COMPLEX RESISTANCE OF TOBACCO PLANTS TO HEAVY METAL IONS IN VITRO: A FACTOR OF RESISTANCE TO ABIOTIC STRESSES

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Abstract. *Functioning and ontogenesis of plants are carried out under genetic control. The full realisation of the genetic potential is a consequence of genotype/environment interaction and depends significantly on environmental conditions. Reactions to various stresses establish distinctions between stable and unstable genotypes. In this case, reactions of the whole plant and reactions realised at the cellular level are distinguished. To determine the contribution of each component to the overall organismal resistance, their parallel study is necessary. In this regard, the best approach is a comparative analysis of the plant and the cell culture initiated from it. To obtain objective information, it is necessary, firstly, to establish stressors that have a versatile effect on the organism. Secondly, identification of general adaptation reactions to them. These conditions correspond to heavy metal ions (HMI), in particular barium cations, Ba2+, and cadmium cations,* Cd^{2+}*. The toxic effect of these cations is similar to the negative effect of osmotic stresses - salinisation and water deficit. Their comparative analysis revealed the key role of cellular level mechanisms. This provides an opportunity to use these cations in cell selection, a biotechnology involving manipulation of cell cultures. The result can be the selection of forms with an increased level of resistance to abiotic stresses.*

Key word. Ba^{2+} *and* Cd^{2+} *cations, salinity, water stress, resistance, cell selection*

Global climate change and the acute shortage of fresh water, even for drinking, are hindering the cultivation of valuable economic crops. Among the abiotic stresses, osmotic stresses, such as salinity and water shortages, have the greatest harmful impact. Often, they act together, which further reduces the possibility of survival of the organism. There is a need to develop plant forms that combine resistance to various stresses with satisfactory consumer qualities. The needs of the current moment determine the directions of scientific research and stimulate the development of new research methods. Currently, new biotechnological methods are being actively developed that use micro and even nanotechnology. At the same time, the ideology of environmental safety is becoming a priority, both for the approach itself and for the product it produces [18, 29, 31].

One of the most promising ways to solve the problem of obtaining stress-resistant plant forms is cell selection. However, like any technology, this method requires constant improvement. It is especially necessary to take into account various aspects that cause distrust when using this methodology. Mass production of plants requires a comprehensive study of the organism. At the same time, the use of an in vitro system can guarantee constant monitoring of exposure parameters and the adequacy of the object of interest's reactions. The use of cell cultures and the study of their behaviour under various influences will ensure the objectivity of information about the state of cell vital activity throughout the entire development cycle. Cell cultures of plants of different origin, cultivated under normal conditions and under extreme influences, are becoming a promising object of research. In vitro biotechnological methods are now beginning to outpace traditional methods of producing genetically modified plant forms in terms of their popularity. However, like any method, they require constant improvement [8, 27, 32].

In general, the in vitro system is a complex factor that affects the genetic programme of a plant. Obtaining cell cultures (dedifferentiation) from various organs of formed plants or immature tissues, manipulating them and further regenerating plants with new characteristics is a complete research/technology algorithm [8, 27].

It is known that abiotic stresses cause a complex of interrelated reactions that can occur simultaneously or alternately. Therefore, for the primary selection, it would be advisable to choose an agent that has both specific and general damaging effects. If a substance characterised by high toxicity in relatively small quantities and therefore causing significant cellular damage is chosen as a modelling stress agent, cellular selection can become the main method of obtaining plant forms with unique characteristics. Such characteristics are associated with heavy metal ions (HMI), especially the group of HMIs that are toxic in residual amounts and are considered physiologically unnecessary. Such HMI include: Ba^{2+}, Cd^{2+}, Hg^{2+}, Pb^{2+}, VO_3^-, WO_4^{2-}. Despite their exceptional toxicity, these IRMs are characterised by different levels of study. While the ions Cd^{2+}, Hg^{2+}, Pb^{2+} are widely and comprehensively studied, the Ba^{2+} cation is relatively little known. As for anions, which in general are much more toxic than cations, they are the subject of research only in some cases [1, 3, 7, 16, 34, 35].

We proposed and practically tested a hypothesis (using tobacco as a traditional model object) about the possibility of cellular selection from HMI to obtain lines with cross-resistance to simulated osmotic stresses.

MATERIALS AND METHODS

Ions of the following heavy metals were used to create an in vitro model system: Ba^{2+}, Cd^{2+}. The selective concentration for each ion was determined in preliminary experiments. It was considered to be the smallest amount of stressor that stopped the development of the wild-type cell culture. If after returning to normal conditions, the vital activity of the culture was restored, the concentration of the stressor was increased. This prevented the selection of adaptants. If the ion used required special modifications of the medium, such changes were made taking into account the preservation of the qualitative and quantitative composition of the nutrient media. [25, 29, 36, 37].

It was necessary to establish the suitability of this approach in the selection of agriculturally important crops. The test was carried out using soybean cell cultures of a number of genotypes. In accordance with the developed scheme and rules of cellular selection, each stage of the experiment was accompanied by constant monitoring. Since the aim of the experiment was to select comprehensively resistant variants, the culture conditions were always changed during each passaging. The alternation of stress1 - control - stress1 or stress 1 - control - stress 2 was arbitrary. The number of passages on a single medium (cultivation period) was also arbitrary.

In cell selection (for further results), the phenomenon of a stable cell culture is of great importance. A culture that can only withstand stressful pressure when growth processes are completely inhibited and resume proliferation only under normal conditions is often considered resistant. In our experiments, we selected cultures that maintained growth and development throughout the entire period of cultivation. To determine the stability, the relative biomass growth rate (Δm) was constantly monitored. $\Delta m = (m1 - m0) / m0$; where m0 is the mass of cells at the beginning of the passage, m1 is the mass of cells at the end of the passage.

RESULTS AND DISCUSSION

Selection and study of Ba^{2+}-resistant tobacco cell lines (Ba-RCL). Ba-RCL were obtained as a result of primary selection on medium with a lethal concentration of Ba^{2+} ions. After cell biomass growth on the selective selection medium, the callus was simultaneously passaged on the control medium (n.w.) and on selective media with Ba^{2+}, sea water salts, and sodium sulfate. Table 1 shows the relative biomass growth (Δm) of different Ba-RCL of tobacco when cultivated under different stress conditions.

Table 1.

Relative growth of tobacco Ba-RCL biomass during cultivation under stress conditions in vitro

Genotype Ba-RCL	(Δm).			
	B5 – control	B5 + Ba^{2+*}	B5 + sea salt*	B5 + Na_2SO_4*
RCL №3	3,21 ± 0,24	1,91 ± 0,32	2,15 ± 0,15	3,01 ± 0,66
RCL №9	5,01 ± 0,76	2,13 ± 0,45	3,51 ± 0,27	4,28 ± 0,32
RCL №2	4,58 ± 0,33	2,69 ± 0,68	1,99 ± 0,18	2,63 ±0,40
Control	5,72 ± 1,41	Did not grow	Dod not grow	Dis not row

**Note: Concentrations of stress compounds were added in lethal concentrations*

Table 1 shows that Ba-RCLs are characterised by a comprehensive resistance to all modelled stresses. However, the maximum relative increase in cell biomass was observed in the medium with sodium sulfate. It can be assumed that high resistance to sulfate salinity is associated with resistance to barium cations, since the literature shows a higher toxicity of sulfates [10, 20]. It is known that Ba^{2+} ions affect the transfer of K+ ions. Ionic interaction/antagonism has been reported for other cations as well [21, 24, 26, 33]. Thus, salinity resistance may be related to the kinetics of toxic cation transport or the structure of the transporters themselves. It is also possible that cross-resistance to Ba2+ ions and different types of salinity is associated with changes in membrane viscosity caused by changes in the degree of lipid saturation [5, 6, 18, 30]. One of the well-established effects that a heavy metal ion can exert in biological systems is the function of a structure stabiliser. This function is also performed in those simple cases where the orientation effect of ions in a complex compound is meant. And in the case when a metal stabilises the structure of proteins in an enzyme without affecting the catalysis process as such. And in the case when a metal ion forms a catalytic structure with qualitatively new characteristics [12, 15]. However, the latter event is only possible in the presence of a metal ion. In our case, the cross-stability of Ba-SCL is confirmed by the proliferation and growth of the culture on any selective medium. Therefore, this event is most likely due to the compartmentalisation of toxic ions.

This assumption was confirmed by determining the free proline content in resistant cell lines. Table 2 illustrates the dynamics of fluctuations of this amino acid during the cultivation of callus on media with different types of salinity.

Table 2.

The content of free proline in Ba-SCL cells during cultivation under salinity conditions

Genotype Ba-SCL	(Δm).			
	B5 – контроль	B5 + Ba^{2+*}	B5 + sea salt*	B5 + Na_2SO_4*
SCL №3	3,21 ± 0,24	1,91 ± 0,32	2,15 ± 0,15	3,01 ± 0,66
SCL №9	5,01 ± 0,76	2,13 ± 0,45	3,51 ± 0,27	4,28 ± 0,32
SCL №2	4,58 ± 0,33	2,69 ± 0,68	1,99 ± 0,18	2,63 ±0,40
Control	5,72 ± 1,41	Did not grpw	Did not grow	Did not grow

During one passage of subcultivation, any developing cell culture goes through a number of stages. Among them, the most important are the logarithmic growth stage (7-14 days) and the stationary growth stage (14-21 days). During the logarithmic growth stage, biomass increases due to cell division, and the stage is characterised by a maximum of mitoses. The stationary growth stage is characterised by an increase in individual cell compartments due to the redistribution of intracellular components.

During long-term cultivation under saline conditions, plants and cell cultures accumulate significant amounts of toxic sodium ions [12, 20, 26]. This leads to a significant change in the osmotic balance inside the organism. To compensate for this disturbance, free proline, one of the possible compatible osmolytes, accumulates in cells. This is obviously the case in the case of cultivation of Ba-SCL in selective medium with the addition of sea water salts (Table 2). The maximum absolute value of the proline level was observed on day 14. This is a natural process, since proline has the ability to retain the water necessary for cell division and thus stabilise the required degree of cell colloid viscosity. From 14 to 21 days, the proline content decreases (in absolute terms) but remains high relative to the same value measured under normal conditions. Since the relative increase in biomass of the culture growing under these conditions is the lowest, the protective role of proline becomes even more pronounced.

The expected increase in the free proline content was not observed in the medium with sodium sulfate. This is a particularly interesting phenomenon, since the osmotic pressure of culture media with seawater salts and sodium sulfate is almost the same, and the mechanism of Na^+ ion transfer to the cell is common in both cases. The active growth of the resistant culture in the presence of Na_2SO_4 indicates the absence of stress inhibition. Thus, it can be assumed that a different defence mechanism is being implemented in this case: "proline-independent". Such a characteristic phenomenon, combined with salt resistance, has also been observed in Ba-resistant tobacco cell lines [5, 9, 11, 18].

Usually, in salt-resistant cell lines obtained by direct selection on saline media, a common type of osmolyte protector, independent of the nature of salinity, is used (selected). In this case, the Ba-resistant lines under different salinity conditions implement resistance mechanisms that can change each other depending on the type of stressor. Obviously, such plasticity is a guarantee of cross-resistance. This hypothesis can be tested experimentally if there is a reliable target of a specific stressor. Such an object is the enzyme nitrate reductase (C.F. 1.6.6.1). [2, 38].

Nitrate reductase (NR), a homodimer encoded by nuclear genes in higher plants, catalyses the reduction of nitrate to nitrite. The NR enzyme complex consists of two parts that are sequentially involved in the transfer of electrons from NAD(P)H to nitrate. This is the diaphorase part, which contains FAD and catalyses the transfer of electrons from NAD(P)H to cytochrome c or other acceptors. And the terminal (reductase) part, which contains molybdenum and transfers electrons to nitrate [41]. The parts differ significantly. The diaphorase complex is exposed to HMI, sulfhydryl groups, and is thermolabile. The terminal part is sensitive to osmotic stresses and redox transformations [40, 41].

Selection and study of Cd^{2+}-resistant tobacco cell lines (Cd-RCL).

It is known that the water balance in plants can be maintained by a special class of proteins - dehydrins. This class includes LEA (late embryogenesis abundant proteins), proteins of the late stage of embryogenesis. A number of publications have noted that Cd2+ ions have a negative effect on LEA [4, 22, 19]. Therefore, Cd^{2+} was used to obtain tobacco cultivars resistant to water stress. Our idea is based on the nature of the effect of Cd^{2+} ions on the water status of the plant. Cd^{2+} ions significantly inhibit the activity of LEA, one of the groups of dehydrin proteins. These proteins are directly related to the maintenance of the plant's water balance by moving water inside the body and transporting it between individual tissues. Therefore, we assume that cell lines resistant to a lethal dose of Cd^{2+} will have an increased level of resistance to the modelled stress [25, 28].

Figure 1 shows Cd^{2+}-resistant tobacco cells cultured in selective medium with cadmium ions.

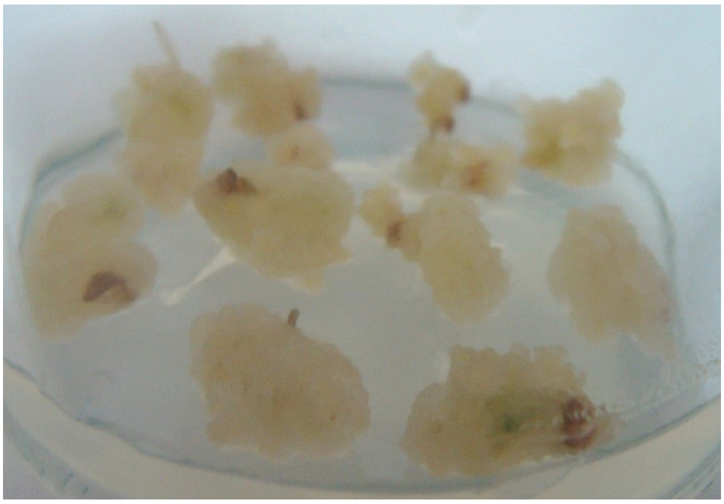

Figure 1. Primary colonies of Cd^{2+}-resistant tobacco cell lines.

After biomass growth for several passages, the callus was divided into parts and grown on medium with a lethal dose of mannitol.

Table 3 shows the relative biomass growth (Δm) of winter wheat callus when grown under different conditions. The complex stability of the selected cellular variants is observed.

Table 3.

Relative increase in biomass of callus induced from tobacco, genotype Dubek, when cultivated under different conditions

Genotipe	Δm, Biomass growth		
	Media B5	B5 + Cd^{2+}	B5 + manitol
Дюбек, control	4,16±0,11	Dies	Dies
Дюбек, Cd^{2+} RCL, №3	5,27±0,99	3,24±0,16	0,98±0,13
Дюбек, Cd^{2+} RCL, №5	3,44±0,23	1,96±0,23	1,22±0,11

In general, there is a linear relationship between the water potential of the medium and the osmotic potential of cells in the stationary growth stage [4, 8, 18, 29, 39]. It is noted that cells adapted to severe water stress do not keep their volume stationary, but change their turgor in proportion to the water potential of the medium. In this way, active osmoregulation is carried out to prevent large-scale dehydration. The process is carried out by increasing the level of endogenous components that reduce the water potential (ψw) and provide a gradient that is optimal for water penetration.

Figure 2 (a, b) shows the growth of winter tobacco cell cultures under different conditions.

Figure 2. Tobacco cell cultures on mannitol medium (a) Cd^{2+}-resistant cell line; b) wild type.

Among the endogenous compounds related to the maintenance of plant water status, free proline plays a special role [11, 13, 14, 17].

Proline, a pyrrolidine-2-carboxylic acid, has a number of properties that make it a nonspecific stress protector. The high hydrophilicity of proline contributes to an increase in the soluble volume of the cell, thereby maintaining the hydration sphere of cellular polymers. The hydrophobic pyrroline ring of the molecule interacts with similarly structured parts of proteins, as a result of which the charge-carrying structures are oriented in the right direction, stabilising the water status.

The content of free proline in cellular variants of winter wheat was analysed (Fig. 3).

Changes in the level of the amino acid were observed depending on the cultivation conditions. Under normal conditions (Hamburg B5 medium), the content of free proline in the callus tissues of genotypes is not significant; it is identical in all cultures. When the cultivation conditions change, the difference between resistant genotypes and the wild type begins to appear. When cultivated under selective conditions (mannitol), the level of proline increases; the increase in absolute value is more significant in resistant cell lines.

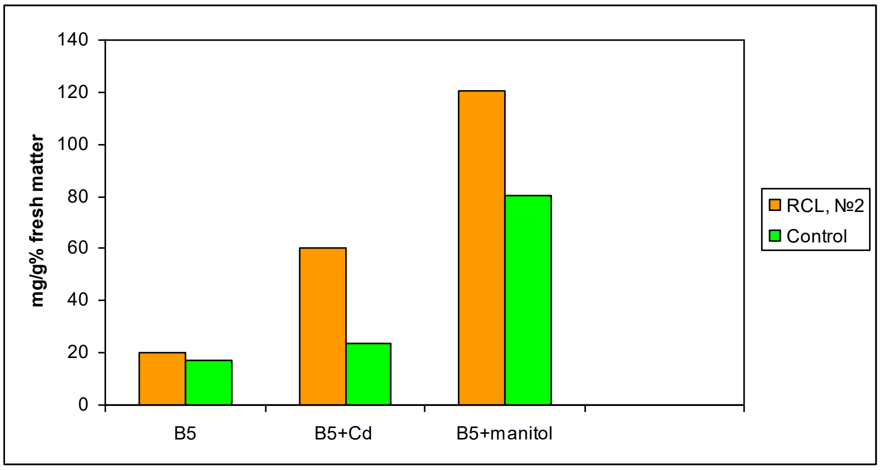

Fig. 3. Free proline content in tobacco cell cultures.

This can be easily explained by the amount of mannitol in the culture medium. The presence of this compound significantly reduced the water potential of the medium. In this case, proline acts as an osmotic active substance that supports the hydration sphere of proteins.

At the same time, the presence of cadmium ions in selective media (lethal dose) can cause toxic damage at significantly lower doses. Therefore, in our opinion, the proline present in the control cultures was the result of the degradation of proline-containing cell wall proteins.

Proline-rich proteins (PRPs) are involved in the formation of cell walls. In their molecules, proline residues are organised into repeating motifs to which hydroxyproline is added. Two PRP subunits (extensins and P/H RGPs) are of great importance in resistance to biotic and abiotic stresses. This is due to stress-induced oxidation, during which inter- and intra-molecular cross-links are formed, which strengthen the structure of the cell wall [11, 13, 14, 17, 18, 20, 26]. In stable cell cultures, the accumulation of free proline occurred due to synthesis. The level of free proline under normal conditions is maintained by its synthesis/degradation systems. However, under stressful conditions, only the synthesis system is active.

Therefore, when obtaining plant forms with an increased level of resistance to osmotic stress, which is coordinated with the accumulation of proline, this should be taken into account.

CONCLUSIONS

Heavy metal ions have a complex effect on living organisms. Resistance to them should cause significant changes that must be genetically determined

When selecting resistant cellular variants, i.e. cells that are characterised by stable growth in the presence of a constant stress factor, it is necessary to investigate the cause of resistance. Obviously, cross-resistance is possible when genetic and epigenetic changes are combined. This is especially important to consider since epigenetic changes are caused by mechanisms that normally operate during cellular differentiation.

The study of cell lines resistant to HMI has shown that cell culture in general and cell selection in particular have not exhausted their potential. As a method of studying the fundamental mechanisms of cell functioning under normal and stress conditions, it is difficult to surpass. As a way to produce resistant plants, it is a promising biotechnological approach. As a research ideology, it is aimed at promoting environmental safety in experimental and production activities.

Plant resistance to osmotic stress is a polygenic characteristic. To achieve success, it is necessary to evaluate the maximum number of vital parameters available. This will create an opportunity to actively influence metabolism.

The latest biotechnologies can be a priority in such experiments.

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