NARDI FUNDULEA, ROMANIA ROMANIAN AGRICULTURAL RESEARCH, NO.27, 2010
www.incda-fundulea.ro Print ISSN 1222-4227; Online ISSN 2067-5720

LIQUID NITROGEN STORAGE OF BARLEY AND TRITICALE CARYOPSES DOES NOT INFLUENCE THE ISOPEROXIDASE PATTERNS DURING THEIR GERMINATION

Monica Şipoş[1], Anca Keul[2], Alina Dora Samuel[1], Cristian Felix Blidar[1]

[1] Biology Department, Science Faculty, Oradea University, Universitatii st.1, Oradea, Bihor County, Romania.
E-mail: siposmonica@yahoo.com

[2] Experimental Biology Department, Biology-Geology Faculty, „Babeş-Bolyai" University, Cluj-Napoca, Cluj County, Romania

ABSTRACT

The germination ability of barley and triticale grains was not affected by preservation in liquid nitrogen (LN) (-196°C) for 5 minutes, 1 hour, 1 day, 1 week or 1 month. Isoperoxidases pattern of germinated caryopses during 44 hours of germination after cryostorage was not influenced by cryostorage, cryostored grain isoenzymes pattern being approximately similar to that of control samples.

Key words*:* barley, triticale, grains, cryopreservation, germination, isoperoxidases.

INTRODUCTION

Research concerning cryopreservation of plant materials has developed because this method assures almost infinity of the germplasm preservation (Engelmann, 2004). In the cryopreservation of plant materials there is no standard protocol. Thus, experiments regarding the optimum protocol of cryopreservation for each type of plantmaterial are required. Anyhow, in cryopreservation technology there are certain common steps: the material preparation, cryoprotection (if it is necessary), the way of cooling, the thawing and the checking of the survival degree, morphological integrity (Hornung et al., 2001), biochemical (Touchell and Walters, 2000), cytological (Mikula et al., 2005) and genetic integrity (Dixit et al., 2003; Gagliardi et al., 2003; Zhai et al., 2003; Harding, 2004).

The orthodox seeds could be kept during tens of years in the gene banks based on classic methods, at -18ºC. Seed banks may meet problems to keep the refrigerating installations and the sample regeneration in experimental field – when the seed sample viability diminishes – it is risky and expensive. National Seed Storage Laboratory, Fort Collins, Colorado, USA, utilizes in parallel the classic methods of plant germplasm preservation and cryopreservation.

Our experiments were concerned with the influence of Liquid Nitrogen (LN) for 5 minutes, 1 hour, 1 day, 1 week or 1 month, on the barley and triticale grain germination ability. The germination ability and the isoperoxidases pattern from germinated grains were determined after cryopreservation, in comparison with the control. The peroxidases were chosen because they are extremely inducible by external stress factors (Van Assche et al., 1986; Wongkaew et al., 1991; Casstillo, 1992; Moerschbacher, 1992; Reuveni et al., 1992) and at the same time are considered biochemical markers for different physiological processes (Gaspar et al., 1991; Greppin and Penel, 1991; Catteson, 1992).

MATERIAL AND METHODS

The germination ability and the humidity of barley (*Hordeum vulgare* L.) and triticale (x *Triticosecale* Wittmack) grains used in our experiments were determined respecting methodological norms recommended by the International Seed Testing Association (ISTA). The seed material selected by us to be used in tests, had a germination ability of 85% in barley and 93% in triticale. The water content of the caryopses was established through gravimetric methods and was 6 and 8% respectively. Barley and triticale grains were

Received January 19, 2010; accepted March 20, 2010

packed in small cotton sacks (containing 50 grains/sack) for LN very easy penetration. The sacks were placed inside LN container and then were submersed directly in -196°C (quick cooling). The experiments were carried out for time variants: 5 minutes, 1 hour, 1 day, 1 week or 1 month of cry storage. After this operation the sacks were extracted from the container compartments and passed to defrosting through their maintenance to room temperature (slow defrosting). Seed samples were thawed at room temperature and later germinated at 23ºC. The germination ability was assessed after 24 hours. The germination percent was established from 300 grains for control, and for each experimental variant respectively.

For electrophoretic analyses caryopses were harvested after 6, 20, 30 and 44 hours from their having been set to germinate. Each time 3 samples were used from non-submersed in LN grains (control lot) and cryopreserved in LN (for 5 minutes, 1 hour, 1 day, 1 week or 1 month) once. Samples preparation was done according to Brinegar and Goudan (1993). The germinated grains were ground with glass particles, on ice and suspended w/v in extraction buffer (Tris-HCl 0.1 M, pH 7.5; ethylendiamintetracetic acid - EDTA 1 mM; $MgCl_2$ 10 mM; KCl_2 10 mM; 10-50 mg/ml solid polyvinyl-pyrolidone-PVP-10). The ground samples were centrifuged to 10,000 rpm, for 10 minutes, at 4ºC. From each sample the supernatant was kept and this extract was used for izoenzymes separation by electrophoresis.

The izoenzymes spectrum was displayed after electrophoresis in polyacrylamide gel (PAGE), with a Consort apparatus. The electrophoresis was realized in discontinuous system, without dodecil sodium sulfate (SDS) (native PAGE), by isoelectric focusing (IEF). The gel concentration was 5%.

The used protocols were according to Murphy et al. (1990) and Acquaah (1992). For the isoperoxidases separation, the following buffers were used: 20 mM NaOH solution and 10 mM H_3PO_4 solution. In the IEF system there is no concentration gel, and the running gel contains: acrylamide/bisacrylamide, ampholine A pH = 3.5-5.0 and ampholine B pH = 3.5-10.0 (in ratio 1:1), distilled water, ammonium persulphate and tetramethylethy-lendiamine (Temed). The gels were run out at 120V for 1.5 hours.

The protocol for the histochemical detection of peroxidases in the gels was the following: sodium acetate 0.05 M, pH 5.0 (50 ml); 10 mg $CaCl_2$; H_2O_2 3%; 250 μl 3-amino-9-ethyl-carbazol (50 mg); dimethylphormamide (5 ml), were dissolved in turn. $CaCl_2$ in the acetate buffer and was added to the oxygenated water. After the separate dissolution of 3-amino-9-ethyl-carbazol in dimethylphormamide, the complete mixing was achieved. Isoperoxidases appear after 30-60 minutes as red-brown color bands. In this period 250 μl H_2O_2 3%, for the color intensification, can be added. If the bands are visible after 30 minute, the gel is fixed with solution of 50% glycerin; the bands color intensification can slowly continue in this solution, too.

RESULTS AND DISCUSSION

Preservation in LN on variable periods of time - from 5 minutes to 1 month - of barley grains with a 7.24% humidity, did not affect the germination ability, keeping their value to 85%.

The pH gradient achieved in gel by using ampholines was between 9 (from the start of electrophoretic migration) and 3 (at bottom of gels) (Figures 1-2). The identified isoperoxidases are in majority basic (B). The basic or cationic isoperoxidases are considered those with an isoelectric point (IP) across 7 and acid or anionic, those with IP below 7 (Mäder, 1992). In accordance to Mäder (1992) classification, we consider isoforms that migrated in alkaline and neutral parts of gels as basic (B) and acid (A), those that migrated in the acide zone (pH below 7).

After 6 hours of germination the first two isoperoxidases, B_1 and B_2, were equally marked out in control barley grains and in the caryopses that had been submersed in LN (Figure 1).

The following isoperoxidases, B_3, B_4, B_5 and B_6, are also basic, but their expressions are not constant, they were visible only to some of the samples, irrespective of the duration of immersion in LN.

The pattern of isoperoxidases from germinated barley grains at 20 and 30 hours were similar (Figure 1). The presence of the first two basic isoeperoxidases, B_1 and B_2 from the start of electrophoretic migration was observed, as being the same after 6 hours from setting to germination. The following basic isoperoxidases, B_3-B_6, were expressed very slightly.

After 44 hours of germination (Figure 1) the control and the cryopreserved caryopses presented the expression at the basic isoperoxidases. Isoforms B_1, B_2, B_3 and B_5 were the best marked for all analyzed samples. At the bottom of gels acid isoperoxidase A_7 was extremely slightly expressed and A_8 isoform was better expressed.

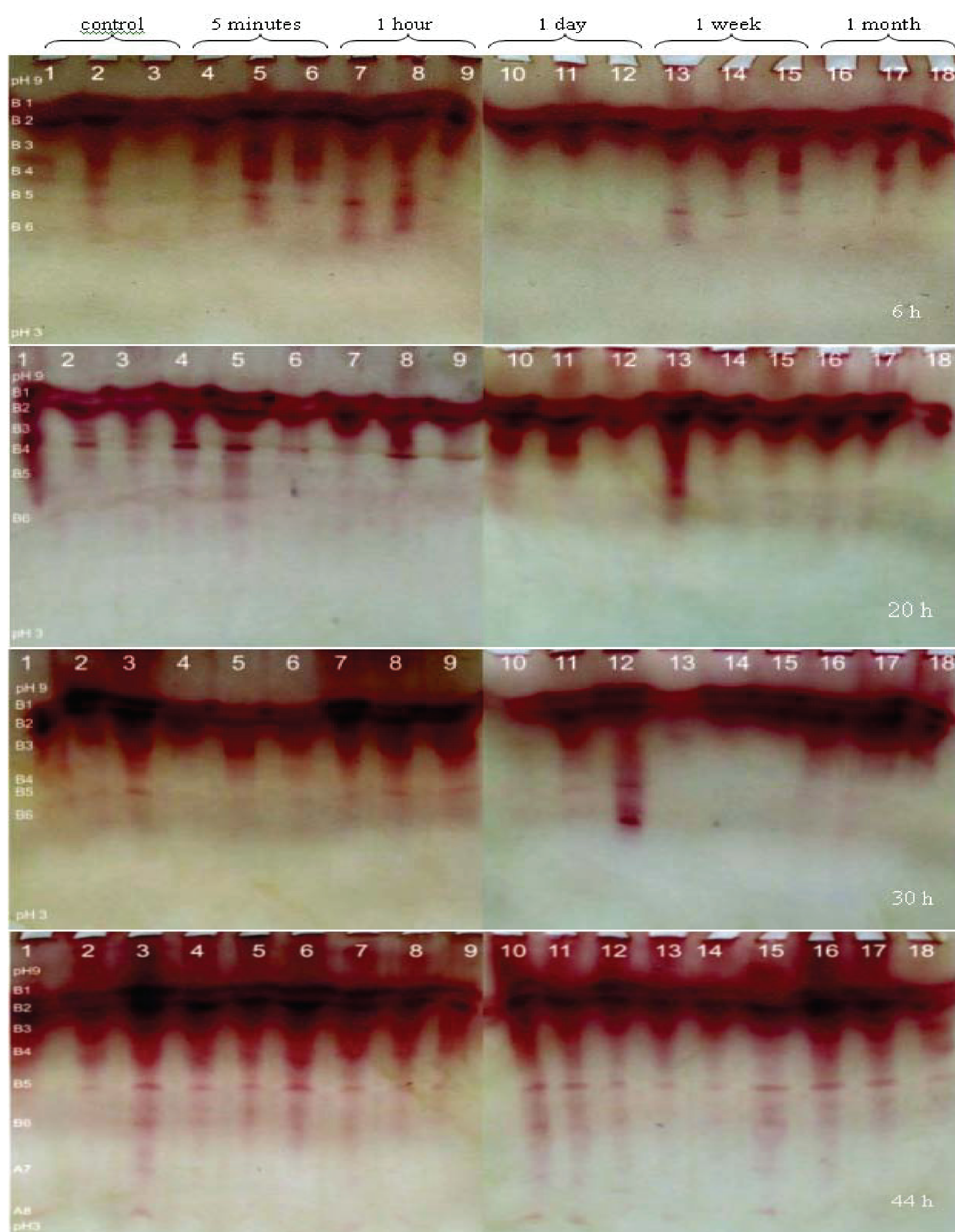

Figure 1. The isoperoxidases zymogrames from germinated barley grains, after 6, 20, 30, 44 hours from their having been set to sprout (1-3 – control; 4-6 – for 5 minutes in LN, 7-9 – after 1 hour in LN; 10-12 – after 1 day in LN; 13-15 – after 1 week in LN; 16-18 – after 1 month in LN); B_1, B_2, B_3 B_4, B_5 and B_6 – basic isoperoxidases; A_7 and A_8 – acid isoperoxidases

The cryopreservation for different periods of time – 5 minutes, 1 hour, 1 day, 1 week or 1 month – of triticale grains (x *Triticosecale* Wittm.) with 6.89% humidity, did not affect the germination ability, maintaining the value of 93%. After 6 hours of triticale grains germination, isoperoxidases pattern expressed (Figure 2) was simpler than that of the barley caryopses (Figure 1). Only two basic isoperoxidases, B_1 and B_2, present in immediate proximity of the start of electrophoretic migration in all analyzed samples were identified. Further, after 20 hours of triticale grains germination, two slight alkaline isoperoxidases (B_4 and B_5) having close isoelectrical points, appeared (Figure 2).

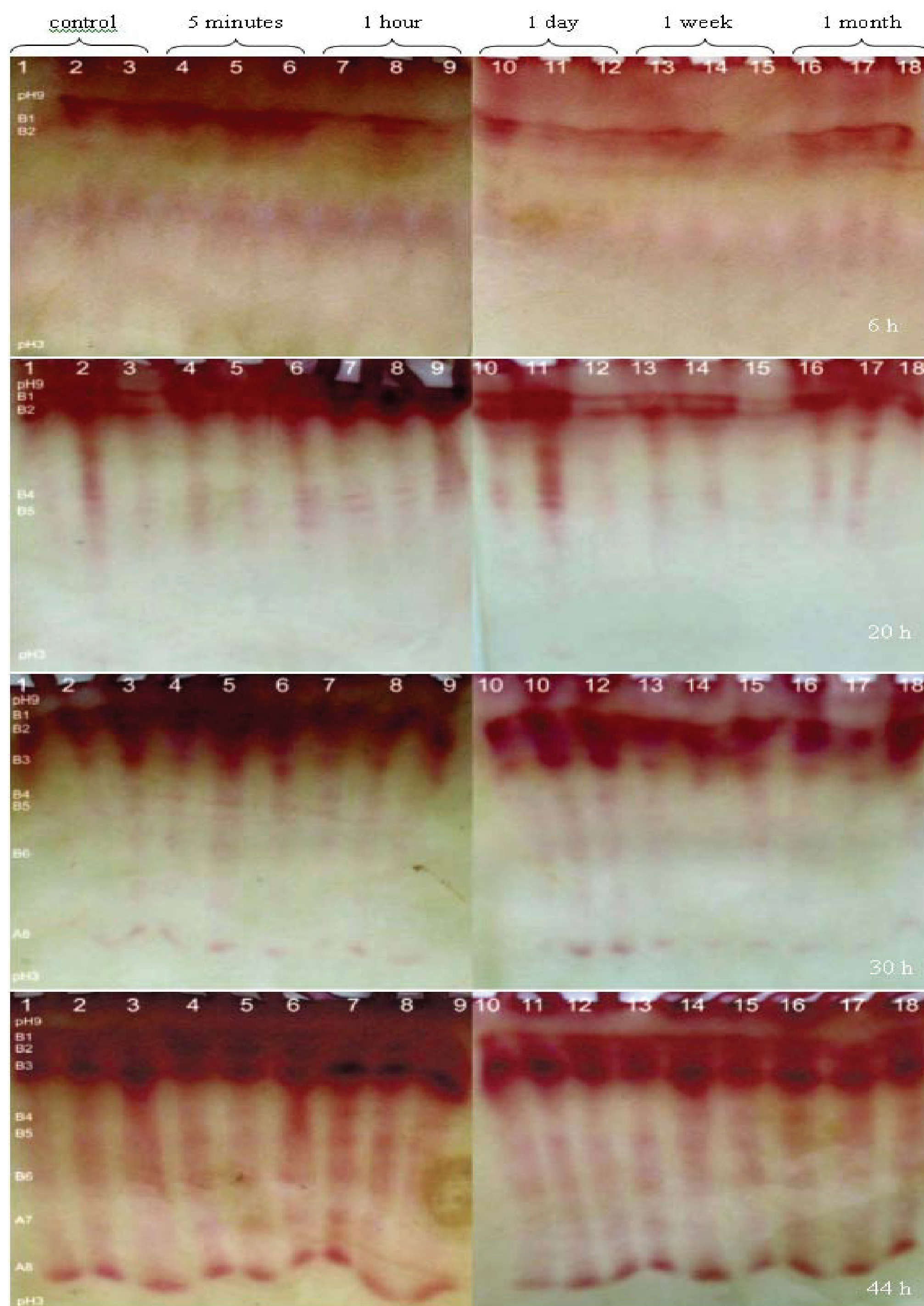

Figure 2. The isoperoxidases zymogrames from germinated triticale grains, after 6, 20, 30, 44 hours from their having been set to sprout (1-3 – control; 4-6 – for 5 minutes in LN; 7-9 – after 1 hour in LN; 10-12 – after 1 day in LN; 13-15 – after 1 week in LN; 16-18 – after 1 month in LN); B_1, B_2, B_3 B_4, B_5 and B_6 – basic isoperoxidases; A_7 and A_8 – acid isoperoxidases

They could be observed very slightly in all analyzed samples, irrespective of being the control or cryopreserved caryopses.

After 30 hours of triticale caryopses germination the next basic isoperoxidase (B_3) was identified (Figure 2). Basic isoperoxidases B_4, B_5 and sixth isoforme B_6, were very slightly expressed. In the acid region, at the bottom part of the gels, the expression of isoperoxidase A_8, was observed.

After 44 hours of germination in all triticale samples, 3 alkaline isoperoxidases: B_1, B_2 and B_3 in the top of the gel and B_4, B_5 and B_6 in neutral region, were identified. Besides, A_8 acid isoperoxidases and a A_7 isoform, were observed (Figure 2).

It can be concluded that preservation in LN did not influence the isoperoxidase patterns in the germinated grains of barley and triticale.

The germination ability of barley and triticale grains was not affected by cryopreservation irrespective of the duration of LN submersing at -196°C. Our results are congruent with those obtained by Lambardi et al. (2004) with recalcitrant seeds of *Citrus sinensis* with the humidity of 16% and those of *C. aurantium* with humidity at 10%. Gonzáles-Benito and Pérez-Garcia (2001) also observed the germination ability after cryopreservation in the case of oleaginous seeds of two species from *Brassicaceae* family and three species from *Asteraceae* family. In all cases the germination ability of cryopreserved seeds was the same as the control lots.

Basic isoperoxidases are involved in scavenging of some noxious radicals produced in metabolic processes started in the first stage of grains germination (Gaspar, 1986; Asada et al., 1993). They have a specific role in growth and in the cellular differentiation (Gaspar, 1986; Gaspar et al., 1991), as well as in defensive mechanism as reaction to stress factors (Gaspar, 1986).

In both studied species we did not observe differences between basic isoperoxidases pattern from the control and from cryopreserved caryopses. Consequently, the exposure to LN temperature (-196ºC) is not a stress factor for caryopses and does not hinder normal growth of the embryos during germination, after grain cryostorage.

With the advance of the germination the enzymatic activities were accelerated. The increase of peroxidasic activity is due to the appearances of new isoenzymes and activation of isoperoxidases preexistent forms (Dencheva and Klisourska, 1976; Cochrane, 1994; Fraignier et al., 2000). With the progressing of the germination of barley and triticale caryopses new isoenzymes appeared at the same in the control and in the grains submersed for variable periods of time in LN. We consider that in the case of our experiments, after 44 hours of grains germination, when at the root level of the small plantlets the absorbent zones are distinguished, acid isoperoxidases were involved in the lignification of the primary wooden tissues. Acid isoperoxidases A_7 and A_8 of barley and triticale, can be considered biochemical markers for the start of lignification process, both in the control plantlets and those which were derived from cryopreserved grains. The fact that the acid isoforms are involved in lignification process of cellular walls is generally accepted (Catteson, 1992).

The fact that in our experiments notable differences between isoperoxidases pattern from the control and cryopreserved caryopses did not appear is very important. It means that during the germination period, after being submersed in LN, the grains quickly restored their physiological natural parameters. As far as we know, this is the first paper treating the subject of the cryopreserved grains germination by means of isoperoxidases markers.

CONCLUSIONS

The germination ability of caryopses with a humidity of 6-8% was maintained around the pre-storage value of 85% in barley and 93% in triticale after cryostorage in LN. The extension of LN storage duration from 5 minutes to 1 month did not influence germination. Irrespective of grain preservation in LN the

isoperoxidase patterns were identical after 6, 20, 30 and 44 hours of germination. Hence, this mode of preservation can be used with success for this plant material storage in a germplasm bank.

REFERENCES

Acquaah, G., 1992. *Practical protein electrophoresis for genetic research*. Dioscorides Press, Portland, Oregon, USA.

Asada, K., Miyake, C., Sano, S., and Amako, K., 1993. *Scavenging of hydrogen peroxide in photosynthetic organisms - from catalase to ascorbate peroxidases*. In: Welinger, K.G., Rasmussen, S.K., Pene, C., Greppin, H. [eds.], Plant Peroxidases: Biochemistry and Physiology: 243-250. Geneva University Press, Switzerland.

Brinegar, C., and Goudan, S., 1993. *Isolation and characterization of chenopodin, the 11 S seed storage protein of quinoa (Chenopodium quinoa)*. Journal of Agricultural and Food Chemistry, 41: 182-185.

Cattesson, A.M., 1992. *Plant peroxidases and cell differentiation: cyto- and histological aspects.* In: Penel, C., Gaspar, T., Greppin, H. [eds.], Plant Peroxidases 1980-1990, Topics and Detalied Literature on Molecular Biochemical and Physiological Aspects: 117-124. Geneva University Press, Switzerland.

Casstillo, F.J., 1992. *Peroxidases and stress*. In: Penel, C., Gaspar, T., Greppin, H. [eds.], Plant Peroxidases 1980-1990, Topics and Detailed Literature on Molecular Biochemical and Physiological Aspects: 187-203. Geneva University Press, Switzerland.

Cochrane, M.P., 1994. *Observation on the germ aleurone of barley. Phenol oxidase and Peroxidase activity*. Annals of Botany, 73: 121-128.

Dencheva, A.V., and Klisourska, D.Y., 1976. *Activity and isoenzyme composition of the peroxidase in the zones of growth differentiation of the cells in shoots of maize*. Comptes Rendus de l'Academie Bulgare des Sciences, 29: 1179-1182.

Dixit, S., Mandal, B.B., Ahuja, S., and Srivastava, P.S., 2003. *Genetic stability assessment of plants regenerated from cryopreserved embryogenic tissues of Dioscorea bulbifera L. using RAPD, biochemical and morphological analysis*. CryoLetters, 24: 77-84.

Engelmann, F., 2004. *Plant cryopreservation: progress and prospects.* In Vitro Cellular and Developmental Biology-Plant, 40 (5): 427-433.

Fraignier, M.P., Michaux-Ferrière, N., Kobrehel, K., 2000. *Distribution of peroxidases in durum wheat (Triticum durum).* Cereal Chemistry, 77: 11-17.

Gagliardi, R.F., Pacheco, G.P., Carneiro, L.A., Valls, J.F.M., Vieira, M.L.C., and Mansur, E., 2003. *Cryopreservation of Arachis species by vitrification of in vitro-grown shoot apices and genetic stability of recovered plants*. CryoLetters, 24: 103-110.

Gaspar, T., 1986. *Integrated relationships of biochemical and physiological peroxidase activities*. In: Greppin, H., Penel, C., Gaspar, T. [eds.], Molecular and Physiological Aspects of Plant Peroxidases: 455-468. Geneva University Press, Switzerland.

Gaspar, T.H., Penel, C., Hagège, D., and Greppin, H.. 1991. *Peroxidases in plant growth, differentiation, and development processes*. In: Lobarzewski, J., Greppin, H., Penel, C., Gaspar, T. [eds.], Biochemical, Molecular, and Physiological Aspects of Plant Peroxidases: 249-280. M. Curie-Sklodowska University, Lublin, Poland and Geneva University Press, Switzerland.

Gonzáles-Benito, M.E., and Pérez-Garcia, F., 2001. *Cryopreservation of lipid-rich seeds: effect of moisture content and cooling rate on germination*. CryoLetters, 22: 135-140.

Greppin, H.R. and Penel, C., 1991. *Peroxidase photocontrol and flowering process*. In: Lobarzewski, J., Greppin, H., Penel C., Gaspar, T. [eds.], Biochemical, Molecular, and Physiological Aspects of Plant peroxidases: 363-368. M. Curie-Sklodowska University, Lublin, Poland and Geneva University Press, Switzerland.

Hornung, R., Holland, A., Taylor, H.F., and Lynch, P.T., 2001. *Cryopreservation of Auricula shoot tips using the encapsulation/dehydration technique*. CryoLetters, 22: 27-34.

Harding, K., 2004. *Genetic integrity of cryopreserved plant cells: a review*. CryoLetters, 25: 3-22.

Lambardi, M., De Carlo, A., Biricolti, S., Puglia, A.M., Lombardo, G., Siragusa, M., and De Pasquale, F., 2004. *Zygotic and nucellar embryo survival following dehydration/cryopreservation of Citrus intact seeds*. CryoLetters, 25: 81-90.

Mäder, M., 1992. *Compartmentation of peroxidase izoenzymes in plant cells*. In: Penel, C., Gaspar, T., Greppin, H. [eds.], Plant Peroxidases, 1980-1990, Topics and Detailed Literature on Molecular, Biochemical and Physiological Aspects: 37-46. Geneva University Press, Switzerland.

Mikula, A., Tykarska, T. and Kuraś, M., 2005. *Ultrastructure of Gentiana thibetica proembryo-genic cells before and after cooling treatments*. CryoLetters, 26: 367-378.

Moerschbacher, B.M., 1992. *Plant peroxidases: involvement in response to pathogens*. In: Penel, C., Gaspar, T., Greppin, H. [eds.], Plant Peroxidases 1980-1990, Topics and Detailed Literature on Molecular, Biochemical, and Physiological Aspects: 91-99. Geneva University Press, Switzerland.

Murphy, R.W., Sites, J.W., Buth, D.G., and Haufler, C.H., 1990. *Proteins I: Izozymes Electrophoresis*. In: Hillis, D.M., Moritz, C. [eds.], Molecular Systematics: 45-126. Sinauer Assoc. Inc., Sunderland.

Reuveni, R., Shimoni, M., Karachi, Z., Kuc, J., 1992. *Peroxidase activity as a biochemical marker for*

resistance of muskmelon (Cucumis melo) to Pseudoperonospora cubensis. Phytopathology, 82: 749-753.

Touchell, D., and Walters, C., 2000. *Recovery of embryos of Zizania palustris following exposure to liquid nitrogen*. CryoLetters, 21: 261-270.

Van Assche, F., Put, C., and Clijsters, H., 1986. *Heavy metals induce specific isozyme patterns of peroxidase in Phaseolus vulgaris L*. Archives of Physiology and Biochemistry, 94: 60.

Wongkaew, W., Meksongsee, L., and Harinasut, P., 1991. *Peroxidases of road trees that survive in polluted atmosphere*. In: Lobarzewski, J., Greppin, H., Penel, C., Gaspar, T [eds.], Biochemical, Molecular, and Physiological Aspects of Plant Peroxidases: 459-462. M. Curie-Sklodowska University, Lublin, Poland and Geneva University Press, Switzerland.

Zhai, Z., Wu, Y., Engelmann, F., Chen, R., and Zhao, Y., 2003. *Genetic stability assessments of plantlets regenerated from cryopreserved in vitro cultured grape and kiwi shoot-tips using RAPD*. CryoLetters, 24: 315-322.