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EX SITU CONSERVATION OF BRYOPHYTES: PROGRESS AND POTENTIAL OF A PILOT PROJECT

Jennifer K. Rowntree & Margaret M. Ramsay

Micropropagation Unit, Royal Botanic Gardens, Kew, Richmond, Surrey, TW9 3AB, U.K.

Abstract: A pilot project was set up to investigate the potential use of *ex situ* techniques for the conservation of bryophytes by the Royal Botanic Gardens, Kew and the U.K. statutory conservation agencies (English Nature, Scottish Natural Heritage and Countryside Council for Wales) in August 2000. Successful protocols have been established for the initiation of plants collected from their natural habitats into axenic culture from sporophytes, gemmae and leafy gametophores. Methods have also been established for the long-term storage of explants in liquid nitrogen (cryopreservation). The existing collection incorporates a number of British Red List species, and includes some European Red List taxa. Current work focuses on expanding the number of species in the collection, both in culture and in cryo-storage. Protocols for the weaning and introduction of bryophytes into their natural habitats after cryopreservation are under development and a priority of the project.

INTRODUCTION

Bryophytes are ubiquitous in the terrestrial environment and play important roles in ecosystem function e.g. sequestering nutrients, retaining water, regulating the soil micro-environment and acting as carbon sinks (Vitt, 2000). Concerns have been growing about the vulnerability of and the increased need to conserve many rare and threatened bryophytes (Hällingbäck & Hodgetts, 2000).

In August 2000, a pilot project for the *ex situ* conservation of endangered UK bryophytes was launched at the Royal Botanic Gardens, Kew with the appointment of a dedicated bryophyte conservation officer. The project, a collaboration between the Royal Botanic Gardens, Kew and the UK statutory conservation agencies (English Nature, Scottish Natural Heritage, Countryside Council for Wales), emphasised the development of *ex situ* techniques as a complement to, rather than a replacement for *in situ* conservation efforts (Ramsay & Burch, 2001).

The initial project phase ran for three years and concentrated on the development of standard methods for the collection, sterilisation, culture and cryopreservation of bryophytic material. The resultant protocols are presented herein. Funding for the work was extended for a further three years in December 2003. The current emphasis of the project is to expand the living and cryopreserved bryophyte collections, with the aim of incorporating more designated European species. Methods for the introduction of species back into their natural environments are also under development.

COLLECTION

Three protocols have been produced for the collection of a) desiccation tolerant mosses, b) desiccation intolerant mosses and leafy liverworts and c) thalloid liverworts and hornworts and disseminated to personnel collecting material for the project. There is an emphasis on limiting detrimental effects on the *in situ* population and collecting representative genetic samples.

General protocol

Collection should only be undertaken by authorised individuals and the appropriate scientific personnel should be contacted prior to collection to ensure that the bryophytic material can be processed as soon as it is received.

Sporophytic material is preferred (mature for leafy bryophytes, immature for thalloid bryophytes), although gametophytic material (gametophores, thalli and gemmae) should also be collected to aid with verification and allow a herbarium specimen to be retained. In addition, protocols have been developed for the sterilisation, and production of protonema from gametophores, thalli and gemmae alone.

Collection of 20 gametophore stems for small plants (<1 cm), less for larger plants, is recommend for leafy species. Stems should be collected from different areas of the cushion or population to minimise the formation of gaps (and potential for invasion into the colony by other species) and to maintain the level of genetic diversity in the sample. Collection of five 1.5-2 cm^2 thalli pieces is recommended for thalloid bryophytes, but smaller pieces can be collected if necessary (CPC, 1991). Sporophytes should generally be detached from gametophores and sent in separate packages, but at least one sporophyte should be sent still attached to the gametophore for retention as a herbarium specimen.

Material should be packaged and sent according to its classification. Desiccation tolerant species should be air dried and wrapped in absorbent paper (e.g. newsprint). If there is a delay greater than seven days between collection and delivery, the packages should be placed in a sealed plastic bag containing a small amount of silica gel. Desiccation intolerant leafy bryophytes should be moistened and dispatched in sealed plastic bags or in a sturdy sealed

container (e.g. old film canister). It is essential that these are processed within three days of collection. Thalloid bryophytes must remain hydrated during transit as regeneration of desiccated samples is severely impaired (J. Burch, unpublished data). These should be placed in a sturdy sealed container with either a small amount of water or a wet tissue to maintain hydration, and protected well during transit.

All material should be sent with the following minimum information: species name, collection site, substratum, donor and verifier names and a six figure grid reference, ideally from a GPS unit. This information allows database records on the species in the collection to be kept as informative and up to date as possible.

INITIATION INTO CULTURE

Bryophytes are grown preferentially in axenic culture in the *ex situ* collection. Although unnatural, axenic culture provides a more uniform and secure method of maintaining plants in a living collection i.e. free from contaminants (Bhojwani & Razdan, 1996). Axenic culture is also an advantage if plants are to be stored long-term in liquid nitrogen as they can be readily overwhelmed by contaminants during recovery (personal observation). Material guaranteed contaminant-free is easier to transport across international borders (The Plant Health (Great Britain) Order, 1993), and has advantages over non-axenic material for use in genetic analyses (R. Cowan *pers. comm.*).

Sterilisation protocols have been developed for a) sporophytes, b) leafy gametophores, c) gemmae and d) thallus tissue using the sterilising agent Sodium dichloroisocyanurate and without the addition of detergents.

General protocol

Material is harvested and washed in deionised water to remove any adhering substratum. All further work is carried out under sterile air on a laminar flow bench. Bryophytic material is submerged in the sterilising solution at room temperature, without the addition of any detergents and agitated for a set period of time. Sporophytes are submerged in 1% Sodium dichloroisocyanurate solution for 3-6 min, leafy gametophores in 0.5% solution for 2-5 min, gemmae in 0.1% or 0.5% solution for 2-8 min and thallus tissue in 0.01% solution for 10-20 min. Material is then removed from the sterilising solution and washed twice in sterile deionised water. Material is dried on sterile filter paper and fragmented onto a 5 cm Petri dish containing standard media. Plates are sealed with micropore tape rather than parafilm, to allow for greater gas exchange. Material is monitored at 2-3 daily intervals initially, for 2-3 months and uncontaminated material is transferred away from any contamination. Species are classed as growing in axenic culture if the material remains clean and is actively growing approximately three months after the initial sterilisation procedure.

All species in the *ex situ* collection are grown on ½ MS, ¼ MS (Murashige & Skoog, 1962) or double concentration Knops minimal media (Basile & Basile, 1988), without sucrose and using Gelrite™ (gellan gum) as the gelling agent (J. Burch, unpublished data). Plants are grown either in a growth room at a temperature of 20º C (±5º C) and at a light intensity between 4-35 $\mu Molm^{-2}s^{-1}$ or in an incubator with a temperature of 16º C (±2º C) and at a light intensity between 3-15 $\mu Molm^{-2}s^{-1}$. Lower concentrations of Sodium dichloroisocya-nurate for longer immersion times are used if sterilisation is initially unsuccessful.

CRYOPRESERVATION

The *ex situ* project aims to provide long-term basal storage of rare bryophyte material for use in future conservation programmes. Material that is continually sub-cultured to maintain a living collection is likely to lose genetic diversity over time and become adapted to growing in culture conditions (Lynch, 2000). This is particularly problematic for material retained for conservation purposes where reintroduction to its natural habitat is a long-term objective. Cryopreservation, the storage of living material at -196º C in liquid nitrogen, has been shown to be useful for the long-term storage of many plants (see Engelmann, 2004; Pence, 2004 and references therein). Genetic diversity of the stored material is easier to maintain as the material is held in a state of suspended animation (Karlsson & Toner, 1996). A standard protocol has been developed for the cryopreservation of protonemal material as sporophytes are not routinely available for all bryophyte species (Hill *et al.*, 1992, 1994). Protocols are under development for the cryopreservation of leafy gametophores.

General protocol

All work is undertaken under sterile air in a laminar flow cabinet. Small (approximately 1 mm^2) plugs of protonemal material are encapsulated in 3% sodium alginate strips, solidified with 100 mM Calcium chloride solution for 10 minutes (see Burch & Wilkinson, 2002 for details). The alginate strips containing the bryophytic material are placed in a Petri dish containing suitable growth media and left in normal growing conditions (see above) to recover for one week. The strips of encapsulated material are then transferred onto growth media containing 10 µM ABA (Abscissic acid) and 5% sucrose to protect the plant tissues during dehydration and freezing (Burch & Wilkinson, 2002). After two weeks the alginate strips are removed from the growth media and dried for 6-7 hours under a sterile air flow. The dried strips are placed into sterile cryovials with sealable lids and frozen rapidly, by immersion in liquid nitrogen (-196º C). Once frozen, the vials are transferred into a Dewar for long-term storage. Survival of all material stored is monitored routinely.

SPECIES	STATUS	WORLD RED LIST	EUROPEAN RED LIST	BRITISH RED LIST
Aplodon wormskjoldii (Hornem.) Kindb.	AX			CE
Bartramia stricta Brid.	AX, CY			CE
Cyclodictyon laetevirens Mitt.	AX		R	E
Ditrichum cornubicum Paton	AX, CY	CE	E	E
Ditrichum plumbicola Crundw.	AX		V	NT
Jamesoniella undulifolia (Nees) Müll. Frib.	NA	V	E	E
Leptodontium gemmascens (Mitt. ex Hunt) Braithw.	AX, CY		R	V
Micromitrium tenerum (Bruch & Schimp.) Crosby	AX		V	CE
Orthodontium gracile Schwägr. ex Bruch, Schimp. & W. Gümbel	AX, CY		E	V
Orthotrichum obtusifolium Brid.	NA			E
Orthotrichum pallens Bruch ex Brid.	AX			E
Seligeria carnicolica (Breidl. & Beck) Nyholm	AX			CE
Tortula cernua (Huebener) Lindb.	AX			E
Weissia multicapsularis (Sm.) Mitt.	AX		E	E
Weissia rostellata (Brid.) Lindb	AX		R	NT
Zygodon forsteri (Dicks.) Mitt.	AX		V	E
Zygodon gracilis Wilson	AX		V	E

Table 1. Number of species and their status in the *ex situ* collection (*Species* after Blockeel & Long, 1998; *World Red List* after IUCN, 2000; *European Red List* after ECCB, 1995; *British Red List* after Church *et al.*, 2001). **AX**: Axenic; **NA**: Non Axenic; **Cy**: Cryo; ; **R**: Rare; **NT**: Near threatened; **V**: Vulnerable; **E**: Endangered; **CE**: Critically Endangered.

FUTURE WORK

The *ex situ* collection is continually expanding (see Table 1 for current species held), and the aim over the next two years is to incorporate more bryophyte species of European importance. Material is being systematically cryopreserved and the development of cryopreservation methods is ongoing. Material from the collection of the moss *Orthodontium gracile* Schwägr. ex Bruch, Schimp. & W. Gümbel is currently undergoing analyses into the genetic diversity of the populations held, and the effects of cryopreservation on the cytoskeleton structure. Trials are also underway to investigate possible methods for the introduction of bryophytic material from the collection into its natural environment.

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