POTENTIAL APPLICATIONS OF WASTES FROM ENERGY AND FORESTRY INDUSTRY IN PLANT TISSUE CULTURE

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Two industrial by-products, deuterium depleted water (DDW) and polyphenolic extract from spruce wood bark (SB) were tested as bioregulators for maize callus cultures. The effects of deuterium depleted water alone or in combination with spruce bark extract on *in vitro* growth and development of callus maize plant were studied. Specific parameters, including biomass accumulation, adventitious roots and aerial adventitious roots organogenesis, formation of new callus, as well as activity of enzymes, such as peroxidase, superoxide dismutase, catalase, and total content of polyphenolic compounds accumulated in callus have been closely monitored. The results have shown that the tested solutions stimulated the fresh biomass accumulation, adventitious roots organogenesis and formation of new callus by comparison with the control sample. The most significant stimulatory effect on callus biomass accumulation and total content of polyphenolic compounds was observed when the deuterium depleted water, combined with spruce bark polyphenolic extract, was used for the performed treatments.

Keywords: deuterium depleted water (DDW), spruce bark polyphenols (SB), callus maize, physiological parameters, enzyme activities, polyphenol biosynthesis

INTRODUCTION

Plant tissue culture refers to the growth and multiplication of cells, tissues and organs on defined solid or liquid media in aseptic and controlled environment. Plant tissue culture technology is being widely used for large-scale plant multiplication.[1,2] Regeneration and development of callus depend on the initiation and growth conditions, but also on the species or vegetative organ from which it arose. The induction of callus cultures, the growth rate, as well as the morphogenetic processes, depend on the type and concentration of the regulators present in the culture medium.[3]

In agreement with literature data,[4] once induced, the callus has the ability to synthesize phytohormones by itself. For example, the callus derived from vegetative fragments of *Daucus carota* was induced on culture media containing polyphenolic extracts separated from the wood bark of beech, oak and spruce.[5] The authors' conclusions have evidenced the combined action of different bioregulators with endogenous capacity of tissue to synthesize phytohormones. There were different results for callus growth and cell differentiation processes depending on the polyphenolic extract used in this treatment. Thus, there is a significant increase in adventitious roots developed in culture media containing extract from spruce bark (0.51 mg/L). The amount of callus accumulation was found to increase by combining the three extracts, in the absence of auxin 2,4-D, in comparison with other experimental variants. In contrast, the number of newly formed shoots was higher for the samples with polyphenolic extracts obtained from beech and oak bark. The extract separated from the spruce bark exhibits a stimulating effect on callus and root development processes.

These results, further developed in the studies of plant cultivation, grafting and bioremediation of vine,[6,7,8] have reconfirmed that the role played by natural polyphenols is similar to that of phytohormones. Therefore, polyphenols obtained from forest and agricultural residues can be used with good results in plant propagation to obtain useful products.

The interest in the secondary metabolites originated from plants has recently increased, not only due to their natural origin, but also to their therapeutic effect.[9] However, the use of plants is not limited to the domain of pharmaceuticals or food, it can be extended to cosmetics and perfumery.[10,11] The production of vegetal secondary metabolites by large-scale culture of plant cells in bioreactors is technically feasible, but it exhibits significant drawbacks from the economic point of view. It may be feasible for some costly products, but unfortunately some of the most important products are produced only in very small amounts and some cannot be produced at all in plant cell cultures.[9]

Recently, many researches have been focused on producing useful compounds from plant tissue cultures.[12,13,10] In this context, there have been remarkable advances in technology, so that a large number of studies based on the usage of cell and tissue cultures for industrial production of plant secondary metabolites are presently carried out.[14,15,9] "In vitro" techniques aim to stimulate the metabolic sequences that result in the synthesis of a desired metabolite. These provide economic benefits by achieving industrial-scale synthesis in bioreactors, as well as through selective routing of a certain biosynthetic pathway depending on the purpose.[3]

Costabel *et al.*[16] realized in 1986 that the synthesis and accumulation of secondary metabolites are dependent on some structural and biochemical processes that occur in tissues cultured "in vitro". It is well known that a series of secondary compounds accumulate in some tissues and organs, such as roots, fruits, shoot etc. For example, in the case of tobacco, nicotine and alkaloids, they are synthesized in the root of the plant, but are transported and accumulated predominantly in the aerial parts of the plant. It is noteworthy that in many cases, both biosynthesis and biotransformation of secondary metabolites are correlated with morphological differentiation, these phenomena being included in the genome of the species.[17] In the case of some species, it was found that when tissues and cells were cultured, the specific biosynthesis of metabolites was necessarily correlated with a certain level of cell and tissue differentiation.[17]

Deuterium depleted water or light water is a microbiological pure distilled water, with an isotopic concentration of 25 ppm, obtained by isotopic distillation in vacuum of natural water with an isotopic concentration of 145 ppm D / (D + H).[18] In Romania, deuterium depleted water (DDW) is obtained in the heavy water plant from Halanga, Caras Severin, in the Western part of Romania, where tons of such water are discharged daily as waste. The changes that occur in normal water characteristics lead to significant changes in the fundamental processes of the cells[19,18] and therefore, DDW was used as tracer to characterize wholetree water transport and storage properties in individual trees belonging to the coniferous species.[20] The use of DDW appears suitable for answering some questions regarding relative differences in water use among trees, water redistribution among neighbours and internal water transport and storage processes in plants.[21] Recent research has shown that spruce bark extract and DDW have a great influence on plant growth and development.[7]

The aim of this study was to evaluate the effect of deuterium depleted water and spruce bark polyphenolic extract as potential bioregulator for maize callus culture and their potential influences on the synthesis of secondary metabolites.

EXPERIMENTAL

Materials

Deuterium depleted water (DDW) was purchased from Romag Prod, Severin (Halanga), a manufacturer of heavy water used for the reactors of the Cernavodă nuclear power plant. Spruce bark was purchased from the Alpine LTD Timber Company, Vatra Dornei. It was dried at room temperature under normal aeration conditions, ground and subjected again to a drying process.

Maize seeds were purchased from Unisem Company, Romania.

Aqueous extraction of spruce bark

5 g of ground/milled dried spruce barks (SB) with particle sizes ranging between 0.5 and 1 mm were extracted with 125 mL double distilled water in a water bath, for 45 min at 80-90 °C. The process was repeated for three times and the extracts were cumulated to a final volume of 500 mL using distilled water.[22] For the DDW + SB variant, a dilution (1:1) of spruce bark extract was made.

Characterization of the extracts

The dry matter content in the extracts was determined by the evaporation of 25 mL extract on water bath and drying at 105 °C until a constant mass is reached, using a porcelain crucible. After that, the crucible was placed into a furnace at 600 °C for 6 hours, with the objective of establishing the dry, organic matter and ash contents.

The Folin Ciocalteu method was used to determine the total polyphenolic content (TPC). The total phenolic content is expressed as the number of equivalents of gallic acid (GAE). The polyphenolic content of the extract was calculated, resulting a value of 52 mg GAE (Gallic Acid Equivalent)/100 g dry mass.[23, 24]

The extracts were characterized from the point of view of total contents of tannins, flavonoids, flavonols and anthocyanins, using selected samples with about the same content in total polyphenols. The total content of tannins was determined using a method based on the precipitation of tannins with casein.[25] The tannins content was established using the FC method and expressed as the difference between the initial content of polyphenols and their content after precipitation with casein.[26]

The total contents of flavonoids and flavonols were determined by the aluminium chloride method, using rutin as a reference compound.[27,26] The method is based on the formation of a flavonoid–aluminium complex that exhibits the maximum of absorption at 510 nm for flavonoids and at 440 nm for flavonols.

The color intensity of the extracts was determined by reading the absorbance at different wavelengths (420, 520 and 620 nm), according to MA-F-AS2-07-CARCHR (2006).

With the aim to determine the total polyphenol content and the composition, the extracts were obtained from spruce bark. 5 g of dried ground material was extracted using distilled water (100 mL) at 80 °C in a water bath. The aqueous extract obtained from spruce bark was concentrated to 10 mL, under vacuum. Before the HPLC determinations, all the concentrated samples were submitted to a fractionation step by successive liquid-liquid extractions with ethyl acetate. The organic phases were evaporated to dryness, diluted in methanol and subjected to HPLC determination.

A reversed-phase high-performance liquid chromatographic technique was used to identify and quantify the phenolic compounds. The HPLC analysis was carried out using a Dionex UltiMate 3000 chromatograph coupled to a PDA detector. Separations were carried out with a Zorbax RX C18 column (4.6x250 mm, particle size 5 µm), operating at 30 °C with a flow rate of 1.2 mL/min and an injection volume of 5 µL. The mobile phase used was 1% acetic acid in water (A) versus methanol (B) for a total running time of 40 min, and the gradient changed as follows: solvent B started at 10% and increased immediately to 40% in 40 min. For quantification, standards for external calibration were used.

Experimental assay

In vitro cultures were developed in the Laboratory of Plant Physiology, Faculty of Biology, "Al. I. Cuza" University of Iasi and enzyme determinations were carried out in the Laboratory of Biochemistry, Department of Chemistry, "Al. I. Cuza" University of Iasi. The used vegetal explant consisted from *Zea mays* plantlets obtained by seed germination under aseptic conditions. Seed germination was performed by a standard procedure.[7] The preparation of the plantlets for inoculation consisted in their chemical sterilization with different reagents according to the following sequences: 70% ethanol for 5 seconds, 3% solution of sodium hypochlorite for 8-10 minutes, sterile water used for repeated washing of the explants in order to remove the previously used reagents.

After the sterilization, the explants, were inoculated under aseptic conditions (in a laminar flow box) on MS basal medium[28] supplemented with an auxine: 1 mg/L of 2,4 D in order to induce callus formation. The induction of callusogenesis was performed using surface cultures on agar medium prepared in the presence of polyphenolic extract derived from spruce bark (SB + 2,4 D/SB - 2,4D) and deuterium depleted water (DDW, DDW + SB). Callus multiplication over several subculturing steps was assessed by periodic weighing of fresh biomass every two weeks during a period of eight weeks. Other parameters that were monitored include: formation of adventitious and adventitious aerial roots, callusogenesis phenomenon, and enzymatic activities of peroxidase, superoxide dismutase, catalase, and total polyphenol content.

Enzyme activity assays

The callus resulted after 8 weeks was suspended in 5 mL of cold 50 mM phosphate buffer (pH 7.8) and sonicated three times for 30 seconds (Ultrasonic Processor CPX130, Cole-Palmer, Instruments, Illinois, USA). The slurry was then centrifuged for 10 min at 5000 rpm. The supernatant was used further for enzyme activity assays.

Peroxidase activity assay

The activity of peroxidase was monitored using hydrogen peroxide as substrate acceptor and *o*-dianisidine as donor. The absorption[29] was recorded at 436 nm.

SOD activity assay

The activity of superoxide dismutase (SOD) was determined by measuring its ability to inhibit photochemical reduction of nitro blue tetrazolium (NBT).[30]

The assay mixture consisting of 100 mM phosphate buffer (pH 7.5), 0.1 M EDTA, 750 µM NBT and 50 µL of enzyme extract were introduced in an Eppendorf vial. Finally, riboflavin (0.12 mM) was added and the tubes were mixed by shaking. One set of tubes was illuminated under a light source (2x5 W) with λ_{ex} = 254 nm for 5 min at a distance of 10 cm and another set of tubes was kept in the dark for 30 min. The mixtures without enzyme extract were similarly kept under light, as well as in the dark, and used as controls. Absorbance was measured at 560 nm. One unit of SOD

activity (U) is defined as the amount of enzyme required to cause 50% inhibition in the rate of reduction of NBT under specified conditions. The results were expressed as unit activity (U)/mg protein of extract.[31]

Catalase activity assay

The activity of catalase was assayed by the method of Sinha.[32] Catalase was allowed to split H_2O_2 for different periods of time. The reaction was stopped at different time intervals by the addition of dichromate and acetic acid mixture and the remaining peroxide was determined by measuring the chromic acetate amount, at 570 nm, after heating the reaction mixture. The activity of catalase was expressed as U/mL.

Quantitative determination of proteins using Bradford assay

In order to calculate the enzymatic specific activities of extracts, the total protein concentration was estimated.

There are numerous methods for quantitative estimation of proteins. Some of these methods depend on the reactions of reagents with peptide bonds or amino acid side chain of protein. The protein concentration can be also measured directly based on UV light absorption of aromatic side chain residues (phenylalanine, tyrosine or tryptophan). Since polyphenols can interfere in this range, this approach was not used in these experiments. Therefore, a dye-binding assay was chosen, in which a differential color change of dye occurs in response to various protein concentration.[33] After protein binding to Coomassie Brilliant Blue G-250, the maximum of absorption spectra shifted from 465 nm to 505 nm.[34, 35]

Statistic analysis

Our results are expressed as mean ± standard error, where n = 3. A comparison of the means was performed by the Fisher least significant difference (LSD) test (PB ≤ 0.05), after ANOVA analysis using program PAST 2.14. Sampling and chemical analyses were carried out in triplicate in order to decrease the experimental errors and to increase the experimental reproducibility.

RESULTS AND DISCUSSION

Characterization of the tested solutions

In our previous studies, the spruce wood bark was used to extract polyphenols using different agents (alcalin solution, aqueous alcoholic solutions or water). In all experiments described in this paper, an aqueous extract was used.

The spruce bark aqueous extract was characterized in terms of dry matter and organic matter, total content of polyphenols, tannins, flavonoids and flavonols, color intensity and pH. The results were summarized in Table 1. Spruce bark extracts contain considerable quantities of bioactive aromatic compounds, especially catechine and vanillic acid. The total contents of polyphenols for each variant were: 130 mg GAE/L (SB+2.4D and SB-2.4D) and 96 mg GAE/L (DDW+SB) (Fig. 1).

The properties of deuterium depleted water are similar to those of normal water (Table 2), excepting the isotopic concentration, which is 25 ppm, compared to 145 ppm for normal water.

Table 1
Characteristics of spruce bark aqueous polyphenolic extract*

Dry matter content, g/L extract	Organic matter content, g/L extract	Total content of tannins, mg/100 g EAG	Total content of flavonoids, mg/100 g RE	Total content of flavonols, mg/100 g RE	Colour intensity	pH (at 25 °C)	TPC, mg GAE/100 g
0.51±0.08	0.42±0.04	164.40±4.07	22.63±1.12	8.13±0.89	0.35±0.05	4.7±0.15	52±1.09

*The results represent average values of triplicate determination (n =3) ±S.D.

Table 2
Characteristics of deuterium depleted water

Isotopic concentration (ppm)	Flash point	Relative density (g/cm^3)	Water solubility	Melting point/freezing point (°C)	pH (at 25 °C)	Initial boiling point and boiling range (°C)
25	not applicable	1.00	completely miscible	0	6.0-8.0	100

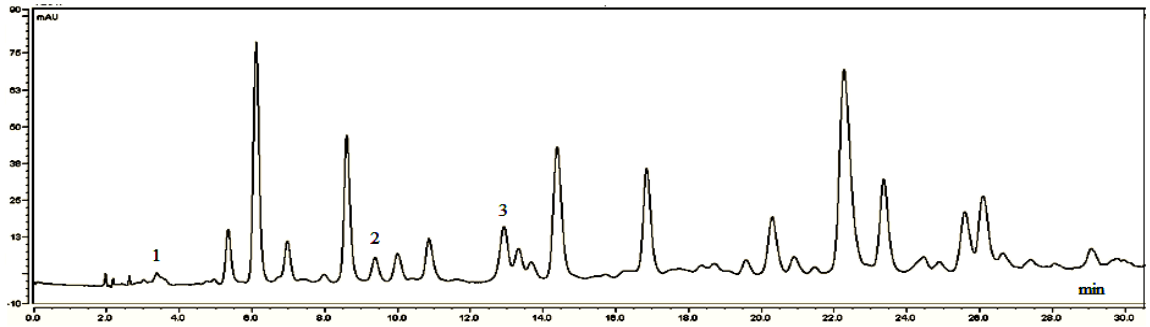

Figure 1: Chromatographic profile of polyphenols from spruce bark aqueous extract: 1- gallic acid (3.19±0.81); 2- catechine (31±1.9); 3- vanillic acid (39.4±0.2)

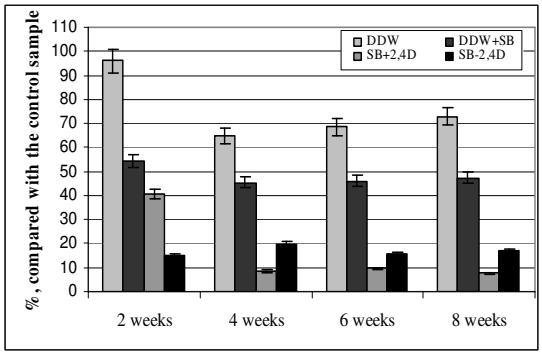

Figure 2: Influence of DDW and polyphenolic extract obtained from spruce barks on the fresh biomass accumulation in the corn/maize callus

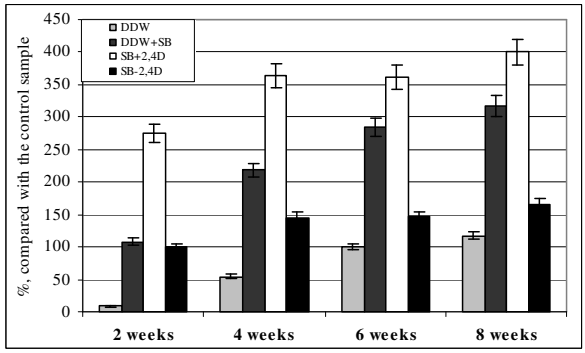

Figure 3: Influence of DDW and polyphenolic extract from spruce barks upon the number of adventitious roots

Physiological analysis

The polyphenols are known not only as potent antioxidants, but also as cell metabolism regulators. In this process, cell-cell communication and signal transduction are very important and some polyphenols can be considered to be essential for plant life. Thus, according to their structure, polyphenols can be involved in plant cell division, plant transportation of auxin, and protection of the growing system against stress factors.

The relationship between the phytohormones composition and the composition of the culture medium was demonstrated by analyzing the organogenesis.[36] The influence of plant growth regulators, especially auxins and cytokinins, on plant cells, during both the elongation and division processes and, generally, on the metabolism[37,12] is well known. The phytohormones influence the cytoskeleton, through the cortical microtubules oriented in different directions, limiting (cytochinines) or increasing (auxins) the cell expansion.[38]

Dynamics of biomass accumulation

The results obtained in the evaluation of biomass growth and accumulation in the radicular callus obtained from maize plantlets monitored over a period of eight weeks are presented in Fig. 2. There is an increase in biomass yield for all the investigated variants, a higher growth rate being observed during the first two weeks.

Comparing the influence of the tested solutions, it was found that after adding DDW in the culture (growth) medium, the amount of biomass increased up to 95% in the first two weeks, compared with the control sample. By combining DDW with the spruce bark extract in a concentration of 130 mg GAE/L (ratio 1:1), the stimulation of biomass accumulation was maintained, but the percentage of stimulation was slightly lower.

Earlier studies carried out by Simionescu *et al.*[5] reveal that the effect of the growth bioregulators feed into the growth medium is combined with the ability of plant tissue to synthesize hormones.

On this basis, it was found that the callus tissue developed in the culture medium containing aqueous extract from spruce barks accumulated a higher amount (9-51%) of biomass, compared with the control sample. It appears that within the first two weeks, the amount of biomass was higher in the variants where the culture medium was supplemented with 2.4 D, in addition to polyphenolic extract. However, in the following weeks, it was found that the wet biomass accumulation was higher in the variants where 2,4 D was not added. Thus, it is likely that 2,4-D is metabolized in the early days, while polyphenolic compounds present in the extract from spruce bark might be further metabolized, acting like phytohormones.

Regenerative capacity of aerial and adventitious roots

The radicular system is fasciculated in the case of maize, being formed by adventitious roots, which come from the underground nodes of the stem and aerial adventitious roots formed from the surface nodes. Aerial adventitious roots appear later in the vegetative stage, their role being related to absorption and fixation for plants.

Analyzing the formation of adventitious and aerial adventitious roots, one may see that the situation is slightly different. For all experimental variants, there was an increase in the number of adventitious roots directly proportional to time (Fig. 3).

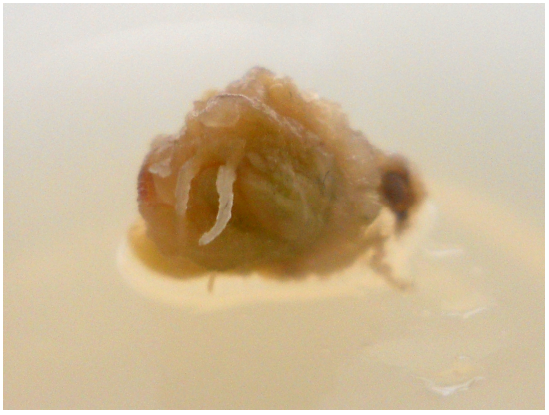

Figure 4: Adventitious root formation for control sample – 4 weeks after inoculation

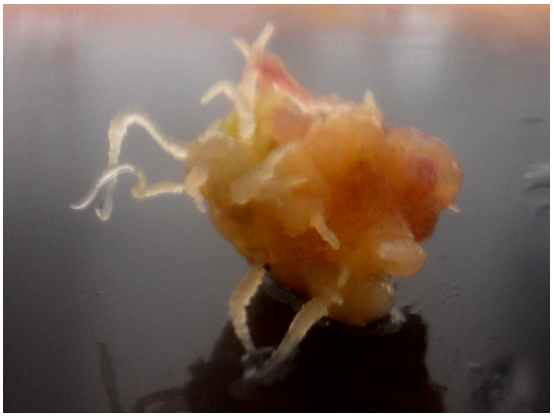

Figure 5: Adventitious root formation for variant DDW+SB – 2 weeks after inoculation

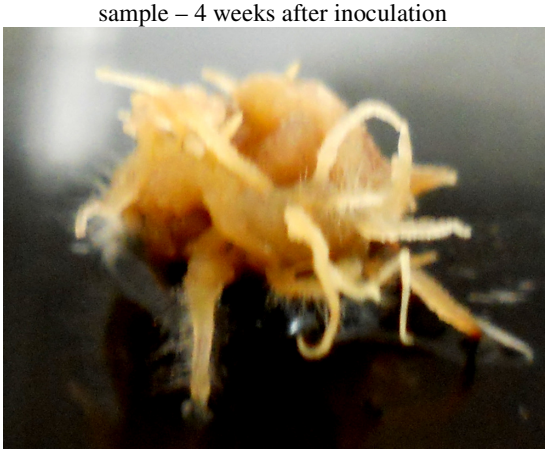

Figure 6: Aerial adventitious root formation for variant SB+2,4 D – 4 weeks after inoculation

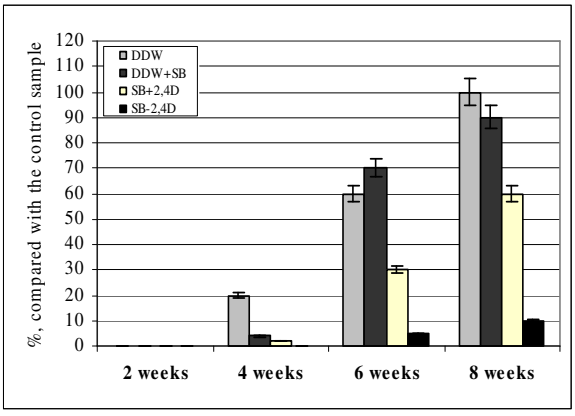

Figure 7: Influence of DDW and polyphenolic extract from spruce barks upon the number of aerial adventitious roots

Figure 8: Callusogenesis phenomenon and aerial adventitious roots in DDW variant – 6 weeks after inoculation

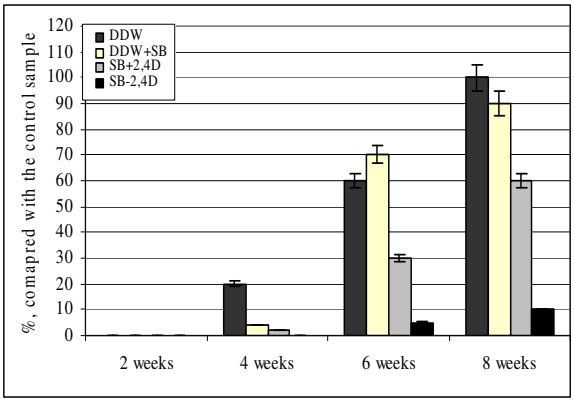

Figure 9: Radicular callusogenesis for maize in different experimental variants

Figure 10: Callusogenesis phenomenon in sample 2 from DDW variant – 6 weeks after inoculation

Figure 11: Callusogenesis phenomenon in DDW variant – 4 weeks after inoculation

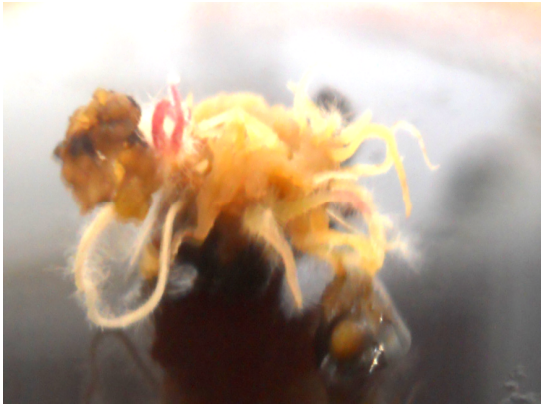

Figure 12: Callusogenesis for DDW+SB – 6 weeks after inoculation

Thus, it was observed that the addition of DDW in the culture medium led to the formation of a larger number of adventitious roots compared with the control sample (Fig. 4), but much lower compared to the other variants. When spruce extract was also added in the culture medium, the number of adventitious root was higher (DDW + SB) (Fig. 5).

The highest number of adventitious roots was recorded in the samples from version SB + 2,4 D (Fig. 6). This observation draws attention to the polyphenolic extract added to the culture medium, which together with 2,4-D may play a role in the

development of maize roots. The difference in the number of adventitious roots, given by the addition of 2,4-D in the culture medium, in which water was substituted by the polyphenolic extract, should be highlighted.

Monitoring the appearance and evolution of aerial adventitious roots (Fig. 7), one may find that they occur after the first two weeks after inoculation. Their good development was recorded in the samples from variants DDW (Fig. 8) and DDW + SB. A higher number of aerial roots (Fig. 7), compared with the control sample, was also recorded within variant SB + 2,4 D (Fig. 6), the percentage of stimulation significantly decreasing in the case of variant SB - 2,4 D. These observations suggest that the culture in which 2,4-D hormone is supplemented with DDW or polyphenolic extract is a medium for cell differentiation, in this case, for root development.

Radicular callusogenesis

After four to six weeks, at the same time with the emergence of adventitious roots, radicular callusogensis was observed (Fig. 9).

Thus, a yellowish friable callus composed of parenchymal cells was formed in adventitious roots (Fig. 10). This process of callusogenesis was observed only for the experimental variants DDW (Fig. 10 and 11) and DDW + M (Fig. 12). In the control samples, the callusogenesis phenomenon was not observed after eight weeks from inoculation. One may conclude that the water of low deuterium content and the polyphenolic extract from spruce bark have an important role in the callusogenesis. The influence of 2,4-D can also be noticed, since, for the variant where it was not added, the callusogenesis is much diminished.

Peroxidase activity

In some previous studies, it was found that the response of lucerne plants to soil acidity consisted in the main root growth inhibition, leading to a distinct change in photosynthesis, but also to the increase of peroxidase enzyme activity.[39] The preliminary results achieved show that there is a genetic variability concerning the tolerance of lucerne genotypes to soil acidity, which provide opportunities for progress in its amelioration. Among the mechanisms related to aluminum tolerance, it has been estimated that lucerne plant presents internal detoxification mechanisms, which result in the increase of activity of peroxidase, an enzyme that acts as scavenger of radicals for plant cells.[39]

By determining the enzyme activity, the behaviour of the enzymatic system in callus tissue in the presence of the solutions applied was investigated. The experiments revealed that peroxidase activity in parenchymal cells of maize callus is different depending on the solution introduced into the culture medium of each variant (Fig. 13). Thus, the most intense peroxidase activity was observed in the presence of DDW (59% higher compared with the control sample) and polyphenolic extract (SB + 2,4 D – 25% higher compared with the control sample).

A decrease in peroxidase activity (28% compared with the control sample) was observed only for the culture medium without 2,4 D.

In conclusion, there is an increased activity of peroxidase enzyme in callus parenchymal cells, which were developed in a culture medium containing DDW or polyphenolic extract supplemented with 2,4 D.

Superoxide dismutase activity

From the recorded data, it was found that the activity of superoxide dismutase (SOD) was increased when a mixture of DDW and an aqueous extract of spruce bark were introduced in the culture medium (Fig. 14). For all the other versions, there was a slight decrease in the activity of superoxide dismutase (by 26% for variant DDW and 15% for variant SB+2,4 D compared with the control sample).

Intensity changes in SOD activity in the presence of DDW and polyphenolic extract from spruce bark show their involvement in the regulation of metabolic processes in cells and the role of the tested solution in maintaining the balance between the free radicals formed and removed.

Catalase activity

The enzymatic activity of catalase decreased in the callus cells that developed in the culture medium prepared with the extract from spruce bark (Fig. 15). By comparing the results obtained for variants SB+2,4 D and SB-2,4 D, one may see that 2,4-D did not significantly alter the enzymatic activity of catalase.

There is a slight increase in catalase enzymatic activity in comparison with the control, in the samples where the medium is prepared with deuterium depleted water. In this respect, the deuterium depleted water most likely induces

gene expression for both peroxidase and catalase. Also, as can be correlated, peroxidase and catalase activity increased with the increase in the amount of biomass.

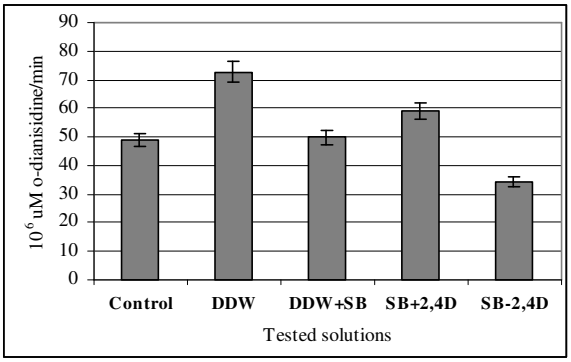

Figure 13: Variation of peroxidase activity in callus cells according to growth conditions

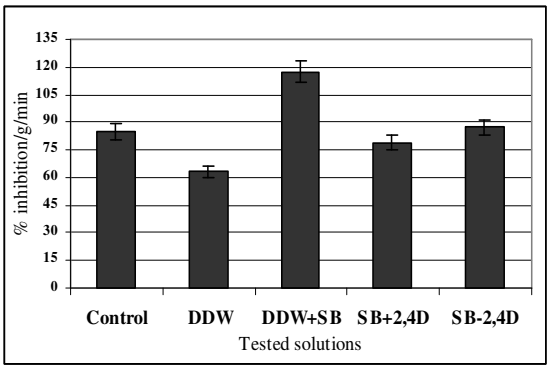

Figure 14: Variation of superoxide dismutase activity in callus cells depending on growth conditions

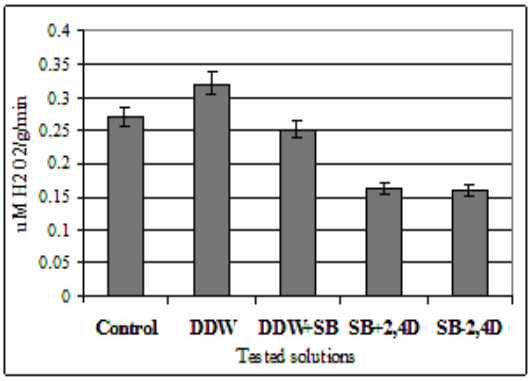

Figure 15: Variation of catalase activity in callus cells depending on the growing conditions

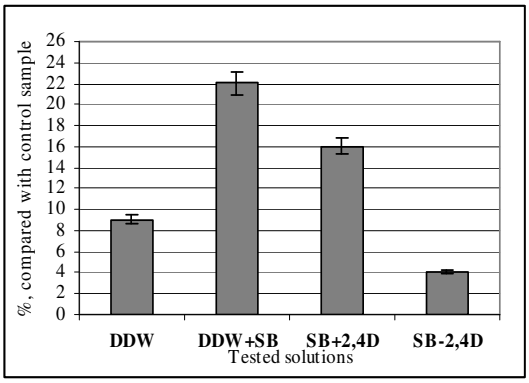

Figure 16: Variation of total content of polyphenols in callus cells depending on the growing conditions

Total content of polyphenols

Since many studies have been devoted to the production of useful compounds by plant tissue culture, remarkable technological progress has been recorded.[40,41] The present study aimed to investigate the effect of the tested solutions (DDW and polyphenols) on the ability to synthesize polyphenolic compounds. Thus, the studies based on cell and tissue cultures performed at a fundamental level could be extended in order to develop the production of secondary metabolites on industrial scale for commercial purposes.[42,43,10,13]

The obtained results have shown that the mixture of deuterium depleted water and polyphenolic extract from spruce bark caused a stimulation of the synthesis of polyphenolic compounds by over 22%, compared with the control sample (Fig. 16). It was also found that the addition of polyphenolic extract from spruce bark in a concentration of 52 mg GAE/100 g led to an increase in the total content of polyphenols in callus cells by 16%, compared with the control. When DDW was applied to the growth medium, the percentage of stimulation of the total content of polyphenols in callus tissue was of 9%, compared with the control.

Therefore, we can appreciate that the metabolic pathways in the callus are influenced by the presence of DDW and polyphenols in the culture medium. The polyphenols can interfere with plant hormones influencing stimulation or inhibition of cell plant development. This aspect will be further investigated to propose a mechanism that could explain these processes.

CONCLUSION

In the present study, the effect of two bioregulators on callus maize plant was investigated. It was found that the callus tissue

developed in culture medium containing aqueous extract from spruce bark and deuterium depleted water accumulated a higher biomass, compared with the control. All solutions used in the culture medium led to an increase in the number of adventitious roots directly proportionally to time. The highest number of adventitious roots was observed when aqueous extract obtained from spruce bark together with 2,4-D were added in the culture medium. It suggests that these solutions can play a role in the root development of maize callus. A culture medium containing 2,4-D hormone and supplemented with DDW or polyphenolic extract is most suitable for cell differentiation. It was also found that water with low deuterium content and the polyphenolic extract from spruce bark have an important role in the callusogenesis.

The enzymatic activity of peroxidase was found to be higher in the callus developed in the presence of DDW and polyphenolic extract. Intensity changes of the enzyme activity in the presence of DDW and polyphenolic extract from spruce bark show their involvement in the regulation of metabolic processes in cells and the role of the tested solutions in maintaining the balance between the resulted and depleted free radicals.

It was found that the mixture of deuterium depleted water and polyphenolic extract from spruce bark caused a stimulation of the synthesis of polyphenolic compounds compared with the control. These results indicate their possible action as bioregulators of physiological and biochemical processes in maize callus under the action of aqueous polyphenolic extract obtained from spruce bark and water with a low deuterium content.

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