Darwin Initiative Annual Report

Important note:

To be completed with reference to the Reporting Guidance Notes for Project Leaders – it is expected that this report will be about 10 pages in length – Submission deadline 30 April 2007

Darwin Project Information

Project Ref Number	14-056
Project Title	Cryo-conservation Centre of Excellence for Sub-Saharan Africa (CCESSA)
Country(ies)	UK, South Africa
UK Contract Holder Institution	Royal Botanic Gardens Kew
UK Partner Institution(s)	Royal Botanic Gardens Kew
Host country Partner Institution(s)	University of KwaZulu-Natal
Darwin Grant Value	£168,852
Start/End dates of Project	April 2005 – March 2008
Reporting period (1 Apr 200x to 31 Mar 200y) and annual report number (1,2,3..)	1 April 2006 to 31 March 2007, annual report 2
Project Leader Name	H.W. Pritchard
Project website	www://ukzn.ac.za/plantgermcons
Author(s), date	P. Berjak, N.W. Pammenter, H.W. Pritchard; 26th April 2007

1. Project Background

The project is located predominantly in the KwaZulu-Natal Province of South Africa. It aims to address the problem that biodiversity conservation of plant species producing recalcitrant seeds is not possible by conventional seeds storage, as recalcitrant seeds are desiccation-sensitive.

2. Project Partnerships

Collaboration between the UK and host country partners is excellent. Through direct and indirect support, RBG Kew are contributing to capacity building in the host institution, assisting in building up a viable and vibrant research group. RBG Kew currently provides financial support for a Ph.D. student who is making outstanding progress. The project leader visited in January for a conference around which numerous informal discussions were held. Additionally, a formal 2 h meeting was held with H.W. Pritchard (project leader), P. Berjak (project partner), N.W. Pammenter (co-project partner), D.J. Mycock, D. Erdey and J. Wesley-Smith (project collaborators) attending. Overall, one could not wish for a better partnership.

3. Project progress

3.1 Progress in carrying out project activities

Satisfactory progress has been made in most of the project activities. During the reporting period the seeds of a further 21 species have been screened, of which 13 exhibit recalcitrant behaviour, four are orthodox and results from the remaining four are pending. *Inter alia*, these studies have demonstrated the recalcitrant nature of the seeds of at least two cycad species, the first such report for cycads. However, successful cryopreservation has been unexpectedly limited. Considerable success has been achieved with many species in the Amaryllidaceae, but little with seeds of dicotyledonous woody species. In many instances cryopreserved embryonic axes will readily produce roots on recovery from the cryogen, but not shoots. As the ultimate objective of the project is the establishment of a cryobank, we do not consider the production of material that will not produce shoots to be successful cryopreservation. Because of the need to use small explants for both the rapid drying and cooling steps of cryopreservation, it is necessary to remove the cotyledons from cotyledonous seeds. It is apparent that the physical damage associated with cotyledon excision is adversely affecting the shoot meristem, preventing shoot growth, even in the absence of partial drying and cryopreservation. Certainly, our data show a burst in the production of reactive oxygen species (ROS) on excision of the cotyledons of *Trichilia dregeana*. Further work on the production of ROS and the activities of anti-oxidant systems is underway in order to identify the cause of shoot meristem damage.

Two approaches are being taken to deal with this problem. The first is to attempt to minimise ROS production consequent on cotyledon excision. This involves excision in anti-oxidant solutions followed by short-term drying and treatment of the sites of excision. These studies are currently underway and data are not yet available.

The second approach to the problem of shoot meristem damage is to use explants other than excised embryonic axes. This approach is also the only feasible one in the case of species that produce seeds with embryonic axes that are too large for rapid drying and cooling. Consequently, considerable effort has been expended on the production and culturing of alternative explants, including somatic embryos, nodal explants and shoot meristems. Currently, alternative explants that may be suitable for cryopreservation attempts have been developed for eight species. Furthermore, cell suspension cultures of a number of coffee species are being established. The homogeneous nature of this material, as opposed to structurally complex embryonic axes, may help assist in our understanding of responses to freezing.

A major problem in the past has been the high incidence of fungal contamination of (especially tropical) recalcitrant seeds, with this contamination often being internal as well as surface. However, judicious use of a number of fungicide 'cocktails', combining systemic and topical fungicides, is promising in the resolution of this problem.

An aspect receiving some attention is the variation between seeds of the same species of different provenances. Seeds of *Ekebergia capensis* from a latitudinal gradient from warm temperate to tropical show differences in chilling sensitivity, and molecular studies show that these are genetically distinct populations, verging on sub-species.

Preliminary molecular studies have also been undertaken to assess the potential for genetic 'selection' by the cryopreservation procedures. It is proving difficult to get clean DNA from the cotyledons of many species, and so the work has been confined to members of the Amaryllidaceae. Initial indications are that cryopreservation procedures do not have any adverse genetic effect.

Studies on growth rates and stress sensitivity of plants derived from explants retrieved from cryopreservation have been undertaken. Many of the steps in the cryotreatment, especially treatment with fungicides, partial drying and cooling to cryogenic temperatures, are themselves potentially injurious, and it has been found that plants produced by embryonic axes retrieved from cryogenic storage are initially of reduced vigour. Whilst not a limiting factor in the development of cryopreservation protocols, this low-vigour phenomenon could impose some constraints on the re-establishment and re-introduction phases of the cryoconservation procedures.

The B.Sc. (Hons) Cryobiology module (see Annex 3) has now been run twice, and with minor modifications to increase the practical component, is ready for presentation to students from elsewhere.

3.2 Progress towards Project Outputs

The major outputs of this project can be summarised as (i) the screening of seeds of sub-Saharan species to establish their storage category, (ii) the development of protocols permitting the cryoconservation of genetic resources of recalcitrant-seeded species, (iii) building capacity in the field of cryopreservation, and (iv) the production of a cryo-manual providing protocols for successful cryoconservation of recalcitrant seeds.

In terms of screening of seeds to establish storage category, the project has exceeded the projected outputs, with the seeds of over 45 species having been screened. Most of the species studied are from South Africa, simply because of ease of access; it is considered that concentrating on local species is acceptable during the development phases of the project. Of the species screened, the majority have been shown to be recalcitrant: it must be emphasised, however, that this does not represent the proportion of recalcitrant-seeded species in the region as sampling was deliberately biased towards species the seed characteristics of which indicated that they may be recalcitrant.

The development of cryopreservation protocols has been more difficult than originally anticipated. Good success has been achieved with the herbaceous monocotyledonous amaryllids, and the project partners had had some success with temperate dicotyledonous species prior to the initiation of this project. However, limited success has been achieved with recalcitrant seeds from tropical dicotyledonous species. This may be partially a consequence of damage associated with cotyledon excision, but there also appears to be considerable variation among species in responses to the steps in the cryoconservation protocol, additional to differences between tropical and temperate species. A further complication is that embryonic axes are structurally

complex, and different tissues may dry at different rates and may respond differently to the various 'insults' associated with the cryopreservation procedures.

Capacity building and training is progressing well at a local level. There are 15 students at the levels of post-doctoral, Ph.D. and M.Sc. As the project has been running for less than two years, it is early too early for these post-graduates to have completed their degrees and to have graduated by this stage. The B.Sc. (Hons) level cryobiology module is running, and over the last two years 14 students have completed the module. External students need to be recruited to this module, but that recruitment is not considered to be the responsibility of the project partners. The following Table lists the Post-doctoral, doctoral and masters students currently involved with the project.

Post-doctoral	Rosa Peran (completed Sept 2006)
	Boby Varghese (started Oct 2006)
	Dalia Matthews Varghese (started Oct 2006)
Ph.D.	Elliosha Hajari
	Sershen Naidoo
M.Sc.	Deshnidevi Govender
	Havendren Chetty
	Melissa Timothy
	Wynston Woodenberg
	Varsha Premsager
	Ashley Subbiah
	Prabashni Naidoo
	Vishal Bahruth
	Ashika Jaimangal
	Meagan Goveia

The problems associated with cryopreservation of tropical recalcitrant seeds have become apparent largely as a consequence of this Darwin Initiative programme. The programme has demonstrated that there is no simple and widely-applicable protocol for successful cryoconservation, and so it is considered premature to consider the production of a cookery-book style cryo-manual. However, the project has produced a recipe-based guideline to the cryopreservation of more desiccation tolerant seeds (see appended publications: Pritchard and Nadarajan, 2007).

3.3 Standard Output Measures

Table 1 Project Standard Output Measures

Code No.	Description	Year 1 Total	Year 2 Total	Year 3 Total	Year 4 Total	TOTAL
Established codes						
1	Ph.D. degree	0	1			
2	M.Sc. degree	1	1			
3	B.Sc. (Hons) degree with seed-associated project	8	6			
4A	Undergraduate students in training		6			
	Training weeks per student		4			
4C	Post-graduate students in training	5	12			
4D	Training weeks per student	40	40			
6A	Visiting scientists from Africa given experience	3	2			
6B	Average training weeks per visitor	3	4			
8	One visit by UK project leader	1	1			
11A	Papers published in peer reviewed journals	3	4			
11B	Papers submitted to peer reviewed journals		4			
14B	Conferences attended		2			
New - Project specific measures						

Table 2 Publications

Type * (eg journals, manual, CDs)	Detail (title, author, year)	Publishers (name, city)	Available from (eg contact address, website)	Cost £
Journal	Ajayi SA, Berjak P, Kioko JI, Dulloo ME and Vodouhe RS (2006) Response of fluted pumpkin (*Telfaria occidentalis* Hook. f.) seeds to desiccation, chilling and hydrated storage.	South African Journal of Botany 72, 544-550	Country partners	nil
Journal	Ajayi SA, Berjak P, Kioko JI, Dulloo ME and Vodouhe RS (2006) Observations on *in vitro* behaviour of the zygotic axes of fluted pumpkin	African Journal of Biotechnology 5, 1397-1401	Country partners	nil
Journal	Berjak P (2006)Unifying perspectives of some mechanisms basic to desiccation tolerance across life forms	Seed Science Research 16, 1-15	Country partners	nil
Journal	Berjak P (2006) The challenge of recalcitrant germplasm cryopreservation	Journal of Horticultural Science and Biotechnology 81, 781-782	Country partners	nil
Published conference proceedings	Berjak P and Pammenter NW (2007) Recent progress towards the understanding of desiccation tolerance	In Seeds: Biology, Development and Ecology. CABI, UK. Pp 17-27	Country partners	nil
Published conference proceedings	Eggers S, Erdey D, Pammenter NW and Berjak P (2007) Storage and germination responses of recalcitrant seeds subjected to mild dehydration	In Seeds: Biology, Development and Ecology. CABI, UK. Pp 85-92	Country partners	nil
Practical guidebook chapter	Pritchard HW, Nadarajan J (2007, submitted),	pp 488-504 in Plant Cryopreservation, Reed, B.B.M. (Ed.), 2008, ISBN 978-0-387-72275-7, due January 2008, Springer Verlag	h.pritchard@kew.org	nil
Newsletter	Berjak P, Pritchard HW	'Science in Africa', SAMARA Issue 12, page 2 (2007); url:	h.pritchard@kew.org	nil

Type * (eg journals, manual, CDs)	Detail (title, author, year)	Publishers (name, city)	Available from (eg contact address, website)	Cost £
		http://www.kew.org/msbp/scitech/publications/samara.htm		

3.4 Progress towards the project purpose and outcomes

See 3.1 and 3.2 above

3.5 Progress towards impact on biodiversity, sustainable use or equitable sharing of biodiversity benefits

Currently not applicable

4. Monitoring, evaluation and lessons

The purposes of this project are basically two-fold – to develop technologies for the cryo-conservation of the germplasm of species producing recalcitrant seeds, and to build capacity in sub-Saharan Africa in this field. Monitoring is thus basically assessing the direct products in terms of species successfully cryopreserved, and the and the number of students undergoing or completed training. The students undertaking the Honours level Cryobiology module were asked to evaluate the module. Their comments were generally positive, but they did suggest more hands-on experience would be useful. This has been incorporated into the module that will be delivered in the second half of 2007 (see Annex 3)

5. Actions taken in response to previous reviews (if applicable)

The only serious criticism in the Y1 report concerned the dissemination of the project and its outcomes. Although there is no formal publicity plan other than the items indicated in the original logframe, in order to respond to this criticism a display was set up at the African Union/Economic Commission for Africa Science and Technology Exposition held in parallel with the African Union Heads of State meeting in Addis Ababa, January 2007 (see point 10, below). However, more does need to be done to raise the profile of the project among African biodiversity and conservation research organisations. In this regard, good contacts have been established with the Tanzanian Tree Seed Centre, the University Ile-Ife, Nigeria and the University of Ghana, Legon.

A request was made for more information on the Honours level Cryobiology module. This is attached as Appendix 3.

6. Other comments on progress not covered elsewhere

7. Sustainability

Three mid-career scientists – Dr J Kioko, Dr J Wesley-Smith and Prof D Mycock are actively involved in the project. From a successor point of view there should be no problems with sustainability. Additionally, contacts have been made with the cryobiology section of the National Zoo with the long term objective of establishing a national cryobank for indigenous and endangered species, plant and animal

8. Dissemination

Four journal articles and two refereed conference papers have been published. Also a newsletter article has been published (see below) and a 'recipe' chapter on seed cryopreservation is accepted for publication in 2008.

The project was given considerable exposure during the 5th International Workshop on Desiccation Tolerance and Sensitivity of Seeds and Vegetative Tissues, Drakensberg, South Africa, January 2007. The screening aspects of the project were managed and presented by Mr Deon Erdey, who was tragically killed in an accident shortly after the Workshop.

The project was highlighted at the African Union/Economic Commission for Africa Science and Technology Exposition held in parallel with the African Union Heads of State meeting in Addis Ababa, January 2007. Details of this are covered in 'Science in Africa', SAMARA Issue 12, page 2 (2007); url: http://www.kew.org/msbp/scitech/publications/samara.htm

9. .

10. OPTIONAL: Outstanding achievements of your project during the reporting period (300-400 words maximum). This section may be used for publicity purposes

We agree for ECTF and the Darwin Secretariat to publish the content of this section

CCESSA, together with the Darwin Initiative and the Millennium Seed Bank Project, were given considerable exposure at the African Union/Economic Commission for Africa Science and Technology Exposition, which was held in parallel with the African Union Heads of State meeting in Addis Ababa, January 2007. In excess of 2000 delegates attended the exposition and visitors to the stand included the South African President, and the South African Ministers of Science and Technology, and of Foreign Affairs, as well as other African leaders. The stand was run by Ms Nomali Ngobese and Ms Ashika Jaimangal, undergraduate and M.Sc. student, respectively. The CCESSA management team were unable to attend because of commitments at the Triennial Desiccation Workshop in RSA, and we are delighted by the professional manner in which the students acquitted themselves and represented the university, CCESSA (DI) and the MSBP. The display was one of a few selected to present a special showing to the representatives of the Gates Foundation. The display and the travel and subsistence costs of the student presenters were sponsored by the South African Department of Science and Technology.

Annex 1 Report of progress and achievements against Logical Framework for Financial Year: 2006/07

Project summary	Measurable Indicators	Progress and Achievements April 2006 - March 2007	Actions required/planned for next period
Goal: *To draw on expertise relevant to biodiversity from within the United Kingdom to work with local partners in countries rich in biodiversity but constrained in resources to achieve* *The conservation of biological diversity,* *The sustainable use of its components, and* *The fair and equitable sharing of the benefits arising out of the utilisation of genetic resources*			*(do not fill not applicable)*
Purpose The establishment of a Centre of Excellence for cryo-banking for sub-Saharan Africa. The development and embedding of 'generic technologies' for *ex situ* collection, storage and utilisation of plant species producing recalcitrant seeds.	Number of requests for research and training placements at CCESSA from African students and from elsewhere. Inward investment (grants) in CCESSA from national and international agencies. Techniques / technologies applied to non-target species by other groups	Presentation at AU/ECA Heads of State Meeting, Addis Ababa, Jan 2007 Several species screened for storage behaviour. Several monocotyledonous herbaceous species of the Amaryllidaceae successfully cryopreserved	Continue attempts to make contact with appropriate AU/NEPAD groups Attempt to reduce damage when cotyledons excised from embryonic axis
Output 1. Recalcitrant-seeded species in cryo-storage (conserved) and utilisable through propagation and 'extension' activities.	Facility up and running and handling > 15 difficult to store (conventionally) species in 3 years, with 5 species reaching the nursery stage *ex vitro*.	Progress on screening for storage behaviour good; some difficulty experienced with dicotyledonous seeds	

Activity 1.1 Screen seeds for storage behaviour		Progressing satisfactorily – in advance of logframe measurable outputs
Activity 1.2, Attempt cryopreservation of embryonic axes of recalcitrant seeds		Some success, but need to attempt to overcome problem of excision damage and to consider use of alternative explants
Output 2. Staff and students (particularly from Africa) trained in cryo-biology (both on 6 week honours and post-graduate courses).	Over 3 years, > 10 post-docs and / or graduate students (MSc to Post-doc) given specialised training (6 training weeks per year) and / or research project guidance (continuous, throughout project).	Training aspect good with respect to local students, but need to attract students from the rest of Africa
Activity 2.1. Establish, run and assess B.Sc. (Hons) Cryobiology module		Module up and running, but needs some minor amendments
Activity 2.2. Attract post-graduate students		Good progress with local students; we are just about at the limit of how many students we can handle
Output 3. Cryo-preservation technologies refined, through research and made available.	(Y3) Cryo-preservation modules released as hardcopy / electronically, following review of market need; (Y2) 4 publications submitted to ISI accredited journals	Difficulties are being experienced with dicotyledonous, non-endospermous seeds so it is premature to consider a recipe-style manual. Course outline and reading matter for the Cryobiology Honours level course can be made available on the web. Not all publications listed acknowledge the Darwin Initiative as the work was completed prior to the award of the grant. Four papers have been submitted on work undertaken with DI funding; these acknowledge the DI

Activity 3.1 Establish other explants as potentially cryopreservable material		Different types of explants from a number of species successfully established
Activity 3.2 Attempt to reduce damage associated with cotyledon excision		Data suggest that this damage is free-radical mediated. Excision techniques using anti-oxidants are being investigated
Output 4 Long term *ex situ* species conservation strategies developed and implemented.	> 45 species collected and evaluated for desiccation tolerance over 3 years; any conventionally bankable species conserved in the Millennium Seed Bank.	Good progress made with screening. Refinement of cryopreservation protocols underway
Activity 4.1 See activities 1.2, 3.1 and 3.2		

Annex 2 Project's full current logframe

Project summary	Measurable Indicators	Means of verification	Important Assumptions
Goal: To draw on expertise relevant to biodiversity from within the United Kingdom to work with local partners in countries rich in biodiversity but poor in resources to achieve • the conservation of biological diversity, • the sustainable use of its components, and • the fair and equitable sharing of benefits arising out of the utilisation of genetic resources			
Purpose The establishment of a Centre of Excellence for cryo-banking for sub-Saharan Africa. The development and embedding of 'generic technologies' for *ex situ* collection, storage and utilisation of plant species producing recalcitrant seeds.	Number of requests for research and training placements at CCESSA from African students and from elsewhere. Inward investment (grants) in CCESSA from national and international agencies. Techniques / technologies applied to non-target species by other groups.	Univ. KZN Annual Report; Independent audit reports, e.g. by IPGRI; NRF Annual Report; RBG Kew / MSBP Annual Report. Peer-review papers and other forms of scientific articles / reports.	Institutional support is sustained, resources are not limiting to delivery, and partnerships continue. New protocols are seen as a valuable component of CBD-related conservation action; students / staff apply knowledge routinely on return to their institutes.
Outputs Recalcitrant-seeded species in cryo-storage (conserved) and utilisable through propagation and 'extension' activities.	Facility up and running and handling > 15 difficult to store (conventionally) species in 3 years, with 5 species reaching the nursery stage *ex vitro*.	University KZN records; database entries, and greenhouse and/or field evaluations of performance of plants established from cryo-preserved explants.	Protocols developed are effective and serve as 'exemplars' for other stakeholders. Sufficient material can be made available to sustainable utilisation projects.
Staff and students (particularly from Africa) trained in cryo-biology (both on 6 week honours and post-graduate courses).	Over 3 years, > 10 post-docs and / or graduate students (MSc to Post-doc) given specialised training (6 training weeks per year) and / or research project guidance (continuous, throughout project).	UKZN Science Faculty handbook; Review of successfully completed student theses.	Wide interest by staff / students across Africa for training. Theses available for consultation.
Cryo-preservation technologies refined, through research and made available.	(Y3) Cryo-preservation modules released as hardcopy / electronically, following review of market need; (Y2) 4 publications submitted to ISI-accredited	Review IPGRI list of publications / Kew - MSBP web site; Consult reprints/ preprints of publications submitted and review journal	Optimisation of methods is possible. Information as presented meets stringent publication requirements.

	journals.	contents pages.	
Long term *ex situ* species conservation strategies developed and implemented.	> 45 species collected and evaluated for desiccation tolerance over 3 years; any conventionally bankable species conserved in the Millennium Seed Bank.	Data entered into project data base and, once verified, into the Seed Information Data base on the WWW.	Data standards are to international standard and information is used by appropriate agencies e.g. IUCN, IPGRI.

Annex 3 onwards – supplementary material (optional)

B.Sc. (Hons): CRYOBIOLOGY

Main areas of research

1. Natural resistance to freezing: hibernation, cold acclimation (e.g. commercially relevant crops)
2. Short or long term conservation of genetics resources
    a. Genetic 'catalogue' or reference
    b. Breeding purposes
3. Pharmaceutical Industry: Stability of freeze-dried products (e.g. antibiotics)
4. Medicine
    a. Storage of blood, organs and embryos
    b. Preservation of cell lines *in vitro*
    c. Cryosurgery in the treatment of e.g. cancer

Course Objectives

1. To develop understanding of how biological cells, tissues, organs respond to freezing temperatures

2. To understand how the cellular environment, and the cooling and warming conditions, can be manipulated to obtain the desired result
    a. Desired Result?
        i. reliable preservation of genetic repertoire (heterogeneous material)
        ii. preservation of 'sufficient' material for later use (e.g. homogeneous / clonal material)
        iii. damage-free preservation (e.g. medicine)
    b. Manipulation
        i. Controlling whether ice forms or not
        ii. Where it forms, and final size attained
            1. Intracellular viscosity: dilute, syrup/rubber, vitreous state
            2. Cooling rate: 'fast' *vs.* 'slow' cooling. (*Relative to what?*)
            3. Sample Properties
                a. size
                b. water content
                c. geometry
                d. developmental status
                e. main type of storage reserve
                f. vacuolation
                g. etc.

3. Assessment of cryopreservation success or failure

4. Applying the lessons learned in one system to another
    a. Empirical *vs.* systematic approaches

SYLLABUS

- Biophysical properties of water and aqueous solutions
    - The unique properties of water
    - Freezing point depression
    - Vitrification
    - Phase diagrams

- Membrane composition and permeability
    - Diffusion in different cell types

- Cooling rates, nucleation and ice crystal distribution
    - The relative meaning of slow, intermediate and fast freezing
    - Supercooling, homogeneous *vs.* heterogeneous nucleation
    - Propagation of ice through cells and tissues; sub-cellular compartments

- Mechanisms of freezing damage in respect of cooling rates
    - Slow-cooling damage; solution effects
    - Intracellular mechanical freezing damage
    - Chilling damage; Phase transitions

- Principles of cryoprotection
    - Freezing sensitivity / hardiness of various cells and tissues
    - Penetrating *vs.* non-penetrating cryoprotectants
    - Loading / off-loading
    - Toxicity

- Recovery from cryostorage
    - Dependence of thawing rates on previous thermal history
    - *In vitro* techniques
    - Assessment of survival
    - Hardening and dissemination of germplasm

- Surviving low temperature in nature
    - Permafrost
    - Chilling injury by snow / frost

PRACTICALS

- Measuring freezing point depression of various cryoprotectants solutions
- Cryo-microscopy:
    - Observing ice propagation in living tissues
    - SEM observation of ice crystal 'ghosts' in freeze-dried material cooled at different rates
- Cryopreservation of plant material at different cooling rates, and assessment of *in vitro* survival after recovery

Checklist for submission

	Check
Is the report less than 5MB? If so, please email to Darwin-Projects@ectf-ed.org.uk putting the project number in the Subject line.	Yes
Is your report more than 5MB? If so, please advise Darwin-Projects@ectf-ed.org.uk that the report will be send by post on CD, putting the project number in the Subject line.	No
Do you have hard copies of material you want to submit with the report? If so, please make this clear in the covering email and ensure all material is marked with the project number.	These will be posted
Have you completed the Project Expenditure table?	Yes
Do not include claim forms or communications for Defra with this report.	OK