PROPAGATION OF CYCAD COLLECTIONS FROM SEED: APPLIED REPRODUCTIVE BIOLOGY FOR CONSERVATION

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ABSTRACT

Propagation of cycads from seed can aid their conservation as it helps reduce the demand for wild-collected plants. Seed-produced plants can be used for reintroduction programmes if the parent plants are from known provenance and care is taken to avoid hybridisation. This paper discusses the techniques required for successful seed propagation of cycads, including pollen collection, storage, viability testing, manual pollination, seed collection, storage and germination.

INTRODUCTION

Propagation of cycads from seed is essential for their conservation as it can help reduce the demand for habitat-collected plants (Kay *et al*., 2011). In the 1980s, for example, over 80 tons of the Mexican cycad *Zamia furfuracea* were extracted from the wild for the horticultural industry (Donaldson *et al*., 2003) but this cycad is now widely cultivated and its horticultural demand is met entirely by nursery-propagated plants. Seed-propagated cycads can help supply traditional and horticultural demand for cycads. They can also be reintroduced to habitats to prevent the genetic erosion or extinction of wild populations (Walters, 1999) if the parent plants are well documented with detailed provenance information, and there is certainty that genetic contamination from other cycads has not occurred in the pollination process.

Most cycad species are believed to be pollinated by host-specific insects, typically curculionid (see Fig.1), eroytilid or nitidulid beetles, but some species are pollinated by other types of beetles, thrips or micro-moths (see Table 1). The presence or absence of these pollinators needs to be known and considered when planning to propagate them by seed. If cycad pollinators are absent seed cones must be pollinated by hand. Conversely, if pollinators are present near a cycad collection, special steps may need to be taken in order to avoid unwanted hybridisation from other cycad species.

The procedures involved in pollinating cycads vary among the ten existing cycad genera because of differences in cone morphology and physiology. This paper covers the various procedures involved in propagating these genera, and includes recommendations aimed at reducing unwanted hybridisations in cycad collections. The intention is that this knowledge will help support cycad conservation by promoting their propagation by seed.

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Genus	Pollinator type	Pollinator genus	References
Bowenia	Curculionid beetle	*Miltotranes*	Wilson, 2002
Ceratozamia	Eroytilid beetle	*Pharaxonotha (or near)**	Vovides, 1991 Pérez-Farrera & Vovides, 2004
Cycas	Curculionid beetle Curculionid beetle Eroytilid beetle Eroytilid beetle Nitidulid beetle Tenebrionid beetle Cosmopterygid moth	*Derelomus (Tychiodes)** *Tychiodes* *Hapalips* *Xenocryptus (or near)** *Carpophilus* *Alphitobius* *Anatrachyntis*	Raju & Jonathan, 2010a Tang *et al*., 1999 Forster *et al*., 1994 Tang *et al*., 1999 Kono & Tobe, 2007 Raju & Jonathan, 2010b Marler, 2010
Dioon	Eroytilid beetle Curculionoid beetle	*Pharaxonotha (or near)** *Parallocorynus*	Vovides, 1991 Vovides, 1991
Encephalartos	Curculionid beetle Cucujoid beetle Cucujoid beetle	*Porthetes* *Metacucujus* *undescribed genus*	Suinyuy *et al*., 2009 Suinyuy *et al*., 2009 Suinyuy *et al*., 2009
Lepidozamia	Curculionid beetle	*Tranes*	Hall *et al*., 2004
Macrozamia	Curculionid beetle Thrips	*Tranes* *Cycadothrips*	Terry *et al*., 2004 Terry, 2001
Microcycas	Eroytilid beetle	*Pharaxonotha*	Chaves & Genaro, 2005
Stangeria	Nitidulid beetle Nitidulid beetle	*Carpophilus* *Urophorus*	Proches & Johnson, 2009 Proches & Johnson, 2009
Zamia	Eroytilid beetle Curculionoid beetle	*Pharaxonotha* *Rhopalotria*	Tang, 1987a Norstog *et al*., 1986 Tang, 1987a

Table 1 Probable pollinators of different cycad genera
*Taxonomy updated based on W. Tang (pers. comm).

DISTINGUISHING BETWEEN POLLEN AND SEED CONES

Cycads are dioecious plants, meaning that pollen cones and seed cones always occur on separate plants. Plants bearing pollen cones are known as male plants, whereas those bearing seed cones are considered female plants. Pollen cones are usually narrower and have smaller and more numerous scales than seed cones. The pollen cone scales, known as microsporophylls, bear sacs of pollen known as microsporangia, whereas the seed cone scales, known as megasporophylls, carry ovules which eventually mature into seeds when pollinated (see Fig. 2). As producing seed cones is presumably more energy intensive than producing pollen cones (Tang, 1993c), male plants generally produce cones more often than female plants (Ornduff, 1996). In *Encephalartos*, *Macrozamia* and *Zamia*, male plants will often produce multiple pollen cones per apex, whereas other genera typically produce only a single male cone per stem (Tang, 1989).

Fig. 1 *Rhopalotria mollis* weevils on *Zamia furfuracea* male cone. Photo: Michael Calonje.

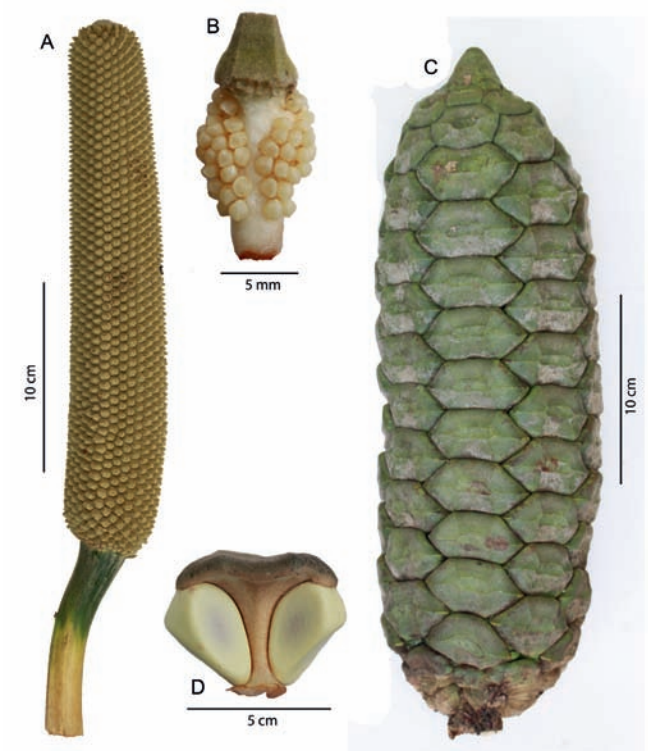

Fig. 2 Reproductive structures of *Zamia encephalartoides*: A) Pollen cone B) Microsporophyll underside, with microsporangia prior to pollen release C) Mature seed cone D) Megasporophyll with mature seeds. Photo: Michael Calonje.

The pollen cones of all cycad genera consist of a peduncle and a cone axis surrounded by spirally arranged microsporophylls which carry pollen-bearing sac-like structures known as microsporangia. While the pollen cones of all cycad genera are quite similar in morphology, seed cones are more varied (see Fig. 3). All cycad genera except for *Cycas* have seed cones made up of a peduncle and cone axis bearing spirally arranged megasporophylls. In *Cycas*, megasporophylls are not borne on a cone axis but rather develop individually from the plant stem much in the way leaves do. The sporophylls are soft and leaf-like in appearance and loosely arranged, forming a more or less open structure often called a pseudocone. Each sporophyll may hold 1–16 ovules whereas the sporophylls of all other genera usually carry only 2 ovules (Grobbelaar, 2002). In *Stangeria* and *Dioon*, scale-like sporophylls are arranged around a cone axis, forming a compact, cone-like shape. The remaining genera have sporophylls that are thick and fleshy.

POLLEN COLLECTION

The process of pollen cone emergence to pollen release will vary in timing and duration between species, and may vary for the same species if grown under different climactic conditions. However, pollen production will usually peak within the same few months each year. When a cone is ready to release pollen it will elongate, the microsporophylls will separate and become lighter in colour and the microsporangia will become visible.

In most species, during pollen release there is a marked temperature increase in the cone accompanied by the volatilisation of fragrances that may function to attract insects (Tang, 1987b, 1993a). The cone temperatures may rise up to 15°C above ambient temperature and this can easily be sensed by hand. The odour released varies among different cycad genera (Tang, 1989). Tang (1989) describes the smell of *Dioon*, *Cycas* and *Microcycas* as "musty and pleasant", the smell of *Bowenia*, *Ceratozamia*, *Stangeria* and most *Zamia* as "fruity and sweet" and the smell of many *Encephalartos* and *Macrozamia* as "resinous".

The shedding process begins once the microsporangia start to break open and this process usually takes from two days to over a week per individual cone, except *Stangeria* which may take up to three weeks to shed its pollen. The life-cycle of an individual pollen cone ends after it sheds its pollen. With the exception of *Stangeria*, the best time to harvest the cone is immediately after the microsporangia begin bursting and releasing pollen. *Stangeria* cones will stop releasing pollen if collected too early, so the cone should be harvested after the first week of shedding. In both cases it is preferable to collect the cone on a dry, non-rainy day to facilitate dry pollination or dry storage. Also, wet pollen is more susceptible to fungal infection (Osborne *et al*., 1992) and can deteriorate quickly. It is important to note that many cycad parts, including pollen, contain potentially dangerous compounds (Norstog and Nicholls, 1997) and may cause allergic reactions that can become more severe with subsequent exposure. It is therefore recommended that a snug-fitting dust mask is used when handling cycad pollen or when assessing male cones for maturity.

Fig. 3 Representative seed and pollen cones for all cycad genera. A) *Bowenia serrulata* seed cone B) *Bowenia spectabilis* pollen cone C) *Ceratozamia decumbens* seed cone D) *Ceratozamia decumbens* pollen cone E) *Cycas couttsiana* seed cone F) *Cycas revoluta* pollen cones G) *Dioon angustifolium* seed cone H) *Dioon angustifolium* pollen cone I) *Encephalartos ferox* seed cone J) *Encephalartos ferox* pollen cone K) *Lepidozamia hopei* seed cone L) *Lepidozamia peroffskyana* pollen cone M) *Macrozamia lucida* seed cone N) *Macrozamia lucida* pollen cone O) *Microcycas calocoma* seed cone P) *Microcycas calocoma* pollen cones Q) *Stangeria eriopus* seed cone R) *Stangeria eriopus* pollen cone S) *Zamia imperialis* seed cone T) *Zamia imperialis* pollen cones. Photos: All Michael Calonje except K: Larry Krauss and N: Irene Terry.

Male cones are harvested by cutting them from the base of the peduncle, taking care not to tip or shake them violently so that no pollen is lost. Excised cones should then be placed on a collection surface such as smooth paper in a cool, dry, wind-free

Fig. 4 Collection of pollen from *Zamia pseudoparasitica*. Photo: Michael Calonje.

environment. The pollen can then be collected every couple of days and should be placed in non-porous paper envelopes labelled with information such as the date of collection, species name and an identifier for the host plant (see Fig. 4).

POLLEN STORAGE

The ability to store pollen is useful when attempting to propagate cycads because fresh pollen of a particular species is not always available when seed cones are receptive. Stored pollen is also useful as a form of germplasm preservation. It assures that even if the parent plant dies, some of its genetic information is preserved and can still be passed on to offspring if used for manual pollination. Stored pollen can be exchanged with other growers or institutions that may need it, and can also be useful for pollen research or for plant breeding or hybridisation experiments. Few studies on the longevity and storage of cycad pollen have been conducted (see Tang, 1986a; Osborne, 1989; Osborne *et al*., 1991, 1992), but all seem to indicate that cycad pollen viability can be extended by reducing its moisture content and then storing it in cold temperatures. However, there does not seem to be an advantage in reducing oxygen content during pollen storage, as an experiment using pollen from four species of *Encephalartos* and *Cycas thouarsii* (Osborne *et al*., 1991, 1992) concluded that pollen retained higher viability in an ambient atmosphere than in an inert nitrogen atmosphere. Cycad pollen can potentially be stored long-term in liquid nitrogen (Osborne, 1989), but it can retain 50 per cent of its viability for at least three years if dried and stored in a sealed container in a cold environment such as a refrigerator or, preferably, a freezer (Osborne *et al*., 1991).

A non-self-defrosting freezer is preferable as it will avoid freeze-thaw cycles that can damage the pollen.

It is important to note that storage methods may affect pollen from different cycad taxa differently. Tang (1986a) noted that pollen from different cycad genera appeared to react differently to cold storage in a refrigerator. Osborne *et al*. (1991, 1992) found that *Cycas thouarsii* pollen exhibited a cyclical pattern in its germinability with alternating phases of dormancy and potential vigour. Mostert (2002) suggests that different species of *Encephalartos* have significantly different cold storage properties.

If pollen is to be stored in a freezer some of the humidity must be removed first, both to inhibit fungal infections and to ensure that the water inside pollen grains doesn't expand and damage the pollen cell membranes when frozen (Osborne *et al*., 1992). Pollen in paper packets can be dried by placing the packet over a desiccant in an airtight container and keeping it in a refrigerator for two days. Several pollen packets can be combined in one jar over desiccant, and the risk of mixing pollen from different taxa can be minimised by using individual small glass vials for each particular taxon. This is also advantageous because the rest of the pollen will not have to be exposed to thawing and refreezing or changes in humidity every time one packet is removed from the jar. The best type of desiccant to use is probably anhydrous calcium sulfate or silica gel with a cobalt chloride indicator. The indicator makes it clear when the desiccant must be replaced or reconstituted because it changes colour when saturated by moisture. After two or more days have passed and the pollen has dried in the refrigerator, the jar can be placed in a freezer with or without the desiccant. Pollen storage experiments have shown slightly better results in pollen viability when pollen is pre-dried before freezing rather than by storing it over the desiccant (Osborne *et al*., 1992). However, if an indicator desiccant is used for freezer storage, it provides the benefit of knowing if the humidity in the container remains appropriate over time.

Although pollen may be stored for a few years by pre-drying and storing in a cold environment, periodic viability testing may be necessary to determine the longevity and cold storage properties for specific cycad taxa.

POLLEN VIABILITY TESTING

Different methods for testing the viability of cycad pollen that have been used in pollen storage experiments or are currently used at botanical gardens are shown below. The pollen used in the assays cannot later be used for pollination so it is important that only a small sample of the available pollen be tested.

Nitroblue tetrazolium assay

Tang (1986a) used a Nitroblue tetrazolium assay (NBT) to evaluate the viability of pollen from cycads belonging to eight different genera. The pollen grains were placed on a

microscope slide with NBT and placed under incandescent lights for 15–20 minutes then examined under a microscope. Browning of the pollen grains was considered a positive reaction but all four species of the genus *Dioon* resulted in an intense false positive reaction, possibly due to unique chemical or structural properties not found in other genera. The accuracy of the test was not evaluated in the field but the results with *Dioon* pollen suggest this technique should not be used for general pollen viability testing.

Safranin stain test

Osborne *et al*. (1991, 1992) evaluated the viability of pollen from four *Encephalartos* species and *Cycas thouarsii* by incubating the pollen for 72 hours at 20°C on a sterile medium of 2 per cent sucrose, 1 per cent agar and 0.01 per cent boric acid, then staining with safranin and examining under a microscope. The accuracy of this test was not evaluated in the field.

Hanging drop method

In Osborne *et al*. (1991, 1992) the viability of *Encephalartos* pollen was assessed by suspending pollen in a hanging drop of solution with 0.005 per cent boric acid and three different concentrations of sucrose (5 per cent, 10 per cent, 15 per cent) and counting the germinated pollen grains after 48 hours at 28°C. The pollen was used in wet pollination of female plants and the viability estimates at the three concentrations were compared with the actual germination rate of the resulting seeds. The 15 per cent solution provided the best estimate of the actual germination rates for seeds and was considered to be a good predictor for the viability of *Encephalartos* pollen.

Aniline blue stain

Montgomery Botanical Center estimates the viability of cycad pollen by staining with aniline blue in lactophenol (Kay *et al*., 2011). A solution is prepared consisting of 20ml of melted phenol crystals, 20ml of 85 per cent lactic acid, 40ml glycerin, 20ml of distilled water and 5ml of a 1 per cent aqueous solution of aniline blue (Hauser and Morrison, 1964). A small amount of pollen is placed on a slide, combined with a couple of drops of the solution and covered with a cover slide. After 12–24 hours the viable pollen will stain a bright blue and can be examined under a microscope to calculate the viability percentage (see Fig. 5).

DETERMINING SEED CONE RECEPTIVITY

For pollination to occur, pollen grains need to travel from male cones to ovules in female cones that are receptive to pollen. The receptivity period for an individual female cone may last anywhere from a few days to a few weeks depending on the

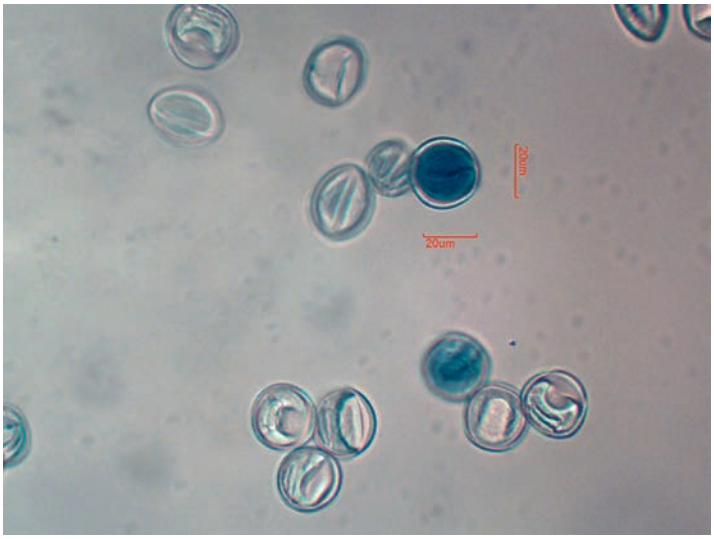

Fig. 5 Aniline blue test for pollen of *Microcycas calocoma*. Photo: Michael Calonje.

species (Tang, 1995; Grobbelaar, 2002) and may vary slightly for individuals of the same species. For example, in a wild *Zamia neurophyllidia* population in Costa Rica, Clark and Clark (1987) found individual females to be receptive anywhere from 7 to 20 days, with the receptivity period lasting longer on cones borne on larger females. In cycads the period of receptivity is generally accompanied by an increase in cone temperature and the release of odours thought to attract pollinators (Tang, 1987b, 1993a). Additionally, a micropylar droplet will form at the tip of receptive ovules within a cone, acting as a stigmatic surface to carry pollen into the ovule and possibly as a germinating medium for pollen (Tang, 1993b). When cycad cones are receptive, the megasporophylls will usually loosen or separate to allow pollinators access to the ovules. Cycad cones of different genera vary morphologically in the way they manifest receptivity (see Fig. 6). Female cones of *Bowenia*, *Ceratozamia*, *Encephalartos*, *Lepidozamia*, *Macrozamia*, *Microcycas* and *Zamia* have sporophylls with a thick and fleshy outer surface (bulla) that may separate evenly or form different opening patterns on the cones such as rows or columns, and even spiral patterns. In many cases the opening is obvious but in a few species openings are minimal and require careful observation or physically touching the cone to feel for sporophyll looseness. In some species, the outside margins of the sporophylls will change slightly in colour when receptive. In cones of *Ceratozamia* the outside margins of the sporophylls may change to an orange or reddish colour when receptive (see Fig. 6b), while in some species of *Encephalartos* they may change from a "dull medium green" to a "bright green or chartreuse" (Whitelock, 2002) and in *Stangeria* they may turn slightly yellow.

Stangeria have scale-like sporophylls and receptivity is not as obvious as it is with genera that have thick and fleshy bullae. When receptive, the sides of the basal sporophylls curve inwards, forming channels to the interior of the cone (Grobbelaar, 2002). However, this may not be as obvious to the untrained eye so feeling the cone may give a better idea of when it is receptive. As the sporophylls of *Stangeria* loosen they can easily be pulled down to about 50 degrees (Broome, 2004).

When *Cycas* becomes receptive, the sporophylls forming the cone will usually loosen. The degree at which they open and reveal their ovules may vary for different species. For example, the sporophylls of *Cycas revoluta* will open up and expose the ovules (see Fig. 6a), while those of *Cycas taitungensis* remain closed and need to be forced open to hand-pollinate. The ovules in most *Cycas* are pea-sized when receptive (Norstog and Nicholls, 1997) and secrete visible micropylar droplets. With *Cycas*, as with *Stangeria*, the best way to determine receptivity may be to feel the cone for sporophyll looseness and look for micropylar droplets forming on the ovules. When female cones of *Dioon* become receptive only the basal sterile sporophylls curve outward,

Fig. 6 Receptivity of seed cones for different cycad genera. A) *Cycas revoluta* B) Ceratozamia sp. C) *Dioon spinulosum* D) *Encephalartos laurentianus* E) *Lepidozamia hopei* F) *Macrozamia lucida* G) *Microcycas calocoma* H) *Zamia cunaria*. Photos: All Michael Calonje except C: Larry Krauss.

revealing the cone's interior (see Fig. 6c). It is easier to determine receptivity in *Dioon* cones by viewing them from above than viewing them from the side. Whitelock (2002) notes that receptivity is easier to notice in *Dioon* species from the east coast of Mexico which have no tomentum on the basal sporophylls, than in *Dioon* species from central and western Mexico which have tomentum on the basal sporophylls that may obscure receptivity.

POLLINATION EXCLUSION AND HYBRIDISATION PREVENTION

If native or introduced pollinators occur near a cycad collection, it may be necessary to protect female cones from them and from wind to prevent accidental hybridisation. This can be done by wrapping the receptive female cone in a breathable fabric such as muslin cloth or polyester mesh cloth and tying it tightly around the peduncle to prevent insects from entering. Additionally, a ring of lanolin paste can be smeared around the peduncle or a solid, long-lasting insect repellent can be included in the enclosure (Grobbelaar, 2002). For cycads cultivated in areas where there are no native pollinators in the vicinity, unwanted hybridisations may be reduced by harvesting male cones from the collection before they release pollen. However, if native pollinators occur in the vicinity, this method may not be as effective because the pollinators may be able to carry pollen from surrounding cycad habitat or nearby gardens. When selecting planting locations for cycads in a collection, it may be useful to learn which species in a collection will produce seed through open pollination and which species in a collection are most likely to hybridise with each other. Species that are likely to produce seed from wind-borne pollen can be planted at a distance from genetically compatible species to avoid the chances of hybridisation. Because of the generally open nature of their cones, species of *Cycas* appear to be the most likely to produce seed by open pollination in areas where no pollinating insects occur. Similarly, *Stangeria eriopus*, a South African species, appears to set seed from open pollination in Hawaii, Florida and California (G. Holzman, T. Broome, and L. Whitelock, pers. comm.). The pollination agents are believed to be ants (G. Holzman, pers. comm.) or fruit flies (Whitelock, pers. comm.). Fortunately *Stangeria eriopus* is the only species in its genus and is unlikely to hybridise with other genera.

Chamberlain (1926) reported creating intergeneric hybrids between *Ceratozamia mexicana* and *Zamia monticola* but no attempts to create hybrids between different genera have been successful in modern times. However, hybrids between species within the same genera are known to occur in habitat and are often created purposefully in cultivation. Vorster (1995) notes that interspecific hybridisation is possible between species of *Macrozamia*, *Cycas*, *Encephalartos*, *Zamia* and *Ceratozamia.* Hybrids within *Stangeria* and *Microcycas* are not possible because they represent monotypic genera. Hybrids within *Bowenia*, *Dioon* and *Lepidozamia* may be possible, although no examples of attempts to produce hybrids were found in the literature.

MANUAL POLLINATION OF CYCADS

Receptive seed cones of cycads can be pollinated by introducing dry pollen or pollen mixed with water into the cone so that it comes into contact with as many ovules as possible. It is best to pollinate cycads on a dry day, and preferably when rainy days aren't forecast in the near future, as rain can wash the pollen away. The best time to pollinate cycads is probably from late evening through mid-morning, as this is when the micropylar droplets that act as stigmatic surfaces form on the tips of the ovules of most cycad species (Tang, 1993b). It is also advisable to repeat the pollination procedure several times during the receptive period, as the individual ovules within a cone may not necessarily be receptive to pollen at the same time.

The efficacy of wet pollination compared to dry pollination has not been scientifically studied and the method used is generally a matter of personal preference. However, a few factors must be considered when deciding which method to use.

The wet pollination method has the advantage that no airborne pollen is released so the person doing the pollinating is less exposed to potentially hazardous and allergenic pollen (Grobbelaar, 2002). The wet method provides better results when very little pollen is available as less pollen is likely to get stuck to the various surfaces it comes into contact with during the pollination procedure, and water helps the pollen travel further down the cone. For wet pollination, pollen is mixed with water to form a cloudy solution and a drop of dish soap may be added as a surfactant. It can then be squirted, injected or poured into cone openings using a variety of devices including plastic wash bottles, syringes, eye droppers or turkey basters. It is best to begin by injecting the solution to the top openings and then injecting it into as many other openings as possible. However, some cycad taxa are not suitable for wet pollination, as cones may rot from the extra moisture. For this reason, it is recommended to avoid using the wet method for *Ceratozamia*, *Macrozamia* and *Encephalartos* species, which have woolly cones, and blue-leaved *Encephalartos* such as *Encephalartos lehmanii*.

Dry pollination works very well with *Cycas* because the ovules are generally quite exposed during the receptive period (see Fig. 7e), so simply tapping a pollen cone over the megasporophylls can result in adequate pollination. For dry pollination the pollen can be placed in a variety of devices such as a hand compression tool, a syringe with a rubber ball (see Fig. 7d) or a dust gun, and the pollen can either be poured or forced into the cone with air in order to pollinate as many ovules as possible.

The genus *Dioon* requires a special procedure for pollination, as only the sterile basal openings open up during the receptive period (see Fig. 6b). When a *Dioon* seed cone is receptive, it is possible to remove the sterile top portion of it by cutting it with a knife or by working the fingers into the top of the cone and pulling the top off manually (see Fig. 7a). The pollen can then be blown in with a hand tool or poured in over the top until some of it makes its way out of the bottom of the cone (see Fig. 7b). The top of the cone can then be replaced where it was severed and may stick to the cone axis because of the mucilage released. Although the removal of the cone apex is most useful in *Dioon*,

Fig. 7 Cycad pollination. A) Removal of cone apex of *Dioon mejiae* B) Wet pollination of *Dioon mejiae* C) Wet pollination of *Encephalartos laurentianus* with cone apex removed D) Dry pollination of *Microcycas calocoma* using syringe and bulb E) Dry pollination of *Cycas maconochiei*. Photos: A, B & D: Larry Krauss, C: Michael Calonje and E: Chad Husby.

the technique may also be used in other species that are fairly closed during the receptive period, such as *Encephalartos ferox* (Tang, 1986b).

THE PROCESSES OF POLLINATION AND FERTILISATION IN CYCADS

Pollination occurs when pollen grains land on the micropylar droplet formed by a receptive ovule and are carried into the pollination chamber. Once in the chamber the pollen grains germinate and form pollen tubes. The pollen tubes grow for three to seven months, after which they enter into the fertilisation chamber (archegonial chamber) and release flagellated spermatozoids which rapidly fertilise the eggs contained in the chamber (Norstog and Nicholls, 1997).

EMBRYO DEVELOPMENT AND SEED AFTER-RIPENING

The process of embryo development begins when one of the fertilised eggs develops into a proembryo and is pushed into the megagametophyte by a helically coiled organ called a suspensor. The proembryo matures into an embryo which eventually grows to fill the seed longitudinally and the seed becomes ready for germination.

The time required for embryo development varies among different cycad taxa and needs to be considered when collecting and germinating seeds. In all species of *Zamia*, *Dioon* and *Microcycas*, as well as some species of *Encephalartos* such as *E. transvenosus* and *E. manikensis* (Vorster, 1995), the embryos are fully developed and ready to germinate when the seed is released from the cone. However, in *Ceratozamia*, *Cycas* and most *Encephalartos* species, the embryo requires several additional months before it is of sufficient maturity to germinate.

SEED COLLECTION AND VIABILITY TESTING

Seeds can be collected from species of *Cycas* when the seeds begin to drop from the sporophylls. In the cones of most other genera, seeds may be collected when the cone begins to disintegrate. In some species, such as *Encephalartos eugene-maraisii*, *E. middelburgensis* and *E. lanatus*, the cones do not disintegrate naturally but simply dry up (Grobbelaar, 2002), so seeds need to be collected once they begin to separate from the sporophylls.

Ovules of *Encephalartos*, as well as some species of *Macrozamia*, and *Lepidozamia*, are large at the time of receptivity, so even if they are not pollinated, they produce normal looking, though sterile, seeds (Grobbelaar, 2002). In all other genera the ovules do not expand unless they come into contact with pollen.

Ovule expansion may occur regardless of whether actual fertilisation occurs. For example, Tang (1987a) found that *Zamia integrifolia* ovules reached seed size after pollination but before fertilisation. In addition, Vorster (1995) noted that pollen of *Ceratozamia mexicana* stimulated sterile seed development in *Zamia integrifolia*, an incompatible species from a different genus. It is not known how pollen affects ovule growth (Vorster, 1995) but the development of sterile seeds may be the result of using pollen from incompatible species, or perhaps using pollen that is no longer viable. To test whether seeds are viable, they can be immersed in water. In many cases, seeds that float do so because they have desiccated and shrivelled inside the shell, forming air pockets. These seeds are often in poor condition or dead but if removed from the shell and rehydrated some may germinate. However, seeds of certain island *Cycas* species, such as *Cycas rumphii* and *C. seemanii*, will always float because they contain a spongy tissue that allows them to be dispersed over long distances on seawater. Conversely, Grobbelaar (2002) notes that the seeds of certain species, such as *Encephalartos arenarius*, *E. nubimontanus* and *E. transvenosus*, will always sink, regardless of whether or not they have been fertilised.

SEED STORAGE

The embryos within the seeds of many cycad species continue to develop for several months after they are naturally released from their cones, a process known as after-ripening. If the seeds are sown when the embryos are still immature they may die as a result of receiving more moisture or higher temperatures than they are able to tolerate. Consequently, it is necessary to store cycad seeds during their after-ripening period. Cycad seeds can be stored at room temperature or refrigerated during their after-ripening phase. Dehgan and Schutzman (1989) refrigerated *Cycas revoluta* seeds at 5°C for 24 weeks and found embryo development and later germination to be more synchronised than with seeds stored at room temperature (22°C). Storage at room temperature resulted in faster embryo development but only 5 per cent of refrigerated seeds lost their viability compared to 58 per cent of those stored at room temperature, a result attributed to embryo desiccation. However, Vorster (1995) reported no noticeable difference in germination between *Cycas revoluta* seeds that were refrigerated and those stored at room temperature, although the germination in the refrigerated seeds appeared more synchronised in this study as well. Witte (1977) observed improved germination of *Zamia floridana* after one year of storage at 5°C.

It appears that in at least a few species of cycads cold storage is not detrimental and may in fact be beneficial. The slower embryo maturation may allow for longer-term storage than at room temperature. However, as long as seeds are not allowed to desiccate refrigeration is not necessary. The key to cycad seed storage is to maintain a proper balance of moisture. If seeds are kept too dry they may desiccate, whereas if they are kept too moist they may swell and break the outer shell (sclerotesta), exposing the embryo to fungal infections. It is best to store the seeds in a breathable open container such as a nylon mesh bag to inhibit the possibility of fungal attack. Seeds of *Cycas* and *Zamia* are best stored without removing the fleshy outer layer (sarcotesta) but those of other genera are best stored with the sarcotesta removed (Grobbelaar, 2002). To minimise risk of fungal infection seeds can be treated with a broad-spectrum fungicide prior to storage. To keep appropriate humidity in stored cycad seeds, Broome (2001) recommends soaking them in water for a couple of hours every two weeks, while Grobbelaar (2002) recommends soaking them for 48 hours every month.

The following holding times are suggested for seeds of different cycad genera:
Bowenia – 1 to 3 months
Ceratozamia – 3 to 6 months
Cycas – 4 to 12 months
Dioon – 0 to 2 months
Encephalartos – usually 6 months
Lepidozamia – 3 months
Macrozamia – 3 months
Microcycas – sow immediately

Stangeria – 1 to 3 months
Zamia – 0 to 2 months

CYCAD SEED GERMINATION

When attempting to germinate cycad seeds it is of critical importance that the embryos are fully developed, as the same heat and humidity that encourages germination in ripe seeds may result in the death of seeds with immature embryos. The removal of the outer fleshy covering (sarcotesta) from the seed is recommended before attempting to germinate them, as at least in certain species it appears to inhibit germination. In a wild population of *Zamia erosa*, Negrón-Ortiz *et al*. (1996) noted that germination did not occur until a minimum of 17 to 20 days after the sarcotesta was removed. Smith (1978) reported improved germination in *Zamia integrifolia* after removing the sarcotesta. Because the seeds may have dehydrated during storage, soaking them in water for a day before sowing is also recommended.

Different techniques have been used to speed up the process of germination and several authors have, for instance, suggested that better germination results can be attained by scarifying cycad seeds manually or with sulphuric acid (H_2SO_4). Smith (1978) reported that the rate and percentage of germination in seeds of *Zamia integrifolia* and *Zamia furfuracea* could be improved by mechanical scarification. Dehgan and Schutzman reported better germination in *Zamia furfuracea* (1983) and *Cycas revoluta* (1989) by chemically scarifying the seeds with concentrated H_2SO_4 and then soaking them in gibberellic acid (GA_3). However, Pérez-Farrera *et al*. (1999) noted that the best results for *Dioon merolae* germination could be obtained by mechanical scarification alone. In this case, exposure to GA_3 appeared to have a detrimental effect on seed germination. It should be noted that while scarifying or removing the sclerotesta may speed germination of some cycad species (by removing a physical barrier to water absorption), the sclerotesta protects the seed during embryo development (Vorster, 1995) and removing it will leave the seed more exposed to damage. It is widely believed that seed germination in most cycads can be improved by germinating under higher than ambient temperatures. Vorster (1995) reports that optimum germination of cycad seeds can be attained at 30°C, while Whitelock (2002) recommends a temperature of 27°C. Grobbelaar (2002) recommends a temperature between 27°C and 35°C, and Tang (1995b) recommends a range between 27°C and 43°C. However, optimum germination temperatures have rarely been tested and probably will vary depending on the species in question. For example, Grobbelaar (1999) found the optimum temperature for germination of *Encephalartos transvenosus* to be 20°C, which is much cooler than the temperatures recommended above. Cycad seeds should be germinated in a sterile medium such as sand or perlite to minimise the chance of fungal infection. They are usually buried halfway in the medium, although Grobbelaar (2002) reports no difference in germinating *Encephalartos* seeds above the sand or buried in it. The seeds can be placed sideways as they would most likely land after dropping from a cone, or one of

the tips can be buried in the sand. The chalazal end of the cycad seed is where it was connected to the sporophyll and usually it will have a scar where it was attached. The opposite end contains a small opening from which the seed sprouts.

Grobbelaar (2002) notes that burying the chalazal end of the seed makes it easy to see when the seed has sprouted and is ready to be transplanted. Broome (2001) mentions that this also diminishes the chance that a fungus will contaminate the seed, and has greatly improved his germination rate for *Encephalartos* seeds. Once seeds have started to germinate they can be removed and placed in individual pots. Deep pots are recommended because cycad seedlings form long tap roots.

In this paper we have presented knowledge about propagating cycads by seed based on information gained from published papers as well as years of practical experience of cultivating these extraordinary plants at Montgomery Botanical Center. The techniques related to seed propagation of cycads, including the collection, storage and viability testing of pollen, determining when seed cones are receptive, manual pollination, seed storage and germination, are all essential in order to increase the availability of these rare plants and reduce the demand for wild-collected plants. However, there is still much to learn and it is essential that this knowledge is discovered and disseminated quickly because it is this type of practical applied biology that is so vital for plant conservation.

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