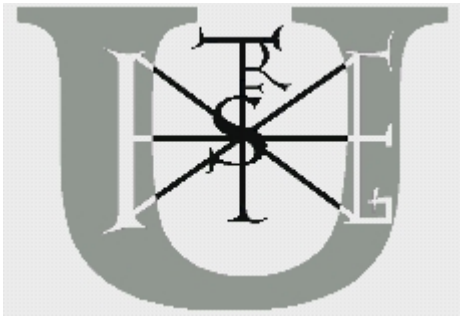

SZENT ISTVÁN UNIVERSITY

CRYOPRESERVATION OF *IN VITRO* SOMATIC CELLS - AND EMBRYOS

Summary of PhD thesis

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1. INTRODUCTION AND OBJECTIVES

1.1. Introduction

Parallel with the development of traditional – and molecular plant breeding techniques the importance of cryopreservation, i.e. conserving *in vitro* cultured plant cells, tissues and organs in under cryogenic conditions has been increased. Freeze-preservation methods recently are specialised towards two directions. A part of experiments published reports a true – to - type preservation of germplasm as a tool for gene-banking. Another part of papers however applies cryopreservation as a special technique of plant tissue culture, allowing to maintain certain cell - cycle and differentiation in various explants. Plant biotechnological practises, such as cell suspension originated protoplast isolation, foreign pollen stimulated haploid induction, or *in vitro* fertilisation mediated hybridisation are based on the use cells or tissues, showing a well characterised level of cell - cycle and differentiation. This requires the continuous maintenance of a large number of basic culture and donor plant. Above methods could be however practised independently from glasshouse or laboratory limitations, provided, if reliable preservation methods for cell suspension, embryo or pollen - based on liquid nitrogen -, are available.

The prerequisite to obtain survival explants from cryogenic conditions is, the earliest appearance of amorf ice during the water-ice transition, while crystal phase remains at the lowest level. This occurs as a result of the decrease in homogenous ice nucleation temperature (Th) and of the increase in glass formation temperature (Tg) by the concentrating aqueous liquid in dehydrating cells. In the practice of cryopreservation the amount of intracellular ice crystals could be minimised, if supercooled explants are transferred from Th temperature (approx. –30 - -40 °C) directly into liquid nitrogen. Intracellular liquids will be solidified into amorf stage - after this transfer - within several seconds. Another requirement for the survival is, the avoidance of the adverse affect of concentrating cell sap, which might cause functional disorders of water stressed membranes. In other words: the increase of cell liquid concentration is the prerequisite of obtaining survival explants, but after a certain extend it will cause membrane damage. These two conflicting phenomena

could be harmonised by the control of cell dehydration and by the maintenance of membrane stability.

1.2. Objectives

Cryopreservation of cultured somatic cells, tissues and organs was chosen for the subject of this theses.

The main aims were – through the modification of different steps of cryopreservation – either to gain recovering explants from the deep-frozen cultures, or to increase the survival rate.

Experiment were set up to prove, that

(1) an optimisation of the freezing program (freezing rate, transfer temperature, holding time) will result in surviving cells in suspension culture,

(2) freeze tolerance could be achieved by pure abscisic acid (ABA), sucrose or proline treatments or in combinations in somatic embryos and in cultured somatic sells,

(3) ABA and stress proteins have an important role in the increase of desiccation tolerance and thus in the creating cryo-tolerance

Answers were looking for whether,

(4) slow prefreezing could be substituted by heat shock treatments,

(5) ABA and alginate gel decrease the toxicity of PVS-2 vitrification solution in somatic embryos,

(6) sugar and sugar alcohol treatments create cryo-tolerance in different cell suspensions,

(7) *afp* (anti-freeze protein) gene transformation in transgenic clones synthesising the anti- freeze protein results in an increased cryo-tolerance,

or not.

2. MATERIALS AND METHODS

2.1. Testplants

Experiments were setting with the use of cell suspension cultures and somatic embryos. Saltmarsh grasses [*Puccinellia distans* (L.) Parl. and *Puccinellia limosa* (Schur.) Holmbg. (Genbank, Tápiószele)], Arabidopsis [*Arabidopsis thaliana* (L.) Heynh. (ecotypes: C24, Erecta (LER), Wassilewskija (WS) (Nottingham, *Arbidopsis* Stock Center)] and the maize [*Zea mays* (L.) (MSCE/79 line and *afp* transgenic clones: 23, 24, 37, 44 and wild type)] were frozen in cell suspension, while horse-chestnut [*Aesculus hippocastanum* (L.), (gödöllő ecotype] and kiwi [*Actinidia deliciosa* (A.) Chev (self- pollinating variety, Tulipa nursery, Gödöllő)] were frozen as somatic embryos.

The maize MSCE/79 line, and *afp* transgenic clones as well as the horse-chestnut somatic embryos were kindly provided respectively by Sándor Mórocz, (GKI, Szeged), Prof. Dénes Dudits (SZBK, Szeged) and József Kiss (SIU Gödöllő).

2.2. Cryopreservation

Treatments having an influence on the survival rate from the cryogenic temperature zone were analysed. Protective dehydration and the preserved membrane integrity –as determining factors in the creation of cryo-tolerance - were established by slow precooling (1); by abscisic acid (ABA) treatments, combined with desiccation, heat shock and vitrification (2) and by cryoprotectan carbohydrates (3) (sucrose, sorbitol, mannitol).

2.2.1. Preculture

Cryoprotectant compounds

Cells suspensions and somatic embryos for freezing were harvested from the exponentially growing phase and transferred in liquid medium containing cryoprotectants for 4 days. With the exception of its cryoprotectant content, the preculture medium was identical with the standard maintaining medium. Cryoprotective compounds in the preculture phase were studied as follows:

-*ABA* (0.75-75 μM) alone and in ABA-sucrose (21%), ABS-proline (5 %) treatment combinations for the horse-chestnut and kiwi somatic embryos and maize cell suspensions,

-sugar and sugar alcohol (sucrose, mannitol, szorbitol) for Arabidopsis and maize cell suspensions.

Media containing abscisic acid were sterilised by filtration, while those containing sugar and sugar alcohol additives were sterilised in autoclave.

Desiccation and heat shock

Desiccation was carried on horse-chestnut somatic embryos; heat shock treatments were applied to horse-chestnut somatic embryos and to maize cells.

Desiccation was accomplished at room temperature (70% relative humidity) in opened, empty Petri dishes (∅ 9 cm), which were placed in the airflow of a laminar cabinet for 2-4 h duration. Horse-chestnut somatic embryos (∅ 1.5-2.0 mm) were transferred in the Petri dishes (3-4 ml = 50-60 embryos), containing filter paper discs (Ø 5 cm). Desiccated embryos were then placed into 2 ml polypropylene cryo-vials (approx. 20 embryos/vial) and were frozen without liquid by directly plunged into liquid nitrogen (quick freezing). Water content of embryos was compared to the fresh weight of untreteated control (100 %), as determined after 48 h desiccation in an oven at 104 °C.

Heat shock treatment was achieved by the immersion of Erlenmeyer flasks containing liquid medium into water bath (+ 40 °C) for 30-90 min duration. Explants were frozen in WK cryoprotectant mixture (DMSO 0.5 M, glycerol 0.5 M, sucrose 1.0 M, proline 1%, pH 5.8) in the polypropylene vials either slowly, or quickly after the heat incubation.

Cryoprotectants

Explants frozen in liquid were placed into 2 ml screw cap polypropylene cryo-vials (0.70-0.75 ml cell suspension, or 12-15 somatic embryos/vial) and filled with 600 μl of different cryoprotectant mixture:

- *Dimethylsulfoxide (DMSO)*: (12.5%, or 15 % in AA2 medium, pH 5.8) in experiments scheduled for the freezing program optimisation of cell suspensions of saltmarsh grasses,
- *WK cryoprotectant mixture* in MS, N6M and E1B5 medium after ABA pretreatment in horse-chestnut somatic embryos and after sugar and sugar alcohol pretreatments in Arabidopsis and maize cell suspensions.

Cryoprotectants prepared always freshly before freezing were sterilised with a 0.2 μm pore sized membrane filter (MILLIPORE), adjustable to hypodermic. Treatment was accomplished in an ice – bath for 1 h before freezing.

PVS- 2 vitrification mixture

Leave originated kiwi somatic embryos in the cothyledonary stage (∅ 2-3 mm), after a pretreatment on medium containing ABA, were capsuled in alginate gels and incubated in 500 μl PVS-2 vitrification mixture (sucrose 30%, DMSO 15%, ethylene -glycol 15%, pH 5.8) at both 0 °C and 25 °C for up to 90 min in plastic vials (6-7 capsuled embryos/vial).

PVS-2 mixture was sterilised by filtration and used fleshly. Treated somatic embryos were plunged into liquid nitrogen without precooling.

2.2.2. Freezing, storage and thawing

Cryoprotective effect of freezing program was analysed in the mutual context of freezing rates, transfer temperatures, and holding times during the cryopreservation of cell suspensions of saltmarsh grasses. Freezing was carried out in a programmable biological freezer (Snowball-1400, SY-LAB). Cultures were frozen in cooling rates of 0.5, 0.75, 1.0, 2.0 °C/min and 1000-2000 °C/min down till –10, -50 °C transfer temperatures, from which temperature after a 0-30 min holding time incubation cells were plunged into liquid nitrogen (LN).

Arabidopsis and maize cell suspensions, as well as horse-chestnut somatic embryos were frozen identically down to – 35 °C in a 1 °C/ min cooling rate regime, then the explants were immersed into liquid nitrogen after holding them at this temperature for 40 min.

Explants after desiccation, heat shock and vitrification treatments were frozen rapidly without precooling (direct immersion into LN from the temperature of pretreatment).

Cultures were stored in liquid nitrogen in the range from 1 to 7 days.

Thawing was accomplished by placing the vials in a water bath at + 40 °C, till ice just melted (50-90 sec).

2.2.3. Post- thawing and assessment of viability.

Explants from the cryo-vials were transferred onto soiled culture medium in Petri dishes (∅ 9 cm). Cell suspension cultures were passed on filter paper discs (∅ 5.5 cm) on the surface of the solid medium, horse-chestnut somatic embryos were placed on agar medium without filter paper and without washing them. Vitrified kiwi embryos were washed for 5 min in liquid culture medium containing 1.2 M of sucrose, which was followed by the passage to solid medium.

At the beginning of the recovery phase explants were kept in the dark during 5 and 14 days for embryos and cell suspensions respectively. Explants were transferred onto fresh medium on the 3rd and 14th day after thawing. Post - culturing - with the exception of Arabidopsis cells - after two weeks were continued under light

conditions (16/8 h light/dark, 44 $mEm^{-2}s^{-1}$ light intensity). Thawed Arabidopsis cell suspensions were cultured under dark conditions.

Original cultures were re-established, or plants were regenerated from the survival explants.

Surviving was assessed by using the TTC staining method, by measuring the fresh-weight changes, or by counting the ratio of somatic embryos showing development. In the course of TTC – test the optical density of ethanol extractable formazane was determined at 490 nm with the use of a spectrophotometer (JENWAY UV/VIS). Samples (50 mg fresh weight) were taken from the cultures at the 1^{st}, 5^{th}, 10^{th} and 15^{th} days after thawing, then incubated in 1,5 ml, 0.6 % TTC concentration, prepared in 0.05 M phosphate buffer (pH. 7.4) over night.

Fresh weight of the cell cultures was measured weekly carried out in a laminar flow cabinet.

Viability of somatic embryos was calculated from the number of greening embryos following either direct or adventitious embryogenesis and from the number of died, brown embryos on the 6^{th} weeks after thawing.

For TTC test 12 samples, for viability assays based on the fresh weight changes or embryo development, explants of 5 Petri dishes were used respectively per treatment. Data were analysed by using the multifactorial analysis of variance statistical method.

3. RESULTS AND CONCLUSIONS

3. RESULTS

3.1. Effect of freezing program on the survival rate of Saltmarsh grasses cell suspension cultures

Experiments were carried on saltmarsh grass and on reflexed saltmarsh grass cell suspension cultures maintained in liquid AA2 medium. In the course of analysing the elements of freezing programs, freezing rates (0.5, 0.75, 1.0, 2.0 °C /min, 1000-2000 °C /min) and the different holding time regimes (10, 20, 25, 30 min) were studied as the function of transfer temperatures (-10, -20, -30, -40, -50°C). No cryoprotectants were added, when freezing rates were tested. The survival rates of the frozen cells were determined by the TTC – test, which was completed with plant regeneration.

Maximal survival was obtained among the tested freezing rates at 1 °C/min, so at the next stage of the experiments only this freezing rate was used. The ration of survival cells increased by the effect of DMSO. Highest survival was found, when the explants were immersed from 40 °C into liquid nitrogen. The viability showed further improvement, if the cells were incubated at the transfer temperature for 10-40 min followed by the immersion into LN. The survival rate of cells plunged from higher transfer temperatures showed significant increase, after incubating them for shorter holding time periods already. The most viable explants were observed, if the cells were kept for 30 min at – 40 °C, before immersed them into liquid nitrogen, with both of the tested DMSO concentration.

Survival cell suspensions were reported with slow precooling followed by a direct plunge from the transfer temperature into LN mainly, when the particle size of the cell suspension was fallen in the “fine sized” category. When the particle size was somewhat larger, - with an immediate immersion from the transfer temperature - conditions for the occurrence of protective dehydration are available only in the surficial cell layers. An extended period of holding time at the transfer temperature however dehydrates the deeper cell layers also. Saltmarsh grass cultures used for cryopreservation consisted of cell aggregates with 2-3 mm in diameter. We suppose therefore, that the significant increase in the survival rate observed in our

experiments after incorporating holding time periods in the freezing program show that an adequate level of protective dehydration occurred in the inner part of aggregates as well.

Survival *Puccinellia distans* cells went thorough a successful plant regeneration process, the regenerants were however albinos without exception. When the cryopreservation was repeated with saltmarsh grass cell suspensions, selected for green calli, a high level of survival was observed with aggregates cryoprotected in DMSO, frozen in a 1 °C/min freezing rate –20 °C- transfer temperature and 30 min holding time cooling regime, but the regenerants remained albinos again.

3.2. Effect of ABA on the survival rate of horse-chestnut somatic embryos and maize cell suspensions

Experiments were adjusted on filament originated horse-chestnut somatic embryos, proliferating by adventitious embryogenesis, maintained in liquid E1B5 medium and on immature embryos originated maize cell suspensions (MSCE/79), cultured in liquid N6M medium. Horse-chestnut embryos were precultured in medium containing ABA alone and ABA - sucrose and ABA - proline combinations, for 4 days before cryopreservation. Maize cells were subjected to preculture medium for 4 days containing ABA only. Both type of explants were frozen in WK cryoprotectant mixture slowly and quickly. Survival was determined by the TTC – test.

The survival of frozen horse-chestnut embryos without preculture and WK cryoprotectant, increased after all of the preculture applied. Embryos cultured on medium containing a standard sucrose level (3 %) and 0.75 μM of ABA showed the highest survival rate, when frozen slowly. In this freezing regime however, by the effect of higher ABA concentrations, than 0.75 μM decreased the survival assayed by TTC – test. A preculture on medium containing 5 % of proline without ABA resulted in only a limited increase in the survival rate. The 7 fold (21 %) sucrose concentration neither alone, nor in combinations did lift the survival significantly.

Based on relevant papers, the abscisic acid mediated cryoprotection correlates to the induction of stress proteins. ABA dependent and ABA independent pathways are reported for the control of stress genes, expressing under water deficit

conditions, which is a common effect of freezing. Acquired freezing tolerance in horse-chestnut probably follows the abscisic acid mediated pathway.

In our experiments maize cells showed only a limited increase of the cryotolerance by the effect of abscisic acid. This could be explained with the natural frost sensitivity of maize. It was evidenced that ABA pretreatment results in freeze tolerance in species which species could acquire the freezing tolerance by cold acclimation also. Proline pretreatment however has conferred cryoprotection in maize cells. According to our hypothesis the acquired cryoprotection in species showing an *ob ovo* freezing tolerance follows the ABA dependent signal-pathways, while in the naturally frost sensitive species, the ABA independent ways are dominating.

3.3. Effect of desiccation on the survival rate of horse-chestnut somatic embryos

Horse-chestnut somatic embryos were used for the experiments. Embryos were pretreated on solid E1B5 medium containing ABA, then after incorporating 2, 3 and 4 h duration desiccation periods in the air flow of a laminar cabinet were immersed directly into LN without cryoprotectants.

Thawed embryos placed on the surface of solid medium showed brown discoloration at the 1^{st}, 2^{nd} days after melting, independently of the preculture regime. Seemingly died, brown embryos however continued their development in the 2-5 week of postculture, therefore the survival was determined based on the differentiation observed at the 6^{th} week after thawing identically.

The survival rate of embryos after an ABA pretreatment and desiccation combination increased significantly, compared to those frozen without ABA and desiccation. ABA treatments did not affect the water content of the embryos, but the 2, 3 and 4 h desiccation periods decreased their fresh weight to 53 %, 22 % and13% respectively. Parallel with reduction in the water content, the viability of unfrozen embryos reduced, but a viability increase was observed in frozen embryos. Highest survival was found after using a 4 h desiccation period, which decreased the fresh weight to 13 %. This desiccation regime (4 h) resulted in a large number of embryos shoving differentiation independently from the concentration of ABA (0.75-75.0 µM)

in the preculture medium. Desiccation periods shorter, than 3 h, coupled to higher fresh weight, than 22 % did not bring satisfactory level of survival.

Zygotic and somatic embryos were successfully cryopreserved in a number of species based on the desiccation freezing. In those reported experiments explants were precultured on medium containing sucrose before desiccation. The effect of sucrose was explained with the increase of desiccation tolerance.

The role of ABA in the acquiring of desiccation tolerance was proved however in somatic embryos of alfalfa and celery. Presumably the significant cryoprotective effect of ABA observed in our experiment, similarly to the effect of sucrose preculture correlates to the enhancement of water stress tolerance.

3.4. Effect of heat shock on the survival rate of horse-chestnut somatic embryos and maize cell suspensions

Experiments were adjusted on horse-chestnut somatic embryos, and on maize cell suspensions (MSCE/79). Cultures were pretreated on medium containing 0.75 µM ABA for 4 days, then immersed in a +40 °C temperature water bath and shaked in the Erlenmeyer flasks for 30, 60 and 90 min. Heat shock treatments were followed by an hour incubation in WK cryoprotectant, then eplants were frozen either slowly, or rapidly. The survival rate was determined by using the TTC- test.

Heat shock treatments without ABA preculture did not elevated the survival rate neither in the applied freezing regime. TTC reduction increased however in both the horse-chestnut embryos and the maize cells, when heat shock was combined with ABA treatments.

Heat shock did not affect the survival of slowly frozen horse-chestnut somatic embryos. On the contrary heat shock significantly increased the survival of embryos frozen quickly. Explants after 60 min heat shock treatment, which was followed by quick cooling, reached as much survival as were found in embryos frozen slowly.

Survival rate of MSCE/79 maize cells was highest after 30 min heat shock period when frozen slowly, while the survival was not affected, if the heat shock teartments were combined with quick cooling.

An increased drought - and freeze tolerance were reported in *intact* plants in *in vitro* cultures by the effect of heat shock. The survival of quickly frozen tobacco cells preteated with a mannitol-heat shock combination was found higher, than those were

found in cells, frozen slowly.

It is supposed, that cryotolerance of tobacco cells were developed through a synergetic effect of proteins, induced by either the slight osmotic stress of mannitol or by heat shock. Our results on the cryopreservation of horse-chestnut somatic embryos and maize cell suspensions can encourage above observations.

Synergetic effect of ABA and heat shock induced proteins could be explained by the chaperon function of HS proteins. We suppose, that ABA inducible proteins confer a direct cryoprotection, while HS proteins are functioning mainly as indirect protectants. Heat shock induced cryotolerance could explain with the restoration mechanism of HS proteins, renovating the proteins denatured during the freeze-thaw trauma.

3.5. Effect of vitrification mixture and alginate gel on the survival of kiwi somatic embryos

Cotyledonary kiwi somatic embryos, maintained in liquid WPM medium complemented with 2.5 mg BA, were harvested for freezing. Prior to cryopreservation, explants were cultured in standard solid WPM, containing 0.75 µM ABA for 4 days. After this pretreatment, embryos were capsuled into alginate gel and were subjected to PVS-2 vitrification mixture at 0 °C and 25 °C for up to 90 min before plunging them into liquid nitrogen. Thawed explants were then washed in standard liquid WPM containing 1.2 M sucrose for 5 min. Postculture continued on the original, solid WPM medium.

PVS-2 alone damaged the survival of the embryos. An ABA preculture however reduced its toxicity, correlating to the temperature and duration of PVS-2 application, either in the capsuled, or not capsuled embryos.

The largest number of developing embryos were found among the ABA pretreated, capsuled explants, followed by PVS 2 treatment for 50 min at 0 °C, before the immersion into liquid nitrogen. When the PVS 2 was added to the capsules at 25 °C, the maximum survival were observed after 30 min incubation.

Concluding: kiwi somatic embryos are sensitive to the PVS 2 toxicity, but ABA preculture and capsulation could develop it.

3.6. Effect of sugar and sugar alcohol on the survival rate of *Arabidopsis* and maize cells

Experiments were carried on 3 ecotypes (C24, LER, WS) of *Arabidopsis* cell suspensions, cultured in liquid MS medium containing mannitol in a concentration range of 0.25 -1.0 M, and on the MSCE/79 maize cells, maintained in liquid N6M medium complementing with sucrose, mannitol and sorbitol (0.5-0.75 M). Suspensions were pretreated for 4 days on the sugar and sugar alcohol preculture media, then incubated in WK cryoprotectant mixure containing 1 % of proline for 1 h in an ice bath, which was followed by slow cooling before the immersion inti liquid nitrogen. The survival was determined either by the TTC test carried on the 1st, 5th and 10th days after thawing, or by measuring the fresh weight changes on the 1st and 20th days of post -culture period.

The highest TTC survival were recorded in *Arabidopsis* cells always after a preculrure on medium containing 0.63 M mannitol. Mannitol concentration higher than 0.63 damaged the survival observed. Among the 3 tested ecotypes, cells of C24 showed the maximum level of survival.

In *Arabidopsis* cells data of fresh weight changes showed a good correlation with the results of TTC test. All the tested ecotype reached their maximum fresh weight growth after pretreated on mannitol 0.63 M. Other mannitol concentration, than 0.63 M decreased the survival rate based on the fresh weight changes.

On the 20th day of postculture period, the cell suspensions were re-established from the recovering colonies. Newly initiated cell suspensions, based on morphological observation did not show any deviation from original suspension.

The highest survival was found in MSCE/79 maize cells based TTC test and fresh weight changes after a preculturing them on medium containing sucrose. Significant differences were however observed in the survival percentage, if TTC was carried out at the 1st, or at the 15th day after thawing. Viability was overestimated when accomplished at the 1st day of postculture period. These data reduced down to its half, when the TTC was performed at the 15th day after thawing. Data from the fresh weight measurement encouraged the accuracy of 15th day TTC %.

Cryopreserved maize cells showed the maximum level of survival after a preculture on medium containing sucrose in 0.63 M.

Cryoprotective effect of sugars has been explained with the osmotic activity of the molecules. In maple cells surviving the cryogenic temperature was evidenced , that a mannitol preculture decreased the size of cells and vacuoles also. The sucrose preculture in our experiments resulted in a significantly higher level of survival, than observed after mannitol, or sorbitol pretratments. The sucrose ability to metabolise another type of sugars in plat cells is well known, while there aren't similar records from mannitol and sorbitol. It could be presumed therefor, that sucrose carries another effect also, than it could be conducted from its osmolarity. It is reported for instance, that sucrose stabilises the cell membrane by bounding to the polar groups of the membrane phospholipids and decreases its permeability.

In the experiments freezing *Arabidopsis* cells, the survival rate determined by the TTC test was comparable to the data calculated from the fresh weight changes. In MSCE/79 cells however the value of TTC reduction showed significant deviations as a function of days after thawing. TTC originated formazane signals the activity of redox enzyme systems of the tissues. Longer or shorter duration of enzyme activity however could be detectable - depending on the species or the culture type - even in died tissue. Maize cell suspensions apparently are more sensitive for the correct timing of TTC test, than *Arabidopsis* suspensions. TTC performed on the 1st day of postculture was too early for this cultures. Therefore the survival rate of maize cells could be expressed more reliably timing the TTC test on the 15th day after thawing.

3.7. Effect of cryopreservation on the morphogenetic potential of maize cells

MSCE/79 maize cell suspension culture used for cryopreservation has lost its morphogenic character for 17 years. Following the cryopreservation however root meristem differentiation was observed on colonies transferred on hormone free solid N6M medium, after 4 weeks of culture in an average of 2-3 roots/ 350-400 mg callus. No similar differentiation was experienced in unfrozen clumps either were treated, or not treated on carbohydrate medium before freezing. The type, or concentration of sugars did not influenced the appearance of roots, but the liquid nitrogen stress had a crucial effect.

It is evidenced that different environmental stresses induce the program of embryogenesis in plant cells. Among these tresses slow precooling might activate the process of ontogenesis presumably with its associated dehydrating effect. Although recovered MSCE/79 cells failed to regenerate hole plants, but the ontogenesis was partly switched on with the reactivation of the cells root regeneration ability.

3.8. Effect of *afp* transformation on the survival rate transgenic maize cells

Experiments were scheduled to increase the cryotolerance of maize cells with *afp* gene transformation from polar fishes. Maize cell suspensions synthesising the protein type AFP I. were used for cryopreservation. Applied freezing and preculture regime to different transgenic clones (afp 23, afp 24, afp 37, afp 44) originating from different transformation events and to the wild type (∅) was identical described in previous paragraph. Survival was expressed based on the fresh weight changes.

While the sugar and sugar alcohol pretreatments resulted in viable cells in every clone tested, the survival rate of wild line did not improved significantly. The largest fresh weight increase was observed in the clone afp 44 after plerultured them on medium containing sucrose sorbitol and mannitol in 0.63 M. Cells of the clone afp 24 showed high level of survival, when precultured on 0.75 sucrose and sorbitol, but on 0.5 and 0.75 M mannotol also. Pretreatment on 0.75 M sucrose and 0.63 M sorbitol for afp 37, and 0.5 M and 0.75 M mannitol for afp 23 brought reasonable survival.

Cryoprotective effects of antifreeze proteins have been explained from their melting point depressing and ice re-crystallisation inhibitor characters. Both effects of AFP could result in an increased cryotolerance in cellular systems.

Results obtained with the use of AFPs as cryoprotectant show however significant deviations, which correspond to the size of the molecule. The most frequently used AFP I. might active only in the extracellular tissues, since its relatively large molecule weight doesn’t allow penetrating in the cells. In transformants however, synthesising the antifreeze proteins, above described problem will be excluded. An appearance of significant freeze tolerance was reported in afp transgenic tobacco after cold acclimation. Accumulation of antifreeze proteins

by cold treatment is explained with post-transcriptional control mechanisms. There are no data however concerning the cryotolerance of afp transgenic explants.

Transgenic clones in our experiments after sugar and sugar alcohol pretreatments showed an elevated level of survival compared to the wild type. Preculture on medium containing carbohydrates might create similar effect to the cold acclimation with the slight increase of osmotic pressure. We suppose, that AFP activated through post-transcriptional phosphorilation by the effect of sugars. Our conclusion is, that significantly higher survival of transgenic clones, than the wild line explains the cryoprotective effect AFP.

3.9 New scientific results

1. Reliable cryopreservation methods were developed for the tested species and tissue culture techniques, from which there are no reports on the freeze preservation of the cell suspensions of saltmarsh grass and reflexed saltmarsh grass, C24 and LER ecotypes of *Arabidopsis* and on the somatic embryos of horse-chestnut and kiwi.
2. The role of freezing program in the formation of protective dehydration, - based on slow precooling – in cell aggregates of saltmarsh grasses was proved.
3. Optimal ABA, ABA - proline, ABA – sucrose treatment combinations resulting the highest survival of somatic embryos of horse-chestnut and MSCE/79 maize cell suspensions were determined.
4. Desiccation sensitivity of horse-chestnut somatic embryos, cryopreserved by desiccation techniques was reduced by ABA treatments.
5. In the course of cryopreservation of somatic embryos of horse-chestnut and MSCE/79 maize cell suspensions the slow precooling need was substituted by heat shock treatments, with which an indirect cryoprotective effect of heat shock was evidenced.
6. Somatic embryos of kiwi were cryopreserved successfully by vitrification technique and the toxicity of PVS-2 was depressed with ABA-alginate gel treatment combination.
7. Optimal concentration of sucrose, sorbiol and mannitol was determined for the cryopreservation of cell suspensions of C24, LER and WS *Arabidopsis* ecotypes and MSCE/79, and afp transgenic maize lines.
8. In MSCE/79 cells, which has lost their morphogenetic character, the ontogenesis was partly switched on with the reactivation of the cells root regeneration ability, by cryopreservation.
9. By the cryopreservation of transgenic maize cells synthesising the antifreeze protein, the cryoprotective effect of afp transformation was proved first.

4. RELATED PUBLICATIONS

4.1. Book chapters:

1. **Zs. Jekkel,** G. Gyulai & L. E. Heszky. (**1995**) Cryopreservation of some halophyte grasses (*Puccinellia* species), in: Biotechnology in Agriculture and Forestry 32, Cryopreservation of plant germplasm I. (ed: Y.P.S. Bajaj), pp. 245-255 **Springer**, Berlin, Heilderberg, New York

2. **Zs. Jekkel**, J. Kiss, G. Gyulai, E. Kiss, L.E. Heszky. (2000) Cryopreservation of Horse Chestnut (*Aesculum*). in: Biotechnology in Agriculture and Forestry, Cryopreservation of plant germplasm II. (ed: Y.P.S. Bajaj), **Springer**, Berlin, Heilderberg, New York

4.2. Patent:

3. Gyulai G. L.E. Heszky, E. Kiss, **Zs. Jekkel**, K.T. Lőkös (1988-1991) Eljárás biológiai anyagok auxin , illetve citokinin aktivitásának szelektív meghatározására. **Magyar Szabadalom**, lajstromszám:204360

4.3. Papers:

4. **Zs. Jekkel**, L.E: Heszky & A.H. Ali (1989) Effect of different cryoprotectants and transfer temperatures on the survival rate of hemp (*Cannabis sativa* L.) cell suspension in deep freezing**. Acta Biologica Hungarica** 40 (1-2): 127-136.

5. L.E. Heszky, **Zs. Jekkel** & A.H. Ali (1990) Effect of cooling rate, cryoprotectant and holding time at different transfer temperatures on the survival of cryopreservad cell suspension culture (*Puccinellia distans* (L.) Parl.). **Plant Cell Tissue and Organ Culture** 21: 217-226.

6. **Zs. Jekkel** & L.E. Heszky (**1994**) Towards a plant cryobank in Hungary. **Hungarian Agricultural Research** 3: 12-17

7. **Zs. Jekkel**, B. Raugh &L. E. Heszky(**1995**) Heat-shock induced survival from cryopreserved horse-chestnut somatic embryos. **Plantnet** 1:15.

8. Gyulai G., J. Kiss, **Z. Jekkel**, E. Kiss & L.E. Heszky (**1995**) A selective auxin and cytokinin bioassay based on root and shoot formation *in vitro*. **Journal of Plant Physiology**. 145: 379-382

9. R.C.S. Ribeiro, **Z. Jekkel**, B.J. Mulligan, E.C. Cocking, J.B. Power, M.R. Davey, P.T. Lynch (**1996**) Regeneration of fertile plants from cryopreserved cell suspension of Arabidopsis thaliana (L.) Heynh. **Plant Science**, 115:115--121.

10. **Zs. Jekkel**, G. Gyulai, E. Kiss, J. Kiss, L.E. Heszky (**1998**) Cryopreservation of horse-chestnut (Aesculus hippocastanum L.) somatic embryos using three different freezing methods . **Plant Cell Tissue and Organ Culture** 52:193-197.

11. **Jekkel Zs**., G. Gyulai, S. Mórocz, E. Kiss, D. Dudits &L.E. Heszky (1998) Restoration of root regeneration in a non-morphogenic maize cell line after sugar or sugar alcohol preconditioned cryopreservation. **Plant Science** (közlés alatt)

4.4. Congress presentations :

12. **Zs. Jekkel**, L.E. Heszky, B. Barnabás, D.Q. Binh (1988) Freeze-preservation of Reflexed saltmarsh grass (*Puccinellia distans* (L.) Parl.) cells grown in suspension culture. **XVIII th Congress of the Hungarian Biological Society**. Keszthely, Abstract Book :52.

13. Heszky L., Gyulai G., **Jekkel Zs**., Kiss E., Lőkös K. (1989) Vegyületek hormonaktivitásának megállapítása hormon szelektív auxin-citokinin bioteszttel. **GATE -Chemolimpex Konferencia**, Gödöllő, Előadások : 126--136.

14. **Zs Jekkel** & L.E. Heszky (1991) Cryopreservation of Puccinellia distans susprension cultures :a model system for investigation the critical points of freezing procedure. **EC-Hungary Joint Workshop on Plant Biotechnology.** Gödöllő, Report EUR 13415 EN : 69-76.

15. **Zs. Jekkel** &L.E. Heszky(**1996**) Cryopreservation of germplasm of higher plants. **The 2nd Brazilian-Hungarian Seminar and Training Course in Plant Biotechnology**, Budapest, Course Book :43-50.

16. **Jekkel Zs**. és Heszky L. (1994) Deszikkált szomatikus embriók krioprezerválása. **Növénynemesítési Tudományos Napok '93,** Budapest, Szimpózium I. Előadásai :10.

4.5 Congress posters:

17. **Zs. Jekkel,** L.E. Heszky & A.H. Ali (1990) Transfer temperature dependent cryoprotectant and holding time effects in the survival response of cryopreserved cells (*Puccinellia distans* (L.) Parl.). **VIIth International 15. Congress on plant tissue and Cell Culture,** Amsterdam, Abstracts:376

18. G. Gyulai, L.E. Heszky, K.T. Lőkös, E. Kiss, **Zs. Jekkel** (1990) A novel hormone-selective auxin and cytokinin bioassay. **VIIth International Congress on plant tissue and Cell Culture,** Amsterdam, Abstracts :279

19. **Zs. Jekkel**, J. Kiss., E. Kiss, G. Gyulai, L.E. Heszky (1992) Freeze-preservation of somatic embryos of a recalcitrant seed species:horse-chestnut (*Aesculus hippocastanum* L.). **XIIIth EUCARPIA CONGRESS**, Angers, Abstracts: 428-429

20. **Zs. Jekkel**, J. Kiss, E. Kiss L.E. Heszky (1992) Cryopreservation of horse--chestnut somatic embryos: a comparision of different desiccation methods. **1st Egyptian-Italian Congress of Biotechnology**, Assiut , Abstracts, :73

21. Zs. Jekkel, B, Rauh and L.E. Heszky (1994) Heat-shoch induced survival from cryopreserved horse-chestnut somatic embryos . **VIIIth International Congress on plant tissue and Cell Culture,** Firenze, Abstracts, :269

22. **Zs. Jekkel**, B. Raugh & L.E. Heszky (1994) A hősokk kezelés hatása krioprezervált vadgesztenye szomatikus embriók túlélésére. **A Magyar Genetikusok Egyesülete III. Konferenciája,** Debrecen, Előadás és Poszter Összefoglaló :135

23. Sharma H.C., Benlhabib O., **Jekkel Z.,** Ohm H.W. (1996) Anther culture and concomitant ovule culture in wheat X wheatgrass hybrids and wheat. **88th Annual Meeting of American Society of Agronomy**. Indianapolis, Abstracts: 160

24. **Jekkel Zs**, Gyulai G, Mórocz S, Kiss E, Dudits D, Heszky L. (1998) A morfogenezis reaktivációja krioprezervált kukorica sejtszuszpenziókban. IV. **Növénynemesítési Tudományos Napok**, Budapest , Előadás és Poszter Összefoglaló:43