II.1 Cryopreservation of *Allium sativum* L. (Garlic)

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1 Introduction

The genus *Allium* comprises about 700 species. Several of them are important vegetables, spices and medicinal plants. The most important crop species are onion and shallot, *Allium cepa* L., garlic, *Allium sativum* L., leek, *Allium ampeloprasum* L. s.l., bunching onion, *A. fistulosum* L., chives, *A. schoenoprasum* L., Chinese chives, *A. tuberosum* Rottl. ex Spr., and rakkyo, *A. chinense* G. Don. Many other species are collected from the wild as spices or vegetables, or they are planted as ornamentals. Vegetative propagation is predominant in some of these species either because no seeds are set (garlic, great-headed garlic, rakkyo) or because of tradition, as in shallots. Seed-sterile hybrids are used as vegetables (top onions, gray shallots) or in the breeding of new ornamentals (as in subgenus *Melanocrommyum*). In all such cases, vegetative maintenance of genotypes is necessary. Due to continual vegetative propagation, virus occurrence is common in garlic. Meristem culture allows production of virus-free lines and, hence, is able to circumvent this problem as long as the material is kept permanently in vitro. In vitro storage is, therefore, preferable for this vegetatively propagated material.

Although today's garlic is a clonal crop, it underwent an intensive phase of diversification in the past before it lost its fertility and after that, perhaps, by mutation. Thus, there are some distinctly diverse groups within this species. Discriminating morphological characters are such as the structure of the bulb (arrangement, number and size of the cloves) and the ability to form inflorescences (the height of the inflorescence stalk, position, number and size of inflorescence bulbils and number of flower buds). The most original group has often been considered a separate species, i.e., *A. longicuspis* Rgl., but there are no morphological characters that separate these forms from true garlic. The present results of DNA analyses support these observations. Therefore, the previous opinion can no longer be followed (Maass and Klaas 1995; Maass 1996; Al-Zahim et al. 1997). This recently so-called *longicuspis*-group possesses high stalks with well-developed inflorescences, in which a high number of small bulbils are formed together with many flower buds. This group is located in

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Table 1. Some morphological characters of the garlic groups observed in the Gatersleben collection, being important for the success of *in vitro* culture and cryopreservation (figures represent average values for the resp. accessions)

Subgroup	Number of cloves per bulb	Inflorescence formation character	Number of bulbils per inflorescence	Bulbil size (weight in mg)
longicuspis	3–7	complete	20–150	15–80
pekinense	3–7	complete	50–100	500–800
ophioscorodon	5–7	complete	20–80	150–800
sativum	4–25	no, incomplete, or complete*	20–150; 0–50*	15–80; 300–1500*

* Bulbil numbers and sizes rather heterogeneous depending on the completeness of the inflorescence formation, incomplete inflorescences very often consist of only one or few very large bulbils.

Central Asia, the presumable site of origin of garlic. Some forms of the "*longicuspis*-group" preserved a certain degree of fertility that is now under investigation in order to make garlic a seed-propagated crop. So far, progress is rather limited in this direction, and the overwhelming majority of types remain vegetative. This is true for all other groups such as some small groups of East Asia (the "*pekinense*-group") and Southeast Asia as well as the European groups from which the world's most widespread garlic has developed which lost more or less the ability to form flower stalks or produces reduced stalks with some large or very large bulbils (the "*sativum*-group"). In central Europe, a small and relatively uniform group exists with high stalks, which are curled when unripe, possessing large bulbils (the "*ophioscorodon*-group"). As discussed below, the bulbil size is a factor that influences the success in cryopreservation. Therefore, the mean bulbil numbers and sizes for the different groups are given in Table 1 for the Gatersleben garlic collection together with the numbers of cloves per mother bulb, which is determined by the number of developing axillary meristems, another influencing factor when using basal plates as the explant source for cryopreservation.

2 Storage of Germplasm

Allium has been the target of in vitro approaches since the 1970s. First reports came from experiments on callus (Havranek and Novak 1973) and meristem culture (Havranek 1972). A modified B5 medium (Gamborg et al. 1968), called BDS, possessing increased levels of phosphate and less nitrogen (Dunstan and Short, 1977) was useful for many purposes. In later years, many reports cite micropropagation and other subjects of *Allium* in vitro culture (Bhojwani 1980; Novak et al. 1986; Moriconi et al. 1990; Keller 1992; Mohamed-Yasseen et al. 1994; Nagakubo et al. 1997). Studies on in vitro storage are, however, relatively rare. Slow growth conditions have been used for plant cultures (El-Gizawy and Ford-Lloyd 1987, Viterbo et al. 1994). Bulblets are formed under

in vitro conditions. Their development is triggered by inductive light conditions, in at least some cases (Debergh and Standaert-de Metsenaere 1976; Keller 1991; Kahane et al. 1992; Mohamed-Yasseen et al. 1995). For *Allium cepa*, a high sucrose content in the medium favors storage of in vitro bulblets to temperatures as low as –1 °C (Keller 1993; Kästner et al. 2001). Thus, it can be assumed that in vitro bulblets could also be used for medium-term storage of garlic, similar to that described for microtubers in potato (Thieme 1988-89). It should be emphasized that an obstacle to permanent in vitro culture and other in vitro methods is the relatively high degree of microbial infection in garlic (Fellner et al. 1996), which can lead to outbreaks of visible contamination, even after several years of culture, and may be exacerbated by severe stress such as would occur during cryopreservation.

3 Cryopreservation

Successful reports for cryopreservation of garlic are rare, despite the increasing demand for garlic and other *Allium* species, not only for food but also for medicinal purposes. All reports so far rely on vitrification as a method of cryopreservation. The first article was published by Niwata (1995). Explants from basal plates of post-dormant cloves were treated by the vitrification method using the cryoprotectant solution PVS 2 (0.4 M sucrose + 30% glycerol + 15% ethylene glycol + 15% DMSO, Sakai et al. 1990; Niwata 2000). The regrowth percentage approached 100% in this study. Hannan and Garoutte (1996, 1998) repeated this approach, mainly experimenting with the pre-conditioning phase, and reached up to 71.4% survival. These authors emphasized the problems caused by the latent infection of the source explants.

In contrast to the experiments published by Niwata (1995) and Hannan and Garoutte (1996, 1998), much better results were obtained in our vitrification experiments using PVS 3 (50% sucrose + 50% glycerol in liquid standard culture medium; Nishizawa et al. 1993) instead of PVS 2. This weaker cryoprotectant does not contain DMSO. With bulbil explants survival after PVS 2 was much lower and reached 80% in only one single genotype, whereas survivals were 85%–95% using PVS 3. Even more extreme was the difference of regrowth, which was, for three compared genotypes 0, 10, and 0% with PVS 2 and for the same material 17, 27, and 60% with PVS 3. The penetration behavior of PVS 3 may be different to that of PVS 2 because for the optimum effect longer pretreatments of about 120-240 min were necessary in comparison to those with PVS 2 (15-20 min; Makowska et al. 1999).

Makowska et al. (1999) summarized the results of comparative series of PVS 3 pretreatment times of 0; 60; 120; 180; 240; and 300 min using cloves and bulbils of four accessions with large or medium bulbils. The best mean regrowth percentages have been found for cloves after 240 min (80%) and for bulbils after 120 min (37%).

Subsequently, in this group, bulbils were predominantly used for cryopreservation. These are formed in the inflorescences and, because these organs

are considerably less contaminated than the cloves, this avoids a high percentage loss of explants after cryopreservation due to contamination if the bulbs had come from the soil. Survival and regrowth of bulbil explants depend, however, on the size of the bulbils. This parameter is genotype dependent as described above. Cryopreservation of explants taken from small bulbils is less successful than that of large-bulbil material. In accessions with large bulbils regrowth was 13–100%, with medium bulbils 15–28%, respectively, and in accessions with small bulbils no regrowth was obtained (Makowska et al. 1999; Keller and Senula 2000). Therefore, the best accession (All 290) possessing large bulbils and regrowth of 100% was chosen as standard material for further experiments.

3.1 Effects of Different Parameters on Cryopreservation Success with Standard Material

3.1.1 Material

Bulbils of garlic All 290, belonging to the *ophioscorodon* group, are of the large type (bulbils of 320–570 mg; Fig. 1A) and were harvested at the end of July and stored at 10 °C. Bulbils directly after harvest exhibit dormancy. Therefore, the experiments were performed between December (Fig. 1B) of the harvest year and May (Fig. 1C) of the next one. Because the quality of the bulbils declines towards the end of the storage period, survival and regrowth data are not directly comparable between experiments performed at different times of the year. Two different explant sizes were used (Fig. 1D). The difference consisted in the presence of different numbers of leaf sheaths in the respective explants (Fig. 1E). For comparison, explants from in vitro cultivated shoots (Fig. 1F) of a defined size have been used (Fig. 1G,H). The in vitro donor material is always regenerated via cyclic micropropagation (Keller et al. 1997; Fig. 1I,K).

3.1.2 Methods

The vitrification method was used (Makowska et al. 1999). Bulbils were sterilized by dipping them in 70% ethanol and subsequently placing them in a 3% sodium hypochlorite solution for 15 min. The isolated shoot tips were cultivated overnight on MS medium (Murashige and Skoog 1962) + 0.1 mg/l IAA + 0.1 mg/l kinetin with 0.3 M sucrose at 25 °C in dark. The explants were immersed in a loading solution containing 2 M glycerol + 0.4 M sucrose for 20 min. In the standard technique, apices were then treated with the vitrification solution PVS 3 for 120 min. After pretreatment, the apices were suspended in 1 ml of vitrification solution within a cryotube and were plunged into liquid nitrogen (LN). The control samples were rapidly rewarmed by plunging the samples in a 40 °C water-bath. Explants were then rinsed with liquid medium containing 1.2 M sucrose for 10 min, and placed on solid

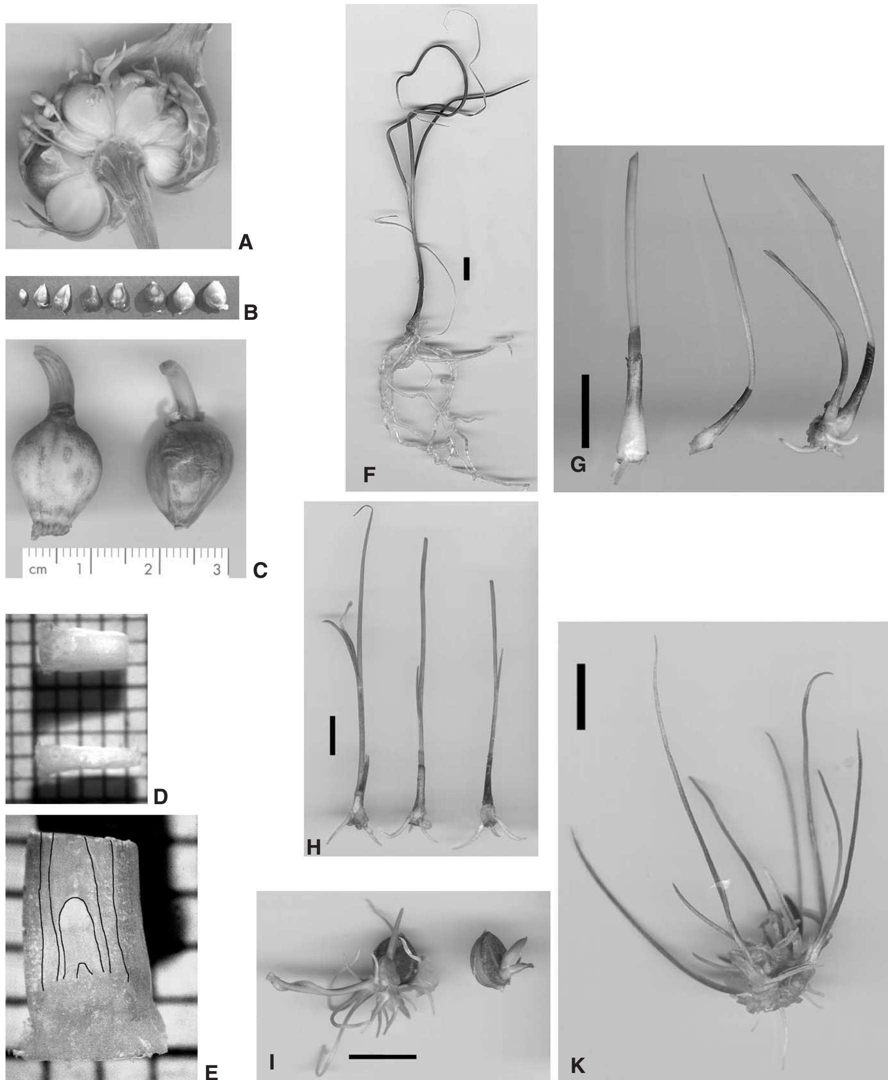

Fig. 1A–H. Explants for garlic cryopreservation. **A** Longitudinal section of an almost ripe inflorescence with bulbils and flower buds; **B** bulbils in the beginning phase of usability (December); **C** bulbils at the end of their usability (May); **D** "large" (*above*) and "small" explants on millimeter paper; **E** scheme of the leaf sheaths in a longitudinally cut "large" explant; **F** in vitro plant in the slow growth phase; **G** early and **H** later phase of in vitro source explants; **I** early and **K** late phase for explant source multiplication

medium containing 0.3 M sucrose in the dark for 1 d. Apices were then transferred to the standard medium MS +80 mg/l adenine sulfate +0.1 mg/l IAA +0.1 mg/l kinetin with 0.1 M sucrose and cultivated at 25 °C in a culture room equipped with fluorescent lamps under a photoperiod of 16 h light/8 h dark

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