PRELIMINARY ADVICE ON FRUIT HANDLING, SEED PRETREATMENT AND 'GERMINATION' OF EMBRYOS OF *PRUMNOPITYS ANDINA*

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Prumnopitys andina is a member of the Podocarpaceae native to Chile and Argentina. It is known to be relatively easy to propagate vegetatively, but germination from seeds is poor and can be spread over at least four years. This paper describes the methods that were used to raise 89 seedlings from 262 seeds (=34%) in less than 1 year. The sequence involves i) completely removing the fleshy sarcotesta; ii) thoroughly washing the seed-coat; iii) 'pretreating' the imbibed seeds by incubating them in moist peat and sand at a daily alternating 10/15°C for several months (to allow 'maturation' or 'after-ripening'/'dormancy breakage' at present we do not know which); iv) carefully cracking the seed-coat in a vice and extracting the embryo; v) culturing clean, firm, healthy (='viable') embryos on moist filter paper at a daily alternating 20/30°C (with lights during the 30°C phase); [vi) where necessary, freeing the cotyledons of all seedlings that become trapped in the female gametophyte]; vii) transplanting seedlings to conventional nursery practice. A further 1008 seeds are continuing 'pretreatment' to investigate whether this will increase the proportion of seedlings per viable embryo or better still lead to a much less labour intensive seedling emergence from intact seeds.

INTRODUCTION

Prumnopitys andina (Poepell. ex Endl.) de Laub. is an evergreen member of the Podocarpaceae native to Chile and Argentina. In Chile, it is distributed between 35°50' to 39°30'S along the Andes (Rodríguez & Quezada, 1995). It crosses the Andes into Argentina at about 38°S (Tortorelli, 1956). There is a little known population in the Cordillera de Nahuelbuta close to Angol (37°50'S 72°50'W) which is rapidly dwindling in size due to land conversion to commercial forestry (Gardner and Lara, 2003). In other parts of its range, populations have been reduced by overgrazing, selective felling and hydroelectric developments. Its conservation status has recently been assessed according to IUCN 2001 categories as Vulnerable [B2a, b(ii-v)] (Hechenleitner et al, 2005).

The germination of podocarps (in general) is very slow and unreliable (Laughton, 1938; Palmer and Pitman, 1972). It has been noted that the germination of some may be hastened by completely or partially removing the fleshy epimatium (Phillips, 1931; Becking, 1965; Noel and van Staden, 1976) and others by 'cautiously cracking the nut'

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(Sim, 1905). Looby and Doyle (1944) have speculated that embryo immaturity at fruit-fall may be 'a common podocarpean feature', and that a period of what they have termed 'after-development' may be important. Clout and Tilley (1992) reported that seeds of *Prumnopitys ferruginea* continued to germinate for over four years – either with, or without passage through a pigeon gut. And Gardner and Lara (2003) also indicate that propagation trials on *Prumnopitys andina* at the Royal Botanic Garden Edinburgh (RBGE) resulted in poor germination, spread over up to four years. Rodríguez (1988) and Rodríguez (2004) observed that chemical and mechanical scarification of the seeds of this species could shorten the germination period.

This paper reports the preliminary findings from a joint study between the British Forestry Commission, Forest Research Agency, Royal Botanic Garden Edinburgh and the Instituto de Silvicultura, Universidad Austral de Chile, Valdivia. The main aim of the study was to obtain as many seedlings as possible from a total of 1270 fruits obtained from 12 different seed accessions. At the same time we attempted to significantly shorten the propagation period.

TERMINOLOGY

Mill *et al.* (2004) have recently published descriptions of the anatomy and morphology of fertile complexes of *Prumnopitys* and *Afrocarpus* species. In this paper we have followed their terminology and also used some practical descriptions of the tissues. This section attempts to clarify our terminology. The fully ripened 'female cones' were approximately 20mm long and 15mm wide, fleshy and olive-green in colour (Plate 1). For brevity they

Plate 1. Foliage and fruit of *Prumnopitys andina*

are loosely referred to as 'fruits'. The olive green, fleshy tissue itself is referred to as 'sarcotesta' (Mill *et al.*, 2004) – believed to be fused integument plus epimatium (*c.f.* epimatium only, of other podocarp fruits). Removal of this tissue (in a process we call de-pulping) revealed the equivalent of a cherry 'stone'. This is the 'seed' (Plates 2a–c). Cracking and removing the hard 'seed-coat' allowed an 'embryo' to be extracted (Plate 2d). The outermost tissues of the embryo are the 'female gametophyte' which enclosed an extremely tiny (sometimes invisible to the naked-eye) 'embryonic axis'.

COLLECTION

1270 ripe fruits (olive green in colour) were collected (usually from the branches of trees) from 12 different locations in Chile in January and February 2004 (Table 1). The numbers of fruits from each tree plus their collection and accession codes are recorded in Table 2, column 1. The fruits from each accession were kept separately for all subsequent treatments.

Table 1. Accession numbers and collection information.

2004 0044	DCI*708	16–Jan–04	Región VIII [Biobío]	Provincia de Biobío	Santa Bárbara	963	37°43'21.7"	71°14'48"
2004 0045	DCI*717	16–Jan–04	Región VIII [Biobío]	Provincia de Biobío	Santa Bárbara	852	37°41'35.1"	71°18'23.6"
2004 0053	DCI*725	16–Jan–04	Región VIII [Biobío]	Provincia de Biobío	Santa Bárbara	777	37°43'07.3"	71°21'39"
2004 0082	DCI*923	25–Jan–04	Región IX [Araucanía]	Provincia de Malleco	Cautín	1,023	38°41'09.7"	71°37'14.8"
2004 0097	DCI*1009	28–Jan–04	Región VIII [Biobío]	Provincia de Biobío	Antuco	868	37°11'19.3"	71°26'28.3"
2004 0098	DCI*1015	28–Jan–04	Región VIII [Biobío]	Provincia de Biobío	Antuco	868	37°12'03.6"	71°26'54"
2004 0099	DCI*1027	28–Jan–04	Región VIII [Biobío]	Provincia de Biobío	Antuco	859	37°13'52.6"	71°26'40.9"
2004 0100	DCI*1036	28–Jan–04	Región VIII [Biobío]	Provincia de Biobío	Antuco	797	37°15'38.2"	71°27'23.6"
2004 0113	DCI*1093	30–Jan–04	Región VIII [Biobío]	Provincia de Ñuble	Pinto	787	36°51'03.0"	71°38'46.6"
2004 0139	DCI*1141	01–Feb–04	Región VII [Maule]	Provincia de Linares	Colbún	906	35°53'27.7"	70°59'32.6"
2004 0140	DCI*1142	01–Feb–04	Región VII [Maule]	Provincia de Linares	Colbún	913	35°53'23.8"	70°59'29.5"
2004 0141	DCI*1155	01–Feb–04	Región VII [Maule]	Provincia de Linares	Colbún	1,000	35°53'03.7"	70°59'19.3"

TEMPORARY STORAGE AND TRANSPORT OF FLESHY FRUITS

Fruits were kept at 4°C or occasionally 15°C in loosely tied polythene bags. During transport they were thermally insulated. Some accessions were stored for up to 4 months at 4°C and this led to the green flesh of some fruits darkening through brown to black with the release of a viscous, sticky fluid – probably a resin.

FRUIT DE-PULPING AND SEED EXTRACTION

The ripe, fleshy, olive-green (or sometimes blackening) fruits were placed in a metal sieve. Flesh was carefully removed by squeezing the sticky fruits between the fingers and against the sieve sometimes using sand as a cleaning agent. Care was taken to remove all traces of flesh from the hard, seed coat. De-pulped seeds were immediately transferred to a beaker, the top of the beaker covered with gauze (to prevent subsequent loss of seeds). Seeds were then washed and agitated in running water at about 15°C for 12 hours – as a means of removing any potential chemical germination inhibitors that might be present. A random sample of 20 seeds was taken from Accession nos. 2004 0097 and 2004 0099 (because they contained the most seeds) and used to a) develop a suitable 'seed cracking and embryo extraction' technique (see below) and b) develop a method for 'viable' seed assessment (see below).

SEED CRACKING, EMBRYO EXTRACTION AND 'VIABLE' SEED ASSESSMENT

Preliminary trials using a nut-cracker and a hammer on the hard-coated seeds either bruised, partially squashed or completely flattened the contents of the seeds! We therefore refined a technique which is frequently used in so-called 'excised embryo' testing, employing a suitably sized vice as an ideal seed cracking tool (Plate 3). This method has also been successfully applied to raise seedlings of *Prumnopitys ferrüginea* (Parratt, pers comm). One seed at a time was inserted into the vice, taking care to hold the longest axis of the seed vertically and the widest profile of the seed across the jaws (Plates 2a & 3). The jaws of the vice were tightened slowly to crack the seed coat and cause as little damage as possible to the contents. The seed case was then carefully prised away from the contents using fingernails and/or a dissecting instrument such as a diamond headed probe. Observations on the seed contents coupled with the principles of a seed viability assessment described by Gosling (2003) were used to categorise seeds as follows:

1. Seeds with less than 50% contents – 'empty'.
2. Seeds containing obviously discoloured, dying, rotting or dead tissues – 'dead'.
3. Seeds with clean, firm, fresh, healthy, apparently live tissues – 'viable' (Plate 2d). Only 'viable' embryos from this technique were 'cultured' (see below).

Plate 2. Seeds of *Prumnopitys andina*. (a) – (c) Different orientations, (d) Extracted embryo.

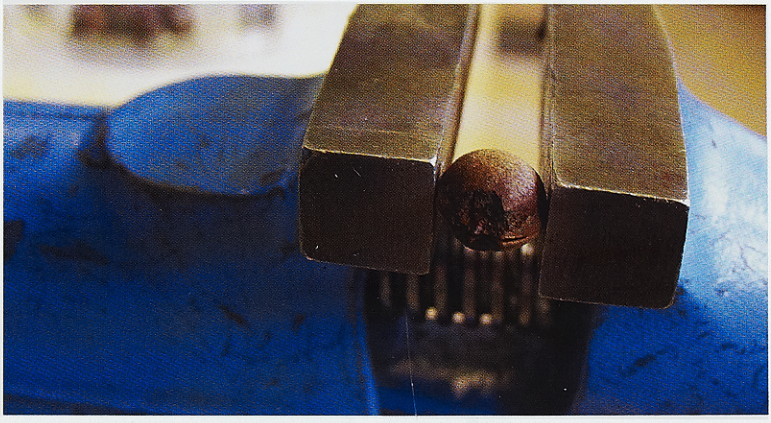

Plate 3. Cracking the hard seed coat of *P. andina* in a suitably sized vice. (Note the orientation of the seed)

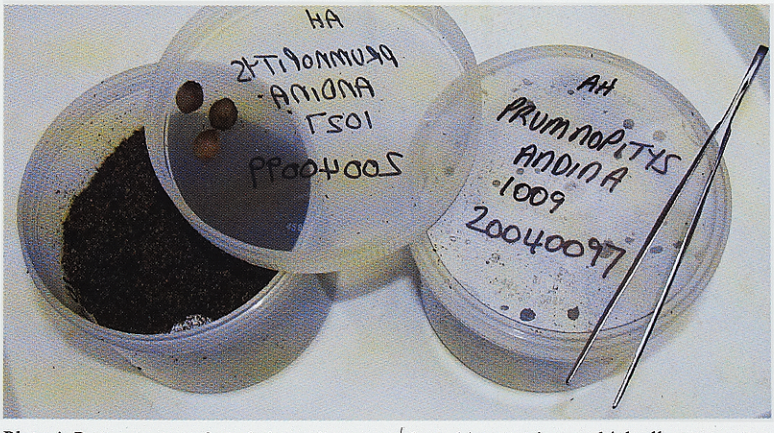

Plate 4. Pretreatment of seeds in moist peat and sand in containers which allow gaseous exchange.

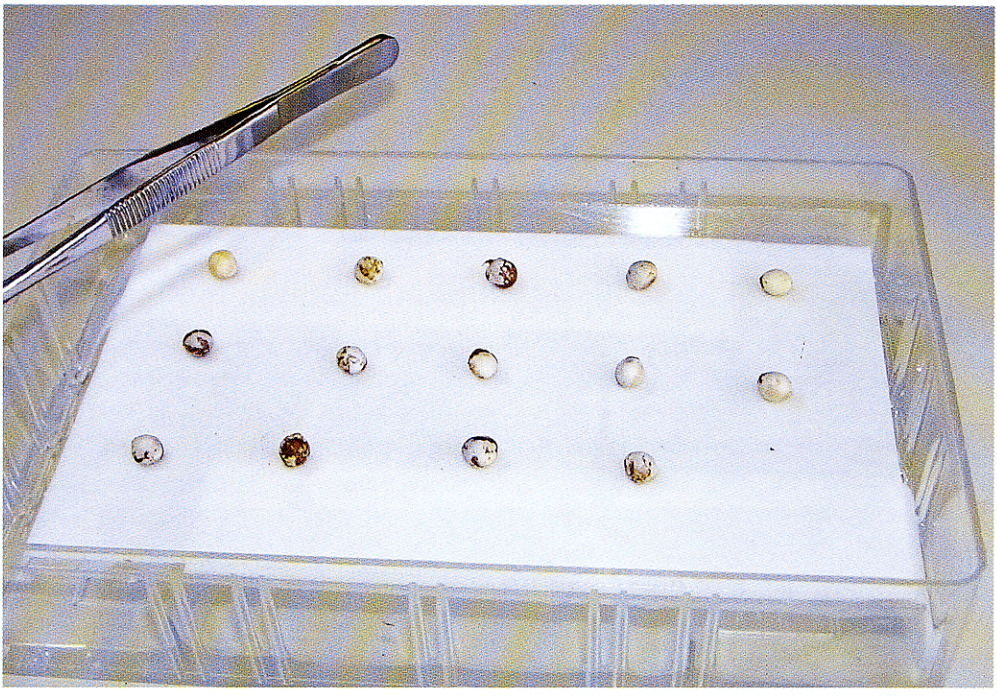

Plate 5. Embryo culture on moist filter paper. Water rises from the reservoir below. Lid is not shown.

Plate 6. *Prumnopitys andina* seedling in a three inch pot. The female gametophyte is to the left of the seedling. The aperture visible indicates where the cotyledons were inserted.

SEED PRETREATMENT

On 18 August 2004, washed, fully imbibed seeds were mixed with 4 volumes of moist peat and sand (1:1 volume to volume ratio) and incubated in the dark at a daily alternating 10–15°C (12h:12h) in a container that minimised moisture loss, but allowed some gaseous exchange (Plate 4). At weekly intervals, the containers were opened (which allowed extra gaseous exchange), and the pretreating seeds inspected. If necessary the medium was sprayed with Reverse Osmosis (R.O.) water (to ensure that the medium remained sufficiently moist) and obviously decaying or dead seeds were removed (to prevent fungal infections from developing or spreading). Any seeds that germinated were 'potted-on' (see below). After 5 and 7 months a small, random sample of seeds was taken from each accession, the seeds cracked, and embryos cultured where possible (Table 1, columns 4 & 5). It is intended to continue this process at 12, 15, 18, 21 and 24 months.

EMBRYO CULTURE

Embryos were transferred to moist filter paper and incubated at a daily alternating 20–30°C (16h at 20°C in the dark / 8h at 30°C in light) (Plate 5). At daily intervals, they were inspected for signs of growth. A protruding root was generally the first sign of seedling emergence. Some emerged within a few days, others took much longer. Most seedlings emerged with 2 cotyledons though some had 3. It is very important to note that the cotyledons of many of these 'forced' seedlings become trapped in the female gametophyte. Whenever this happened, it was essential to very carefully tease the female gametophyte from the seedling. Sometimes this had to be attempted daily over the course of up to a week, but failure to remove the female gametophyte after a couple of weeks could lead to seedling death. Quiescent embryos were only discarded if they succumbed to mould.

POTTING-ON SEEDLINGS

Seedlings that germinated 'naturally' during pretreatment, or those derived from embryo culture were both transplanted into 75mm pots containing peat:perlite:bark (1:1:1 volume: volume:volume ratio). For about two weeks the pots plus seedlings were incubated at a daily alternating 20–30°C and were then transferred to a heated poly-tunnel (Plate 6). Irrigation and fertiliser application were carried out according to conventional nursery practice. It is very important to note that, in common with the embryo culture technique, the cotyledons of many of the naturally germinated seedlings also became trapped in the female gametophyte. However, naturally germinated seedlings could not be freed from the combined female gametophyte plus hard seed coat, and all died.

RESULTS AND DISCUSSION

The main aim of this study was to obtain as many seedlings as possible from the 1270 fruits from the 12 different seed accessions. A secondary aim was to propagate these seedlings in less time than the 4 years currently reported by Clout and Tilley (1992) for *Prumnopitys ferruginea* and Gardner and Lara (2003) for *P.andina*.

It became clear at the earliest stages of the investigation that to achieve either of the above aims required the equivalent of a 'quality assurance' check on the proportion of seeds that were likely to contain embryos with the potential to germinate. Initially, we tried to x-ray the freshly de-pulped, intact seeds, but because the seeds were fully imbibed, the high moisture content rendered them x-ray opaque. Since we did not wish to dry the seeds, we employed a method for cracking them in a suitably sized vice and extracting the embryos without causing physical damage (Plate 3). Just over half the seeds (Table 2, bottom of column 5) contained fresh, healthy, apparently live embryos, but because some contained obviously shrivelled, dead or dying contents and others, nothing at all, we adopted the 'viable' seed assessment of Gosling (2003). This proved very useful for two reasons. First, further dissection of a few the embryos of the 'viable' seeds confirmed the speculations of Looby and Doyle (1994) – that embryo immaturity was a feature of *P. andina*. Second, this observation suggested that if the seeds could be incubated under favourable conditions for growth, then embryo maturation or even after-ripening might take place – leading to better developed, 'viable' embryos suitable for culturing into seedlings. These proved to be the bases for the successful procedures described and illustrated above.

Table 2 shows that although we have only worked on 21% of the 1270 fruits, most accessions have just over 50% of 'viable' seeds and after about 6 months 'pretreatment' almost 66% of viable seeds are capable of producing a seedling. This equates to an overall 34% of seeds used producing a seedling, in less than 1 year since collection.

Clearly, these are already very effective methods of collection, transport, handling, processing and pretreatment – although it is acknowledged that the seed cracking and embryo extraction technique is extremely time consuming and labour intensive.

We have not applied the technique to all of the remaining seeds for two reasons. First, we do not wish to raise all this valuable material to the sensitive 'seedling' stage. Second, because we hope to extend the pretreatment period and observe whether this either increases the proportion of seedlings further or better still leads to much less labour intensive seedling emergence from intact seeds.

It is intended to use the methodology in Chile where it will not only contribute towards conservation but will also play a small part in local poverty alleviation.

Table 2. Numbers and percentages of fruits, seeds, viable seeds and seedlings obtained from each accession of *Prumnopitys andina* at the different stages of handling. (Percentages are based on the number of fruits, seeds, viable seeds at the preceding stage.)

Fruits were collected in February–March 2004 and stored at 4°C; fruits de-pulped April – July 2004 and extracted seeds mixed with moist peat and sand and returned to 4°C; seed pretreatment in moist peat and sand at 10–15°C began on 18 August 2004.

2004 0044	122	4 Aug 04 / 1			0
			8 Mar 05 / 24	1 (4%)	0
2004 0045	110	18 Jan 05 / 1			0
			26 Jan 05 / 18	10 (56%)	5 (50%)
			8 Mar 05 / 21	9 (43%)	4 (44%)
2004 0053	122		8 Mar 05 / 24	3 (13%)	2 (66%)
*2004 0082	49		8 Mar 05 / 10	0	0
2004 0097	172		26 Jan 05 / 6	5 (83%)	5 (100%)
			8 Mar 05 / 27	18 (67%)	9 (50%)
2004 0098	110		8 Mar 05 / 21	17 (81%)	12 (71%)
2004 0099	173		8 Mar 05 / 34	13 (38%)	11 (85%)
2004 0100	100	15 Dec 04 / 2			0
		17 Jan 05 / 1			0
			8 Mar 05 / 16	13 (81%)	5 (38%)
2004 0113	99		8 Mar 05 / 19	17 (89%)	14 (82%)
2004 0139	72		8 Mar 05 / 14	10 (71%)	8 (80%)
2004 0140	85		8 Mar 05 / 17	14 (82%)	10 (71%)
2004 0141	56		8 Mar 05 / 11	5 (46%)	4 (80%)
TOTALS	1270	4	262 (21%)	135 (52%)	89 (66%)

*Accession number 2004 0082 was collected from the ground and probably consisted of the previous year's fruits. This may explain the absence of any viable seeds.

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