1. Problem statement

Cryopreservation is the safest and most cost-effective method to maintain vegetatively propagated germplasm. Garlic falls into this category. So far cryopreservation is relying on bulbils, basal plates of in vivo material and basal parts of in vitro plantlets. In contrast to this a novel source can be used from the bases of unripe inflorescences from in vivo material. The project is aiming at adopting this new method (Kim et al., 2007) to genebank material under the European conditions.

2. Justification and rationale

AEGIS is aiming at integrating European germplasm strategies. Integrated adoption of a new method in cryopreservation will be a good example how to implement integrative strategies of germplasm preservation. The method will be developed for bolting garlic accessions, which will be shared between the three partners. After finalization of the project, a documentation will be elaborated which will enable other garlic collections to apply it and to extent it to all material of bolting garlic. As material will be shared by the partners in course of the project, this example can be used for improving the safety duplication strategy necessary for the European Genebank Integrated System.

3. Background

First garlic cryopreservation was published by Niwata (1995) using clove explants. In IPK, it was first published by Makowska et al. (1999). Here, clove and bulbil explants were used. So far garlic cryopreservation followed the way of stepwise completion of the results. Beginning with one research clone, one target organ and one technique, respectively, later the methods vitrification, DMSO droplet freezing, encapsulation/dehydration and droplet-vitrification were used and compared. Cloves have the disadvantage that they are mostly present in low quantities and are, as organs taken from soil, often severely contaminated. Therefore, other sources were used as bulbils and in vitro plantlets. The disadvantage of the latter is that they need long in vitro multiplication phases; however, these clones cannot be maintained for more than 2 years. Then, the quality of plantlets goes down to a level, causing reduction in cryopreservation results (Keller, 2005). The consequence is the need to frequently establish new in vitro cultures. Most research efforts were investigated by two dissertations (Kim, 2004; Kim et al., 2004; Souch, 2006).
For cryoprotection, most researchers prefer PVS3 (50 % w/v sucrose + 50 % w/v glycerol - Nishizawa et al., 1993), because no (poisonous) DMSO is in it. However, others documented that both PVS3 and PVS2 (0.4 M sucrose + 30 % w/v glycerol + 15 % w/v ethylene glycol + 15 % w/v DMSO - Sakai et al., 1990) lead to good results (Volk et al., 2004). The DMSO droplet method seemingly is also suitable (Keller, 2002). In the latest time, more and more researchers focus on the droplet-vitrification, because of the simple handling and ultra-rapid cooling. It is also preferred in IPK and can be used also with PVS3.

4. Main objective and specific objectives

The main objective of the project consists in the adoption of a new cryopreservation method using new source organs in three shared and additionally three local garlic accessions (one per partner).

Specific objectives are:
- choosing one well-known reference and five new accessions per partner fulfilling the requirements of an MAA and exchanging the three reference accessions amongst the partners,
- technology transfer in the frame of a working meeting prior to the start of the practical activities,
- comparing inflorescences of different developmental stage,
- exploring the best-suited time for cold storage of young inflorescences to prolong the period in which the method will be usable,
- comparing two cryopreservation methods: vitrification and droplet vitrification,
- optimising the pretreatment duration and concentration of the cryoprotecting agents (loading solution and PVS3).

5. Materials and methods

For the implementation of the project the following materials are needed:
Equipment to be provided by the laboratories: Laminar flow bench, dissection microscope, water bath, shaker, Dewar vessels, preparation tools, cryo storage tank, cultivation chamber or incubator
Equipment and consumables to be purchased: Parafilm, additional preparation tools (tweezers, scalpels), cryo tubes (1.8 ml), Petri dishes (6 cm), filter paper, growth media constituents, dehydration pretreatment chemicals, liquid nitrogen
Field plant material of six garlic accessions as the source for experiments

Methods: The basis material for the cryopreservation will be young inflorescences taken from the field culture. For this at least 15 plants per reference accession have to be planted in the field or greenhouses. The inflorescences of this material and the new accessions from the collection fields in the appropriate developmental stage are cut and stored in a refrigerator for variable times. For introduction the inflorescences are sterilized with alcohol and hypochlorite and the spatha is removed. Explants are cut from inflorescence bases and precultured for two days. The final explants are excised and pretreated with loading solution and cryoprotectant mixture. Then, depending on the protocol, the explants are placed on an aluminium foil in cryoprotectant droplets (droplet vitrification) or floated in the cryoprotectant solution in tubes (vitrification). They are rapidly cooled down to liquid nitrogen and stored there. In the experiments the samples are quickly rewarmed by a water bath (vitrification) of via directly plunging in ambient-tempered media (droplet-vitrification). After a washing phase, they are cultivated for 10 days in the light or in the dark and thereafter in light conditions for regrowth. Survival is counted two weeks, regeneration 2 months after rewarming.

6. Expected outputs

The output will consist in a feasibility study on the adoption of a novel source organ under European conditions and the optimized protocol of this method for broader utilization throughout all European garlic collections. Furthermore exemplary experience will be accumulated on safety duplication of cryo-material. After the project the shared accessions will be stored using the elaborated method, thus adding three accessions to the European Integrated System, complementary to the EURALLIVEG project and expanding its options to a new source organ and a new partner institute/country.

7. Benefits and impact

Adoption of new methods is one of the ways to improve a given technology. The new method is suitable for all germplasm of bolting garlic. The explant source is available only for a relatively short period in the year, when the inflorescence bases are still young and not much differentiated. In this time, the basis is still meristematic, and already existing bulbil and flower primordia can be stimulated to continue growth giving rise to new plantlets on artificial media. As an advantage, it was reported and can be confirmed in some preliminary own experiments, that the regenerants come out of the primary explants as bunches of little plantlets thus enabling quick multiplication. As the preculture phase can be skipped which causes relatively long periods prior to the cryopreservation step proper, the entire procedure will by much shorter then when in vitro material is used. Thus, the overall benefit will mainly consist in quicker introduction of material into cryopreservation.

The results of the project will be published in a scientific journal, and a protocol will be elaborated which than can be applied by all genebanks using cryopreservation for garlic germplasm. Further conclusions will be expected towards using the method for other *Allium* materials which develop flower heads with a sufficiently broad inflorescence bases (top onion, some shallot types, great headed garlic, onion types with low seed set etc.).

8. Innovation

The innovation consists in the introduction of a new explant type into routine cryopreservation, which allows to speed up the procedures of cryopreservation by a new protocol.

9. Application of results

The results and conclusions from the project will directly be introduced into the routine cryopreservation of the participating genebanks and will be communicated to other collections. Training of the method for laboratory technicians will be offered.

10. Workplan for the proposed period of the Grant

The activities are planned for twelve months beginning in January 2010.

IPK Gatersleben, Germany

Main activities	**Timeline (months)**											
	J	F	M	A	M	J	J	A	S	O	N	D
A1: Selection reference accessions	■											
A2: Exchange reference cloves	■											
A3: Field culture reference material	■	■	■	■	■	■	■					
A4: Meeting at IPK					■							
A5: Collecting reference and new accessions' inflorescences					■	■						
A6: Cryopreservation proper					■	■	■					
A7: Recovery in vitro culture					■	■	■	■	■			
A8: Regrowth analyses									■	■		
A9: Exchange of results and drawing conclusions										■	■	
A10 Safety duplication of cloves of well regenerating new accessions												■

RIVC Skierniewice, Poland

Main activities	**Timeline (months)**											
	J	F	M	A	M	J	J	A	S	O	N	D
A1: Selection reference accessions	X											
A2: Exchange reference cloves	X											
A3: Field culture reference material	X	X	X	X	X	X	X					
A4: Meeting at IPK					X							
A5: Collecting reference and new accessions' inflorescences					X	X	X					
A6: Cryopreservation proper						X	X	X				
A7: Recovery in vitro culture						X	X	X	X	X		
A8: Regrowth analyses									X	X	X	
A9: Exchange of results and drawing conclusions										X	X	
A10 Safety duplication of cloves of well regenerating new accessions												X

BPGV Braga, Portugal

Main activities	**Timeline (months)**											
	J	F	M	A	M	J	J	A	S	O	N	D
A1: Selection reference accessions	X											
A2: Exchange reference cloves	X											
A3: Field culture reference material	X	X	X	X	X	X	X					
A4: Meeting at IPK					X							
A5: Collecting reference and new accessions' inflorescences					X	X						
A6: Cryopreservation proper					X	X	X					
A7: Recovery in vitro culture					X	X	X	X	X			
A8: Regrowth analyses							X	X	X	X		
A9: Exchange of results and drawing conclusions										X	X	
A10 Safety duplication of cloves of well regenerating new accessions												X

11. Budget

IPK Gatersleben, Germany

- Staff time: Half laboratory technician 3 months 4,200 Euro
- Travel: non
- Meetings: non
- Equipment: laboratory consumables 300 Euro
- Supply/services: non
- Sum 4,500 Euro

RIVC Skierniewice, Poland

- Staff time: Half laboratory technician 3 months 3,500 Euro
- Travel: Collecting material from the field collection 100 Euro
- Meetings: Technical meeting at IPK 500 Euro
- Equipment: Laboratory consumables 400 Euro
- Supply/services: non
- Sum 4,500 Euro

BPGV Braga, Portugal

- Staff time Half laboratory technician 3 months 2,400 Euro
- Travel: non
- Meetings: Technical meeting at IPK 950 Euro
- Equipment: Laboratory consumables 1,150 Euro
- Supply/services: non
- Sum 4,500 Euro

Total

- Staff time: 10,100 Euro
- Travel: 100 Euro
- Meetings: 1,450 Euro
- Equipment: 1,850 Euro
- Supply/services: non
- Total: 13,500 Euro

12. Contributions foreseen by applicant

The applicants will give the working times of the supervising scientific staff as an input in kind together with the running energy costs, working places, field culture costs and the use of the laboratory devices like clean benches, nitrogen tanks etc.

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