PRELIMINARY OBSERVATIONS ON FRUIT HANDLING, SEED GERMINATION AND CHLOROPLAST INHERITANCE OF AN *AMENTOTAXUS* HYBRID ARISING AT THE ROYAL BOTANIC GARDEN EDINBURGH FROM *A. ARGOTAENIA* (F) × *A. FORMOSANA* (M)

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ABSTRACT

Seed quality of *Amentotaxus* species is generally considered to be poor. This paper describes some general observations on the collection, processing and germination of seeds of an *Amentotaxus* hybrid (*A. argotaenia* [F] × *A. formosana* [M]) that originated at the Royal Botanic Garden Edinburgh in 2005. It also includes the details of five different methods for incubating/culturing the hybrid seeds – three of these methods resulted in almost 100% germination. DNA analysis confirmed the hybrid origin of the seeds and in common with many conifers, chloroplast inheritance was shown to be via the paternal side.

INTRODUCTION

Amentotaxus is an unusual genus of conifers in the family Taxaceae (Farjon, 2001; Gadek *et al.*, 2000) that is currently restricted to eastern Asia. All species are generally dioecious shrubs or small trees up to 20m (Rushforth, 1987) although large trees up to 40m tall and with 1m diameter trunks have been reported from Vietnam (Hiep *et al.*, 2004).

Amentotaxus argotaenia (Hance) Pilg. has a wide but fragmented distribution across southern China, northern Vietnam and Lao PDR while four of the other five species are narrow endemics in Assam (*A. assamica* D.K. Ferguson), southeastern Taiwan (*A. formosana* H.L. Li) and Vietnam (*A. hatuyenensis* T.H. Nguyen & Vidal and *A. poilanei* (Ferré & Rouane) D.K. Ferguson). The fifth species, *A. yunnanensis*, is known from southeast China, northern Vietnam and adjoining areas of Lao PDR (Thomas *et al.*, 2007). In general, their currently known distributions are allopatric except in Vietnam, close to the border with China, where *A. yunnanensis* and *A. hatuyenensis* have been reported from the same general locality (Luu & Thomas, 2004; Hiep *et al.*, 2004). Although the genus itself is known from 60 mya Paleocene fossils, the extant species are very similar in morphology and are thought to be recently evolved (Ferguson, 1992). They are generally distinguished on the basis of differences in the width of the stomatal bands, leaf shapes and internal leaf structures (Ferguson, 1992).

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Amentotaxus formosana has been assessed by the IUCN as critically endangered due to its small population size and very restricted distribution. *Amentotaxus yunnanensis* is regarded as endangered for similar reasons, while the remaining species have been assessed as vulnerable due to their restricted distribution or fragmentation of their habitats (IUCN, 2007). Various conservation strategies including establishment of special reserves and *ex situ* conservation have been proposed. However, several authors have noted the need for a better understanding of each species' reproductive and regeneration biology to enhance conservation strategies (Pan & Yang, 1996; Hiep *et al*., 2004; Ge *et al*., 2005; Wang *et al*., 2005).

Since 1976, the Royal Botanic Garden Edinburgh (RBGE) has maintained accessions of *A. argotaenia* and *A. formosana* under glasshouse conditions. Until 1995, all clones represented female plants and, although female cones had been produced, the absence of males meant that they never developed. In 1995 and again in 2000, the RBGE received a range of new clones of *A. formosana* via the Smith College in the USA. These included a male. In 2002, staff noticed that female cones on the original clones of both *A. formosana* and *A. argotaenia* were developing and maturing. In March 2004, a male clone *A. formosana* was observed; when the pollen cones were shedding, the male plant was shaken over receptive females of *A. argotaenia*. In August 2005, 107 fruits were collected from this controlled cross between *A. argotaenia* (F) × *A. formosana* (M) which, in the rest of this paper, will be referred to as *Amentotaxus* hybrid. At the time this paper was being prepared, male cones on *A. argotaenia* had still not been observed.

There is little information about the fruits or seeds of *Amentotaxus* species. Chen & Wang (1984) describe early embryo development and starch distribution in relation to taxonomy. Ferguson (1992) has described pollination. Luu & Thomas (2004) report that seeds are difficult to obtain, that the embryos are not fully developed at dispersal and that at least 12 months post-dispersal maturation is required before they are sufficiently developed to germinate. Ge *et al.* (2005) report low germination percentages and poor seedling survival and attribute these characteristics to possible inbreeding.

This paper reports the preliminary findings from a joint study between the Forestry Commission Forest Research Agency (FCFRA) and RBGE. One aim of the study was to obtain as many seedlings as possible from the 107 fruits derived from the *A. argotaenia* × *A. formosana* cross. A second aim was to verify the hybrid status of the derived seedlings from the *A. argotaenia* × *A. formosana* cross and to infer chloroplast inheritance in this cross.

MATERIALS AND METHODS

Collection, temporary storage and transport of fleshy fruits

The 107 fruits were collected on 17 August 2005 from a single *A. argotaenia* plant at the RBGE following a controlled cross between *A. argotaenia* (F) × *A. formosana* (M). On the day of collection, the fruits were placed in a loosely tied polythene bag and cooled

to 4°C (which retarded drying, whilst allowing respiration), wrapped in a thermally insulated crush-proof container and dispatched to FCFRA. They were received on 18 August 2005 and stored at 4°C until 22 August 2005 (see Fig. 1).

Fruit and seed anatomy

One typical fruit was dissected to examine its gross anatomy, assess practical means of fleshy tissue removal for the remaining fruits and develop a suitable seed extraction procedure.

Moisture content determination

The moisture content of one seed was determined using the destructive 'low temperature oven method' (103°C +/− 2°C for 17 +/− 1h – ISTA, 2005) and expressed on a percentage fresh weight basis.

Fruit de-pulping, seed extraction and washing

The ripe, fleshy, bright red fruits were placed in a metal sieve. The majority of the flesh was removed very carefully by squeezing the fruits between the fingers and against the sieve (in a process we call 'de-pulping'). The flesh was very slippery to the touch yet adhered quite persistently to the seed coat and fingers. Care was taken to remove all traces of flesh from the seed coat by using running water and a nail-brush for several minutes – until the seed coat no longer felt slippery. At this stage 25 de-pulped, scrubbed and intact seeds were left without further washing – 'unwashed'. The remaining 80 were immediately transferred to a beaker, and the top of the beaker was covered with gauze (to prevent subsequent loss of seeds). Seeds were then washed and agitated in running water at about 15°C for either 1 or 12h – as a means of removing any potential chemical germination inhibitors that might be present.

The above processes resulted in 25 unwashed seeds, 40 washed for 1h and 40 washed for 12h.

Intact seed, de-coated seed (embryo plus megagametophyte), embryo, seedling and 'embling' culture

At the experimental planning stage it was envisaged that culture method 1 (below) would be applied to de-coated seeds (and possibly excised embryos) and culture method 2 (below) would be applied only to intact seeds. Also that delicate 'emblings' (germinating embryos), 'chitted seeds' (intact or de-coated seeds showing the very first signs of radicle emergence) and seedlings (intact or de-coated seeds with more than 2mm of radicle extension) would be moved sequentially through culture methods 3, 4 and 5 as they became progressively hardier.

Culture method 1. Incubation on moist filter paper at 20°C

The seed coat was removed from five unwashed seeds by making a small incision in the coat with a diamond headed probe (causing as little damage to the embryo within as possible) and then prising and peeling the seed coat away from the outside of the megagametophyte. The five de-coated seeds were transferred to moist filter paper and incubated at a constant 20°C (12h:12h light/dark) from 24 August 2005, as described in Gosling *et al.* (2005). At weekly intervals, they were inspected for signs of growth. After 44 weeks (7 July 2006) none showed any signs of chitting (the first signs of radicle emergence) or germination. Therefore, one de-coated seed was carefully dissected to observe whether there had been any growth or development of the embryo within the megagametophyte tissue.

(This technique was intended to promote the 'germination' of de-coated seeds and was never intended for the incubation of intact seeds.)

Culture method 2. Incubation in moist peat and sand at daily alternating 10/15°C

Twenty 'unwashed' plus 40 '1h washed' plus 40 '12h washed' seeds were mixed with 4 volumes of moist peat and sand (1:1 volume to volume ratio) and incubated at a daily alternating 10/15°C (12h:12h) in the dark, in plastic, screw-top containers. At weekly intervals, the containers were opened (which allowed extra gaseous exchange), and the pretreating seeds inspected for signs of radicle protrusion. If necessary the medium was sprayed with Reverse Osmosis (R.O.) water (to ensure that it remained sufficiently moist) (see Gosling *et al.*, 2005 for details). Some germinants were transferred to method '3' or '4' (below) at the first signs of radicle emergence (chitting); others were allowed to grow until the combined radicle plus hypocotyl length was anything up to 120mm.

(This technique was intended to act as a pretreatment regime to promote the maturation, growth, development, after-ripening and/or dormancy breakage of seed. In practice it was later applied to the ungerminated, de-coated seeds (above) and to allow sprouted radicles plus hypocotyls to elongate to at least 50mm.)

'Embling', chitted seed and seedling culture techniques

Culture method 3. Incubation on moist filter paper at daily alternating 20/30°C
Seeds (from culture method 1 and/or 2) that had 'chitted' or grown radicles of any length up to 50mm were transferred to high humidity conditions and incubated on moist filter paper at a daily alternating 20/30°C (16h at 20°C in the dark / 8h at 30°C in light) as described in Gosling *et al.* (2005). At daily intervals, their growth / health / survival was monitored until they were considered large enough and apparently vigorous enough to be transferred to culture method 4.

(This technique was intended to provide interim, warmer conditions (20/30°C) and higher humidity (moist filter paper over a reservoir of water) to encourage the growth

of chitted seeds, seedlings and 'emblings'. Unfortunately, as will be seen later, it led to cotyledons becoming entrapped by the megagametophyte. It therefore became necessary during this phase to monitor (and if necessary assist) the shedding of the seed coat and/or megagametophyte.)

Culture method 4. Chitted seed/seedling culture in peat:perlite:bark at daily alternating 20/30°C

Seeds (from culture methods 1 and/or 2 and/or 3) that had either just 'chitted' or had radicles of any length up to 50mm were transferred to 75mm pots containing peat:perlite:bark (1:1:1 volume:volume:volume ratio) and incubated at a daily alternating 20/30°C. At daily intervals, their growth / health / survival was monitored until they were considered large enough and apparently vigorous enough to be moved to culture method 5.

(This technique was intended to provide interim conditions between the high humidity environment of culture method 3 and the ambient humidity conditions of culture method 5.)

Culture method 5. Incubation of seedlings in pots in heated poly-tunnel

This stage was intended for completely normal, healthy, autotrophic seedlings or 'emblings'. Potted seedlings (from stage 4) that were assessed as being healthy and well established were transferred to a heated poly-tunnel where they were tended as described in Gosling *et al.* (2005).

DNA extraction and chloroplast analysis

To confirm the hybrid origin of seedlings from the putative *A. formosana* and *A. argotaenia* cross, fresh needles were collected from the putative parent plants (*A. formosana* and *A. argotaenia* grown at RBGE), and five seedlings raised from the *A. argotaenia* mother. DNA was extracted from the freshly collected needles using a Qiagen DNeasy Plant kit. The chloroplast region *trn*L was amplified using the c and d primers of Taberlet *et al*. (1991). PCR products were sequenced using DTCS Quickstart Mix (Beckmann Coulter) and analysed using a Beckmann Coulter CEQ 8800 genetic analyser and the program 'Sequencher'.

RESULTS AND DISCUSSION

Fruit and seed anatomy

Fig. 1 shows the fully ripened 'seed cones' which were approximately 40mm long and 15mm wide, fleshy and bright red in colour. For brevity they are loosely referred to here as 'fruits'. In common with the fruits of most Taxaceae, the apex of the seed protruded slightly beyond the fleshy red covering. Removal of the fleshy tissue (in the process we call 'de-pulping') revealed a cigar shaped seed c. 35mm long × 10mm wide.

Fig. 1 Freshly collected, ripe, bright red fruits of *Amentotaxus argotaenia*, one with flesh removed ('de-pulped'). Photo: Peter Gosling.

Fig. 2 shows that the seed coat was a little thicker than paper, quite fibrous and relatively easy to remove by making a small incision (causing as little damage to the embryo within as possible) and then prising or peeling. The embryo within was composed of a very large female gametophyte enclosing a tiny, 'embryonic axis' slightly less than 1mm long. It was deduced that in common with the seeds of many trees, even when the

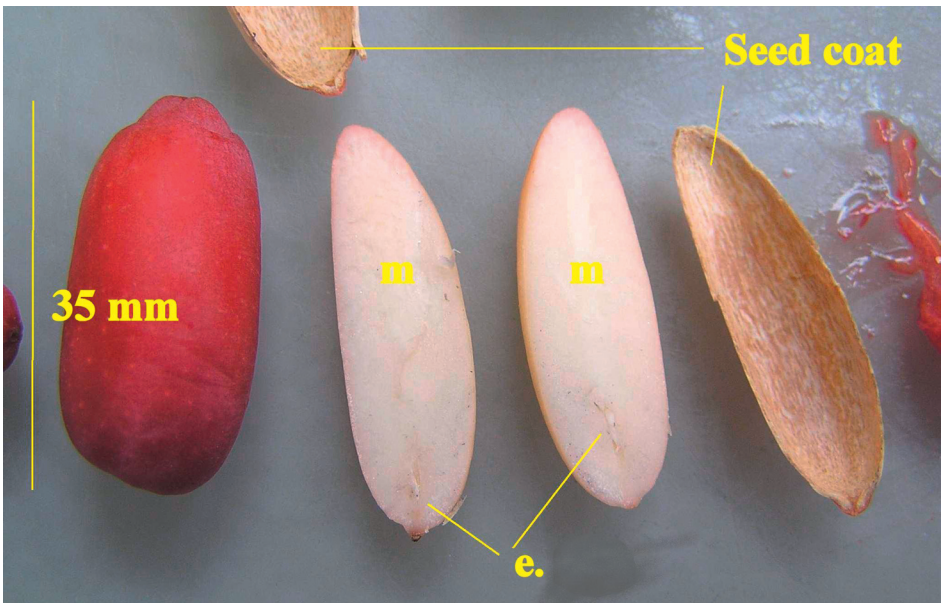


Fig. 2 Ripe *Amentotaxus argotaenia* fruit and longitudinally sectioned seed showing large megagametophyte (m), tiny, immature embryo (e) and seed coat. Photo: Peter Gosling.

fully ripened fruits are ready for dispersal the embryonic axis is still probably extremely immature and in need of a significant period to allow development and maturation to be completed.

Moisture content

The fresh weight, dry weight and moisture content (mc) (on a fresh weight basis) of the single, freshly extracted seed were 2.1g, 0.87g and 59%. When most pine and spruce seeds are freshly extracted from their cones, their mc is c. 15–20% and yew seeds are at about 30% mc. The *Amentotaxus* hybrid seeds therefore appear to have a high mc at shedding compared to other conifers.

Intact seed, de-coated seed (embryo plus megagametophyte), embryo, seedling and 'embling' culture

Fig. 3 shows that intact seeds began to germinate after approximately 30 weeks incubation in culture method 2 and that by 60 weeks germination had plateaued at almost 100%. The intended 'pretreatment' regime (i.e. incubating seeds under moist conditions at a daily alternating 15/10°C) had been very effective at allowing not only maturation, growth, development, after-ripening and/or dormancy breakage, but also germination.

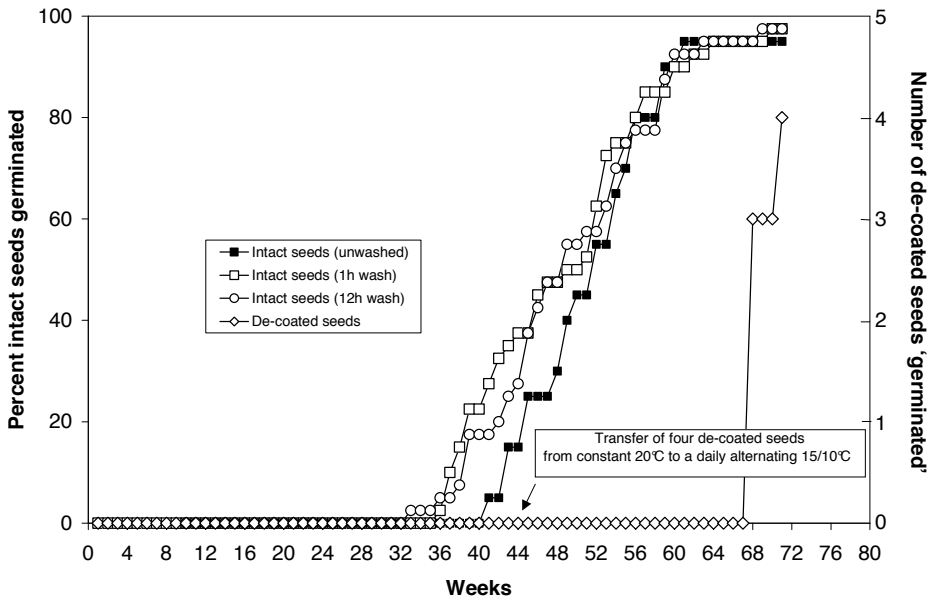


Fig. 3 Course of germination of intact *Amentotaxus* hybrid seeds (in moist peat and sand at a daily alternating 15/10°C) and de-coated seeds in the same conditions (after 44 weeks incubation on moist filter paper at a constant 20°C).

Fig. 4a Surgical removal of most of the megagametophyte tissue from a 'chitted' seed (after 40 weeks at a daily alternating 15/10°C) revealed an embryo with greening cotyledons; pink, growing hypocotyl; and protruding radicle. (N.B. Incubation for 60 weeks led to almost 100% radicle protrusion, i.e 'chitting'). Photo: Peter Gosling.

Fig. 4b Surgical removal of most of the megagametophyte tissue from a quiescent de-coated seed (after 44 weeks at a constant 20°C) revealed an embryo which had undergone little growth or development. Photo: Peter Gosling.

It can also be seen (Fig. 3) that washing had little effect on either germination speed or final germination. Either there had been no germination inhibitors present or they had been removed with the pulp during the initial scrubbing. Despite earlier literature describing the slow maturation (Luu & Thomas, 2004) and poor germination (Ge *et al*., 2005) of *Amentotaxus* seeds, the germination percentage of almost 100% attained by this seed-lot could hardly have been higher.

Fig. 3 also shows that at week 44 a significant percentage of the intact seeds had germinated in culture method 2 (moist peat and sand at a daily alternating 15/10°C). However, in comparison, none of the de-coated seeds at a constant 20°C had shown any signs of growth or emergence. An embryo was therefore very carefully, surgically removed from one intact seed showing the first signs of radicle emergence (chitting) and one ungerminated de-coated seed. Fig. 4a shows that the embryo of the former had not only undergone significant swelling, elongation and colour change, but that the cotyledons had also grown considerably within the megagametophyte. In comparison, the embryonic axis of the latter is shown in Fig. 4b and had undergone very little growth and was still only c. 2mm long.

The successful surgical excision of the two embryos (one chitting and one quiescent) prompted us to transfer them both to moist filter paper and incubate them at a daily alter-

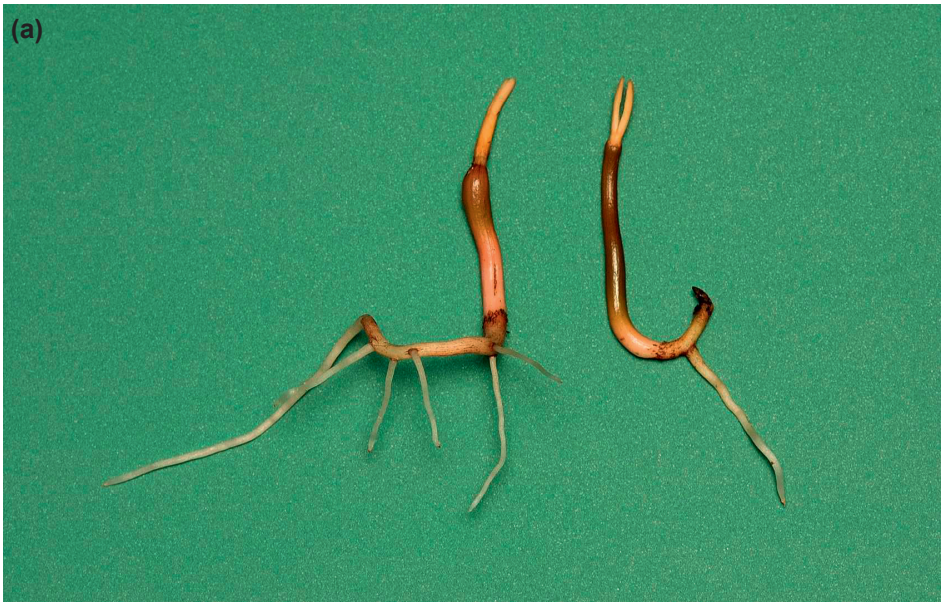
Fig. 5 Germinating seeds after 44 weeks at 15/10°C in the dark – note splitting seed coat; pink, swollen hypocotyl; root curvature and that there is still no sign of cotyledon exposure where the hypocotyl enters the megagametophyte. This would be too early to pot-on. Photo: Peter Gosling.

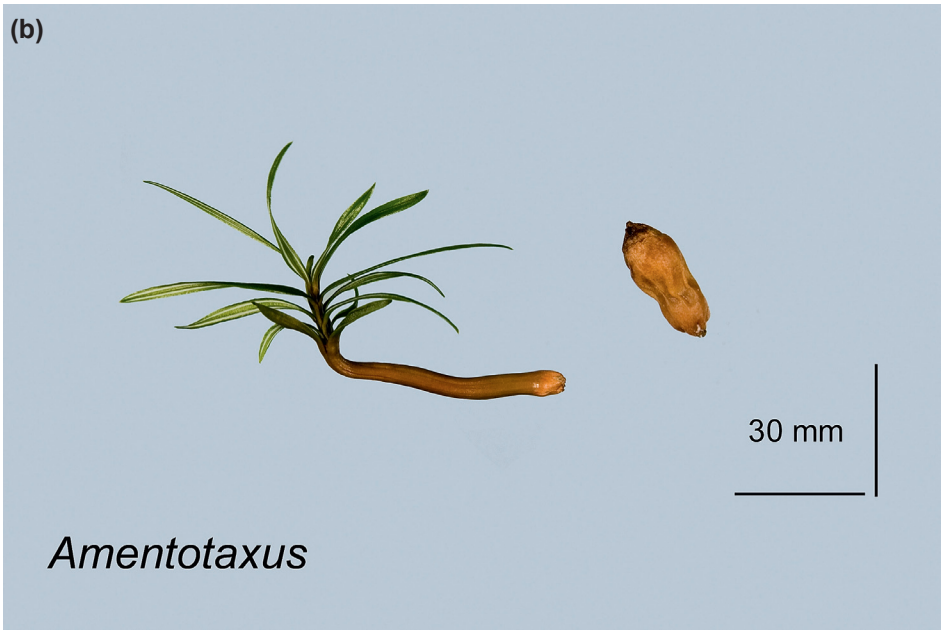
nating 30/20°C (8h at 30°C in light plus 16h at 20°C in the dark). Unfortunately, neither was capable of sustained autotrophic growth and both had died within 7 days.

Finally, in relation to Fig. 3, it can be seen that the four remaining de-coated seeds which had remained quiescent for 44 weeks at 20°C were transferred to the same conditions as the intact seeds (moist peat and sand + daily alternating 15/10°C). It is interesting to note that after a further 26 weeks under the new conditions all four had germinated. Apparently, incubation at a constant 20°C did not permit either embryo maturation, or growth or germination, whereas incubation at a daily alternating 10/15°C was very effective at allowing all three, in both intact and de-coated seeds.

Seed-coat and/or megagametophyte removal

At the experimental planning stage it was envisaged that culture method 1 would lead to the comparatively rapid 'germination' of de-coated seeds; and that culture method 2 would provide a means of 'pretreating' intact seeds to promote their maturation, growth, development, after-ripening and/or dormancy breakage – but not be conducive to germination. It was not envisaged that method 1 would fail to promote even embryo





Figs. 6 a+b Seedling abnormalities. 6a – Removal of the megagametophyte before the greening cotyledons plus first needles were naturally exposed usually led to continued root growth and development but no further cotyledon growth, greening or development. 6b – The roots of 3% of apparently normal, healthy, vigorous seedlings suddenly shrivelled and died for no apparent reason. These pictures were taken 6 months after each type of seedling first showed its abnormality. Photos: George Gate.

growth and that de-coated seeds would have to be transferred to method 2. It was also not anticipated that method 2 would lead to germination of both intact and de-coated seeds *in situ*.

When the first few intact seeds (and later de-coated seeds) began to germinate in culture method 2 they were initially left to elongate until their radicles reached 20–30mm (see Fig. 5) before they were moved on to culture method 3. The warmer temperatures (20/30°C) and higher humidity (moist filter paper over a reservoir of water) were intended to provide favourable conditions for root elongation, cotyledon development and successful shedding of the megagametophyte. Unfortunately, under the high humidity conditions of method 3 the dependence of the cotyledons on their megagametophyte food source appeared to increase, the two tissues fused more intimately and the cotyledons became firmly entrapped. It was then necessary to make daily attempts to carefully tease the megagametophyte away from the cotyledons. But by the time this worked, the seedlings had often elongated to c. 150mm and outgrown their container. Worse still the artificially released cotyledons and recently formed first needles were extremely sensitive to any change from this environment. It proved very difficult to pot these seedlings on and not lose seedlings at subsequent stages!

Initially, we tried to overcome these difficulties by transferring seeds with the first signs of chitting in method 2 directly into method 4 and sowing them half-buried in peat and sand – unfortunately, this led to fairly rapid root decay and seedling death. We therefore re-introduced method 3 and tried to overcome the problem by attempting to remove the megagametophyte earlier. However, Fig. 6a shows that premature removal frequently induced a severe and irreversible abnormality where the cotyledons showed no further growth, greening or development. Although seedlings with albino cotyledons were otherwise healthy for 6–9 months, they all eventually died.

Finally, it was discovered that leaving intact or de-coated seeds in method 2 (moist peat and sand at 10/15°C) until they had sprouted a combined radicle plus hypocotyl length of between 30–50mm (sometimes up to 120mm) was usually satisfactory. Germinants with these characteristics could completely miss out culture method 3 (moist filter paper at 20/30°C) and be transferred directly from method 2 to 4 (75mm pots containing peat:perlite:bark at a daily alternating 20/30°C). In fact, within 7–14 days of transfer to culture method 4, the megagametophyte would often dry, decay, shrivel and need very little human assistance to effect removal. It was therefore ultimately much easier and more successful to completely avoid the high humidity growth phase on top of moist filter paper at 20/30°C! Occasionally slightly delayed megagametophyte removal resulted in deformed or distorted first needles, but these seedlings invariably recovered. There were also a couple of interesting exceptions when culture method 3 had to be used to resurrect otherwise deteriorating seedlings.

Fig. 6b shows a further example of how unpredictable *Amentotaxus* seedling growth could be. In addition to the human-induced cotyledon abnormalities described above, apparently normal, healthy roots could also shrivel spontaneously, without any obvious cause. In common with the cotyledon abnormality, a stunted root could also persist for

Fig. 7 Normal healthy seedling after 6 months growth. Note luxuriant first needles, pair of withering cotyledons, and on surface of peat and sand the shrivelled megagametophyte within seed coat c.f. similarly aged abnormal seedlings in Fig. 6. Photo: George Gate.

several months. However, unlike the albino cotyledons, secondary roots sometimes developed after several months and a successful seedling resulted.

Chloroplast analysis

In total, four base pair sequence differences were identified in the *trn*L region between the studied *A. formosana* and *A. argotaenia* plants. The DNA sequences obtained from each of the five seedlings matched that obtained from *A. formosana*. As the seedlings were raised from seeds collected from a single *A. argotaenia* plant, and chloroplasts are considered to be paternally inherited in conifers, this data infers that *A. formosana* was the paternal parent, that the seedlings are of hybrid origin and that the chloroplast genome was inherited paternally in this cross.

CONCLUSIONS

It is concluded that this seed-lot of *Amentotaxus* hybrid had a very high viability (almost 100%), and that neither washing, nor seed-coat removal, was necessary to promote germination speed or final percentage. Incubating intact seeds under moist conditions, in darkness, at a daily alternating 15/10°C was very effective at allowing embryo after-ripening, maturation and growth to take place, plus subsequent germination. Incubation

at a constant 20°C did not permit either embryo after-ripening, maturation, growth or germination.

Growing seedlings were reluctant to shed their megagametophyte when incubated under a combination of high humidity (moist filter paper over a water reservoir) and warm alternating temperatures (20/30°C), and premature, human-assisted removal of the megagametophyte was often fatal. The same alternating temperatures at a lower humidity overcame this problem.

Despite the destruction of one seed for moisture content determination and two seeds for anatomical studies and the loss of 26 *Amentotaxus* seedlings to abnormalities, a total of 78 normal seedlings were raised from the initial 107 fruits (see Fig. 7).

Conservation implications

Amentotaxus formosana is thought to be more closely related to *A. yunnanensis*, *A. poilanei* and *A. hatuyenensis* than to *A. argotaenia* and *A. assamica* (Ferguson, 1992; Hiep & Vidal, 1996; Ge *et al*., 2005). The creation of an artificial hybrid between *A. formosana* and *A. argotaenia* gives a strong indication that managers of *in situ* reserves and *ex situ* collections that are intended to conserve individual species should take care to prevent hybridization if other *Amentotaxus* species are grown in the same locality, even though it is still too early to tell whether the hybrid will produce viable offspring.

ACKNOWLEDGEMENTS

Many thanks are due to Andrew Ensoll, Senior Horticulturist at the RBGE, for tending the parent plants and carrying out pollination and also to the Science Technical Support at RBGE. At FCFRA we would like to thank Jon Davey for monitoring and maintaining laboratory equipment, Steve Coventry for his help in raising the seedlings and George Gate for Figs. 6a & b and 7. Additional thanks also go to Roger Trout, Bill Mason and Helen McKay for their many helpful comments, suggestions and additions to the manuscript.

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