industrial Plants in Poland

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Abstract

This work focuses on the achievements of Polish researchers in the field of vegetative reproduction of plants under in vitro conditions. For more than 50 years, micropropagation methods have been used in Poland whenever vegetative reproduction is necessary. Most perennial horticultural crops, such as fruit plants, the majority of ornamental geophytes, and some vegetables (e.g., rhubarb and horseradish), require clonal reproduction owing to their high heterozygosity, i.e., their offsprings when reproduced from seeds, do not repeat the parental characteristics. Various goals have been pursued in the development of regenerative and in vitro propagation systems for each of the aforementioned groups of plants, such as pathogen elimination, reproduction of healthy plants, rapid multiplication of newly obtained valuable breeding lines and cultivars, and breeding to obtain polyploids, haploids, and doubled haploids. Owing to the growing interest of researchers and plant producers in environmentally friendly technologies, one of the sections is devoted to the issue of biotization of micropropagated plants.

Keywords

biotization; commercial micropropagation; historical overview; planting material; virus eradication

1. Introduction

Vegetative propagation of plants under in vitro sterile conditions on synthetic media has a long history that began in Graz, Austria, when Gottlieb Haberland was the first to maintain mesophyll tissue cultures in vitro (Gautheret, 1985). Haberland published his results in 1902. He was the first to hypothesize that all plant cells are totipotent and can regenerate a whole plant from a single cell under certain conditions. Subsequently, a great achievement in the development of in vitro plant cultures was obtained in 1934 by White for long-term in vitro root cultures of tomato. Two French workers, Roger Gautheret from Paris and Pierre Nobécourt from Grenoble, who had been attempting to culture plant tissues for several years, reported potentially unlimited growth of carrot tap root tissue when the growth substance indole-3-acetic acid (IAA) was incorporated into the culture medium.

In vitro cultures were first used for basic research on the physiological and morphogenetic processes. In the 1960s, with the development of a medium for in vitro culture of tobacco calli by Murashige and Skoog (MS) (1962), in vitro cultures began to be used for various scientific and commercial purposes, including mass

plant propagation. The basic composition of the MS medium, including the mineral salts, vitamins, and sucrose, with minor modifications and enriched with various growth regulators appropriate for a given plant material, is now most commonly used for in vitro culturing of various organs, tissues, and cells of many plant species.

A historical overview on the development of plant tissue culture in Poland was presented by M. Zenkteler and Zenkteler (2013) from the Institute of Experimental Biology, Adam Mickiewicz University (AMU), Poznań, Poland – the scientific center where it all began in the country. Pioneers in Polish research on plant tissue culture were scientists from AMU: Jerzy Czosnowski and Janina Rogozińska, who gained experience with Roger Gautheret at Sorbonne University (Paris, France), and Alicja Szweykowska, who worked with Folke Skoog at the University of Madison, Wisconsin, USA. During her postdoctoral research, Janina Rogozińska participated in the discovery of cytokinins and explained their role in plant morphogenesis (Rogozinska et al., 1964). The discovery of cytokinins was a milestone in research on plant tissue culture, especially vegetative propagation in vitro. In turn, at Poznań Agriculture University (PAU), the first Polish laboratory dedicated to releasing carnations free from viruses was established (M. Zenkteler & Zenkteler, 2013). Carnations were very popular at the time and were massively grown in greenhouses for cut flowers. This laboratory, which was founded by Władysław Oszkinis after moving to Owińska near Poznań, gave rise to the first commercial laboratory in the country for plant tissue culture. Thanks to the achievements of these researchers, the Poznań laboratories of AMU and PAU became the centers for plant in vitro culture, influencing other scientific institutions in Poland.

One of the conditions for success in the cultivation of in vitro-propagated plants is a high health status and good quality of planting material. Diseases caused by viruses, viroids, and phytoplasmas, transmitted by insects, mites, and nematodes, and during vegetative reproduction, are major problems in vegetatively propagated perennials (Cieślińska & Malinowski, 2002; Górecka & Lehmann, 2001; Madhavan et al., 2021). Owing to the lack of effective methods for chemical control of the above-mentioned pathogens, special attention was paid to the health status of the planting material. A brochure was recently published by a research team from the National Institute of Horticultural Research, Skierniewice, Poland (NIHR), which presented recommendations for obtaining high-quality planting material (Wojtania, Markiewicz, Wójcik, et al., 2020). The authors have described a step-by-step quality control system for in vitro propagated plants based on three pillars: (***i***) health control, (***ii***) assessment of plant growth and development and their physiological condition, and (***iii***) verification of genetic identity and stability. During micropropagation, it is essential to control the presence of both pathogenic organisms and saprophytic species of bacteria and fungi, which can behave like "vitropathogens" under the specific conditions of in vitro culture (Orlikowska et al., 2017). Treatments limiting the occurrence of infections in in vitro cultures include preculture of donor plants under strict sanitary conditions, chemotherapy and thermotherapy, surface disinfection of explants, indexing of initial explants using microbiological media, frequent monitoring of cultures and elimination of primary and secondary contaminants, and the use of microbiological media or classical biochemical methods for the detection of microorganisms inhabiting explants asymptomatically. In vitro propagation of plants under controlled, sterile conditions means that the plants are devoid of their natural microbiome. This may result in slower growth and increased susceptibility to pathogen infection. Recently, an increasing number of researchers have focused on the biotization of in vitro-propagated plants, i.e., the colonization of plants with beneficial bacteria or fungi (Orlikowska et al., 2017). Owing to the growing interest of researchers and plant producers in environmentally friendly technologies, one of the sections is devoted to the issue of biotization of micropropagated plants.

A condition for the successful use of in vitro techniques for mass production is the maintenance of the genetic stability of a given genotype. It is well known that micropropagation may be accompanied by somaclonal variations resulting from permanent changes (genetic variation, e.g., point mutations or changes in the number or structure of chromosomes) or transient changes (epigenetic variability,

changes in DNA methylation pattern) (Miler & Zalewska, 2014; Pawełkowicz et al., 2021). The degree of variation depends on the regeneration system, culture conditions, and genotype. The authors emphasize that to avoid mutations, it is optimal to use axillary shoots during the multiplication stage, to use growth regulators (especially cytokinins) at the lowest possible concentrations, and to use in vitro culture renewals every year. Propagule production via somatic embryogenesis, adventitious regeneration of shoots or bulbs, and especially using calli, may increase the probability of mutation. Most of the changes at the DNA level are not expressed phenotypically. Determining the actual degree of genetic variation in plants obtained by micropropagation as compared to the mother plant is possible using molecular methods. The most frequently used markers are random amplification of polymorphic DNA (RAPD), simple sequence repeat (SSR), inter simple sequence repeat (ISSR), and amplified fragment length polymorphism (AFLP) (Martínez, 2018). All of them have been successfully used for years as tools to identify genotypes, test genetic identity, and determine the stability of micropropagated plants, as described in the following sections.

Micropropagation methods have been used in Poland whenever vegetative reproduction was necessary (M. Zenkteler & Zenkteler, 2013). Most perennial horticultural crops, such as fruit plants, the majority of ornamental geophytes, and some vegetables, such as rhubarb (Wojtania & Mieszczakowska-Frąc, 2021) or horseradish (Górecka, 1992), require clonal reproduction owing to their high heterozygosity, i.e., their offsprings when reproduced from seeds do not repeat the parental characteristics. Various goals have been pursued in the development of in vitro propagation systems for each of the aforementioned groups of plants, such as pathogen elimination, reproduction of healthy plants, and rapid multiplication of newly obtained valuable breeding lines and cultivars. This work focuses on the achievements of Polish scientists in the field of vegetative propagation of plants under in vitro conditions.

2. Ornamental Plants

Commercial-scale micropropagation using apical and axillary buds was successfully applied for ornamental plant propagation in the early 1970s. In Poland, the first studies refer to in vitro propagation of virus-free carnations (Dąbski et al., 1979; Oszkinis et al., 1974; Weryszko & Hempel, 1979). The first commercial in vitro culture laboratory was established in the Owińska Greenhouse Enterprise. During the period 1968–1983, this laboratory was involved in virus eradication from carnations through meristem culture, and through micropropagation in chrysanthemum, anthurium, and gerbera. Initially, the position of scientific director at the laboratory was held by Krystyna Kukułczanka (M. Zenkteler & Zenkteler, 2013). A significant contribution to the horticultural industry through the development of new methods for ornamental plant propagation was made at the Research Institute of Pomology and Floriculture (currently known as NIHR) in Skierniewice, Poland, by a group of researchers, including M. Hempel, T. Orlikowska, E. Gabryszewska, and M. Podwyszyńska. Research on the in vitro propagation of ornamental plants has been carried out for several decades at various scientific and commercial laboratories in Poland.

The annual world production of ornamental plants by in vitro cultures (approximately 160 genera) over the past 10 years has increased from 800 million to 2 billion. Of 140 commercial in vitro laboratories in Europe, 20 are situated in Poland, producing 70 to 100 million microplants annually, 80% of which are ornamentals (Gabryszewska, 2013; Kulus, 2015). Most of them (80%) are exported to Holland, the USA, Great Britain, Spain, Israel, Hungary, the Czech Republic, Turkey, Lithuania, Ukraine, and Russia (Ilczuk et al., 2013; Purohit et al., 2011). The four main groups of ornamentals propagated under in vitro conditions are: (***i***) perennials (e.g., *Lilium*, *Hosta*, *Heuchera*, *Hemerocallis*); (***ii***) trees, shrubs, and climbers (e.g., *Rhododendron*, *Rosa*, *Hydrangea*, *Magnolia*, *Syringa*, *Cottinus*); (***iii***) potted plants (e.g., *Alocasia*, *Calathea*, *Ficus*, *Maranta*, *Syngonium*, *Yucca*, *Santpaulia*, *Musa*, *Neohrolepis*, *Phalenopsis*, *Pelargonium*, *Chrysanthemum*);

and (***iv***) cut flowers (*Anturium*, *Lilium*, *Rosa*, *Gypsophila*, *Limonium*, *Zanthedeschia*) (Gabryszewska, 2013).

2.1. Ornamental Geophytes

2.1.1. Tulipa *spp.*

Tulips are one of the leading cut flowers produced worldwide (Orlikowska et al., 2018). However, tulip breeding is time-consuming and expensive. From the selection of the first seedlings to the propagation of 10,000 bulbs of a new cultivar, its introduction to the market can take 20–25 years. This period can be shortened by using the in vitro propagation method. Extensive studies have been undertaken by a team from NIHR to improve the micropropagation methods of tulip (Podwyszyńska & Marasek, 2003; Podwyszyńska et al., 2014; Podwyszyńska & Sochacki, 2010). As a result of their research, cyclic multiplication of adventitious shoots of this geophyte was made possible using a medium supplemented with thidiazuron (TDZ) in combination with cytokinin isopentenyladenine (iP). Moreover, in the last micropropagation stage, the formation of microbulbs was significantly improved, which is very important because only bulbs are capable of rooting and further growth in the soil. Thus, prolongation of the last multiplication subculture prior to cooling, combined with growth retardant and methyl jasmonate (MeJA) treatments following the low-temperature treatment significantly enhanced the shoot's bulbing capacity.

To mitigate the impact of severe viral infection on tulip production, an in vitro method of virus eradication from plant material was developed. The method is based on the application of ribavirin for in vitro chemotherapy (Sochacki & Podwyszyńska, 2012). This novel method has enabled quick, virus-free multiplication of several Polish tulip cultivars. Recently, an effective method for tulip regeneration via somatic embryogenesis (SE) was developed (Podwyszyńska & Marasek-Ciolakowska, 2020). Successful results in tulip SE have also been reported by a team from the University of Agriculture in Cracow (Bach & Ptak, 2001; Maślanka & Bach, 2016; Ptak & Bach, 2007). Maślanka and Bach (2014) also made a large contribution to the development of the tulip micropropagation method. Their method is based on the direct regeneration of adventitious shoots on the seedling explants of *Tulipa tarda* and could be used to reproduce endangered wild tulip species.

2.1.2. Narcissus *spp.*

Narcissus is one of the major ornamental bulbous crops. As a perennial geophyte, *Narcissus* bulbs are prone to viral accumulation. To obtain potyvirus-free *Narcissus* plants, a team from NIHR developed an in vitro method for virus eradication and *Narcissus* micropropagation (Sochacki & Orlikowska, 2005; Sochacki & Podwyszyńska, 2012). In this method, buds are regenerated in vitro on explants taken from the bulbs of infected plants, and developing shoots cyclically multiplied and tested for viruses. This method is recommended for the development of high-quality reproductive stocks of *Narcissus*. An innovative protocol for large-scale production of *Narcissus* 'Carlton' somatic embryos was developed based on repetitive somatic embryogenesis (Malik & Bach, 2017). This procedure was established as a stepwise process beginning with primary somatic embryogenesis in ovarian explants, followed by secondary somatic embryogenesis (SSE) and continuous repeated cycles of SSE in the presence of 6-benzylaminopurine (BAP) and amino-3,5,6-trichloropicolinic acid (picloram) or 2,4-dichlorophenoxyacetic acid (2,4-D). On regeneration medium containing BAP and 1-naphthaleneacetic acid (NAA), it was possible to obtain more than 20 embryos per 100 mg of callus.

2.1.3. Hemerocallis *spp.*

Daylilies are becoming increasingly popular as garden perennials owing to the high decorative value of their flowers and their low soil requirements. For many years, daylily cultivars have been propagated in vitro via plantlet regeneration from calli. In the method developed at the Institute of Pomology and Floriculture in

Skierniewice, various types of initial explants were used, including shoot tips, fragments of flower buds, and other parts of the inflorescence (Gabryszewska & Wojtania, 2005). The use of TDZ in combination with BAP, 6-furfurylaminopurine (kinetin), and iP in the medium significantly accelerated shoot regeneration and increased the multiplication efficiency. This method is used for the multiplication of daylily cultivars on a commercial scale, as well as in breeding works for the propagation of selected genotypes and production of plant material for breeding purposes.

2.1.4. Paeonia *spp.*

Peony is grown as a garden and medicinal plant. *Paeonia lactiflora* and *P. officinalis* are the most commonly cultivated species. Over the last two decades, there has been a marked increase in the popularity of peonies as cut flowers. Gabryszewska (1998) developed an in vitro propagation method for herbaceous peony 'Jadwiga,' in which axillary shoot formation was significantly enhanced by adding TDZ at a very low concentration (0.01 mg L^{-1}) to MS medium containing a mixture of BAP, iP, and kinetin (each at 1 mg L^{-1}). Subsequent studies showed that formation of the renewal buds and shoots depended on the physiological status of shoots (cooled and non-cooled) (Gabryszewska, 2006), the glucose level in the medium, and the type of explant (with or without leaves) (Gabryszewska & Kawa-Miszczak, 2010).
The highest rooting frequency was observed in the medium without auxin, at temperatures of 15 or 20 °C. However, during acclimatization to ex vitro conditions, the plants showed a strong tendency for premature dormancy. This was manifested by starch accumulation, stunted growth, and dormant bud development.

2.1.5. Other Ornamental Geophytes

Other ornamental geophytes grown domestically as cut flowers or pot plants have been the subject of research aimed at developing micropropagation methods for nursery material production and breeding purposes. Efficient in vitro methods of vegetative reproduction have been developed, inter alia, for *Helleborus niger* (Gabryszewska, 2015; Matysiak & Gabryszewska, 2016), *Hippeastrum* (Ilczuk et al., 2005; Sochacki et al., 2018; Witomska et al., 2008), *Fritillaria imperialis* (Witomska & Łukaszewska, 1996), *Gloriosa rothschildiana* (Kozak, 2002a, 2002b) and *Zantedeschia rehmannii* (Kulpa, 2016).

2.2. Ornamental Trees and Shrubs

Woody plants are known to be difficult to propagate in vitro. In many trees and shrubs, low activity of axillary buds, tissue browning, and the subsequent death of cultured explants, as well as a low rooting ability, have been observed. There is a growing interest in the micropropagation of ornamental trees and shrubs. However, for many species, efficient methods that can be used in commercial production are still lacking (Gabryszewska, 2013).

2.2.1. Rhododendron *spp.*

Rhododendron was one of the first ornamental shrubs to be micropropagated on a mass scale. Its production has increased from zero in 1985 to 1.4 million plants within 10 years (Hutter & Schneider, 2019). Large-scale micropropagation of *Rhododendron* has been carried out in the USA and Europe (Belgium, Germany, and Poland). Bojarczuk (1996) was the first to develop an in vitro propagation method for different *Rhododendron* species and cultivars in Poland. She achieved the best shoot development or regeneration when initiating shoot cultures from vegetative and floral buds collected in February and March, cultured on 50% Anderson's (1975) medium supplemented with IAA and iP. Microcuttings rooted best under ex vitro conditions in a non-sterile medium (peat and perlite 3:1) treated with *Trichoderma viride*. Recently, Pacholczak et al. (2020) reported the efficient shoot multiplication of *Rhododendron* 'Ken Janeck' in the presence of iP and zeatin, and rooting in vitro on a medium with indole-3-butyric acid (IBA). Research conducted by Jesionek et al.

(2016) on the micropropagation of *R. tomentosum* is also worth noting. In this method, shoots are initiated from leaf explants and multiplied on Hildebrandt (SH) medium containing iP and TDZ, then elongated and rooted using perlite substrate saturated with 50% woody plant medium (WPM) supplemented with 1% sucrose and IBA. The high level of genetic stability of these micropropagated plants was confirmed by RAPD analysis. Considering that most species and cultivars of *Rhododendron* are characterized by poor growth in the soil with high content of calcium ions and high pH, Giel and Bojarczuk (2002) studied the effect of high concentration of calcium salts [$CaCl_2$, $CaSO_4$, $Ca(NO_3)_2$, and $CaCO_3$] in a medium on the growth of *Rhododendron catawbiense* 'Grandiflorum' microcuttings to determine their sensitivity to such stress conditions. The results of these studies were the basis for planning further studies on the selection of variant genotypes that are more tolerant to high levels of calcium in the soil.

2.2.2. Rosa *spp.*

Roses are some of the most important commercial crops used in ornamental horticulture. Some genotypes also contain bioactive and pro-health compounds in fruits, flower petals, and leaves; therefore, they are used as raw materials in food and herbal industries. In vitro propagation method has been developed in Poland for different rose genotypes, including miniature roses 'Starina' and 'White Gem' (Podwyszyńska & Goszczyńska, 1998; Podwyszyńska & Olszewski, 1995); the naturally occurring wild species in Poland, viz. *R. canina*, *R. dumalis*, *R. rubiginoisa*, and *R. tomentosa* (Pawłowska, 2011); rootstocks *R. multiflora*, *R. indica*, and *R. manetti* (Kucharska et al., 2006); as well as hiproses *R. pomifera* 'Karpatia' (Kwaśniewska et al., 2017) and rose 'Konstancin' (Wojtania & Matysiak, 2018). Most rose genotypes showed the best growth on MS medium supplemented with BAP alone (Kucharska et al., 2006) or in combination with gibberellic acid (GA_3) (Pawłowska & Szewczyk-Taranek, 2014; Wojtania & Matysiak, 2018). Podwyszyńska and Olszewski (1995) modified the mineral composition of standard MS medium by adding 1.5-fold higher levels of Ca^{2+} and Mg^{2+}, and twofold higher levels of Fe^{2+} and Mn^{2+}. This resulted in the elimination of shoot tip necrosis and leaf yellowing. Wojtania and Matysiak (2018) improved shoot multiplication efficiency in rose 'Konstancin' by lowering the sucrose concentration in the medium to 20 g L^{-1}. The authors observed that the ethylenediamine di-2-hydroxy-phenyl acetate ferric acid (Fe-EDDHA) form was superior to ferric ethylenediamine-tetraacetic acid (Fe-EDTA) in producing higher-quality, longer microcuttings that were easier to root. Malik et al. (2018) developed an efficient method for the micropropagation of *Rosa canina* using a temporary immersion system.

2.2.3. Magnolia *spp.*

Magnolias (Magnoliaceae) are deciduous or evergreen trees and shrubs, prized worldwide for their majestic form and the beauty of their flowers. Because of the high popularity of these species and the problems with propagation by traditional techniques, the method of micropropagation of different magnolia species has been developed in NIHR in collaboration with researchers from the Franciszek Górski Institute of Plant Physiology of the Polish Academy of Sciences in Cracow (Wojtania et al., 2015, 2016). These studies showed that the main factor inducing shoot formation is the cytokinin BAP, but the activity of axillary buds and shoot quality depend on the levels and ratio of cytokinin, sucrose, and nitrogen salts in MS medium. Similar to other woody plant species, shoot rooting is one of the most critical stages in the in vitro propagation of magnolia. The best root formation was obtained in a medium containing high levels of IBA (6 mg L^{-1}) alone or in combination with NAA (Wojtania, Dziurka, & Skrzypek, 2020; Wojtania et al., 2019). Wojtania, Dziurka, and Skrzypek (2020) found a clear relationship between the over-accumulation of chlorogenic acid and coumaric acid in the late phase of rooting and the low rooting response of recalcitrant cultivars such as 'Yellow Bird' and 'Butterflies.' All these findings contributed to the development of a relatively effective micropropagation method for magnolias.

2.2.4. Clematis *spp.*

Clematis is a climbing vine or herbaceous perennial plant with beautiful, colorful flowers. It offers endless possibilities for enhancing gardens and urban landscapes. Micropropagation is used to accelerate the introduction of new cultivars in the market and for the multiplication of species and cultivars of *Clematis* that are difficult to propagate traditionally by cuttings. Studies on the in vitro propagation of *Clematis* vines were carried out at the Institute of Pomology and Floriculture in Skierniewice (Gabryszewska et al., 2008) and the University of Life Sciences in Lublin (Parzymies & Dąbski, 2012). Most *Clematis* species and cultivars have a strong apical dominance. Therefore, it is difficult to achieve axillary bud development in vitro. Parzymies and Dąbski (2012) showed that the branching and shoot quality of *C. integrifolia* and *C. viticella* were higher in MS medium containing iP or kinetin, and that the shoot tips needed significantly higher cytokinin levels for efficient shoot multiplication compared to nodal explants. Gabryszewska et al. (2008) improved the shoot propagation rate of *C. pitcheri* using MS medium supplemented with 0.2 mg L^{-1} *meta*-topolin (mT) and nitrogen reduced by half.

2.2.5. Cotinus coggygria *Scop.*

Smoke bush is a small tree or shrub with maroon-red foliage and purplish-red inflorescence. This makes several of its cultivars attractive for gardens and landscaping, including rooftop gardening, where it has shown to exhibit mild cold tolerance (Teixeira da Silva et al., 2018). Smoke bush is dioecious; thus, generative propagation results in the production of male plants (devoid of the attractive inflorescence) in addition to female plants. Traditional vegetative propagation of some cultivars is difficult because of the very low rooting capacity of the cuttings. Protocols for the efficient micropropagation of *C. coggygria* 'Royal Purple' were developed by Podwyszyńska et al. (2012). In these methods, efficient multiplication and improved shoot quality were achieved on MS medium with meta-methoxytopolin (1–2 mg L^{-1}), whereas a higher rooting percentage was obtained on MS with the nitrogen reduced by half and supplemented with 3.0 mg L^{-1} IBA. Research conducted at the Warsaw University of Life Sciences (SGGW) showed enhanced shoot formation of *C. coggygria* 'Royal Purple' on MS medium with BAP and NAA (Jacygrad et al., 2012).

2.2.6. Other Trees and Shrubs

Tissue cultures have been used for the propagation of lilac (*Syringa vulgaris* L.), the most common species widely planted in parks and gardens, and cultivated for cut flowers. Gabryszewska (2011) reported that the multiplication rate and morphology of lilac plantlets were significantly affected by the sucrose/nitrogen ratio in MS medium. She obtained the highest number of axillary shoots in the presence of iP on a medium with a low level of sucrose (5 g L^{-1}). For *S. vulgaris* 'Katherine Havemeyer' and 'Sensation,' Ilczuk and Jagiełło-Kubiec (2015) reported the best shoot formation on medium containing mT, and the shoot rooting was the highest in the presence of IBA. Studies on micropropagation of ninebark (*Physocarpus opulifolius* L.) are also noteworthy (Jagiełło-Kubiec et al., 2021). Ninebark is an attractive flowering plant of the family Rosaceae, native to eastern North America. It has recently gained enormous popularity and is massively planted in green areas.

2.3. Cut Flowers, Potted, and Bedding Ornamental Plants

2.3.1. Gerbera jamesonii *Bolus*

Gerbera is an important cut flower in the global floricultural industry. Micropropagation is the main system used to clonally propagate gerbera in vitro, resulting in the production of millions of plantlets each year. Numerous explants and protocols for micropropagation have been established and used for this species. In Poland, research on the in vitro propagation of gerbera has been pioneered by the Research Institute of Pomology and Floriculture in Skierniewice (Hempel et al.,

1984, Soczek & Hempel, 1986). Shoot cultures were initiated from shoot tips and then multiplied on MS medium supplemented with 5–7 mg L^{-1} of kinetin. Pawłowska et al. (2018) reported that the morphogenesis of *G. jamesonii* in vitro strongly depended on the spectrum of light emitted by the LEDs. The highest shoot formation was achieved under a higher proportion of red light.

2.3.2. Chrysanthemum

Chrysanthemum is globally the second most economically important floricultural crop after rose. Micropropagation plays an important role in the commercial propagation of *Chrysanthemum grandiflorum* (Ramat.) Kitam. (Teixeira da Silva & Kulus, 2014). Studies on the in vitro propagation of 11 cultivars of *C. grandiflorum*, representing the Lady group, were conducted at the University of Science and Technology in Bydgoszcz and West Pomeranian University of Technology in Szczecin. The shoots were regenerated from shoot tips, leaf explants, ovaries, and ligulate florets either by organogenesis or somatic embryogenesis (Lema-Rumińska & Niedojadło, 2014; Miler et al., 2021; Tymoszuk & Zalewska, 2014; Tymoszuk et al., 2014; Zalewska et al., 2007, 2011). Miler and Zalewska (2014) obtained new genotypes of *C. grandiflorum* using calli induced from leaves and internodes cultured on MS medium with BAP and IAA. Owing to the research conducted by a group of scientists from Bydgoszcz, chrysanthemums are currently propagated commercially in several Polish laboratories.

2.3.3. Alstroemeria ×hybrida

Successful micropropagation of different *Alstroemeria* cultivars has been reported by Gabryszewska and Hempel (1985) and Gabryszewska (1995). In their method, efficient rhizome multiplication was achieved on an MS medium supplemented with 2.0 mg L^{-1} BAP and 0.5 mg L^{-1} NAA. In the Polish cultivar 'Juanita,' Podwyszyńska et al. (2000) reported enhanced root formation after adding paclobutrazol (0.1 mg L^{-1}) to NAA containing medium. Furthermore, pretreatment of the in vitro rhizome cultures at 20 °C and 16-hr photoperiod for 10 days, prior to a low-temperature treatment, resulted in superior rooting in vitro, better plant survival in a greenhouse, and positively influenced flowering.

2.3.4. Orchids

Orchids such as *Cymbidium*, *Dendrobium*, *Oncidium*, and *Phalaenopsis* spp. are marketed globally, and the orchid industry covers approximately 8% of the world's floriculture trade. Tissue culture techniques have helped orchids occupy one of the top positions for potted and cut flowers (Szot et al., 2015). Since the 1970s, exotic orchids have been prioritized in studies conducted by Professor Kukułczanka at the Botanical Garden in Wrocław. These studies showed the possibility of in vitro propagation of *Cymbidium*, *Cattleya*, *Phalaenopsis*, and *Dendrobium* from apical meristems, root tips, and rhizome segments on modified Tsuchiya's medium containing BAP and solidified with peptone (Kukułczanka & Kromer, 1984; Kukułczanka & Paluch, 1971). Induction of protocorm-like bodies, protocorms with occasional root- and shoot primordia, or calli from the sections of green capsules and young ovaries of five orchid species (*Cypripedium calceolus*, *Dactylorhiza maculata*, *Epipactis helleborine*, *Goodyera repens*, and *Gymnadenia conopsea*) on MS medium with 0.3 mg L^{-1} NAA, 1.0 mg L^{-1} BAP, and 1% activated charcoal (AC) has been reported by Pindel and Pindel (2004). Poniewozik, Parzymies, et al. (2021) and Poniewozik, Szot, and Parzymies (2021) developed a relatively efficient in vitro propagation method for *Paphiopedilum insigne*. They obtained higher shoot branching and quality on 1/2 MS medium supplemented with 5 mg L^{-1} kinetin, 1 mg L^{-1} BAP, and 2 g L^{-1} AC.

2.3.5. Pelargonium *spp.*

Geranium cultivation has constituted an important part of greenhouse potted and bedding plant production for almost a century. In Poland, a dynamic increase in the

production of geraniums occurred during the mid 1990s. Currently, geranium cultivation accounts for 50% of the total production of bedding and balcony plants, and is estimated to be 15 million plants per year. Healthy planting materials are important for mass production. *Pelargonium* species are affected by several bacterial, viral, and fungal diseases. The most destructive disease of geraniums is bacterial blight, caused by *Xanthomonas campestris* pv. *pelargonii* (*Xhp*) (Wojtania & Puławska, 2010). Studies on the in vitro propagation of *Pelargonium* cultivars for obtaining pathogen-free plants have been undertaken in NIHR. Wojtania (2010) reported an in vitro propagation method for *P. hortorum* and *P. hederaefolium* cultivars in which efficient shoot multiplication from shoot buds was achieved on MS medium supplemented with mT. The use of mT instead of BAP allowed large-scale multiplication of pathogen-free plant material of many cultivars of *Pelargonium* species that were considered difficult to micropropagate. A method of adventitious shoot regeneration from petiole explants via organogenesis and embryogenesis has also been developed (Wojtania et al., 2004).

3. Fruit Plants

The interest in either in vitro mass propagation of fruit plants or studies on their in vitro cultures started with strawberries and highbush blueberries – the small fruit planting materials sought in the market (Orlikowska, 1986; Sobczykiewicz, 1980). Such works were first undertaken at the NIHR. References to various aspects and purposes of in vitro vegetative propagation of fruit plant species are presented in Table 1.

3.1. Small Fruits

3.1.1. Fragaria ×ananassa *Duch.*

In the case of strawberries, at the end of the 1970s, the biggest problem was the viral infection of the plantations, which resulted in a reduction in fruit quality and yield. Therefore, researchers sought to develop effective methods for eliminating viruses from planting materials and obtaining virus-free plants, which would then be used to establish elite nursery stocks. The in vitro method was determined to be the most appropriate method for this purpose. Sobczykiewicz (1980) confirmed the high suitability of plant thermotherapy combined with in vitro meristem culture for eliminating strawberry mottle virus, strawberry crinkle virus, and strawberry latent A virus from strawberry plants. Healthy plants were then used for reproduction. In the following years, studies on in vitro propagation of strawberries focused on increasing the efficiency of micropropagation, especially during the rate-limiting steps, which include acclimatization of in vitro plantlets to ex vitro conditions and intensification of the growth of young plants in a greenhouse. Studies on replacing traditional in vitro rooting, which is laborious and costly, with direct rooting in a non-sterile substrate were initiated at the Institute of Pomology and Floriculture in Skierniewice by Borkowska (2001). Her studies showed that plants rooted in vitro had larger root systems, and during subsequent growth, they produced more than twice as many runners as in vitro-rooted plants.

An essential element of micropropagation is the field performance of strawberry plants produced in vitro. The growth evaluation of in vitro-derived plants (through axillary and adventitious shoot organogenesis) and plants propagated conventionally by runners was conducted at the University of Rzeszów (Litwińczuk, 2004) and University of Life Sciences in Lublin (Żebrowska, 2015). Plants obtained in vitro produced larger and better quality fruits, resulting in higher yield during the first and second growing years (Litwińczuk, 2004). In the third year, however, a reduction in yield and fruit quality was observed compared to the control. Similarly, Żebrowska et al. (2015, 2019) reported that despite the phenotypic changes observed in in vitro-derived plants, their agronomic value was equal or superior to that of conventional plants. The authors concluded that micropropagation could be safely recommended for strawberry production.

Table 1 Aspects and purposes of in vitro propagation of fruit plant species.

Species	Aspect and purpose	References
Amelanchier alnifolia	Patented micropropagation method	Kucharska (2016)
Fragaria ×ananassa	Virus elimination using thermotherapy and meristem cultures	Sobczykiewicz (1980)
	Direct rooting ex vitro	Borkowska (2001)
	Mycorrhization of microplants at acclimatization to ex vitro conditions	Borkowska (2002)
	Field performance of in vitro derived plants	Litwińczuk (2004); Żebrowska et al. (2019); Żebrowska (2015)
	Long-term storage of in vitro shoot cultures	Lisek & Orlikowska (2008)
	Selection of somaclones resistant to *Verticillium* using pathogen mycelium	Sowik et al. (2001, 2015, 2016); Żebrowska (2010, 2011)
	Agronomical cultivar traits as predictors of micropropagation efficiency	Żebrowska (2015)
Lonicera caerulea var. *kamtschatica*	Improved of micropropagation method	Dziedzic (2008)
	Optimization of ex vitro rooting and confirmation of genetic stability using molecular markers, AFLP and ISSR	Wojtania, Markiewicz, & Góraj-Koniarska (2020)
Malus ×domestica	Improvement of in vitro rooting efficiency of rootstocks P2 and M.9	Orlikowska (1992a, 1992b)
	Improvement of in vitro rooting efficiency of newly obtained autotetraploids of scion cultivars	Podwyszyńska & Cieślińska (2018)
	Long-term storage of in vitro shoot cultures	Orlikowska (1992a)
	Producion of apple tetraploids for breeding purposes using the in vitro leaf and shoot cultures	Podwyszyńska et al. (2017)
Prunus cerasus	Sudies (using radioactive $^{45}CaCl_2$) on calcium uptake and transport in shoots rooted in vitro	Borkowska & Michalczuk (1989)
	Optimization of sugar type and concentration at shoot multiplication stage and sugar metabolism	Borkowska & Szczerba (1991)
Prunus domestica	Development of micropropagation method	Małodobry (1986)
	Optimization of in vitro method by replacing auxins with natural supplements	Wiszniewska et al. (2016)
	Sugar uptake and utilization and balance between sugar and inorganic nitrogen in adventitious shoot regeneration	Nowak et al. (2004, 2007)
	Field performance of in vitro derived plants	Faber et al. (2002)
Prunus sp.	Optimization of micropropagation method	Borkowska & Litwińczuk (1993)
	Optimization of micropropagation cherry rootstocks	Dziedzic & Małodobry (2006)
Ribes grossularia	Optimization of micropropagation method	Kucharska et al. (2020)
Ribes nigrum	Development of in vitro propagation method	Orlikowska (1984)
	Autotetraploid production using in vitro shoot cultures	Podwyszyńska & Pluta (2019)
Rubus idaeus	Virus elimination using thermotherapy and meristem cultures	Sobczykiewicz (1992)
	Optmization of micropropagation method	Dziedzic & Jagła (2012); Zawadzka & Orlikowska (2006)
	Confirmation of genetic stability of micropropagated plants using molecular markers, AFLP and ISSR	Wójcik et al. (2021)
Vaccinium corymbosum	Optimization of micropropagation method	Litwińczuk (2012); Litwińczuk & Wadas (2008); Orlikowska (1986)
	Direct rooting of shoots in ex vitro condition	Pacholczak & Nowakowska (2015)
	Field performance of in vitro derived plants	Litwińczuk et al. (2005)

An important aspect of the micropropagation of a given species is the determination of its long-term storage conditions on media without cytokinins or with a low concentration of cytokinins in order to reduce the likelihood of mutations occurring due to cyclic shoot multiplication in the presence of cytokinins. Using 'Senga Sengana' in vitro shoot cultures on cytokinin-free medium, Lisek and Orlikowska (2008) showed that the maximal storage period at 23 °C in light was 12 months, while at 4 °C in darkness, it was 24 months.

In vitro cultures of micropropagated shoots have also been used in studies on resistance breeding aimed at selecting strawberry genotypes more resistant to *Verticillium* wilt (Sowik et al., 2001, 2015; Żebrowska, 2010, 2011). Since the filtrates of the fungal cultures and homogenated mycelia proved useless (Sowik et al., 2001), the authors used in vitro shoot cultures inoculated with mycelium homogenate and selected somaclones of strawberry 'Merton Dawn' (Żebrowska, 2010) and 'Elsanta' (Sowik et al., 2015, 2016) which were more genetically resistant to infection by *Verticillium dahliae* and their resistance was confirmed in field conditions.

The micropropagation efficiency of a particular strawberry cultivar is difficult to predict and can be determined only after several cycles of in vitro shoot multiplication. In mass propagation, it would be useful to predict the outcome of in vitro regeneration on the basis of easy-to-assess plant traits that could be associated with efficient in vitro propagation. Żebrowska (2015) (University of Life Sciences in Lublin) via path coefficient analysis revealed that plant height, the number of runners per plant, fruit yield per plant, and pollen viability had the highest positive direct path effects on the number of microcuttings produced per explant. These parameters were the best predictors of the efficiency of in vitro regeneration, and could be used as selection criteria to facilitate the identification of strawberry genotypes for which micropropagation would be the most effective.

3.1.2. Vaccinium corymbosum *L.*

In the 1980s, there was great interest in establishing highbush blueberry plantations. Its fruits belong to the elite group of the eight healthiest "healthy berries," due to the high content of bioactive compounds. To meet the growing interest in this species, in NIHR-Skierniewice, a method of micropropagation of highbush blueberry was developed (Orlikowska, 1986). The material obtained using Orlikowska's method was used to establish the first plantation. This method was further improved and optimized for new cultivars by Litwińczuk (2012) at the University of Rzeszów. Thus, micropropagation of blueberry was carried out through a routine method based on subculturing of shoot explants or shoot clumps on Anderson (1975) medium supplemented with IAA (4 mg L^{-1}) and iP (10–15 mg L^{-1}). Litwińczuk and Wadas (2008) showed that IAA stimulates the formation of undesirable adventitious shoots, enhancing the probability of somaclonal variation. Replacement of IAA with IBA facilitated the micropropagation of highbush blueberry 'Herbert' through axillary shoots (Litwińczuk, 2012; Litwińczuk & Wadas, 2008). Pacholczak and Nowakowska (2015) from Warsaw University of Life Sciences – SGGW contributed to increasing the efficiency of blueberry micropropagation by developing a system for direct rooting under ex vitro conditions using auxin (IBA 50 mg L^{-1}) or the commercial rooting powder Rhizopon containing 1% IBA. All the treatments increased the degree and percentage of rooting. Observations of field performance revealed that the culture age had a more significant influence on phenotype variation than shoot type (adventitious or axillary), indicating the necessity of frequent establishment of new in vitro cultures of highbush blueberry through a limited number of subcultures (Litwińczuk et al., 2005).

Poland is currently the second-largest producer of highbush blueberries in Europe (USDA Foreign Agricultural Service, 2021). The interest in establishing new plantations is not diminishing, and Polish producers of in vitro plants are meeting the growing demand for planting materials. Such plants are generally accepted and produced on a large scale by several Polish commercial laboratories for the domestic market and for export.

3.1.3. Other Small Fruit Plants

Poland is also a world leader in the production of black currants (*Ribes nigrum* L.) and raspberries (*Rubus idaeus* L.), with an annual production of 112 and 104 thousand tonnes, respectively, in 2021 (Statistics Poland, 2021, section "Owoce z krzewów owocowych i plantacji jagodowych" [Fruits from fruit bushes and small berry plantations]). The production of other berries, such as the saskatoon berry (*Amelanchier alnifolia* Nutt.) and blue honeysuckle (*Lonicera caerulea* var. *kamtschatica* Pojark.) is developing along with the growing acreage of crops. For many years, the NIHR has been implementing a breeding program for raspberries, strawberries, saskatoon berries, black currants, and gooseberries. In Poland, Sobczykiewicz (1992) was the first to develop a scheme for in vitro propagation of virus-free planting material of raspberry, which included thermotherapy of mother plants, isolation of meristems, testing of the obtained plants using ELISA, mass propagation of virus-free plants on MS medium with BAP and IBA, and rooting in the presence of IBA.The experiments on the optimization of micropropagation of small fruit plants have been continued at the Skierniewice Institute. Zawadzka and Orlikowska (2006) modified a medium for raspberry cultures by replacing the standard iron source, FeEDTA, with FeEDDHA at the stages of shoot multiplication and adventitious regeneration from leaf explants. Protocols for the efficient micropropagation of raspberry and black currants were published by Dziedzic and Jagła (2012) from the Agricultural University (Cracow). In their methods, shoots were multiplied on MS media containing optimal concentrations of BAP, IBA, and GA_3 for each species, with the stage of in vitro rooting in media with IBA combined with IAA. Orlikowska (1984) was the first in the world to publish the results of the research on black currant micropropagation using BAP for shoot multiplication and IBA for rooting in vitro. Kucharska et al. (2020) improved the micropropagation of a related species, gooseberry (*Ribes grossularia*), by replacing BAP with mT. This resulted in the elimination of explant necrosis and leaf yellowing. *Meta*-topolin enabled shoot elongation as well as efficient rooting and acclimatization. The ISSR and AFLP analyses showed that the method of in vitro propagation of gooseberry used in their research was suitable for obtaining plants with high genetic fidelity (Wójcik et al., 2021). Application of mT enabled the initiation and propagation of shoots in in vitro cultures of black currant for polyploidization and then for the propagation of the obtained tetraploids (Podwyszyńska & Pluta, 2019).

Dziedzic (2008) developed an in vitro propagation method for blue honeysuckle, in which efficient shoot multiplication was achieved on modified MS medium with salt reduced by 1/4, while the best shoot rooting was obtained on WPM medium supplemented with 2.0 mg L^{-1} IBA and 5.0 mg L^{-1} IAA. Recently, Wojtania, Markiewicz, and Góraj-Koniarska (2020) reported an optimized ex vitro rooting system. The high level of genetic stability of the micropropagated plants (97.7%–99.5%), has been confirmed by AFLP and ISSR markers. Research on the micropropagation of saskatoon berries conducted by Kucharska (2016) is also worth noting. The method developed by the team was patented.

3.2. Fruit Trees

Studies on in vitro cultures of fruit trees have mainly been undertaken to obtain virus-free rootstocks and scion cultivars, as well as for physiological or genetic investigation and breeding purposes, such as genetic transformation and polyploidy induction (Table 1).

3.2.1. Malus ×domestica *Borkh.*

Orlikowska (1992b) significantly enhanced the rooting efficiency of vegetative dwarf apple rootstocks P2 and M9 microcuttings by the addition of 200 mg L^{-1} arginine to IAA-containing medium with low or no inorganic nitrogen. The author also optimized the conditions for long-term storage of apple rootstock shoot cultures at 4 °C, providing useful information for in vitro plant producers and for in vitro

preservation of genetic resources (Orlikowska, 1992a). She showed that it is possible to store shoot cultures for 2 years without transferring shoots onto a fresh medium. For apple scion cultivars, the conditions for rooting of micropropagated plants were further optimized to establish autotetraploids of six apple cultivars in a greenhouse (Podwyszyńska & Cieślińska, 2018). However, apple tetraploids have extremely low rhizogenic capacity. A significant improvement was achieved owing to the use of a two-step rooting system: 6–7-day induction of rhizogenesis in the dark, at elevated temperatures up to 25 °C, on a medium containing IBA combined with IAA and arginine or putrescine, and then planting the shoots ex vitro. Furthermore, the quality of microcuttings and their rooting capacity were significantly increased by replacing BAP with mT during the multiplication stage. This improved micropropagation method has been used to produce apple tetraploids for breeding purposes (Podwyszyńska et al., 2017).

3.2.2. Prunus cerasus *L.*

Shoot tip necrosis and leaf yellowing are undesirable phenomena, often found in in vitro cultures of woody plants, including fruit trees with strong apical dominance (Podwyszyńska & Goszczyńska, 1998). They are related to the limited uptake of calcium and magnesium due to high in vitro humidity and, therefore, reduced transpiration stream. Experiments by Borkowska and Michalczuk (1989), conducted with the use of radioactive $^{45}CaCl_2$, contributed significantly to tracing calcium uptake and transport in sour cherry shoots rooted in vitro. Borkowska and Litwinczuk (1993) investigated the activity of thidiazuron in in vitro shoot cultures of various *Prunus* spp. (*P. cerasus* L., *P. avium* L., and *P. mahaleb* L.) and found that replacement of BAP with thidiazuron (0.2 mg L^{-1}) had no positive effect. In vitro shoot cultures of sour cherry were the subject of studies on sugar metabolism when different sugars (sucrose, glucose, fructose, and the sugar alcohol sorbitol) were used as carbon sources (Borkowska & Szczerba, 1991). For each sugar investigated, invertase activity was significantly higher at 2% sucrose than at 3% (w/v) sucrose. The optimal concentrations of all carbon sources were 2% and 3% (w/v), and sucrose and glucose favored shoot production. Based on these results, many researchers showed that sugar at the lower concentration (2%) rather than the standard concentration (3%) favored the maintenance of a high shoot multiplication rate, keeping the shoots at a higher degree of juvenility (e.g., Gabryszewska, 2011, 2015; Wojtania et al., 2015). The micropropagation of vegetative cherry rootstocks was optimized by Dziedzic and Małodobry (2006). Their work contributed to the popularization of the in vitro method for mass propagation of virus-free cherry rootstocks.

3.2.3. Prunus domestica *L.*

Research on in vitro cultures of plum trees was concentrated at the Agricultural University of Cracow, starting with the development of a micropropagation method, which was the subject of a doctoral thesis (Małodobry, 1986), through optimization of the regeneration of adventitious shoots on leaf explants (B. Nowak et al., 2004, 2007), to the assessment of growth and yield of plum trees derived from in vitro cultures (Faber et al., 2002). Thus, the trees of seven plum cultivars originating from in vitro started fruiting in the third year after planting, and their cropping was similar to that of the plants grafted on the 'Wangenheim Prune' rootstock (Faber et al., 2002). To meet the growing restrictions on the use of chemicals in plant production, Wiszniewska et al. (2016) investigated the possibility of replacing auxins with natural supplements such as pineapple dialyzate and green alga preparations. The authors, in a framework of cooperation between the Agricultural University of Cracow, University of Gdańsk, and Cracow Institute of Plant Physiology of the Polish Academy of Sciences, found that during rooting of the difficult-to-root plum cultivar under reduced levels of exogenous auxins, the presence of phytoactive supplements positively affected the rooting frequency. It was concluded that pineapple dialyzate could be regarded as a source of antioxidants, mainly phenolic acids, which counteract auxin oxidation and inactivation. B. Nowak et al. (2004, 2007) studied sugar uptake and utilization as well as the effect of total inorganic

nitrogen and the balance between its ionic forms on adventitious shoot formation from leaf explants of 'Węgierka Zwykła' plum (*P. domestica* L. subsp. *italica*). The authors reported that on media with total nitrogen equal to 1/2 MS, explant regeneration increased significantly and was highest on media with 1:2 or 1:4 of NH_4^+:NO_3^- ratio. With increasing concentration of sugars, the efficiency of organogenesis decreased, and this relationship was more evident for media with glucose. These published results have been widely cited in the literature and have contributed to the development of many improved methodologies for the regeneration of adventitious shoots from leaf explants of various plant species that are then used, inter alia, for physiological studies and breeding purposes.

3.3. Summary

Owing to the research carried out at Polish universities and institutes, micropropagation of selected plants free of viruses has become a standard procedure in the production of fruit species. Micropropagation methods are currently being applied for the rapid multiplication of new cultivars and the establishment of healthy elite plants for further reproduction. The production of such planting material was conducted at the Center of Elite Nursery Materials of NIHR, Ltd. in Prusy near Skierniewice and other commercial laboratories.

4. Industrial Plants and Potato

In the 1990s, industrial plants were added to the list of species propagated in vitro. The interest in *Miscanthus* or *Sida* species stemmed from their potential use in renewable energy production (Malepszy & Burza, 2010). On the other hand, the development of tissue cultures of hops and potatoes enabled the elimination of viruses, viroids, and bacteria from the plant material and allowed the production of healthy propagation material (Grudzińska et al., 2006; Sekrecka & Michałowska, 2015). The in vitro industry has searched and is still searching for efficient methods of cannabis micropropagation that enable the production of a large number of genetically homogeneous plants used for the production of valuable cannabinoids.

4.1. *Humulus lupulus* L.

Common hop belongs to the order Rosales, family Cannabaceae, and is closely related to species of the family Rosaceae. It is a perennial plant that can be grown on the same site for several decades (Szczepaniak et al., 2019). Viruses and viroids pose a threat to hop cultivation because there are no suitable chemical means of controlling them. During the first years after plantation establishment, their presence in the plants does not cause any visible symptoms, but over time, it contributes to a reduction in yield and in the content of secondary metabolites in the cones (Przybyś et al., 2019; Ziegler et al., 2014). In the 1990s, high levels of hop mosaic virus (HpMV), hop latent virus (HpLV), and apple mosaic virus (ApMV) were detected in most hop plantations in Poland. This was mainly due to the vegetative propagation of plants and the use of infected root cuttings to establish new plantations. Works on obtaining healthy nursery stock of Polish hop cultivars was started by Cajza et al. (1996) from the Institute of Plant Protection in Poznań. They tested plant regeneration from apical meristems (0.2–0.3 mm long) subjected previously to thermotherapy at 38–40 °C for 14 days, and then at 30 °C temperature. The method developed was characterized by high explant mortality but was extremely effective in eliminating viruses, including carlaviruses. The composition of the media for the in vitro culture of hops was simplified to the maximum (thermolabile components were completely removed from the media for mass cloning of plants). The final result of this study was the establishment of plantations from healthy plantlets in the Wielkopolska Region. The technique of plant regeneration from apical meristems combined with high-temperature therapy proved to be ineffective in eliminating another dangerous hop pathogen, hop latent viroid (HLVd). Therefore, in the following years, research on the elimination of this viroid was undertaken by Grudzińska and Solarska (2004) from the Institute of Soil Science and Plant Cultivation – State Research Institute in Puławy. The authors

eliminated the negative effect of high temperature on apical meristems in vitro by introducing long-term cooling of starting materials in vivo, followed by the regeneration of plants from apical meristems in in vitro cultures. They were successful in obtaining a number of HLVd-free plants.

The improved methods for the elimination of viruses and viroids from hop plants were implemented in agricultural practice during the years 2004–2008 (Doroszewska et al., 2008; Grudzińska et al., 2006). In their method, shoot apical meristems of the four most popular hop cultivars at that time 'Magnum,' 'Sybilla,' 'Iunga,' and 'Lubelski' were used; they were cultured on MS medium containing IBA, BAP, and GA_3, and then, on MS medium supplemented with BAP alone to stimulate shoot organogenesis. To obtain healthy plant materials, smaller meristems without leaf primordia were isolated. Finally, regenerated plants were again tested for viruses and HLVd using DAS-ELISA and RT-PCR, respectively, during ex vitro growth. In the next stage, the pathogen-free plants were propagated in vivo using single-node stem cuttings. More than 330,000 hop plantlets were obtained and used for replanting hop yards. In the years 2012–2015, studies on virus and viroid elimination continued, extending the research to two new cultivars, 'Magnat' and 'Puławski' (Skomra, 2018). A higher efficiency of pathogen elimination was observed after the use of apical meristems between 0.1 and 0.2 mm in length as compared to that observed after the use of meristems exceeding 0.3 mm. As a result, almost 170,000 healthy hop plantlets were obtained and implemented into agricultural practices, making it possible to seed 77 ha of hop yards. Moreover, it was shown that cones of healthy hop plants contain more alpha acids (from 13.3% to 72.7%) than cones of infected plants.

4.2. *Miscanthus*

Miscanthus species are tall perennial grasses characterized by their fast growth and high biomass yields, and used in construction, agriculture, paper industry, and the production of biofuels. The species most useful for these purposes is *Miscanthus* ×*giganteus* (Greef et Deu.), which has been cultivated in Europe for approximately 50 years (Lewandowski, 1997). It is an interspecific hybrid of *M. sinensis* (Anderss.) and *M. sacchariflorus* (Maxim.), which does not produce seeds (Deuter & Jeżowski, 2002). Its multiplication is carried out mainly by the division of rhizomes or mother clumps, but the multiplication rate with this method is relatively low. The establishment of *Miscanthus* plantations is expensive because of the high cost of the traditional planting materials. An alternative method for *Miscanthus* reproduction is in vitro propagation. In Poland, the studies on micropropagation of this species were first undertaken in the late 1990s at the Institute of Plant Genetics (IPG) and the AMU in Poznań. Głowacka et al. (2004) determined the optimal culture conditions for the micropropagation of *M.* ×*giganteus* from fragments of immature inflorescences and mature spikelets. Initially, explants were maintained in the dark on MS medium supplemented with different combinations of auxins, 2,4-D, 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), cytokinins (BAP, kinetin, zeatin), and cysteine for callus induction. For the induction of embryogenic calli of *Miscanthus*, young inflorescences were far better initial explants than mature spikelets.
In contrast, the highest number of callus-regenerated plants was obtained in the presence of BAP combined with 2,4-D. Studies on the improvement of the micropropagation of *Miscanthus* were continued at the Institute of Plant Genetics in Poznań (Głowacka et al., 2007). The effect of genotype, degree of explant maturity, and medium composition on the micropropagation efficiency of three genotypes of *M. sinensis* and one *M.* ×*giganteus* was studied (Głowacka et al., 2010).
The unflagging domestic interest in energy grasses and their propagation in vitro, prompted a collaboration between scientists at the University of Life Sciences and the Polish Academy of Sciences in Cracow. The experiments showed that replacing sucrose in the MS medium with another carbon source, such as honey and banana pulp, resulted in a higher number of somatic embryos (from 54% to 84%) and regenerated plants of *M.* ×*giganteus* (Płażek & Dubert, 2010). The resistance of plantlets obtained under in vivo and in vitro conditions to low temperatures was also compared, taking into account the hardening stage. It was found that plants obtained from in vitro cultures showed higher tolerance to low temperatures than in vivo

produced plants (Płażek et al., 2011). The tissue culture conditions, somaclonal variation, and their effect on biomass yield, cellulose, hemicellulose, and lignin content in *M.* ×*giganteus* plants were the subject of the research conducted by a large team of scientists from research centers in Cracow, Katowice, and Radzików. The researchers showed that micropropagation of this species favors somaclonal variation, which may be used for the selection of plants with improved functional traits (Płażek et al., 2015).

The development of ecological programs combining phytoremediation of sites contaminated with heavy metals by cultivating energy crops and their conversion to energy has resulted in a growing interest in the energy grass planting material in Poland in the second decade of the twenty-first century. In response to market demand, a new and very effective in vitro regeneration system of *M. sinensis*, *M.* ×*giganteus*, and *M. sacchariflorus* was developed at the IPG, Polish Academy of Sciences, Poznań and AMU in Poznań (Ślusarkiewicz-Jarzina et al., 2017). The initial explants, such as whole immature inflorescences, inflorescence axes, and nodes, were used. Callus cultures were initiated in the dark on the media: MS with 30 g L^{-1} sucrose or C17 with 90 g L^{-1} maltose, supplemented with a combination of growth regulators: 2,4-D and BAP or 2,4-D with kinetin. The calli obtained were transferred to MS or 190-2 shoot regeneration media differing in the combination and concentration of cytokinins (BAP and kinetin) and auxin (NAA). The applied technique enabled the mass production of plantlets of three *Miscanthus* species. The production potential of this technique was estimated to be between 1,500 and 2,000 plants from a single donor clump. Such a high micropropagation rate allowed a 50%–80% reduction in the cost of the planting material compared to those produced from rhizomes.

4.3. *Sida hermaphrodita* L.

Virginia mallow is a perennial species of the Malvaceae family used for energy purposes in the pulp and paper industry (Tworkowski et al., 2014) and also for soil protection against erosion. The vegetative propagation of *Sida* is time-consuming and involves significant financial investment. In turn, *Sida* seeds generally show a low germination capacity and sometimes require stratification. The first Polish studies on the in vitro reproduction of *Sida* were undertaken at the University of Warmia and Mazury in Olsztyn by Przyborowski et al. (2004). Callus cultures were initiated from leaf discs and petiole fragments placed on MS medium supplemented with different combinations of auxins (IAA, 2,4-D) and cytokinins (kinetin, BAP, TZD, and iP). Initially, the cultures were maintained in the dark, followed by exposure to light under16/8-hr (day/night) photoperiod conditions. A callus was produced, which regenerated the roots and embryoid structures. Shoot regeneration was achieved only after placing the callus on a medium devoid of phytohormones. Przyborowski et al. (2006) evaluated the effects of 2,4-D, dicamba, and iP on the induction of somatic embryogenesis; however, the majority of embryos produced were malformed. Then, optimization of culture conditions and plant regeneration of *Sida* was investigated by Mańkowska et al. (2013) from the Institute of Natural Fibers and Herbaceous Plants in Poznań. Finally, a highly efficient micropropagation method was developed, making it possible to produce Virginia mallow microcuttings for commercial purposes.

4.4. *Cannabis sativa* L.

Fiber hemp originates from Central Asia, where it has been cultivated for years on a large scale for use in textile, paper, food, and pharmaceutical industries (Brzyski & Fic, 2017). At the turn of the twentieth and twenty-first centuries, there was an increased interest in technologies enabling extremely efficient multiplication of selected cannabis genotypes to obtain large batches of raw material with the desired cannabinoid composition. Studies have also been undertaken on genetically-modified hemp for use in the production of biopolymers, among other applications. This has resulted in an increased interest in the micropropagation of this species. In Poland, significant progress in hemp micropropagation was achieved

at the Institute of Plant Genetics in Poznań, when Ślusarkiewicz-Jarzina and her team (2005) obtained plant regeneration from callus of five hemp cultivars: 'Silesia,' 'Fibrimon-24,' 'Novosadska,' 'Juso-15,' and 'Fedrina-74.' Attempts at the mass propagation of hemp through in vitro propagation were also made at the Institute of Natural Fibers and Medicinal Plants by Pawłuszewski et al. (2006). The authors reported a micropropagation technique based on somatic embryogenesis (SE), which resulted in the production of three Polish monoecious hemp cultivars. An important contribution to the development of micropropagation techniques for this species was made by Wielgus et al. (2008), who used DARIA medium for the initiation of in vitro cultures that had been used for the in vitro propagation of woody plants. The authors demonstrated that cotyledons and stem fragments are better explants for callus induction and shoot regeneration than roots. However, the efficiency of the protocols for the micropropagation of fiber hemp was still insufficient for commercial applications. A significant improvement in the efficiency of in vitro multiplication of cannabis was achieved by modifying the previously used method by cutting off the shoot tips of the microcuttings in order to stimulate the growth of axillary buds and to obtain a large number of lateral shoots (Dreger et al., 2019; Wróbel et al., 2020). In further experiments, the obtained lateral shoots were divided into single-node microcuttings. The new lateral shoots were cut again, the shoot tips were rooted, and the nodal fragments were used for further propagation. This procedure significantly increased the propagation rate from 5.4 to 7.9 explants per primary shoot (Dreger et al., 2019). Their method enabled the faster propagation of breeding lines with the desired cannabinoid content. The micropropagation of cannabis was investigated by researchers from the Institute of Natural Fibres & Medicinal Plants – National Research Institute in Poznań, who evaluated the shoot rooting ability of 'Epsilon 68' cultivar on auxin- and non-auxin-containing media and determined the effect of various cytokinins (BAP, TDZ, and mT) on the initial explant's ability to regenerate shoots. In addition, they demonstrated a more than two-fold increase in cannabis multiplication efficiency using the modified multiple explant cutting technique compared to the propagation procedure in which TDZ was added to the medium. The novel method of micropropagation of cannabis is an alternative to techniques where cytokinins, which might increase the frequency of somaclonal variation, are applied.

4.5. *Solanum tuberosum* L.

Potato is a vegetatively propagated plant. Therefore, it is affected in successive cropping years by a number of viral, fungal, and bacterial diseases, contributing to a reduction in its yield. Tissue culture techniques play an important role in maintaining healthy plant materials and the high production potential of this species. The intensive development of potato breeding in Poland using in vitro cultures has been reported since the 1980s. In the Department of Potato Protection and Seed Production in Bonin, Institute of Plant Breeding and Acclimatization-PIB (IHAR-PIB) in Radzików, Zaklukiewicz et al. (1995) developed and implemented a potato micropropagation method based on single-node microcutting cultures. Plants obtained from micropropagation can be directly planted in the field or used for the production of microtubers under cover. This method of potato micropropagation has been widely applied in practice and is currently used by three commercial laboratories: Zamarte Potato Breeding, Pomeranian-Mazurian Potato Breeding in Strzekęcin, and its branch in Szydlak. In turn, in vitro potato cultures combined with thermotherapy have been used to obtain plants free of potato virus Y (PVY), potato leafroll virus (PLRV), potato virus M (PVM), and potato virus S (PVS) (Zaklukiewicz & Sekrecka, 1994). Currently, at the IHAR-PIB branch in Bonin, research is being conducted on the effectiveness of chemotherapy using ribavirin, malachite green, and azacitidine to eliminate viruses from potato plants (Downar-Zapolska & Sekrecka, 2017). Plant materials obtained by micropropagation, in the form of in vitro plants, microtubers, and apical meristems, are widely used for the long-term storage and conservation of potato genetic resources (Michałowska & Sekrecka, 2014).

5. Biotization of In Vitro Cultures

Microorganisms have always been associated with plants and are considered the determining factor in the evolution of autotrophic organisms for donating organelles that enable photosynthesis (Rosenberg et al., 2010). Plants are colonized by numerous systematically diverse microorganisms, and only a few of them have been classified as pathogens. The functions of many microorganisms associated with plants are still unknown; only a small fraction is found to be beneficial for plants, while the remaining ones are considered neutral, although many of them probably affect the functional characteristics of plants. Some microorganisms colonize internal tissues (endophytes), whereas others remain only on the surface (epiphytes). In vitro cultures have long been considered microbe-free by the definition that they are aseptic. It has been believed that surface sterilization frees the initial explants from bacteria and fungi. In the past, bacteria that appeared sometime after in vitro plant culture initiation were mostly considered a "malpractice," and, if possible, such cultures were removed, but, in commercial production, they were accepted as accompanying bacteria. Some bacteria have been shown to promote plant growth. The first ones that proved useful were denitrifying bacteria on legumes, which provided the plant with atmospheric nitrogen. For the first time, at a conference in Gembloux (Belgium) in 1985, results of the research on the use of beneficial bacteria in plant production in vitro were presented. Rooted microcuttings of rose, hydrangea, and pelargonium were acclimatized under sterile conditions in cellulose plugs, and *Pseudomonas fluorescens* and *P. putida* were added to the medium (Digat et al., 1985). These bacteria were harmful to rose, neutral to hydrangea, and beneficial to pelargonium. In the following years, there were more reports on the use of beneficial bacteria in in vitro plant cultures. In 1990, Herman suggested the future use of bacteria in plant tissue culture. Soon after, the bacterium *Burkholderia phytofirmans* (initially designated as *Pseudomonas* sp.) made its debut. This bacteria was shown to significantly improve micropropagation in potatoes and other plants (Frommel et al., 1991; Orlikowska et al., 2017). Nowak of Nova Scotia Agricultural College, Canada, introduced it to in vitro propagation (J. Nowak, 1998). He also popularized the term "biotization."

At NIHR, *B. phytofirmans* was used to improve the rooting of *Helleborus*. In vitro, this plant multiplies better at 15 °C than at 22–25 °C, increasing the cooling costs. We hypothesized that *Burkholderia* spp. could reduce the stress from overheating. It turned out that the inoculation of the microshoots with this bacterium positively influences the quality of the root system, increasing the number of roots three times and their length twice, thus improving acclimatization (Orlikowska et al., 2015). However, this bacterium showed no effect on raspberry cultures, possibly because of its inability to colonize this particular species.

Experimental studies on the optimization of micropropagation methods for various horticultural plants have often faced the problem of bacterial contamination. This has been described in two reviews (Orlikowska et al., 2010; Orlikowska & Zawadzka, 2006). Generally, bacteria appearing immediately after culture initiation are eliminated along with the initial explants. However, contaminants appearing in older cultures, especially during the multiplication stage, often affect the efficiency of multiplication and acclimatization, and their population should be limited. However, some bacteria did not appear harmful and even had a positive effect on plant multiplication. The problems with bacteria in plant tissue culture, both the contaminating ones and those used for bacterization of plant tissue cultures, were described by E. Zenkteler (2009). Three of such bacteria, two from raspberries and one from hosta shoot cultures, were isolated. These have been identified as *Curtobacterium pusillum*, *Methylobacterium extorquens*, and *Paenobacillus glucanolyticus* (Zawadzka et al., 2013). We tested their influence on in vitro explants of 'Ludo' chrysanthemum, 'Kormoran' gerbera, 'Paradigm' hosta, and 'White Gem' rose. None of the investigated bacteria caused symptoms of hypersensitivity or vitropathy in the shoot explants at the multiplication and rooting stages. *Curtobacterium pusillum* stimulated axillary shoot formation in all the studied plant genotypes. Furthermore, our experiments indicated bacterial/host specialization. Only *C. pusillum* affected all four species in the same way, but *M. extorquens* had a

positive effect only on hosta and gerbera, and *P. glucanolyticus* only on chrysanthemum. All the assessed bacteria were able to assimilate atmospheric nitrogen, and *M. extorquens* and *P. glucanolyticus* were able to produce IAA.

Another microorganism investigated at the NIHR was the endophytic mycorrhizal fungus, *Serendipita indica* (syn. *Piriformospora indica*). It is a root-inhabiting microorganism that stimulates plant growth and increases plant resistance to biotic and abiotic stresses (Oelmüller et al., 2009). This fungus was introduced into in vitro cultures during the rooting of *Rhododendron* in perlite with a liquid medium (Trzewik, Orlikowska, et al., 2020). It inhabits the roots, but has an unfavorable effect on the growth and quality of microplants in vitro. However, the colonized plants adapted better in a greenhouse and grew faster than the untreated plants. The results confirmed other reports indicating the ineffectiveness of using this endophyte during multiplication and rooting in vitro, and above all, aiding in the acclimatization of microplants to ex vitro conditions. Microplants, after removal from a sterile medium, have no microbial protection, and the supporting and protective role of *S. indica* is important during this period. In subsequent experiments, this endophyte was administered to the substrate during acclimatization of the microplants. With such application, *P. indica* enhanced the resistance of *Rhododendron* and blueberries against *Phytophthora cinnamomi* and *P. citricola* (Trzewik, Maciorowski, et al., 2020; Trzewik, Marasek-Ciołakowska, & Orlikowska, 2020). The use of mycorrhizal fungi in vitro is ineffective because they are difficult to obtain in a non-contaminated state. Their use after the removal of microshoots from sterile conditions has been widely appreciated. Matysiak (2001) reviewed the importance of microorganisms obtained as a result of in vitro multiplication. Their use after planting in a greenhouse is primarily based on obtaining photosynthetic autonomy faster (Matysiak, 2007). Using greenhouse experiments, Borkowska (2002) and Borkowska et al. (2008) investigated the effect of mycorrhization on the growth and drought stress tolerance of strawberry plants derived from micropropagation. They observed that mycorrhization strongly affected growth and tolerance to water deficiency. Water deficiency negatively influenced photosynthetic parameters in untreated plants, whereas, in mycorrhized plants, the parameters did not change. Plants colonized by mycorrhizal fungi fully recovered their photosynthetic activity when watering was restored. Sowik et al. (2016) reported the effect of mycorrhizal symbiosis in suppressing *Verticillium* wilt in strawberry plants propagated in vitro. Bojarczuk et al. (2015) discovered that mycorrhization of *Populus* microshoots with ectomycorrhizal fungi facilitates the adaptation of microplants to metal-polluted soils; therefore, they can be used for afforestation and phytoremediation.

Today, although we still do not know most of the relationships among the microorganisms in the soil and in the plants, as well as between the microorganisms and the plant, we often use them in agricultural practice, with good results. The basis for future biopreparations supporting the protection of crops against biotic and abiotic stresses is the collection of microorganisms. Such a collection, called SymbioBank, has been established at the NIHR (Sas-Paszt et al., 2012). Moreover, Goryluk-Salmonowicz et al. (2018) and Pylak et al. (2020) collected native microorganisms from herbal plants and wild raspberries, respectively.

6. Summary

In vitro propagation is the main system of vegetative reproduction in many crops because of the much higher multiplication rate, the possibility of reproduction regardless of the season, rapid introduction of new cultivars into production, the possibility of long-term storage of plant material at a low temperature (gene banks), and facilitation of trade turnover (Kulus, 2015). In vitro culture is also an excellent tool for obtaining virus-free plants. This method is used to produce elite (pre-basic) nursery materials, which are then used to establish nurseries for the production of a certified planting material free from pathogens. Micropropagation is also used to produce plant cuttings intended for direct planting in field or greenhouses. Owing to studies conducted by scientists from Polish research centers in collaboration with each other and with colleagues from foreign research centers, methods of in vitro propagation have been adopted for the production of many economically important

horticultural and industrial plants, including elite plants of high unit value and plants in which reproduction using traditional methods is difficult or inefficient.

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