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Experimental investigations into the feasibility of *ex situ* preservation of palm seeds; an alternative strategy for biological conservation of this economically important plant family

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Given the widespread belief that the conservation of palms, especially the large-trunked species, is only accomplished through *in situ* preservation or in plantations, this paper explores the feasibility of a third approach, i.e. cryogenic preservation of their seeds *ex situ*. Seeds of the following palm species were subjected to air-drying to assess their tolerance of dessication: *Washingtonia filifera* (L. Linden) H. Wendl.; *Sabal mexicana* Mart. (syn. S. texana; Zong, 1990); *Schippia concolor* Burret; *Orbignya cohune* (Mart.) Dahlgren ex Standley; *Acoelorraphe wrightii* (Griseb. & H. Wendl.) H. Wendl. ex Becc.; *Desmoncus orthacanthos* Mart.; *Attalea crassispatha* (Mart.) Burret; *Zombia antillarum* (Desc.) L.H. Bailey; *Pinanga malaiana* (Mart.) Scheff.; *Pinanga aff. polymorpha* Becc.; *Daemonorops verticillaris* (Griff.) Mart. Of these, only two (*W. filifera* and *S. mexicana*) survived drying to moisture contents around 5% (fresh weight basis). Seeds of the remaining spp. would be difficult or impossible to conserve *ex situ* in seedbanks or cryostores. Data are presented to show that the response of *O. cohune* embryos to drying is similar to other recalcitrant (dessication intolerant) seeds, while seeds of *A. wrightii* may belong to an intermediate seed storage category with limited tolerance of drying. The results are discussed in relation to the inadequacy of current knowledge as a basis for decisions on the broad scale *ex situ* conservation of palm germplasm.

Keywords: palms; seeds; preservation; desiccation; survival

Introduction

Palms are amongst the most economically important plant families throughout their extensive distribution in the tropics and subtropics. As well as the familiar examples such as coconut and oil palm, currently important in commerce, there are hundreds of other palm species of economic utility (Balick and Beck, 1990). The conservation and effective utilization of palm germplasm is beset by the same problems facing other tropical families. *In vivo* collections, both in the wild and in repositories, are subject to diseases, bud mutations, human encroachment and poor management, as well as being expensive in terms of management inputs and space. The application of *in vitro* methods of *ex situ* conservation has several associated technical problems, including the relatively frequent

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need for subculture and the fact that a single protocol is unlikely to be useful to diverse genotypes (Towill and Roos, 1989).

The storage of seeds at low moisture contents (air-dry) and at low (sub-zero temperatures offers a convenient and cost-effective means of conserving plant genetic resources in the medium and long term, and is the basis of the operation of a number of seed banks worldwide, in which useful seed storage lives are expected to be of the order of at least many tens and probably many hundreds of years (Roberts, 1989). Success of this technology depends upon the ability of seeds to withstand drying to quite low moisture contents (< 10% on a fresh weight basis). Low seed moisture content not only removes the possibility of damage due to freezing injury in subsequent deep-freeze (−20° C) or cryogenic (e.g. liquid nitrogen, −196° C) storage, but also itself promotes, along with reduced temperature, increased longevity in orthodox seeds (Roberts, 1973).

Another group of plants, including several important tropical plantation species, bear seeds classified as recalcitrant (Roberts, 1973). The main characteristics of such seeds is their inability to withstand any degree of desiccation and so they cannot be kept at sub-zero temperatures. Their lifespans are relatively short and cannot be extended by the methods presently employed to preserve germplasm in seedbanks or cryogenic stores.

It seems that many palm species are propagated primarily by seed (Broschat and Donselman, 1988); and, although Corner (1966, p 183) maintains that palm seeds in general cannot withstand any degree of drying, the seeds of several species (e.g. date palm) are amenable to storage at freezing temperatures in the air-dry state (Al-Madeni and Tisserat, 1986). However, there are also examples, such as coconut, of recalcitrant seed behaviour among the Palmae (Chin and Roberts, 1980), as well as at least one instance of apparently recalcitrant seeds (oil palm) being subsequently shown to be desiccation tolerant under certain conditions (Grout *et al.*, 1983; Ellis *et al.*, 1991). This paper presents data from a study aimed at classifying seed storage behaviour in a wide range of palm species, mostly having little or no previous work reported, with a view to obtaining a reliable estimate of the proportion having seeds amenable to seed-banking or cryogenic storage.

Materials and methods

Seeds of eleven palm species were collected from sites in Central and South America and South-East Asia, as shown in Table 1, and sent to Wakehurst Place for investigation. The opportunistic nature of the collections meant that samples were often low in numbers of seeds, which were sometimes also in poor condition on arrival. Thus, the difficulties (including sporadic fruiting, obtaining export permits and the time taken to transport seeds to the laboratory) inherent in working with tropical trees precluded a rigorous experimental and quantitative analysis of all the palm seed samples received.

On arrival, fruits and seeds were stored moist in ventilated polythene bags at 16° C for up to one week before experimental treatments were begun. Where necessary, fleshy pericarps were removed by washing in tapwater. In *Orbignya cohune* the substantial hard endocarps were removed by progressive and careful application of pressure in a large engineer's vice, with further pressure splitting the seed to permit removal of the embryonic axis.

In the control lots, seeds or embryos were set to germinate without any drying, i.e. at the relatively high moisture contents at which they arrived. Seeds or embyros were dried

Table 1. List of palm species whose seeds were air-dried to low moisture contents, together with the geographical sources of the seed samples

Species	*Source*
Acoelorraphe wrightii (Griseb. & H. Wendl.) H. Wendl. ex Becc.	Hattieville, Belize District, Belize
Attalea crassispatha (Mart.) Burret	Haiti
Daemonorops verticillaris (Griff.) Mart.	Kepong, Selangor, W. Malaysia
Desmoncus orthacanthos Mart.	Cayo District, Belize
Orbignya cohune (Mart.) Dahlgren ex Standley	Cayo District, Belize
Pinanga malaina (Mart.) Scheff.	Pahang, West Malaysia
Pinanga aff. polymorpha Becc.	Pahang, West Malaysia
Sabal mexicana Mart. Becc.	San Antonio, Texas, USA
Schippia concolor Burret	Cayo District, Belize
Washingtonia filifera (L. Linden) H. Wendl.	San Antonio, Texas, USA
Zombia antillarum (Desc.) L.H. Bailey	Fairchild Tropical Garden, Florida, USA

to low moisture content in a room at 15% relative humidity and 15° C, in monolayers for periods up to four weeks. Throughout this paper moisture contents are quoted on a % fresh weight (f.wt.) basis; and they were measured gravimentrically, by weighing samples (whole seeds were quartered) before and after drying at 103 ± 2° C for about 17 h. Equilibrium relative humidities were measured using a Michel S-4020 dewpoint hygrometer (Michel Instruments Ltd, Cambridge, UK).

Germination tests consisted of incubating seeds (and embryos in the case of *O. cohune*) on 1% (w/v) distilled water agar in either 9 cm polystyrene Petri dishes or polystyrene sandwich boxes; in incubators maintained at 26° C, or fluctuating diurnally (33/19° C) with a 12 h thermoperiod, illumination on a 12 h photoperiod being provided by 'warm-white' (Sylvania) fluorescent tubes. Incubation was continued until it was obvious that no further germination would or could occur. Palm seed germination is often quite protracted (Loomis, 1958; Ellis *et al.*, 1985) and in this work incubation periods varied from five weeks (*W. filifera*) to more than one year (*A. wrightii*). The tests were monitored at regular intervals and germinated seeds (embryo extension > 2 mm) counted and removed. Whenever incipient drying out made it necessary, seeds were re-sown on fresh substrate.

In vitro techniques were also applied to embryos or seeds of *O. cohune*, *D. verticillaris* and the two *Pinanga* species. After extraction from fruits, seeds were disinfected for 2 min in 70% ethanol, followed by 50% bleach for 20 min. They were then rinsed five times in sterile distilled water and embryos were excised under aseptic conditions, and individually cultured on 1 ml of MS (Murashige and Skoog, 1962) medium containing 0.6% agar and 0.25% activated charcoal, in glass tubes sealed with polypropylene covers secured by rubber bands. For disinfection of excised embryos of *O. cohune*, a weaker (10–20%) solution of bleach was used. Cultures were incubated at 26° C in darkness. After 40 days germinating seeds or embryos were transferred to fresh medium and incubated at 29° C with a 12 h photoperiod.

For topographical tetrazolium staining of excised *O. cohune* embryos, they were imbibed on paper towel wetted with distilled water for 24 h at laboratory temperature

(20–22° C) and then incubated in buffered 1% solution of 2,3,5-triphenyl tetrazolium chloride (Moore, 1973) for 24 h at 31° C in darkness. Embryos were scored as viable when they showed an overall even carmine red staining.

Results and discussion

Orbignya cohune

A sample of 36 kg of *O. cohune* fruits yielded about 600 seeds (usually one seed per fruit, but occasionally two and rarely three). The extraction technique using an engineer's vice gave over 90% undamaged seeds and 70–80% undamaged embryos, and was regarded as successful, bearing in mind the toughness of the mesocarp and endocarp.

About half the fruits appeared quite green, presumably having been harvested either at an earlier stage of maturity or closer to despatch than the remainder. Of the seeds from these fruits, the embryos had mean moisture contents of 69%, whereas the endosperm moisture contents were much lower (27%). Nevertheless, the equilibrium relative humidities of both tissues were found to be 97–98% at those moisture contents, indicating markedly different moisture sorption characteristics. Due to the the difficulties involved, initial germination tests were not carried out on the seeds, but tetrazolium staining of a small sample (n = 10) of excised embryos indicated high (100%) viability. The remaining fruits were already brown and the mean moisture contents of embryos and endosperms were 25% and 11% respectively (equilibrium relative humidity for both, 70%), with the fruit tissue at 12%, and vital staining of embryos indicated only 60% viability.

The majority of the fruits (of both ripeness levels) were dried to different levels by holding them in the drying room for periods varying from 1–28 days, giving a range of embryo moisture contents from 69% to 20%, with endosperms (and pericarp tissues) ranging from 27% to 5%. As the embryo moisture content barely fell over the first 7–10 days of drying, it is likely that they were being protected to an extent from desiccation, probably by the surrounding endosperm and fruit tissues; e.g. the embryos from green fruits took 28 days to reach 28% moisture content. With germination tests on whole seeds expected to be protracted and difficult, the excised embryo test (Ellis *et al.*, 1985) was used, in which the survival of healthy isolated embryos under non-sterile conditions is supposed to give a comparatively rapid indication of seed viability. In fact, all embryos rapidly succumbed to microbial infection and this method proved inadequate as a means of assessing viability over the range of moisture contents investigated. However, tetrazolium tests were run on small numbers of embryos (n = 10) at the same time. The results of these (Fig. 1) showed that viability remained unaffected (at 100%) by a reduction in moisture content from 69% to 46%. Below the latter, further reduction in moisture content led to a progressive reduction in viability, until at 20% moisture content it was only 50%.

The remaining green fruits were used in an attempt to monitor *in vitro* growth of isolated embryos (as described in Materials and methods). Of 20 non-dried (45% moisture content) embryos, 17 (85%) showed some extension after seven days incubation, with hypocotyl expansion and some greening following, but no further growth after 41 days. Embryos were dried rapidly (< 24 h) in the dry room to 6% moisture content and of 29 dried embryos, none showed any sign of growth after 142 days of *in vitro* culture. These

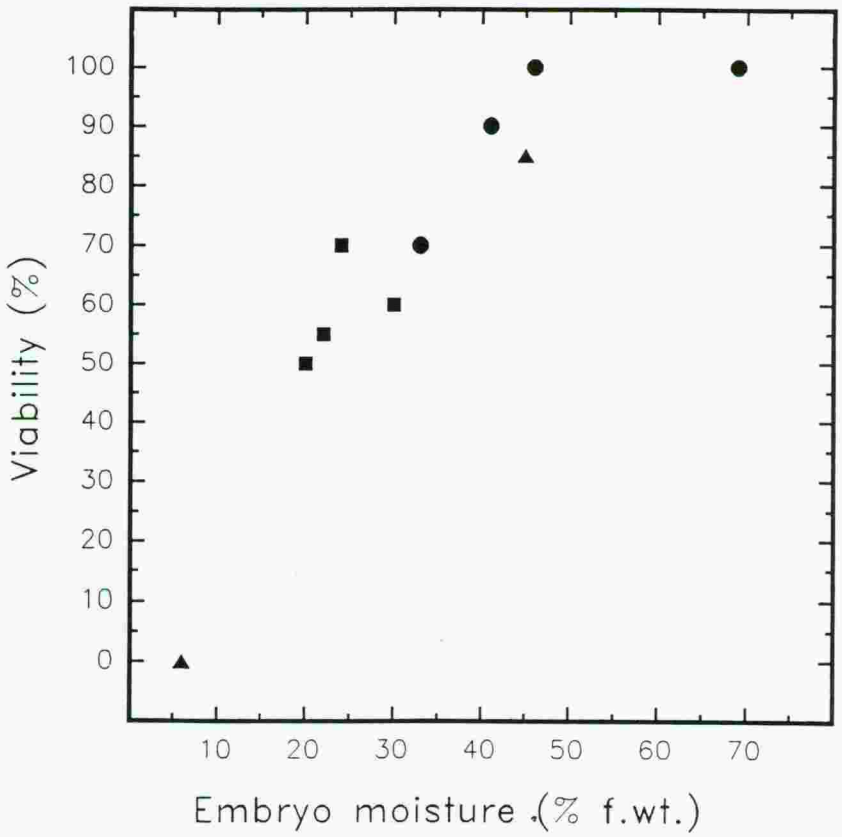

Figure 1. The effect of moisture content (% freshweight (f.wt.)) on the viability (%) of *Orbignya cohune* embryos, as measured by tetrazolium staining of embryos from green (●) or brown (■) fruits, or by *in vitro* survival and growth (▲).

results are also indicated in Fig. 1, for comparison with those obtained by drying whole fruits.

It appears that *O. cohune* axes, and therefore seeds, are not tolerant of drying to the low moisture contents required for successful cryopreservation or seedbank storage. While it is unsafe to rely on data from vital staining alone, and it would be desirable to assess survival at intermediate moisture contents quantitatively using the *in vitro* technique, the overall response to drying by *O. cohune* embryos shown in Fig. 1 is very similar to those of recalcitrant seeds (e.g. Dickie *et al.*, 1991), with reduction in moisture content below a critical value of about 45% leading to viability loss.

Sabal mexicana and Washingtonia filifera

Seed viability in the samples of both these species was high (90–95%) on arrival (with moisture contents of 12–13%), and it was not significantly reduced after drying to moisture contents as low as 4.5%. Moreover, storage at 6° C or −20° C, at any moisture content investigated, did not result in significant losses in viability, except that a small reduction (ca 10%) did occur in *S. mexicana* seeds at 12% moisture content and −20° C,

possibly due to freezing injury. The responses to drying and low temperatures of seeds of these two species were in accord with those reported for *W. filifera* and four other *Sabal* spp. by Al-Madeni and Tisserat (1986); they appear to be orthodox and should pose no problems for seedbank or cryo-storage. Indeed, it appeared from rapid ageing experiments not described here that seeds of *W. filifera* would be relatively long-lived under such conditions; as they showed no significant decline in viability over storage periods which should have removed viability completely from short-lived orthodox seeds, such as those described by Tompsett (1986).

Acoelorraphe wrightii

Whole seeds were either sown on arrival, or after drying to < 5% moisture content for 28 days in the dry room. In both cases germination was sparse, erratic and spread over 15 months. Of 100 fresh seeds, 19 germinated and a further 60 remained healthy and firm at the end of the test (presumed alive, but dormant); while 21 rotted during the test (presumed dead). Control viability may thus be regarded as 79%. Of the 90 dried seeds, only eight germinated, with a further seven presumed dormant, and 75 rotted during the test. Total viability of seeds after drying to 5% moisture content was thus 15/90, or 17%. While there was obviously some survival and germination after drying in seeds of *A. wrightii*, it was significantly reduced in comparison with non-dried seeds and the response to desiccation in this species needs further quantification, possibly employing *in vitro* techniques on excised embryonic axes to speed up viability testing. Seed banking and cryopreservation are likely to be problematical because of the losses inherent in drying seeds prior to freezing.

Attalea crassispatha

After removal from fruits, by careful use of a hacksaw, seeds germinated within two weeks of sowing. Only 14 seeds were available for investigation and of seven sown without further drying, four germinated. The remaining three rotted during the test, as did all seven dried for two weeks, and all were presumed non-viable. It seems likely that no seeds of *A. crassispatha* would survive drying to the moisture content (4.9%) reached in the drying treatment and they would not be appropriate subjects for seed banking or cryopreservation. The viability of the non-dried seeds (57%) was relatively low, and whether this was as a result of drying during shipment (moisture content on arrival, 13.7%), or some other cause is not clear.

Desmoncus orthacanthos, Schippia concolor and Zombia antillarum

Numbers of seeds received of each of these were 20, 16 and 34 respectively. Neither dried or non-dried seeds of any of them germinated, even after many months in test and all eventually rotted. It is not possible from these very limited observations to classify any one of them as recalcitrant, but it seems clear that straightforward field collection, drying and low-temperature storage is unlikely to be successful.

Daemonorops verticillaris

Cleaned whole seeds were sown *in vitro* and of 44 non-dried sown and in good condition after 14 days, 31 germinated over the next 62 days, indicating 70% viability. Of 59 seeds sown after drying to 5% moisture content, none germinated over a period of 84 days.

This result strongly suggests a lack of tolerance low moisture contents in seeds of *D. verticillaris*.

Pinanga malaiana and P. aff. polymorpha

In very small samples of seeds and excised embryos of these two species sown *in vitro*, survival and germination was only observed in freshly arrived non-dried seeds. Drying to 5% moisture content was apparently detrimental.

Concluding remarks

Of eleven species of the Palmae examined so far in this investigation, the seeds of only two (*S. mexicana* and *W. filifera*) appear to be immediately amenable to seedbank storage or cryopreservation as a means of *ex situ* conservation of genetic resources. This finding supports the commonly held view (J. Dransfield, personal communication) that considerable difficulties would attend attempts to conserve most palms *ex situ* as seeds. However, it is in contrast to data extracted from a recent literature search by Hong (1991), in which, of 21 palm species referred to, sixteen were reported as having orthodox seeds. Extrapolating, it could be estimated that only about 24% of palm species would bear seeds difficult to store (cf. 82% from the present study); but the work reported in the literature appears biased towards species from dry habitats (e.g. *Sabal* spp. and *Phoenix* spp.). Indeed, the results of the present study suggest that it is only those species regarded as belonging to dry habitats (*S. mexicana* and *W. filifera*) that bear truly orthodox seeds, easy to store at low temperatures in the air-dry state. In contrast, the remaining species examined here are characteristic of comparatively moist habitats and it appears that the seeds of none of them would be easy to preserve at low temperatures, largely due to their inability to withstand desiccation. Of the difficult seeds some would be truly recalcitrant, while others may belong to an intermediate category, in which a certain level of desiccation is tolerated, but below which loss of viability occurs. Ellis *et al.* (1991) have demonstrated this type of behaviour in seeds of at least one palm (*Elaeis guineensis* N. Jacq.). The work presented here has not allowed clear differentiation between recalcitrant and intermediate seed storage behaviour in the species examined, although the evidence available might point to the seeds of *A. wrightii* being intermediate and those of *O. cohune* being recalcitrant.

The information on seed storage behaviour generated in the present study, together with that compiled by Hong (1991) represents only 31 from a total of over 2600 palm species. As well as being very small, the sample under-represents species from moist habitats, which probably make up the great majority of palm species. Clearly, more work is needed to establish an adequate and unbiased database of palm seed storage characteristics, which could be used to assess the utility of *ex situ* seed storage in individual palm species conservation programmes. In the meantime it may be possible to suggest a rule of thumb, whereby those species of definitely dry habitats are highly likely to bear seeds that are amenable to dry, cold storage, whereas those from relatively moist habitats are likely to be difficult or impossible to store. Even the latter group will contain species with intermediate seed storage behaviour (Ellis *et al.*, 1991), which will allow medium-term preservation of viability in optimum environments. Also, the work of Chin *et al.* (1988) raises the possibility that for species whose seeds are difficult to store, there

are nevertheless *ex situ* conservation possibilities in the cryopreservation and *in vitro* culture of their excised embryos.

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