International Cryobank of the genus *Allium*

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Summary: The maintenance of plant genetic resources in living plant collections causes too many risks, the collections are exposed to natural disasters, attacks by pests and pathogens, and moreover, the costs of technical personnel and consumables are very high. Cryopreservation has become an alternative, safe and the most effective method for long-term conservation of germplasm of vegetatively propagated species. Within the frame of the EURALLIVEG project financed by the European Commission a tripartite garlic cryobank was organized, initially formed by three partners: the Czech Republic, Germany and Poland. For the creation of this cryobank, cryopreservation by vitrification was applied. Garlic shoot tips isolated from bulbils or *in vitro* plantlets were used. The explants were dehydrated by osmotically effective cryoprotectant solution PVS3. At present, 220 garlic accessions are maintained in the cryobank, including 202 accessions originated from the European Core Collection.

INTRODUCTION

In the Research Institute of Horticulture in Skierniewice, Poland, genetic resources of the *Allium* genus have been maintained for many years and represent garlic, shallot, onion and wild species. Polish garlic collection (*Allium sativum* L.) has been maintained since 1986. At the time of this study, the Research Institute of Horticulture maintains 517 garlic accessions in a field collection. In this collection, two forms of garlic: bolting (281 accessions) and non-bolting (236 accessions) are distinguished. The plant material was gathered on collection missions in various parts of the world. This vegetatively propagated collection is planted in the field each autumn and spring. The collection must be replanted each year, because most of the left-over bulbs do not survive storage conditions until the next planting season. Genetic resources of garlic maintained in an open field are particularly vulnerable to loss during winter and are susceptible to fungal, bacterial, and viral infections (Volk et al., 2004).

In genebanks, four main strategies are used to maintain garlic germplasm. The first method is field growing, the second is seed storage, the third option is *in vitro* storage, and the fourth is cryopreservation. Cryopreservation is a tool which could really help to reduce the risk of infections and abiotic factors (flood, drought, cold winters), and also reduce the cost of staff, equipment and consumables (Keller et al., 2013). Cryoprocedures (vitrification procedure – Niwata 1995; Makowska et al. 1999; Keller 2002; Keller 2005; droplet vitrification – Kim et al. 2004 a, b; Kim et al. 2005; Baek et al. 2003; Volk et al. 2004; Ellis et al. 2006) have been developed for garlic germplasm (Reed, 2008). Shoot tips from cloves, bulbils and *in vitro* plantlets or young inflorescence bases of bolting garlic could be successfully preserved in liquid nitrogen.

Garlic plants do not produce fertile seeds, the maintenance of a field collection is laborious and expensive, *in vitro* storage of garlic germplasm does not seem to be very promising in the long term (accumulation of

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endophytes), therefore cryopreservation has proved to be the ultimate option for long-term storage (Keller et al., 2013).

MATERIAL AND METHODS

Plant material

The plant material used in this study originated from the Polish genebank's collection of the Research Institute of Horticulture in Skierniewice and from the EURALLIVEG project partners (Czech Republic, France, Germany, Italy). The Polish collection contains 517 accessions, which have been collected since 1986. Garlic plant material has been gathered during expedition missions mainly in Poland but also in the bordering countries, in Central Asia and in other parts of the world. For methodical reasons, it is not possible to consider specifically the special requirements of all these diverse materials. Therefore, an experimental set of accessions out of the garlic core collection was used (Keller et al., 2005).

For this investigation, garlic cloves of bolting and non-bolting accessions were planted in 2007-2011. Bolting garlic accessions were usually planted in the middle of October, and non-bolting accessions in April. Bulbs were harvested from the end of June till mid-July of each year, and left to dry in a ventilated store room. In the case of bolting accessions, bulbs were harvested together with the flower stalks and bulbils. For the cryopreservation of bolting garlic accessions, shoot tips from bulbils were used, while in the case of non-bolting accessions (do not produce flower stalks) shoot tips from *in vitro* plantlets were used. Garlic bulbils were appropriate plant material from October to April. After that time, most of the apices had sprouted and had a low survival rate after cryopreservation (Kim et al., 2005). *In vitro* cultures of non-bolting garlic accessions were precultured for 8 weeks under the following regimen: day-length 16 hours, illumination 60-80 μmol.m^{-2}.s^{-1}, alternating temperature of 25°C during the day and -1°C at night.

Within the framework of the EURALLIVEG project, the Polish genebank received from the Italian partner virus-free *in vitro* cultures of the most important French, Italian and Polish accessions. The Polish partner's task was to multiply this plant material to obtain 150 plantlets per accessions and to preserve this material in liquid nitrogen.

Sterilization and shoot tip isolation

Garlic inflorescences of bolting accessions and bulbs of non-bolting accessions were divided into individual bulbils or cloves, the skins were removed and the bulbils or cloves (150 bulbils or cloves per accession) were sterilized in 70% ethanol by short dipping and shaking for 30 seconds. The main disinfection was carried out by using 3% sodium hypochlorite solution with 2 drops of Tween 20 and gently agitating for an additional 20 minutes. Bulbils/cloves were rinsed 3-4 times with sterile distilled water. The meristematic base of garlic cloves was used to initiate *in vitro* cultures. Shoot tips extracted from bulbils or *in vitro* plantlets, which were approx. 1 mm in diameter and 2-3 mm in length and consisted of the basal plate and 1-2 leaf primordia, were preconditioned at 25°C and 16 h illumination until the next day on Petri dishes containing solidified MS medium (Murashige, et al. 1962) with 10% (w/v) sucrose, 0.1 mg/L α-naphthalenacetic acid (NAA), 0.5 mg/L 2-(γ,γ-dimethylallylamino) purine (2i-P) and 1% (w/v) agar.

Cryopreservation method

Cryopreservation of garlic shoot tips was performed by the vitrification method. The vitrification procedure consists of one-day preculture in a medium with 10% sucrose after explants preparation (150 explants per accession), followed by loading in a standard liquid medium with 0.4 M sucrose and 2 M glycerol for 20 minutes and dehydration in PVS3 (50% w/v sucrose + 50% w/v glycerol in the standard liquid MS medium) for 120 minutes. After that, the explants were placed in cryo-tubes containing 0.5 ml of the PVS3 solution in the standard liquid medium. These tubes (10 explants per tube) were plunged directly into liquid nitrogen. Rewarming was also fast by plunging the tubes into a water bath at 40°C for two minutes. Then, the PVS solution was removed and the standard liquid medium with 1.2 M sucrose was added. After ten minutes,

fifty explants (control) were deposited on filter paper and placed onto standard 1% w/v solid agar MS medium with 3% w/v sucrose, 0.1 mg/L α-naphthalenacetic acid (NAA), 0.5 mg/L 2-(γ,γ-dimethylallylamino) purine (2i-P). The rest of the explants (100 explants) were stored safely in a tank. Further cultivation was in the dark for one week, followed by standard long-day conditions (Keller et al., 2005).

RESULTS AND DISCUSSION

During the project period it was necessary to prepare, multiply and maintain the *in vitro* cultures of 50 garlic accessions. Among these accessions, 16 were bolting and 34 non-bolting accessions, two accessions came from France and six came from Italy, the rest of the accessions were from the Polish genebank's collection. Of the 34 cryopreserved non-bolting accessions, 20 fulfilled EURALLIVEG safety standards for long-term storage in liquid nitrogen. These standards are as follows:

a) regeneration percentage of the control explants is equal or higher than 30% – this means that 100 explants are a representative set to be put into liquid nitrogen as safety duplicate,
b) regeneration percentage of the control explants is between 10-30% – it is necessary to put into liquid nitrogen 200 explants as safety duplicate,
c) regeneration percentage of the control explants is lower than 10% – such accession is excluded from the project.

In routine cryopreservation of bolting garlic accessions, 38 of 43 accessions fulfilled EURALIVEG standards.

The survival rate of garlic explants was determined two weeks after re-warming. Survival was characterized by the green colour still remaining in the explant, the swelling and elongation of the leaf bases which were originally present in the explant after preparation immediately before the cryogenic treatment. Regrowth in *Allium sativum* L. is clearly defined by the appearance of new normal (not hyperhydric) leaflets and/or roots from the explants, which give rise to a normalization of the *in vitro* plantlets. For this, the time of six weeks after re-warming is considered to be the best one (Keller, 2005). In the case of non-bolting accessions, the time of eight weeks after thawing was the best for observing the regeneration rate.

The survival rate of explants of bolting garlic accessions ranged from 0.0% to 100%, whereas the regeneration rate ranged from 0.0% to 94%. In the case of the survival rate of non-bolting accessions, it was from 0.0% to 84%, whereas the regeneration rate ranged from 0.0% to 40%.

The establishment of the European Tripartite Cryobank of vegetative alliums was one of the main goals of the EURALLIVEG project. The creation of the cryobank was made possible by having efficient and repetitive procedures of cryopreservation. The garlic germplasm cryobank was established on April 1, 2011, and it is the first garlic cryobank in Poland. The European safety-duplicated cryocollection hosted in the genebanks of three countries is legally fixed by the Consignment Agreement between the three partner institutions (Czech Republic, Germany, Poland). This means that one cryopreserved garlic accession is maintained in two locations, one set is in the host genebank and the other set is in the partner's genebank. Furthermore, it will be possible to arrange safe duplicate exchanges between three participating institutions in the Czech Republic, Germany and Poland. At present, 147 garlic accessions are maintained in the Polish cryobank: 51 accessions received from the Crop Research Institute in Prague, Czech Republic; 34 accessions from the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany; 6 accessions from the Dipartimento di Scienze dei Sistemi Colturali, Forestali e dell´Ambiente, Università degli Studi della Basilicata (UNIBAS), Potenza, Italy; 2 accessions from the National Institute for Agricultural Research (INRA), France, and 54 accessions from the Polish genebank. At the same time, the Research Institute of Horticulture sent 31 garlic accessions to IPK, Germany, and 27 accessions to CRI, Prague.

Cryopreservation is now a viable option for storage of plant cells, tissues, seeds and embryos. At present, we have the possibility to use modern techniques (*in vitro* regenera-

tion and cryopreservation) for *ex situ* conservation of more than 200 species (Reed, 2008). In the case of the genetic resources of garlic, shoot tips from bulbils (Olas-Sochacka et al., 2010), cloves (Ellis et al., 2006), or *in vitro* plantlets (Keller et al., 2005) are cryopreserved. Successful cryopreservation of garlic shoot tips using PVS2 and/or PVS3 solutions has been reported by laboratories in several countries (Kim et al., 2005). Currently, cryopreservation offers the only safe and cost-effective option for long-term conservation of genetic resources of garlic from different living collections in the world. In Korea, the national garlic field collection, which is maintained in the field at the RDA Genebank in Suwon and the Garlic Experiment Station in Danyang, includes over 700 accessions (Baek et al., 2003). In the Czech Republic, in the Crop Research Institute, Olomouc, a collection of 648 accessions of garlic genetic resources is maintained (Smékalová et al., 2010), while in Germany, in the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, a collection of 481 accessions is maintained (Keller et al., 2008). More than 200 garlic accessions are maintained at the Western Regional Plant Introduction Station (WRPIS) in Pullman, USA, as part of USDA's National Plant Germplasm System (NPGS) (Volk et. al., 2004).

Three cryobanks of garlic germplasm exist in the world, as follows:

1) Cryobank of the National Agrobiodiversity Center, Republic of Korea. A total of 1158 accessions of garlic, as well as some *Allium* species, have been cryopreserved during 2005-2010 using the droplet-vitrification technique. These results open the door for large-scale implementation of cryostorage and for simplifying international exchange for clonal *Allium* germplasm (Kim et. al., 2012).
2) Cryobank of the National Center for Genetic Resources Preservation (NCGRP), Fort Collins, USA, where 27 garlic accessions are stored (Keller et al., 2008).
3) European Tripartite Cryobank in Skierniewice maintains 220 garlic accessions, including 202 accessions originated from the European Core Collection, and it is the first cryobank of garlic germplasm in Poland.

In Poland, there are three cryobanks. The oldest one (since 1991) is the Cryobank of the Polish Academy of Sciences Botanical Garden – Centre for Biological Diversity Conservation in Powsin, where seeds of 83 species of protected and endangered plants from 315 populations and dormant buds of 105 old varieties of apple, 10 varieties of pear, and 5 varieties of cherry are maintained in liquid nitrogen. The second one (since 1996) is Kostrzyca Forest Genebank, in which plumules of 14 455 accessions of *Quercus robur* L. and *Quercus petraea* (Mattuschka) Liebl.; embryonic axis of 2200 accessions of *Fagus sylvatica* L.; seeds of *Prunus avium* L., *Tilia cordata* Mill. and 23 accessions of herbaceous plants are stored. The third, and the youngest, cryobank in Poland is the European Tripartite Cryobank, which was established on April 1, 2011 in the Research Institute of Horticulture, Skierniewice (Mikuła et al., 2013). Currently, there are 147 garlic accessions maintained in Polish cryobanks. A Cryobank Network has been established by the three project partners: the Czech Republic, Germany and Poland. This system ensures safe storage of plant material by adopting a safety duplicate strategy (Blattner et al., 2012).

CONCLUSIONS

Cryopreservation proved to be the most effective method to preserve many vegetatively propagated collections in liquid nitrogen. It is a very important tool needed to conserve plant biodiversity in many scientific institutions and cryobanks. The EURALLIVEG project was a model for further activities in cryopreservation in European genebanks, and measures to disseminate the *Allium* experience to other crops are proposed. This bank is seen as being an initiating structure open to be joined by other cryopreservation groups to form a network of European *Allium* cryobanks.

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REFERENCES

Baek H.J., Kim H.H., Cho E.G., Chae Y.A., Engelmann F. 2003. Importance of explant size and origin and of preconditioning treatments for cryopreservation of garlic shoot apices by vitrification. CryoLetters 24(6): 381-388.

Blattner F.R., Kik C., Stavělíková H., Zámečník J., Esnault F., Kotlińska T., Zanke C.D. 2012. EURALLIVEG: Establishment of a European Core Collection by Cryopreservation and Virus Elimination in Garlic. In VI International Symposium on Edible Alliaceae 969: 319-327.

Ellis D., Skogerboe D., Andre C., Hellier B., Volk, G. 2006. Implementation of garlic cryopreservation techniques in the national plant germplasm system. CryoLetters 27(2):99-106.

Keller E.R.J., 2002. Cryopreservation of *Allium sativum* L. (garlic). In Cryopreservation of Plant Germplasm II: 37-47. Springer Berlin Heidelberg.

Keller E.R. 2005. Improvement of cryopreservation results in garlic using low temperature preculture and high-quality *in vitro* plantlets. CryoLetters 26(6): 357-366.

Keller E.R., Kaczmarczyk A., Senula A. 2008. Cryopreservation for plant genebanks- a matter between high expectations and cautious reservation. CryoLetters 29(1): 53-62.

Keller E.J., Zanke C.D., Senula A., Breuing A., Hardeweg B., Winkelmann T. 2013. Comparing costs for different conservation strategies of garlic (*Allium sativum* L.) germplasm in genebanks. Genetic Resources and Crop Evolution 60(3): 913-926.

Kim H.H., Cho E.G., Baek H.J., Kim C.Y., Keller E.R.J., Engelmann F., 2004a. Cryopreservation of garlic shoot tips by vitrification: Effects of dehydration, rewarming, unloading and regrowth conditions. CryoLetters 25: 59–70.

Kim H.H., Kim J.B., Baek H.J., Cho E.G., Chae Y.A., Engelmann F., 2004b. Evolution of DMSO concentration in garlic shoot tips during a vitrification procedure. CryoLetters 25: 91–100.

Kim J.B., Kim H.H., Baek H.J., Cho E.G., Kim Y.H. Engelmann, F. 2005. Changes in sucrose and glycerol content in garlic shoot tips during freezing using PVS3 solution. CryoLetters 26(2): 103-112.

Kim H.H., Popova E., Shin D.J., Yi J.Y., Kim C.H., Lee J.S., Engelmann F. 2012. Cryobanking of Korean Allium germplasm collections: results from a 10 year experience. CryoLetters 33(1): 45-57.

Makowska Z., Keller J., Engelmann F., 1999. Cryopreservation of apices isolated from garlic (*Allium sativum* L.) bulbils and cloves. CryoLetters, 20(3): 175-182.

Mikuła A., Makowski D., Tomiczak K., Rybczyński J.J. 2013. *In vitro* culture and cryopreservation for biodiversity conservation – genebank standards (Kultury *in vitro* i krioprezerwacja w zachowaniu różnorodności roślin – standardy dla banku genów). Polish Journal of Agronomy 14: 3-17.

Murashige T., Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.

Niwata E., 1995. Cryopreservation of apical meristems of garlic *(Allium sativum* L.) and high subsequent plant regeneration. CryoLetters 16:102-107.

Olas-Sochacka M., Kotlińska T. 2010. Cryopreservation of genetic resources of bolting garlic (*Allium sativum* L.) (Krioprezerwacja zasobów genetycznych czosnku pospolitego (*Allium sativum* L.) tworzącego pędy kwiatostanowe). Nowości Warzywnicze 50: 69-74.

Reed B.M. 2008. Plant cryopreservation: a practical guide. New York: Springer.

Smékalová K., Stavelíková H., Dušek K. 2010. Distribution of viruses in the garlic germplasm collection of the Czech Republic. Journal of Plant Pathology 92(1): 273-274.

Volk G.M., Maness N., Rotindo K. 2004. Cryopreservation of garlic (*Allium sativum* L.) using plant vitrification solution 2. CryoLetters 25(3): 219-226.

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