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REVIEW in POLISH BOTANY CENTENNIAL

In Vitro Culture as a Tool for Studying Plant Developmental Processes at the Physiological Level in Poland

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Abstract

Over the last 40 years, in vitro tissue culture has developed dynamically and has become a popular technique for scientific research in the field of biology. Initially, studies were carried out to develop procedures to obtain callus cultures, cell suspensions, and protoplasts of various plant species. Over time, these cultures have been used to analyze the course of processes and mechanisms that occur at the cellular level, including the course of embryological development, formation of cellular structures, polyploidization, signal transduction, gene expression, and responses to various stress factors. In a minireview, different nutritional, hormonal, atmospheric, and light conditions occurring in in vitro cultures, which are stressful conditions compared to those in ex vitro plant culture, were discussed. In this review, some examples of physiological studies conducted on in vitro culture by Polish scientists are presented, including studies carried out to optimize the composition of media that induce callus and plant regeneration; determine the use of in vitro culture for the preservation of endangered plant species; understand the mechanisms of resistance responses to pathogens, salinity, nutritional stress, and low temperatures; and determine the potential production of plants with different chemical compositions. The potential of sterile plant culture is large and beyond the scope of its current use. Therefore, the number and variety of applications of these cultures will be significantly greater in the future.

Keywords

abiotic and biotic stresses; callus; cell suspension; nutritional stress; regenerants; protection of endangered species; protoplasts

1. Introduction

Sterile culture methods for cells, tissues/organs, and whole Sterile culture methods for cells, tissues/organs, and whole plants, also known as in vitro plant culture methods, were introduced in the last century and have revolutionized many directions of plant physiology research. Such progress was possible as plant cultures exhibit quantitative and qualitative changes in growth and metabolism, and developmental and morphogenetic processes. Surprisingly, plant cells/tissues react violently to a seemingly simple experimental procedure, such as the provision of sterile plant culture conditions and exogenous supply of nutrients and regulatory substances. However, the above experimental activities introduce new stress factors into the cultural environment, such as:

- Opposite direction of trophic gradient. In plants, the nutrients produced by photosynthesis flow from the organs of producers (donors) to the acceptor organs (sink), generally from the aboveground organs to the roots (i.e., in the

direction of gravity). In in vitro cultures, nutrients flow from the nutrient solution to the cultures, which is against the direction of gravity.
- The modified direction of light rays from the top or sides; for transparent medium and multi-level cultures, from the bottom obliquely upwards. The spectrum of this radiation depends on the type of light source, and the spectral properties of the material of culture vessels.
- Different gradient directions of regulatory substances. In plants, phytohormones are synthesized in specific organs, such as auxins in the apical meristems, and their concentration gradient decreases vertically. The remaining phytohormones are also formed in other, but specific regions, and move to other tissues of the plant, following their gradients. Accordingly, specific ratios of the concentrations of hormones are formed in each part of the plant, which defines the so-called morphogenetic potential of individual plant organs and regions. Contrary to natural conditions, phytohormone concentration gradients in in vitro cultures are stable because they mainly depend on the size of the flux of these substances from the medium.
- The occurrence and concentration gradients of auxotrophic substances added to the cultures of various plant species to induce specific responses, and some relatively stable parameters, such as the gas composition of the atmosphere inside the vessels (content of oxygen, nitrogen, and water vapor, or substances such as ethylene, propylene, or styrene, as monomers of plastics from which the vessels are made).

At the XIII Overall Polish in vitro Culture and Plant Biotechnology Conference "Plant cell – The objective of genetic and physiological manipulations" of the Polish Botanical Society and International Association for Plant Tissue Culture (IAPTC) in 2012, the role of gradients in different stages of the evolution of the molecular world and the world of living matter was presented (Dubert & Płażek, 2012). Further, the mechanisms of the modifying effects of stresses as states of imbalance and the homeostasis that oppose them (i.e., the balance between objects (inorganic and organic molecules, cells, and organisms) and their surroundings) were discussed. We hypothesized that evolution only occurs during the object's equilibrium state and the more efficient the process, the shorter this period. However, in the state of homeostasis, the direction of processes that would favor further changes cannot be clearly indicated as the state of homeostasis has already been reached. Therefore, changing the gradients of environmental parameters can accelerate evolution by selecting objects with individual abilities to more quickly overcome the stresses caused by these gradients.

Processes that occur in in vitro culture differ from those that occur in planta conditions:

- In tissue culture, callus tissue is produced, which indicates that the culture conditions enabled a unique process of restoring mitotic cell division in organ tissues in plants.
- In cultures, organogenesis occurs (i.e., the formation of organ buds and their subsequent growth). In some cells of an organ, buds, leaves, shoots, etc., a development program is launched in various versions, such as root growth, the formation and development of new shoots, and even plant regeneration through embryogenesis. In contrast, the simplest development program based on the formation of chloroplasts (the tissue turns green) may also occur.
- Cells can be transformed into protoplasts by enzymatic lysis of the cell wall. Protoplasts are unique biological objects owing to their fusion ability, which results in so-called heterokaryons or crossing at the cellular level. Heterokaryons can have a complete number of chromosomes, summed based on the number of parental cells, or incomplete chromosomes, due to the rejection of some chromosomes or parts of chromosomes during mitosis. Melchers et al. (1978) obtained the first hybrid of tomato and potato, and later studies revealed that protoplasts do not have non-crossing barriers (cell wall). Thus, any cross-species can theoretically be obtained to increase cell ploidy.

- Protoplasts can be used to select single cells with higher resistance to harmful or toxic compounds, plants of medical importance, and cells with increased active substance content. Such experiments enable more stringent selection.
- Protoplasts can be genetically modified. These modifications can be carried out by treating protoplasts with foreign DNA deposited on a "heavy gold" core. If such transformants are regenerated, genetically modified plants can be obtained. Such genetic modifications can also be obtained by another method, such as electroporation or chemical "poration," usually with PEG. DNA molecules are also used in this method and due to their negative electric charge, these molecules move in an electric field and can penetrate the protoplasts.
- Genetic transformations can be carried out using vectors of biological objects that can penetrate plant cells, such as bacterial plasmids (e.g., from *Agrobacterium tumefaciens* or *A. rhizogenes*), viruses, or artificial creatures called "cosmids."
- Sterile cultures allow the cultivation of embryos that are few days old, which accelerates the succession of generations and avoids the often long-term stratification of seeds.
- In vitro cultures enable haploid plants that produce double haploids (i.e., fully homozygous plants that are valuable in plant breeding) to be obtained.

Of note, Professor Maciej Zenkteler discussed the possibility of distant crossing using in vivo fertilization in flower pistils by introducing pollen into the pistil in the vicinity of the ovulum. This method requires in vitro cultivation of very young embryos, which allows crossing barriers to be overcome.

In the 1970s and 1980s, most studies conducted on in vitro culture focused on the optimization of media composition to obtain morphogenic calli and regeneration of various plant species. Indeed, these studies began with model species (e.g., tobacco) (Raveh et al., 1973); however, for many other plant species, in vitro cultures were very difficult to perform and many experiments were required. After a procedure was developed to derive callus tissues, cell suspensions, or protoplasts, studies on the many physiological, biochemical, and genetic processes occurring at the cellular level could be performed. Of note, the response of plants to many abiotic or biotic factors proceeds differently in terms of signal transduction at the tissue or plant level compared to the cellular level. The potential use of protoplasts to study cellular responses to stresses has been debated as protoplasts do not have a cell wall, which is an important component of signal transduction (Gilliard et al., 2021). Although the researchers cited the results of many experiments performed on protoplasts, whether the lack of a cell wall disrupts the signaling pathway remains unknown. Thus, only callus and cell cultures offer new opportunities to discover the mechanisms of plant responses to biotic and abiotic factors. As the response at the cellular level is not always similar to the degree of resistance observed in whole plants, such studies are faced with criticisms.

2. Parameters Determining the Morphogenic and Regeneration Ability of Callus

Filek et al. (1998) showed that the regenerative capacity of wheat callus cells is conditioned by the distribution of ions (mainly calcium ions) within the cell and the electric potential on the surface of the plasmalemma. Owing to these results, many studies devoted to analyzing the influence of the electric potential of cells on the regenerative abilities of other plant species have been performed (Filek et al., 2005, 2010; Hura et al., 2015; Płażek et al., 1999). An analysis of the electrical potential of the plasmalemma of callus cells of timothy (*Phleum pratense* L.) roots, shoots, and leaves was performed by Płażek et al. (1999). This analysis revealed that in the cells of non-morphogenic calli, this potential was the lowest, ranging from 0 to −4 mV. The cells regenerating root-derived calluses had membrane potentials from −5 to −49 mV, while those regenerating shoot-derived calli had potentials from −40 to −59 mV. The cell-regenerating leaves had the highest potential of −60 to −100 mV. The electrical potential of membranes depends on the quantitative and qualitative composition of phospholipids, saturated and unsaturated fatty acids, steroid content, and unequal distribution of ions on both sides of the membrane. The reasons for the

secretion of many pectin compounds into the medium by the cell suspension of Timothy were also described. The culture of Timothy cells in liquid Murashige and Skoog (1962) medium with the most commonly used auxin [i.e., 2,4-D (dichlorophenoxyacetic acid)] resulted in disturbances in polysaccharide metabolism, which inhibited plant regeneration. However, these effects were not observed in the presence of dicamba (3,6-dichloro-2-methoxybenzoic acid).

Żur et al. (2000) found that the ability of tissue cultures of *Brassica napus* L. var. *oleifera* to differentiate between leaves and roots is correlated with high metabolic activity based on the respiration and heat emission rates. In this study, callus activity was measured using an LCA-2 infrared CO_2 analyzer and an isothermal microcalorimeter. In another study, Żur et al. (2002) showed that quantitative changes in galactolipids and plasmalemma sterols were not correlated with the process of morphogenesis in callus cells of this plant species.

Another achievement was the elaboration of a procedure to obtain morphogenetic calluses and regenerants of giant miscanthus [*Miscanthus* ×*giganteus* (Greef et Deu)] plants (Płażek & Dubert, 2010). *Miscanthus* ×*giganteus* is a sterile triploid (hybrid of diploid *M. sinensis* and tetraploid *M. sacchariflorus*) that naturally reproduces vegetatively from rhizomes. The main problem in the tissue culture of this species is the secretion of very large amounts of phenolic compounds owing to tissue injury during explant sourcing. The best source of explants for obtaining calluses of this plant species is very young inflorescences. Cutting them into smaller fragments causes severe oxidative stress, manifested as tissue blackening and the release of phenolic compounds into the culture media, resulting in cell death. Different compounds that inhibit the phenolic pathway were added to the culture media, such as chitosan, cysteine, and natural sources of polyphenol oxidase inhibitors, such as banana pulp and honey. In this study, a lower degree of callus tissue darkening on the medium containing honey and a significantly higher rate of callus induction and plant regeneration on the media containing banana pulp and honey compared to the other combinations were found (Płażek & Dubert, 2010).

3. Regenerants as a Source of New Plant Forms

Plant breeders have already successfully used genome resources to develop new cultivars with desirable characteristics, such as high yield and resistance or tolerance to biotic and abiotic stresses. Plant genetic diversity can be increased using in vitro cultures and mutagenic agents (Patade et al., 2008; Szarejko et al., 2017). In tissue culture, tissues are cultivated in media with hormones at concentrations markedly higher than those under natural conditions (Larkin & Scowcroft, 1981). The variability found in tissue cultures is called somaclonal variation (Ahloowalia, 1998). Somaclonal changes may result from polyploidization, interchromosomal translocations, inversion, and chromosomal duplication.

In vitro cultures have been used to determine the possibility of obtaining fertile plants of triploid giant miscanthus. *Miscanthus giganteus* is a sterile triploid with 57 chromosomes, with a basic number of chromosomes of 19. Miscanthus is a plant with low soil requirements, is suitable for strengthening slopes and coasts, and takes up heavy metal ions; hence, this plant is used for phytoremediation. Long-term plantations can efficiently store carbon dioxide in rhizomes, which is of great ecological importance in the current fight against global warming. As C4 plants produce a large amount of biomass, which is used for cellulose production, shoots are used to produce small garden architecture or construct thatches (Clifton-Brown et al., 2008, Lewandowski, 2006). Owing to its sterility, miscanthus has a low genetic variability. For this species, classical breeding methods to improve economic characteristics cannot be applied. Hexaploid *M.* ×*giganteus* plants were reported in some studies (Głowacka et al., 2009, 2010; Melnychuk et al., 2020); however, owing to a lack of reports on the use of genotypes with double the number of chromosomes in breeding and industry, further research on the polyploidization of this species has been encouraged. Kopeć (2017) attempted to obtain fertile plants of *M.* ×*giganteus* using antimitotic compounds. Morphogenic callus tissue and regenerants obtained in vitro were used. Both types of plants were exposed to colchicine, caffeine,

trifluralin, and oryzalin at different concentrations and treatment times. Changes in the number of chromosomes in callus cells, leaves, and roots of the obtained plants; the percentage of surviving plants; and number of new shoots produced by plants after mutation were determined. The callus cells demonstrated a variable number of chromosomes, from 18 to 58, regardless of the mutagen type and exposure time. All regenerants obtained from the calli were triploids. For regenerants, two hexaploid plants were obtained only after treatment with 1,252 μM colchicine for 18 hr. Unfortunately, after 3 months of cultivation, both plants died. Callus cells naturally show large variations in the number of chromosomes; however, only euploid cells (57 chromosomes) regenerate plants. For the examined *M.* ×*giganteus* plants with 114 chromosomes, miscanthus may serve as a disadvantageous phenomenon that either results in the lack of development of plants from cells with a doubled number of cells or the death of the hexaploid plants themselves.

Studies conducted on *M.* ×*giganteus* enabled the observation of another phenomenon, which involves obtaining plants with altered chemical composition. Miscanthus plants obtained via in vitro cultures have higher hemicellulose and lower cellulose and lignin contents than plants propagated in vivo (Płażek et al., 2015). These results clearly confirm that plants regenerated from callus cultures can be a source of new forms with altered biochemical compositions compared to the initial plants.

4. Study on Plant Frost Tolerance

One of the problems in field cultivation of *M.* ×*giganteus* is its sensitivity to frost during the first winter. Podleśny (2005) reported that regenerants obtained in vitro were more resistant to frost than those obtained by rhizome division. Płażek and colleagues (2011) sought to explain this phenomenon. Briefly, the researchers compared the degree of frost resistance of plants obtained in vivo and in vitro after prehardening and frost-hardening procedures. In vitro regenerated plants were more resistant to frost during the first year of cultivation, despite the presence of smaller rhizomes. However, these plants produced more shoots than plants obtained in vivo by rhizome division. In the second year of cultivation, the degree of frost resistance in both plant groups was similar. Analyses of abscisic acid levels in the leaves and rhizomes, activity of antioxidant enzymes, and content of low-molecular-weight phenolic compounds did not reveal differences between plants obtained in vivo and in vitro. In contrast, rhizomes were demonstrated to be divided very intensively by commercial companies for plant propagation; hence, plants obtained from initial shoots from rhizomes have a low content of assimilates, which might be the reason for their poor condition during the first winter. The greater number of shoots produced by the regenerants may provide greater production of assimilates transported during autumn from the leaves to the rhizomes, which survive the winter.

5. Solving Problems of Endangered Species Using In Vitro Cultures

The populations of many plant species are endangered owing to the limited potential of generative reproduction and low rate of seed germination. Previously, the positive effect of scarification on *A. penduliflorus* seed germination was demonstrated, thereby confirming that the seeds show physical dormancy owing to the impermeable seed coat (K. Dziurka, Skrzypek, & Dubert, 2019). Breaking the seed dormancy of *A. penduliflorus* increased the seed germination rate up to 100%; however, nearly half of the seedlings were infected. Therefore, to increase the number of healthy seedlings obtained, seeds should be disinfected, mechanically scarified, and germinated under in vitro conditions (K. Dziurka, Skrzypek, & Dubert, 2019). Healthy seedlings of *A. penduliflorus* were used as the starting material for in vitro culture. The medium previously used by Skrzypek (2001) to induce and regenerate *Vicia faba* L. ssp. *minor* was successfully used for the micropropagation of *A. penduliflorus* from the shoot apical meristem.
The propagated single shoots were rooted in medium containing half of the macro- and micro-elements of the standard MS medium, 1.5% sucrose, and 4 mg of NAA.

Rooted plants were acclimatized and planted in the experimental garden. Various biotechnological techniques, including micropropagation, in vitro seed germination, and regeneration from callus, are very helpful for plant germplasm conservation, especially for species with very limited or impossible generative reproduction.

The preservation of endangered species through in vitro culture was discussed in other studies. *Osmunda regalis* is a fern species under strict protection in Poland. In fact, *Osmunda regalis* is one of the few fern species that form spores that do not tolerate desiccation and age fast. Spores are believed to be equivalent to recalcitrant seeds. By using in vitro cultures, Mikuła et al. (2015) demonstrated how the spores of this species age and the disturbances that can occur during their germination. These spores were revealed to tolerate some degree of dehydration and could be stored for up to 1.5 years under certain conditions. Before being placed in liquid nitrogen, these spores do not require additional drying, which is an important element in the process of depositing biological material in a gene bank.

6. Role of Phytohormones in the Vernalization Process

Phytohormones are vital elements in the regulation of plant development, including flowering, which is the main physiological process that enables plant propagation. Winter wheat requires vernalization, exposure to cold, and long days to enable transition to flowering. Exogenously applied plant regulators can only partially substitute vernalization in time- and dose-dependent manners. Marcińska et al. (1995, 1996) carried out noteworthy studies on the flowering process of plants that require vernalization. The researchers investigated the vernalization process and the ability for heading of plants obtained from callus of winter wheat (*Triticum aestivum* L.). Plant regeneration via callus tissue enables the study of physiological processes in winter wheat, such as differentiation, growth, and generative development (Dubert et al., 1989; Marcińska et al., 1995, 1996). Cultures obtained from winter wheat seedlings were used as the plant material. Immature wheat embryos are the most widely used source for the induction of calluses capable of plant regeneration. Low temperatures stimulate the induction and differentiation of calli; however, it is not optimal to produce a generative stadium when calli are obtained from immature embryos (Marcińska et al., 1995, 1996, 1999).
The surprising effect of heading in regenerated plants was obtained from immature inflorescences of winter cultivars under growth conditions, excluding their renewed vernalization. The callus tissue derived from the explant is suggested to produce regenerants, which is interesting as the immature embryos of winter wheat cultivars and regenerants obtained from the scutellum of these embryos via callus have been shown to require vernalization before flowering. In immature inflorescences, only few days before flowering, cells developing into new plants do not require renewed vernalization. However, immature embryos isolated approximately 10 days after fertilization develop into plants that cannot head without renewed vernalization (Dubert et al., 1993). This observation implies that the effects of generative thermoinduction disappear during the period shortly before gamete development to few days after fertilization. Hence, the repression of genes responsible for the state of generative induction of winter wheat plants can be assumed to occur in the course of gamete formation during meiosis, or during fertilization.

Interesting experiments were conducted to determine the role of the fungal toxin, zearalenone [6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-β-resorcylic acid lactone)], in plant metabolism. Zearalenone (ZEN) is mainly produced by fungi of *Fusarium* species. Some studies have reported that this toxin can act as a hormonal substance and has a favorable effect on the development of plants and animals. Biesaga-Kościelniak and Filek (2010) studied the influence of ZEN on the development and regeneration of winter rape calli and showed that treatment with this toxin affected callus proliferation and cell differentiation, which was similar to the activity of auxins in in vitro cultures, which may confirm the hormonal properties of ZEN in plants. Zearalenone possesses high activity and shortens the flowering period during vernalization. The aim of the next study was to determine whether the effect of ZEN, which leads to accelerated flowering in winter wheat, is followed by a specific hormonal balance during vernalization (M. Dziurka,

Dziurka, et al., 2019). Experiments were performed under in vitro conditions. Isolated winter wheat embryos were grown on MS medium with and without ZEN (control). Both cultures were subjected to vernalization for 3, 6, 9, 12, and 15 days. Thereafter, the regenerants were transferred to the vegetation tunnel. After 120 days, the degree of apical meristem development was determined, and targeted phytohormone profiling of the apexes was conducted. Among the control plants, only 10% achieved the generative developmental phase. In contrast, plants grown from embryos vernalized on medium with ZEN reached the generative phase at 80% after 6 days of cooling, whereas after 15 days, all plants were generative. ZEN activity, as a promoter of generative winter plant induction, consists of the specific determination of the mutual proportions of gibberellins, cytokinins, auxins, abscisic acid, jasmonates, and ethylene.

7. Nutrient Stress Studied in In Vitro Conditions

Controlled conditions in in vitro cultures allow studies on plant responses to a lack or excess of certain nutrients. This technique has been used to assess the weak yield of common buckwheat (*Fagopyrum esculentum* Moench). Common buckwheat is a valuable species owing to its very good chemical composition, which is similar to that of cereal grains. Moreover, common buckwheat has a very good amino acid composition, fiber, and rutin, and does not accumulate gluten. Buckwheat produces numerous flowers during the entire vegetative season (it is not self-finishing), but only approximately 10% of these flowers produce seeds. Many flowers and already-set embryos are aborted. The reasons for the poor yield include the high sensitivity to frost, cold, drought, and high temperatures occurring during the flowering period, and the flowering biology of this plant species. Common buckwheat produces two types of flowers with long and short pistils, which must pollinate each other owing to their strong self-incompatibility. Single buckwheat flowers can be pollinated for only one day. Additionally, buckwheat experiences a high percentage of developmental abnormalities in the ovules, which are specific to individual genotypes. A disadvantage of this species is the very long flowering period and overproduction of flowers, which compete for assimilates transported to the already set seeds. To determine whether nutritional stress is significant in the process of yielding, an experiment was established, in which flower buds of two buckwheat cultivars were placed on MS medium with a full composition of nutritional compounds (macro- and microelements, vitamins, amino acids, and sugar) – control, medium with nutrients depleted by half, and medium with 2/3 nutrients relative to the control (Hornyák et al., 2020). The same amounts of hormones and vitamins in the control were added. The cultivars selected for the study, 'Panda' and 'Korona' (preregistration name of the cultivar – strain PA15), differ in their degree of embryo sac degeneration, which occurred under in planta conditions. The floral buds cut from inflorescences were cultivated on the media for 10 days at a constant temperature of 20 °C, relative humidity of air 50%–60%, 16-hr photoperiod, and 300 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD). The embryological development of the embryo sacs was then analyzed. The results confirmed differences in the responses of the studied cultivars to the content of nutrients in the medium, as well as a significant increase in the number of degenerated embryo sacs and oocytes on media with a poorer nutrient composition. Thus, poorer access to nutrients, especially 2/3 compared to the control, had a significant effect on the embryological development of the studied species.

A reduced nutrient solution of the medium was used for in vitro multiplication of gametophytes of *Osmunda regalis* (Makowski et al., 2016). The effect of Knop's medium (1865) and various concentrations (1/2, 1/4, 1/8) of mineral salts in MS basal medium, in the presence or absence of ammonium nitrate and a full complement of vitamins, on gametophyte proliferation and sporophyte production was determined. Maximum gametophyte proliferation (89%) was observed on the ammonium nitrate- and vitamin-free MS medium with 1/2 or 1/4 strength mineral salts. The maximum sporophyte production was 30 plantlets per gametophyte clump, which was obtained on 1/8 MS without ammonium nitrate or vitamins.

Flow cytometric analysis confirmed that sporophytes were obtained via sexual reproduction.

8. Plant Tolerance to Heavy Metal Ions

In vitro cultures have been used in research on the defense responses of plants to the toxic effects of heavy metal ions. Direct contact of tissues with metal ions added in strictly defined concentrations to the media, lack of a sorption complex that absorbs ions, and exclusion of complex interactions between compounds of various elements in the soil solution enable precise defining of the defense mechanisms triggered in plant tissues under the influence of heavy metal ions. Muszyńska et al. (2018) investigated the response of metallicolous (M) and nonmetallicolous (NM) *Alyssum montanum* ecotypes to multi-metal stress evoked by Pb, Cd, and Zn ions under in vitro conditions. In this study, the ability to detoxify heavy metal ions and the role of the antioxidative system in the defense response of these two ecotypes to this stress were investigated. The researchers concluded that the enhanced tolerance of the M ecotype was due to heavy metal detoxification in trichomes and intracellular leaf compartments, and the balanced accumulation of reactive oxygen species (ROS). Notably, the peroxidase-flavonoid system played a key antioxidative role in this response.

These two ecotypes were used in further in-depth research on the role of phenolic compounds and phytohormones in the defense mechanism against heavy metal ions that damage the photosynthetic apparatus (Muszyńska et al., 2021). For the M ecotype, these studies showed that jasmonic acid and flavonoids, which are involved in ROS scavenging, play an important role in the protection of photosystems, and the rearrangement of electron transport in thylakoid membranes. However, in the NM ecotype, abscisic acid causes the closure of the stomata, which reduces transpiration and the uptake of water with metal ions.

9. Investigation of Plant Resistance to Pathogens

Tissue cultures have been used for many years to assess the degree of resistance of crop plants to pathogens, particularly fungal pathogens. Callus tissue or cell suspensions have been cultured in media containing specific fungal toxins or a spectrum of metabolites secreted by fungi into culture media (Chawla & Wenzel, 1987; Hunold et al., 1992; Rines & Luke, 1985). Such studies enable potential analysis of the mechanisms of plant resistance to pathogens at the cellular level. Lepoivre et al. (1986) proposed the use of "two-layer cultures." The fungus was grown in the first medium layer, and its metabolites diffused into the upper medium layer, supplemented with a fungicide that stops the mycelium from overgrowing. On the upper layer the callus develops in contact with the metabolites. This method provides more information on the resistance of plant tissue to a pathogen than a single toxin, because each metabolite produced by the fungus can cause different disturbances in the metabolism of plant tissue (Wojciechowski et al., 1996). Plants regenerated from calluses selected using this method may, in practice, show greater resistance to the disease. Using this technique, Płażek (1994) obtained seven meadow fescue genotypes with increased resistance to infection by *Drechslera dictyoides* (Drechsler) Shoem. spores. In another experiment, meadow fescue genotypes regenerated from calli were selected based on metabolites of *Bipolaris sorokiniana* (Sacc.) Shoem. and showed greater resistance to this fungus over two years in field cultivation compared to control plants (Płażek, 1997).

Studies on cell suspensions or callus cultures allow us to follow pathogenesis within the first hours after elicitation. Płażek et al. (2000) showed that in callus culture of barley, within the first hour after elicitation with metabolites of *B. sorokiniana*, a significant increase in respiration rate and a decrease in heat emission occurred. These effects may indicate a high mobilization of the tissue within the defense response to the pathogen, including an increased synthesis of energy necessary for the synthesis of phenolic compounds or specific proteins, such as pathogenesis-related (PR) proteins. This study also demonstrated a differential

response of calli to metabolites compared to the response of leaves to spore inoculation with this fungus.

Płażek and Niemczyk (2000) conducted a study to compare the response of callus tissue of meadow fescue, which is more resistant to *B. sorokiniana*, and barley, which is markedly more sensitive to this pathogen. These studies demonstrated that the response to this stress occurs in two phases. In the first phase, between 1 and 24 hr after elicitation, a remarkable decrease in fructose and glucose levels was noted in the callus of both plant species compared to that of the control. The second phase of response occurred after 24 hr (the whole experiment lasted 168 hr) and the examined species were differentiated in terms of susceptibility to this fungus. Fescue callus showed a gradual increase in the content of the studied sugars, which would indicate the mobilization of cells to eradicate the disease, whereas barley callus showed significantly lower amounts of these sugars than the control tissue. The time between 24 and 168 hr can be called "recovery" and this second phase determines the resistance capacity of the tissue.

Hura et al. (2015) studied the response of calli of oil winter rape (*Brassica napus* L. var. *oleifera*) to cold (2 °C), temperature changes in the ranges 2/20 °C and 20/2 °C, and biotic stress evoked by secondary metabolites of *Lepthospheira maculans* (anamorph *Phoma lingam*). Temperature change was identified as the factor that most strongly affected the metabolism of the winter rape callus tissue. According to Hura et al. (2015), catalase (CAT) activity and respiration intensity can be considered reliable indicators of callus response to elicitation with secondary metabolites of *L. maculans*. A reduction in CAT activity results in increased levels of ROS, which are key molecules that induce the defense response to pathogens. In another study, Płażek et al. (2005) investigated whether chitosan or pectinase, used as elicitors, could initiate the response of oilseed winter rape calli, similar to the response of that tissue to metabolites obtained from liquid culture of *P. lingam*. The estimated total amount of phenolics and the activity of phenylalanine ammonia-lyase (PAL) and CAT were 24, 48, and 72 h after elicitation. Overall, changes in CAT activity, PAL activity, and phenolic accumulation evoked by pectinase were found to be similar to the pattern of changes caused by *P. lingam* metabolites.

Double-layer cultures (Lepoivre et al., 1986) have been used in studies of the interactions between roots of *Populus nigra* var. 'Italica' and *Populus ×canescens* and the ectomycorrhizal fungus, *Paxillus involutus*, and arbuscular fungus, *Rhizophagus irregularis*. In this type of experiment, in vitro culture is indispensable as the stages of inoculation with mycorrhizal fungi can only be observed on seedlings obtained under sterile conditions; this is because seedlings obtained in vivo may already be infected with other species of this type of fungi. Kulczyk-Skrzeszewska et al. (2018) proved the protective role of mycorrhizal fungi under osmotic stress induced by salinity (salt concentrations from 0 to 150 mM NaCl were added to the medium) in vitro. The ectomycorrhizal fungus, *Paxillus involutus*, was also found to promote the growth of *Populus ×canescens* seedlings, whereas the arbuscular fungus, *Rhizophagus irregularis*, had no effect.

10. Conclusions

In the present review, only few examples of studies conducted by Polish researchers using in vitro techniques in the field of plant physiology are presented. The procedures for obtaining tissue cultures developed dynamically in the eighties and nineties of the twentieth century. These procedures have become the basis for the progress of many studies in the broad field of biological sciences.

Callus cultures, cell suspensions, and protoplasts are tools commonly used in the study of extremely complex processes occurring in plants at the cellular level, the maintenance of biodiversity, and production of wild, crop, and ornamental plants. In most cases, it is difficult to separate the typical physiological functions from genetic or biochemical experiments. Herein, the authors mainly focused on typical physiological studies, and demonstrated that the above-mentioned research examples are often closely related to experiments on other issues, such as secondary plant metabolites, signal transduction, increasing ploidy, obtaining haploids or

dihaploids, androgenesis, or gynogenesis. Owing to dynamic climatic and environmental changes, increasing studies on plant physiology under in vitro conditions and the effects of environmental abiotic and biotic stresses on plant physiology under in vitro conditions are expected. The potential of sterile plant culture is surprisingly large and beyond the scope of its current use. Therefore, the number and variety of applications of these cultures will be significantly greater in the future.

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