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CADMIUM IONS IN CELL SELECTION FOR OBTAINING WHEAT CELL FORMS TOLERANT TO WATER STRESS

Introduction. *Water deficit significantly decrease the plant development and crop production. Genetic effects that increased the genotype tolerance abilities are the aims of various investigations. Cell selection is the appropriate biotechnology for obtaining plant forms that challenged abiotic stresses. It is known that Cd^{2+} cations significantly destroy various plant compartments and tissues. There was detected that Cd^{2+} injures the water status of the organism.*

Purpose. *The aim of the investigation was the promotion of cell selection with Cd^{2+} cations for obtaining wheat cell lines tolerant to water stress.*

Methods. *Selective systems with lethal doses of cadmium ions (Cd^{2+}) for obtaining wheat cell forms tolerant to water stress are proposed and elaborated. The minimum Cd^{2+} concentration that eliminates wild type cell population was established as lethal doses.*

The water stress was conducted by the addition of manitol. Manitol is usually used for simulation water deficit in vitro.

*Callus and suspension cultures were initiated from immature embryos of winter wheat, (*Triticum aestivum* L.), cv. Favoritka. Cell suspension (wild type) was placed on agar cultural B5 medium with the addition of lethal doses of cadmium ions (“plating procedure”). Such doses were deduced during preliminary tests. Only Cd-resistant cell survive under lethal ion stress pressure.*

In Cd^{2+} resistant cell lines relative fresh weight and free proline levels were estimated.

Result. *Resistant cells formed primary minicolonies. Such colonies are considered to be wheat resistant cell lines (Cd-RCL). Cd-RCL grew at Cd^{2+} ions presence during 3 passages. Then callus was cut and transferred to fresh media: basal medium (normal conditions) and selective media (stress conditions). There were established two variants of selective systems: medium with the addition of Cd^{2+} cations, (stress I); cultural medium with the addition of manitol (stress II). Cd-RCL maintained their viability under any stress pressure. Genetic basis of Cd-RCL combined stress resistance was confirmed via media rotations. The changes were: normal conditions → stresses I, II; stresses I, II → normal conditions; stress I → stress II or other way roads. The type of cultural medium and the number of passages were always free. As proliferation marker calli relative fresh mass growth (RFW, Δm) was used. It was always positive. This parameter measured under normal conditions exceeded (40-45%) biomass RFW estimated under manitol pressure. But normal data were lower (more than three times) than data measured during calli cultivation at Cd^{2+} presence. It is assumed that such events are the exhibitions of combined resistance.*

The levels of free proline (pro) were estimated in Cd-RCL. Under normal conditions wheat cell cultures accumulated pro in various amounts. In wild type callus the proline level was the highest. In Cd-RCL cultivated at manitol presence pro contents increased. We suppose that elevated pro levels in Cd-RCL under water stress were due to activity of system of its synthesis. The wild type cell cultures eliminated at the end of any passage. Proline levels were lower than level of determination.

Conclusion. *Cell lines of winter wheat with combined stress resistance were obtained via cell selection with Cd^{2+} cations. Cd^{2+} -resistant cell lines tolerated both lethal ion and water stresses. Under water stress pressure callus RFW of Cd^{2+} -resistant cell lines was lower and under Cd^{2+} affect was higher than normal parameters. The growth under water was cooperated with free proline accumulation. Cell selection with heavy metal ions is the perspective approach for obtaining cell variants with higher tolerance to osmotic stresses.*

Key words: *winter wheat, cell selection, Cd^{2+} ions, water stress tolerant cell lines, proline*

The problem setting. *Drought is hard limiting factor of crop production that adversely impacts the agricultural production all over the world. Water deficit, declining*

drinking water quality are problems which are becoming more actual. Multiple events promote the development of various scientific strategies for obtaining tolerant plant forms. The success of the investigation always relies on selection producer.

Heavy metal ions (HMI) belong to group of the most toxic pollutants because their stress pressure can form vast alterations in different tissues of plant organism. Usually HMI act together with abiotic stresses and their joint stress pressure is more hazardous. From another hand the resistance to HMI may be associated with analogous reaction to abiotic stresses.

Plant cells developed various highly regulated metabolic networks that involved in their water stress tolerance. Water homeostasis is concerned with several proteins. Dehydrins, large group of proteins are among them. LEA (*late embryogenesis abundant proteins*) as a dehydrin version gain special interest [1-3]. LEA is detected in nuclei, cytoplasm, mitochondria. It assumed that LEA like chaperons can hinder molecules denaturation during drying. The histidine presence promotes the binding of heavy metal ions (HMI) [4]. At the same time some cations essentially repress LEA [5].

There are a lot of HMI harmful at trace concentrations. Cadmium (Cd^{2+}) ions are extremely toxic because their effect expanded towards various plant compartments and tissues. Monitoring of the publications shows that Cd^{2+} is studied actively on both cellular and plant levels [6-8]. Considerable interest over decades is based on the elevation of Cd^{2+} environmental injury from industrial, agricultural and municipal sources.

Plant cell culture protocols have potential opportunity for selecting cells tolerant to number simulating situations/compositions of cultural media. Various biotic and abiotic stresses are established *in vitro*. E. Lestari, (2006), as appropriate approach suggested the screening within somaclonal variations [9]. (When plants are regenerated from cell cultures they are not always identical to primary form from which cultures were initiated. This event is called somaclonal variation that is the result of calli cultivation). Somaclonal variation would give advantages if it is increases genetic variation particularly the feature which is not obtained at the parent form. To enhance genetic changes both physical and chemical treatments there were applied. E. Lestari noted that PEG and manitol were chemicals useful for drought tolerance, fusaric acid or filtrate were suitable for fusarium wilt, $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ was used for Al^{3+} tolerance.

New plant forms that challenge water stress were obtained via cell selection. Some of them combined tolerance to osmotic stresses (salinity, water deficit).

Common idea is declared that breeding for resistance to osmotic stresses cannot be divorced from breeding for various other traits of mineral metabolism. Since it is known that Cd^{2+} destroys the water status of the organism we decided to use such feature for obtaining new plant forms with higher tolerance to water stress [10]. We have established the feasibility of selection at the cellular level. We obtained tobacco cell lines with combined resistance to Cd^{2+} ions and water stress. Regenerated plants and R1 seed progeny demonstrated tolerance to water stress *in vitro* and *in vivo*.

Cereals in many countries suffer from water deficit. Irrigation is recognized to be a main task in arid and semi-arid areas. At the same time in those regions agriculture faces a danger from secondary salinization. Plant breeding aims are the creation screening techniques for selecting drought-tolerant forms. Various physiological and biochemical reactions are choosing as a marker of selection. On cellular level stress tolerance is defined as biomass growth under stress pressure [11-13].

The aim was to expand our approach for obtaining wheat cell lines tolerant to osmotic stress via cell selection with Cd^{2+} cations.

The research methodology and organization

Callus was induced from immature embryos of (14 days after artificial pollination) winter wheat, (*Triticum aestivum L.*), cv. Favoritka. Seeds surface were disinfected in 96% ethanol for 10 minutes, 30% (v/v) commercial bleach Belizna for 30 minutes and rinsed three times with sterile water.

For callus induction and proliferation nutritional medium with B5 Gamborg (1968) inorganic salts and organic compounds was used [14]. The medium supported 30.0 g/l sucrose and (mg/l) 1.0 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.2 kinetin. Callus induction occurred in 5-7 days. Calli were sub cultivated 3 – 5 passages (passage duration 30 – 35 days). Those cultures (control, wild type) were bases for cell selection.

Suspension cultures were established by replacement calli to liquid shake medium of the same contents for pellet disaggregation. Wheat suspensions were grown in 300 ml flasks containing 100 ml culture per flask. The ratio cell biomass/medium (w/v) was 1:2. The viability level of cell population was estimated according to [15]. The suspension density for plating manipulation was counted: 10mg of individual cell sediment per 100ml of liquid medium.

Medium for cell selection was elaborated by the addition to B5 medium the lethal for wild type cell cultures doses of cadmium cations. It was the minimal Cd^{2+} dose that stopped the cells growth. Under normal conditions such calli never again restored their development. There was the elimination of wild cells population. Such Cd^{2+} doses were deduced prior the applying “plating” procedure.

Medium with lower osmotic potential for evaluation the water stress tolerance level of selected cultures was made by the addition lethal doses of manitol to B5 medium. So both selection and investigations were conducted under lethal stress pressure.

The “plating” procedure means cell suspension uniform arrangement between two layers of selective (Cd-containing) solid media. 0.5 ml of wheat cell suspension was displayed at Petri dishes at 16-hr photoperiod at 25°C. Dishes were monitored for appearance of primary microcolonies. These colonies are considered to be ion-resistant cell lines. Ion-resistant colonies were cultivated for obtaining sufficient calli biomass during 3 – 4 passages; the duration of average passage was 30-35 days. Further cultivations were performed under changed conditions: normal nutrition, B5 medium; stressed environment, B5 medium with the addition of toxic matters – Cd^{2+} cations, (stress I), manitol, (stress II). Besides, the medium rotations were arbitrary.

As proliferation marker calli relative fresh mass growth (RFW, Δm) was used. Δm means: $(m_f - m_i)/m_i$, where m_i – initial biomass weight at the start of the passage; m_f – final biomass weight at the end of the passage [10, 15]. Data of biomass production are statistically analyzed. Data shown are the average of 3 replicates \pm SE. Free proline levels in wheat cell cultures were estimated according [16].

The results and their discussion

A number approaches for obtaining tolerant to water stress cell variants are developed. We propose simple one that does not require long chain selection protocol. The chromosomal instability of cultures *in vitro* even under normal conditions promotes the increasing the variability within the material being screened. The selective pressure and especially its level minimized the cell population. Under lethal stress pressure only peculiar forms survive. Our method provides the selection of genetically changed cell variants.

On selective media resistant single colonies were obtained. (The frequency of appearance was 10^{-6}). These colonies later formed Cd-resistant cell lines (Cd-RCL). During 3 – 4 passages calli were cultivated under normal or stress I (+ Cd^{2+} cations) conditions. The variants viability is regularly controlled. The positive relative fresh mass growth (RFW)

indicated the common effectiveness of development. When cell biomass exceeded 1.0g callus was cultivated on medium with manitol addition (stress II). RFW of wheat cultures estimated under normal conditions varied in Cd-resistant cell lines №3, №5 (table). Both cultures differed from control but differences were not essential. We assume that this event is the result of distinctive metabolism feature of any genotype.

Wheat Cd-RCL tolerated lethal water stress. Data exhibited that normal calli RFW exceeded this parameter measured under stress pressure. Does this event a result of a stress inhibition? P. Hasegawa et al. (2000), explained analogous features of salt-adapted cells as a realization of protective mechanisms. Cells with little inner volume could accumulate enough amounts of compatible solutions for maintenance their osmotic status under salinity [17]. Cd-resistant cell lines challenged lethal water stress. So we can assume that its low RFW was the exhibition of their peculiar characteristics.

The biomass RFW, measured during cultivation on media with addition of Cd²⁺ exceeded normal amounts in both Cd-RCL №3 and Cd-RCL №5 more than three times. This event we observed during investigation tobacco Cd-resistant cell lines [10]. Our assumption is partly explained by free proline analysis (table).

Free proline (*pro*) level highly increases in plants in response to various biotic and abiotic stresses including salinity, water deficit, high/low temperatures, heavy metal ions, plant pathogens attack [17, 18]. Most explanations that support *pro* contribution to stress tolerance rely on *pro* ability to promote osmotic adjustment, stabilization of sub cellular structures. *Pro* can take part in recovery processes as additional source of nitrogen and carbon [17, 18].

We measured *pro* contents in wheat calli cultivated under contrast conditions (table).

Table 1
Relative fresh mass growth (RFW) and free proline levels in wheat calli cultures

Wheat cell cultures	B5		B5 + manitol	
	RFW	Proline, mg/g FW	RFW	Proline, mg/g FW
Favoritka, Cd-RCL №3	1.75 ± 0.23	0.26 ± 0.04	0.79 ± 0.09	0.66 ± 0.03
Favoritka, Cd-RCL №5	2.27 ± 0.18	0.35 ± 0.07	0.92 ± 0.13	0.75 ± 0.09
Favoritka, callus, wild type	1.96 ± 0.14	0.41 ± 0.03	Elimination	Elimination

Under normal conditions wheat cell cultures accumulated *pro* in various amounts. In wild type callus the proline level was the highest. *Pro* content measured in both Cd-RCL was coordinated with appropriate RFW. In plants under normal conditions the *pro* level is controlled by systems of synthesis/degradation/transport. On cellular level *pro* transfer is realized within distinct cell. A key enzyme of *pro* biosynthesis is delta-pyrroline-5-carboxylate synthetase (P5CS). The process is carried out in cytoplasm. Under normal conditions *pro* is produced from either glutamate or ornithine. *Pro* oxidation is carried out in the mitochondria. The degradation is catalyzed by proline dehydrogenase (ProDH). Table data reflected normal relations between two systems of *pro* metabolism.

In Cd-RCL cultivated at manitol presence *pro* levels increased. There are experimental data about the reciprocal manifestation of P5CS and ProDH genes under abiotic stress condition [19]. Analysis of transcription during abiotic stress and subsequent recovery periods showed that levels of P5CS transcripts are elevated during stress and gradually decrease during recovery. While levels of ProDH are gradually reduced within several hours of abiotic

stress and immediately increased after release from stress. We suppose that elevated *pro* levels in Cd-RCL under water stress were due to activity of system of its synthesis. The role of increased *pro* contents in promoting plants more tolerant to osmotic stresses is under discussion. We fixed the stable proliferation of selected wheat cultures under any type of stress conditions. Positive RFW was accompanied by higher free proline contents. Both those figures reveal the viability of experimental cultures. The wild type cell cultures eliminated at the end of any passage. Proline levels were lower than level of determination.

The mechanisms which provide higher osmotic tolerance to cultured *in vitro* cells and molecular processes by which cells maintain viability under stress pressure are not completely understood. Somaclons that appears within cell population are apparently caused by gene amplification, the alteration of a basic couple, transposing migration, methylation transform, chromosome instability, translocation, ploidy change, restricting or restructuring [9]. The direct selection towards obtaining plant forms with good tolerance to osmotic stresses does not satisfy investigators. Experimentally obtained cell cultures manifested higher levels to salinity or water stress. At the same time regenerants from those variants have no preferences during cultivation under stress conditions *in vitro* and *in vivo*. The success of such approach in breeding tolerant forms requires the availability of: a) vast cell variability; b) adequate selective agent in its concentration; c) easy but trustworthy regeneration method of tolerant cell lines and d) the inheritance of desired character. Addition of appropriate selective agent to culture media is advantageous.

The selective system with Cd²⁺ is a new approach for obtaining plant cell lines tolerant to water stress. Tobacco cell lines, regenerated plants and R1 seed progeny demonstrated tolerance to water stress *in vitro* and *in vivo* [10]. Today we have no achievements in plant regeneration from Cd-resistant wheat cell lines. However, if the aims of research for water stress tolerance in plant cell cultures are to create tolerant crops then the availability of genomes containing the information for integrated cellular reaction to stress, must represent a valuable genetic basis. We suppose the cell selection with heavy metal ions makes contribution to agricultural plant breeding. The knowledge of plant stress tolerance will enrich.

Conclusion

Cell lines of winter wheat with combined stress resistance were obtained via cell selection with Cd²⁺ cations. Cd²⁺-resistant cell lines tolerated both lethal ion and water stresses. Under water stress pressure callus RFW of Cd²⁺-resistant cell lines was lower and under Cd²⁺ affect was higher than normal parameters. The growth under water was cooperated with free proline accumulation.

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Анотація. Л.Є. Сергєєва, Л.І. Броннікова Клітинна селекція із іонами кадмію для отримання клітинних форм пшениці, стійких до водного стресу.

Проблематика. Водний стрес суттєво погіршує розвиток рослин і знижує урожайність. Генетичні зміни, котрі спрямовані на підвищення толерантності генотипів, є метою чисельних досліджень. Клітинна селекція є перспективною біотехнологією отримання форм рослин із підвищеним рівнем стійкості до абіотичних стресів. Відомо, що катіони кадмію здійснюють широку шкодо чинну дію на тканини та компартменти рослин; у тому числі порушують водний статус.

Метою дослідження було створення селективної системи із катіонами Cd^{2+} для отримання стійких до водного стресу клітинних ліній пшениці

Методи дослідження. Створено селективну систему із іонами Cd^{2+} . Іони застосовували у летальних для клітинних культур дикого типу дозах. Летальною вважалась концентрація, яка викликала елімінацію клітинної популяції дикого типу. Вижили окремі клітини, які утворювали мікроколонії, а в подальшому – Cd -стійкі клітинні лінії. Відбір стійких варіантів здійснювався в масиві суспензійної клітинної культури дикого типу.

Із незрілих зародків пшениці сорту Фаворитка отримано калусну та суспензійну культуру клітин (культура дикого типу).

Генетично змінені клітинні варіанти виділяли методом «плейтингу», що полягає у рівномірному розподілі суспензії між шарами селективного середовища.

Дослідження осмотостійкості Cd -стійких клітинних ліній здійснювали за умов прямої дії маніту. Маніт є речовиною, яку застосовують для моделювання водного стресу *in vitro*. Концентрація маніту також була летальною.

У стійких клітинних ліній, культивованих за умов водного стресу вимірювали вміст вільного проліну.

Основні результати дослідження. На селективних середовищах із катіонами кадмію отримано стійкі клітинні лінії пшениці. Дані варіанти відзначались комплексною стійкістю, а саме проявляли толерантність до летальної концентрації маніту. При культивуванні за умов водного стресу в калусі стійких варіантів зростає рівень вільного проліну.

Висновки. Методом клітинної селекції з іонами Cd^{2+} отримано клітинні лінії пшениці із комплексною стійкістю до токсичного іону селекції та водного стресу. За прямої дії водного стресу у клітинах зростає рівень вільного проліну.

Ключові слова: пшениця озима, клітинна селекція, іони Cd^{2+} , клітинні лінії, стійкі до водного стресу

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