Progress report of the AEGIS model crop: *Allium*
July 2008

1. Introduction

In 2001 the *Allium* WG held an *ad hoc* meeting on vegetatively propagated *Allium* at Gatersleben to discuss the conservation of the vegetatively propagated *Allium* crops. The WG recognized the benefits of cryopreservation for the long term conservation of vegetative germplasm and recommended that work on garlic and shallot should be progressed. In the development of the AEGIS project *Allium* was chosen as one of the 4 crops to be studied as exemplars for the development of European collections. The AEGIS *Allium* group chose to concentrate their efforts on the development of an AEGIS strategy for the vegetatively propagated *Allium* taxa and in particular on garlic. A majority of the AEGIS *Allium* group are also ECPGR National *Allium* Coordinators and members of the ECPGR *Allium* Working Group, a low priority rated WG in Phase VII. The WG partners agreed to use a majority of the WG funds in support of a meeting to discuss and make recommendations on the report of the AEGIS *Allium* group. These priorities fitted well under the ECPGR Phase VII priority areas for the use of documentation and characterisation/evaluation to improve the conservation of European germplasm.

The *Allium* WG recommendations and AEGIS *Allium* group strategy led to the development of an EU GENRES project, EURALLIVEG, to assess clonal duplicates in collections using the European *Allium* database; to fingerprint the accessions in order to define unique clonal material as European Accessions; to use *in-vitro* culture to remove viral infection from accessions and produce the material required for cryopreservation; develop an European collection of garlic in cryopreservation. The EURALLIVEG proposal was successful and started work in April 2007. The EURALLIVEG project partners are the curators for 50% of the garlic collections in ECPGR, thus the project will provide significant impetus to the development of a European collection. The European *Allium* Database was rebuilt in 2007 to support the objectives of the EURALLIVEG project. EADB2007 contained 3251 accessions of garlic in 18 countries including Israel and Russia. EURALLIVEG partner collections (Czech Republic, France, Germany, Italy, NGB and Poland) contained 1549 garlic accessions.

The first step was to identify historic safety duplicates in the EADB2007 collections. The total safety duplicates identified was 358 mainly in the Czech Republic, Germany and Spain. Sufficient funds are available in EURALLIVEG to molecular fingerprint (Array-On) all EURALLIVEG partner accessions to identify duplicates & MAAs. In order to expand this genepool we have proposed a Project for ECPGR Phase VIII to fingerprint selected material from Portugal and Spain (see below).

2. Establishing selection criteria for the identification of the Most Appropriate Accessions (MAAs)

The process for evaluating the selection criteria was initiated at the first EURALLIVEG project meeting in April 2007. The meeting was attended by Jan Engels, where he presented the draft selection criteria for identifying MAAs. The

project partners reviewed the selection criteria in the context of the objectives of the EURALLIVEG project and concluded the following:

Primary Criteria

1. In the public domain (i.e. Annex I material that is in the Multi-lateral System and non-Annex I material designated to AEGIS by governments or any other holder)

Allium **is a non-Annex 1 genus, but EURALLIVEG partners, their collections all being in the public domain, agreed to use the sMTA for any transfer of material.**

2. Genetically unique (i.e. genetically distinct accessions; assessment based on available data and/or on the recorded history of the accession)

For clonal crops, such as garlic, this is the key criterion.

3. Agronomically (including research material) and/or historically/culturally important

The AEGIS members agreed with the relevance of this criterion.

4. Plant Genetic Resources, including medicinal and ornamental species, and crop wild relatives (i.e. excluding forest genetic resources; non-plant agro-biodiversity species, etc.)

The AEGIS members agreed with the relevance of this criterion.

5. European origin or introduced germplasm that is of actual or potential (breeding/research) importance to Europe

Garlic and shallot originated in Central and southwest Asia. Both crops are of significant importance to European agriculture, commerce and health.

Comments on draft priority selection criteria

We did not find the draft priority selection criteria of particular relevance to the objectives of the working group in considering the development of MAAs for vegetatively propagated material.

Recommended secondary selection criteria

1. Maintained in “country of origin”

Garlic and shallot originated in Central and southwest Asia. At the accession level no selection pressure is applied in vegetative maintenance, therefore, the country of origin is not critical.

2. A known origin (collected and/or bred; pedigree data!?)

Having a known origin is generally a useful characteristic because origin is an indicator of certain characteristics (e.g. daylength requirements). In some circumstances material with no passport data is maintained because of special characteristics making it of high importance.

3. Comprehensiveness of passport information

Passport data are extremely useful, the value increasing with quality and volume.

4. Number of regeneration/multiplication cycles

Garlic and shallot have to be grown annually making this criterion of no relevance.

5. Health status (i.e. is the germplasm disease free?)

The health status of vegetatively propagated germplasm is an extremely important criterion.

6. Existence of morphological/molecular characterization data

In the context of vegetative material and the objectives of the AEGIS *Allium* group, the use of morphological and molecular characterisation is essential.

7. Existence of (agronomical) evaluation data

This is useful, but not essential.

8. Validated accession name (particularly relevant for perennial clonal crops, where the same name can be attributed to different accessions; history of individual accessions is important; special attention to be paid to synonyms and homonyms)

The validation of accession name is of limited use for garlic and shallot.

<u>General observations and comments on the process of developing the criteria and lessons learnt for other crops</u>

The method we have developed would be relevant to other vegetatively propagated crops.

3. Establishing the list of MAAs

The procedure followed and lessons learned

As described in the introduction the objectives of the ECPGR *Allium* working group and the EURALLVEG project are identical. In support of EURALLIVEG, the European *Allium* Database was rebuilt in 2007. It contained 3251 accessions of garlic in 18 countries including Israel and Russia. The EURALLIVEG partner collections (Czech Republic, France, Germany, Italy, NordGen and Poland) contained 1549 garlic accessions.

Our discussions have covered the economic implications of implementing the AEGIS cryobank proposal and in part therefore the establishment of MAAs.

Currently in EURALLIVEG there are three institutions involved in the cryopreservation of garlic, namely the Czech Republic, Germany and Poland (Tripartite Model CGP). We are aware that other institutions/countries (NordGen and Portugal) are interested in developing cryopreservation capabilities. The expertise in cryopreservation developed in EURALLIVEG is available to assist in the establishment of other cryo programmes and we would hope in the future to expand the cryopreservation network. AEGIS *Allium* assumes that the initial costs of any new programme developments will be the responsibility of national programmes and the NordGen.

The AEGIS *Allium* model would require coordination funds for the development, management and administration (database management and funds for meetings) of a cryo-network including the identification of duplicates by molecular analysis, identification of MAAs, integrated collection management (safety duplicate systems), quality assurance training, cleaning of material from pathogens, cryo training and establishment of network-wide protocols, etc.

Molecular fingerprinting is the main criterion to be used in the selection of garlic and shallot MAAs. The EURALLIVEG project (2007-2011) will provide significant information and technical expertise in the development of MAAs for a European collection of garlic and shallot based on the results of the molecular analysis. A significant limitation is that EURALLIVEG involves only a proportion of European collection curators. The AEGIS *Allium* group propose that the molecular method and analysis resulting from EURALLIVEG will provide the tools for screening other ECPGR garlic and shallot collections to identify unique MAAs.

We considered the cost implications (potential cost savings and additional costs) of the implementation of AEGIS for vegetative alliums.

In our discussions there was the assumption that national programmes will support the routine financial inputs for the maintenance of European cryobanks including facilities, equipment, consumables and staff.

There are two major cost implications (molecular fingerprinting and the preparation and transfer of MAA accessions into cryopreservation) in the development of a European cryobank systems for garlic and shallot. The AEGIS *Allium* group coined the phrase "Activation energy" to describe the financial inputs required to provide the labour necessary to introduce newly identified MAA accessions into cryopreservation. This "Activation energy" will be an additional cost for each cryobank. However, once material is cryopreserved the maintenance costs per accession are almost negligible. Additionally there is potentially a reduction in field maintenance costs available to national programmes.

The AEGIS *Allium* group has developed a 3 step work plan towards the creation of a European garlic collection in cryopreservation, whereby each phase will require some financial inputs in order to achieve total success.

The first step is the current EURALLIVEG project. The fingerprinting (SNP) of 1600 garlic and 550 shallot accessions will be carried out to identify MAAs. The Tripartite Cryobanks (Czech Republic, Germany & Poland) have labour resources sufficient to cryopreserve 200 garlic accessions. Therefore, any number of garlic MAAs identified over this 200 limit for garlic and the total of shallot MAAs will require additional labour inputs (Activation energy) to achieve complete cryopreservation of even the EURALLIVEG MAAs.

The second step is outlined in the ECPGR *Allium* Working Group project proposal for ECPGR Phase VIII to extend the fingerprinting of garlic collections to those in Portugal and Spain. The funds available in Phase VIII will only permit the fingerprinting of a proportion of both collections. Therefore at its most successful this will provide indications of the extent of unique genotypes in these 2 collections.

Additional funding will be required to fully screen the collections in order to identify MAAs. The national programmes will have to develope their own cryo capability or agree with their material being transferred to a cryobank in a 2nd country. As in step 1 additional labour inputs (Activation energy) will be required to achieve complete cryopreservation of the MAAs in these 2 national programmes.

The third step looks to the future, although almost anything is achievable if the relevant funds became available. In the longer term it will be possible to further extend fingerprinting of other ECPGR garlic and shallot collections (Bulgaria, Greece, Hungary, Israel, Lithuania, Macedonia, Romania, Russia, Slovakia, Turkey and Ukraine) providing the funding can be found. As with steps 1 and 2 above, the Activation energy funding will be required to transfer any MAAs identified into cryopreservation.

Once MAAs (unique genotypes) have been identified we will inform National Coordinators of our recommended list of MAAs and European safety duplicates. Furthermore, we will identify a group of MAAs being the most frequently ordered accessions and request the national programmes to ensure that these accessions be maintained additionally in field culture as an active collection to ensure quick delivery. In AEGIS terms this work plan will reduce the field maintenance costs for the national programmes to this limited number of frequently used accessions, which could provide a significant financial saving. The non-MAA accessions in a national programme will be genotypic duplicates of MAAs and European safety duplicates in European cryobanks. Each national programme can then decide whether they wish to maintain these other national accessions in the field culture or to discard them to maximize cost saving.

There is a potential additional cost factor derived from needs to clean the plants from viruses, which is dependent on the level of actual quarantine requirements.

4. Establishing the AEGIS quality system (AEQUAS)

The ECPGR *Allium* group is fortunate that the EURALLIVEG Project has established quality standards for their work based on the IPK Gatersleben QMS system. These EURALLIVEG standards have been adopted by the AEGIS group.

Comments on the proposed principles and elements of the AEQUAS

The main thrust of the objectives is to establish a European cryobank network and, therefore, a majority of the standards are relevant to this area. However, there is a requirement for national programmes to maintain a number of frequently used MAAs under field conditions with associated quality standards.

Recommendations on crop specific technical standards

Field maintenance of garlic and shallot

Due to the specific environmental conditions at different sites around Europe the material has to be grown using best local practice to maximise health and quality.

The most frequently requested MAAs will be maintained as field collections in national programmes, in addition to being stored in cryopreservation. The field maintenance of an active collection permits the quick delivery of material. For these

most frequently requested MAAs, a minimum of 40 clonal plants, initially derived from one mother bulb to be replanted every year.

For some shallot accessions, which produce orthodox seeds, it is also possible to follow seed reproduction and seed storage standards as for onion. However, this seed material must be clearly identified as a population because cross pollination leads to the loss of the original clonal genotype.

***In vitro* culture for medium-term storage**

In vitro storage is not considered to be a basic method for the maintenance of garlic and shallot germplasm because these species are subject to deterioration in quality after 1-2 years in culture. This method should be used in special cases only (e.g. transient storage of material to be distributed or exchanged).

Quality protocols exist for this *in vitro* method as reported below.

Pretreatment:
Material is taken from the field, where it is treated according to the regular plant protection and management program. No special pretreatment for in vitro donor material is requested.

Collection of materials:
Bulbils and cloves are harvested in summer and stored until use, maximally until March (cloves) to April (bulbils) of the following year. Cloves are used only if no bulbils available, because of less material per accession. An alternative option (efficient but time-limited) is use of inflorescence bases in summer (exact time depends on the local conditions).

Cleaning
The cleaning procedure consists of

- washing in running tap water
- shaking in 70% ethanol for 10-20 s
- sterilising in sodium hypochlorite (3% active chlorine) + 1 drop Tween 20 per 100 ml sterilizing solution for 20-30 min
- washing 4-5 times with sterile water

Explant preparation
Explant preparation should be done by using dissection microscope.

Sample size
minimum 10, maximum 50 in vitro plants per accession

Culture medium
Garlic: MS (Murashige & Skoog 1962) + 3% sucrose
Shallot: MS + 8% sucrose

The application of hormones depends on the special situation. If needed (e.g. for speeding up the multiplication in the initial phase), three options are used and should be compared because of possible genotype effects:

- MS + 0.5 mg/l 2-iP + 0.1 mg/l NAA (Bhojwani, 1980)
- MS + 0.1 mg/l KIN + 0.1 mg/l IAA (Moriconi *et al*., 1990)
- MS + 2 mg/l BA + 0.2 mg/l NAA (Kahane *et al.,* 1992)

Multiplication phase (including initial phase)
Culture in tubes
Visual check monthly, bacteria test on liquid medium if required
20-25 °C, 16 h light 60-80 $\mu mol \cdot cm^{-2} \cdot s^{-1}$, fluorescent light

Storage phase
Culture in glass jars or Erlenmeyer flasks (Magenta boxes not recommended)
2 °C or 10 °C
Low light intensity storage time (0.5)-1-(2) year(s)

Cryopreservation of vegetative material

For shallot, cryopreservation is still in development and applicable methods need to be finalised in the future.

We have accepted the EURALLIVEG protocol and standards for the cryopreservation process for garlic.

Cryopreservation of Garlic
Method: Vitrification
All activities have to take place under sterile conditions using sterile instruments and media in a laminar flow box.

Prior to placement into the cryo tank the tubes must be marked unambiguously with accession number and date of introduction.

i. Preculture of the in vitro donor plants
(This step is not necessary in case of direct use of bulbils or cloves - in this case the procedure starts with the preparation step)

Beginning with 2-4 weeks subculture on one of the media MS [Murashige & Skoog 1962] + (mg/1) *)
- a 0.5 2-iP, 0.1 NAA (Bhojwani 1980)
- b 0.1 Kin, 0.1 IAA (Moriconi et al. 1990)
- c 2.0 BA, 0.2 NAA (Kahane et al. 1992)

*) Medium [a,b,c] means you should use one of these three variants.
At Gatersleben, usually variant a is used. However, if some accessions do not well or you do not have one of the biochemicals available, you are free to use the other variants. You could do also all the variants in parallel.
However, if you decided to use one of the variants, you should go all the other steps also with this medium.

For the next step stages of well developed single plants are used which should have developed roots, avoid to use bunches or clumps of shoots.

At 20-25 °C and 16 h light, a period of 8-10 weeks alternating temperature (optimum day/night temperature 25/-1 °C and 16 h light) is applied as cold preculture.

ii. Preparation
Explants are taken from single, well developed in vitro plantlets.
Take the plantlet out of the vessel and excise explants of about 2 mm thickness and 3-5 mm length.

iii. Explant preculture
Preculture of explants in Petri dishes sealed with Parafilm on one of media [a,b,c] containing 10 % sucrose for 20-24 h at 25 °C and 16 h light.

iv. Loading Phase
Transfer the explants into cryo tubes (10 explants per tube),
Add 1 ml solution A [13.7 % (w/v) sucrose + 18.4 % (w/v) glycerol in liquid medium [a,b,c] to the tubes,
Close the tubes and shake them,
Place the tube at ambient temperature for 20 min,
Remove solution A

v. PVS 3 treatment
Per tube 1 ml PVS 3, solved in one of the liquid media [a,b,c]
Close the tubes and shake them,
Place the tube at ambient temperature for 2 h,
Remove PVS 3 solution

vi. Transfer to liquid nitrogen
Add 0.5 ml PVS 3 solution per tube,
Close the tubes and shake them, then plunge them immediately into a Dewar with liquid nitrogen,
Transfer the cryo tubes to be stored into the liquid nitrogen tank

vii. Rewarming
Control cryo tubes rewarm quickly in a water bath at 40 °C for 2-2.5 min,
Remove PVS 3 solution,
Add per tube 1 ml liquid medium [a,b,c] with 41 % sucrose (=1.2 M),
Close the tubes and shake them,
Place the tube at ambient temperature for 10 min,
Remove liquid medium.

viii. Transfer onto regeneration medium
Place the explants shortly on filter paper,
Transfer the explants into Petri dishes with solid medium [a,b,c] containing 10% sucrose,
Seal the Petri dishes with Parafilm,
Cultivate the Petri dishes in the dark at 25 °C for 20-24 h,
Transfer the explants into Petri dishes with solid medium [a,b,c] containing 3% sucrose,
Cultivate the Petri dishes in the dark at 25 °C for other 6 days,
After that the Petri dishes are further cultivated in 16 h light at 20-25 °C.

ix. Counting the results (growth controls)
First record is to be counted 14 days after rewarming, count the survival of regenerants,
Transfer the explants into culture vessels (tubes, glasses or Erlenmeyer flasks) containing medium (a,b,c) with 3 % sucrose,
Second record is to be counted 7-10 weeks after rewarming, count the regeneration of small plantlets.

x. Introduction into storage
Per accession 100 explants must be introduced into storage. For the growth and regeneration control additionally 50 explants need to be cooled and rewarmed after about 1 h.
When regeneration rates are better than 30 % (calculated in relation to the explants used for the control), the sample is complete.
When regeneration rates are between 30 % and 10 %, a second set of 100 explants needs to be introduced with again 50 explants as control.
When the regeneration rates in the sum of both sets is less than 10 %, do not use this accession for the project.
Split the sample into two equal parts and place them into two different tanks.
One of the parts is dedicated to the final exchange of safety duplicates.

Virus elimination
The following two levels of phytosanitary standards were adopted from the 2002 report of the ECPGR *Allium* WG *ad hoc* meeting on vegetatively propagated *Allium*, held at Gatersleben.

Lower level [without virus elimination]
The material exchanged should be free of visible diseases such as fungi, bacteria and arthropods. Prior to dispatch, the material should be disinfected by a fungicide (e.g. by a 0.2% solution of benomyl). For phytosanitary details, see Diekmann (1997). The recipient of such material would bear the responsibility for the level of quarantine precautions taken when the material is planted, maintained and reproduced.

Higher level [including virus elimination]
Sanitary prerequisites are at least the same as for the lower level.
With respect to the viruses in question, two categories of cleanliness have been formulated:
A. material free of potyviruses, carlaviruses and allexiviruses
B. material free of potyviruses and carlaviruses (allexivirus untested)

(As the virus elimination process is very labour-intensive, it will be a prerequisite for the most important material only. Definition of importance has to be agreed in a later stage)

The method of virus elimination is meristem culture. It consists of isolation of the meristem explants with a diameter of 0.5 mm and cultivation on medium MS + 0.1 mg/l Kinetin + 0.1 mg/l IAA or MS + 0.5 mg/l 2-iP + 0.1 mg/l NAA.

Virus indexing is performed by ELISA tests. Five months after meristem culture, the regenerated plantlets are tested and the virus-free plantlets identified undergo a pre-multiplication phase, after which they will be introduced into cryopreservation.

General comments and observations

Monitoring and Audit

The group views the requirement for quality standards and routine cryonetwork audits as essential components of the AEGIS Agreement with national governments. The cryopreservation network will require all partners to work to an agreed minimum set of quality standards and to accept routine audits of their facilities and the AEGIS samples in cryopreservation. The audit will be carried out by the cryonetwork members or by independent experts depending upon available resources and the independent experts. The audit results will be forwarded to the institute, the national *Allium* coordinator and the ECPGR national coordinator for action where required. The group agreed that the members of the *Allium* cryopreservation network (currently Tripartite) will coordinate the network by consensus.

5. Observations on the framework and tools for the assessment of operational costs for collection maintenance

Following the presentation of Daniela Horna (IFPRI) at the AEGIS Model Crop Curators Meeting the AEGIS *Allium* group considers assessment of operational costs of high importance. It will be useful to review the costs of field and cryobank management in order to assess the relative costs. The development of an economic appraisal form could be used to compare the cost of garlic field conservation in ECPGR national programmes to provide an estimate of potential savings in moving European garlic to a cryopreservation system. Therefore, we express our interest in collaboration in the assessment of operational costs for the conservation of garlic and shallot under field conditions and in cryopreservation. However, there are no labour resources available in IPK Gatersleben to implement this initial economic evaluation. Therefore, in order to progress this economic assessment of an AEGIS model crop system additional funds will be needed to be made available within the AEGIS budget.

6. Proposal on the involvement of all the relevant stakeholders of the European Region in establishing and operating the European Collection for vegetative *Allium* crops (including services to be provided; rationalization aspects; coordination; etc.)

As outlined above the AEGIS *Allium* proposal is scheduled in three steps depending on the availability of financial resources and the involvement of relevant institutions and national programmes. The first step is the EURALLIVEG project where European Commission and national matching funds will support fingerprinting, cryopreservation and virus elimination in six national programmes. The second step is the ECPGR *Allium* Working Group proposal to the Steering Committee for a Phase VIII project to support fingerprinting of garlic in Portugal and Spain. The third step will extend this fingerprinting to other ECPGR garlic collections providing that the relevant funds can be found.

The fingerprinting and data analysis are merely the starting point for the development of a cryopreservation network in that steps 2 and 3 both have to be followed by the Activation energy step to transfer the MAAs identified into cryopreservation. The Activation energy step is only achievable providing that the necessary funds are forthcoming.
Inherent in EURALLIVEG is the intention to disseminate the experience and expertise developed in the project by offering training activities for other genebanks interested to adopt cryopreservation and/or virus elimination. The success of the EURALLIVEG project will provide significant expertise for the development of AEGIS garlic and shallot collections. However, the completion of steps 2 and 3 including the Activation energy (as outlined above) is essential for the implementation of a Europe-wide cryopreservation network system.

7. Proposed "general workplan", whenever possible costed, for the model crop *Allium* Working Group activities

The 3 step work plan of the AEGIS *Allium* group has been described above. For historical reasons the European *Allium* DataBase manager is not a partner in EURALLIVEG. Therefore future inputs into the EADB for all steps in the work plan will require support directly or as inputs-in-kind. The development of steps 2, 3, and the cryopreservation (Activation energy) step will require financial inputs and/or significant defined inputs-in-kind by national programmes to achieve any progress. Support will be needed in the following areas:

- coordination meetings
  Step 2 in the work plan has limited funds available for meetings. For AEGIS to succeed regular (biannual) meetings of the collection curators (Portugal & Spain) and EADB manager will be required with the cryobank network curators. The cost of these meetings will be relatively low if held in tandem with EURALLIVEG meetings. In the longer term such coordination meetings will be required with the step 3 curators of garlic and shallot.
- training courses
  The EURALLIVEG partners have agreed to offer training in cryopreservation techniques for garlic to any institution/national programme interested in developing this technique(s). Such training will inevitably require funds for travel and subsistence depending upon the duration of the individual needs.
- referee test to monitor performance
- national programmes to provide freeze-dried samples for fingerprinting
  This is a relatively small cost providing the garlic and shallot curators have access within their national programmes to freeze drying equipment and expertise.
- fingerprinting
  The SNP analysis of garlic and shallot in EURALLIVEG is carried out by the commercial company ArrayOn. We propose to use this system and company for future fingerprinting and thus can provide accurate costing for numbers of accessions to be screened.
- analyses of the molecular data derived from the fingerprinting
  The analysis of the molecular data will need to be reassessed as data from the fingerprinting of additional collections is carried out to identify new MAAs.
- "activation energy" for the introduction of MAAs into cryopreservation.
  In EURALLIVEG the tripartite cryobanks have a limited capacity of 200 MAA accessions for the transfer to cryopreservation. This limitation in capacity is not in

terms of cryo storage space, but in the labour available to carry out the in vitro preparation work and the transfer of in vitro material into cryopreservation. The Activation energy is the funds required to supply this labour in order to surmount this transfer step. It is possible to calculate the labour required to achieve specific objectives in terms of numbers of MAAs into cryopreservation.

In Phase VII of ECPGR, the *Allium* Working Group concentrated their efforts on the main vegetatively propagated crops. At the Allium Working Group meeting during the Vegetables Network Meeting in Olomouc 2007 we recognised the applicability of the AEGIS concept for all *Allium* genetic resources. In Phase VIII, the VEGNET is proposing a meeting to discuss the application of the AEGIS concept further in which the experiences of the exemplar groups will be presented and discussed by the six vegetable working groups.