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Petroleum flotation technique upgrades the germinability of *Casuarina equisetifolia* seed lots

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Abstract The germination of *Casuarina equisetifolia* Forst seed lots is low (40–50%) even when freshly collected. This study aimed at upgrading the germination performance by removing non-germinable seeds using petroleum flotation technique. Seeds collected from 19 individual trees in seedling seed orchards in India were assessed using X-ray to determine the proportion of different seed lot fractions. The feasibility of petroleum ether as a separation medium was tested. Morphological characters of floating and sunken seeds were further studied using image analysis technique to examine their effects on the separation efficiency. The X-ray analysis revealed a large quantity of shrivelled, empty and insect-damaged seeds, altogether accounting 50%, which were the causes of low percentage germination of un-graded seed lots (48%). Petroleum flotation resulted in 90% germination in the sunken fraction and 4% in the floating fraction. The separation was distinct for most seed lots, except few seed lots that had relatively low germination in the sunken fractions and more than 10% germination in the floating fractions. As a whole, petroleum flotation appears to be a feasible technique to upgrade the germination of *C. equisetifolia* seed lots, and its efficacy is influenced by wing surface area, seed density and wing quotient of filled and empty seeds.

Keywords Empty seeds · Filled seeds · Specific density · Seed lot enhancement

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Introduction

Casuarina equisetifolia Forst, a nitrogen-fixing tree native to Australia, occurs naturally across a broad geographic area along tropical coastlines of northern Australia, throughout Malaysia, Melanesia and Polynesia through the Kra Isthmus of Thailand (Pinyopusarerk and House 1993; Pinyopusarerk et al. 1996; Pinyopusarerk and Williams 2000). It is probably the most extensively planted species outside its natural range in littoral zones of Asia, the Pacific and Africa mainly for soil stabilization and reclamation works, in coastal protection and rehabilitation as well as in agroforestry systems (Pinyopusarerk et al. 1996). In the Indian sub-continent, *C. equisetifolia* is planted extensively in Tamil Nadu, Andhra Pradesh and Orissa States on the east coast, and Maharashtra and Gujarat States on the west coast (Lal et al. 1996). Farmers grow *C. equisetifolia* as a short-rotation (3–4 years) cash crop with an initial density of 10,000 trees per hectare (Prasad and Dieters 1998). In Andhra Pradesh State alone, nearly 50 million seedlings of *C. equisetifolia* are planted annually by farmers (the State government sells seedlings to farmers at a subsidized rates), and the species has a sustained market as fuel, poles and wood chips for the pulp and paper industries (Prasad and Dieters 1998). Apparently, such extensive planting demands a continuous supply of high quality seedlings—the most preferred and suitable way of planting by Casuarina growers. The success of seedling production in nurseries largely depends on the continuous supply of high quality seeds. Thus, tree seed suppliers need to maintain seed lots of high purity and physiological quality to produce the desired quantity of seedlings to meet customers' demands.

The germination performance of *C. equisetifolia* seed lots is reported to be low, 40–50%, even when freshly collected (Jerlin and Srimathi 1997; Anandalakshmi et al. 2001; Umarani and Vanangamudi 2002). The reasons for poor germination were variously contemplated to be emptiness or dormancy (Umarani and Vanangamudi 2002). It is a well-known fact that many wind-pollinated tree species produce a large quantity of empty and shrivelled seeds due to pollination failures or post-zygotic degeneration (Owens et al. 1994; Palupi and Owens 1996; Tangmitcharoen and Owens 1996; Slobodník and Guttenberger 2000). As a wind-pollinated species, the reported low germination performance of *C. equisetifolia* seed lots might be due to the presence of non-germinable seeds, such as empty seeds, insect-damaged seeds and/or dead-filled seeds. These unproductive seeds must be eliminated from the seed bulk during cleaning stage to upgrade seed lot performance. In the past, several attempts have been made to upgrade the germination performance of *C. equisetifolia* seed lots. For example, the South Dakota seed blower was used to grade *C. equisetifolia* seed lots by density and the result showed that high-density seeds, retained at the highest air-speed setting, had a better germination and vigour (Maideen et al. 1990). Attempts made to grade seed lots by size (seed mass) have resulted in better germination for larger (167 mg/100 seeds) than smaller (105 mg/100 seeds) seeds (Umarani et al. 1997), and specific gravity separator yielded better germination for heavier seeds (Umarani and Vanangamudi 2002), which in turn showed significant correlation with progeny growth (Mahadevan et al. 1999). Although these studies have documented some improvement in germination following grading, the separation of empty and filled seeds was not distinct, thus leaving scope for further improvement in seed germination and separation efficiency.

Flotation on liquid media is a widely used technique to separate filled-viable seeds from empty, dead-filled, mechanically and insect-damaged seeds. The method works on the principle that viable and dead-filled or empty seeds are sorted in liquid media based on differences in specific density. For instance, McLemore (1965) used *n*-pentane as flotation medium for separating full and empty longleaf pine seeds (*Pinus palustris* Mill.) while Brown (1967) used ether to remove non-viable seeds of jack pine (*Pinus banksiana* Lamb.), and both of them reported significant improvement in germination. The application of this method to remove empty, dead-filled, insect-attacked, mechanically damaged and embryologically undeveloped seeds has been demonstrated for several seed lots of *Pinus sylvestris* L., *Picea abies* L. (Karst.) and *Larix decidua* Mill. (Simak 1973; Barabin 1983). For broad-leaved species, Falleri and Pacella (1997) and Demelash et al. (2003) reported 12.5% and 22.5% improvement in germination of London plane [*Platanus* X *acerifolia* (Ait.) Willd] and pepper tree (*Schinus molle* L.) seed lots, respectively after specific density separation. The technique has also worked well for *Acacia leucophloea* willd (Raunja) seed lots using water as flotation medium (Mani et al. 2002).

To our knowledge, no documented information exists about the application petroleum flotation technique to *C. equisetifolia* seed lots. However, Umarani and Vanangamudi (2002) claimed that separation of high density seeds of *C. equisetifolia* through water flotation technique is not possible as they are small-sized samaras, feathery and light weighted. It should be noted that the separation efficiency not only depends on seed size variability within a seed lot (e.g., *Cupressus lusitanica* Mill.., Bergsten and Sundberg 1990), but also on inadequacy of the specific density gradient between germinable and non-germinable seeds (e.g., *Albizia schimperiana* Oliv., Tigabu and Odén 2003) or insufficiency of the specific density of the flotation media. Thus, an appropriate flotation media should be sought for any given species. The objectives of the present study were to: (1) determine the cause of low germination performance in *C. equisetifolia* seed lots, (2) test the appropriateness of petroleum ether as a flotation medium for sorting non-germinable seeds, and (3) examine the effects of seed morphological characters (wing surface area, seed density, seed weight, seed volume, and/or wing quotient) on the separation efficiency.

Materials and methods

Seed materials

Seeds were collected from 19 randomly selected trees in seedling seed orchard at Panampalli, Kerala, India. Fully mature cones were collected and dried under sun for 2 days to release the seeds by shaking. Seed lot numbers were given serially for each tree. Seed weights were determined for each seed lot by weighing samples of 100 seeds in eight replications and then converted into 1,000 seed weight (International Seed Testing Association 2003). The seeds were stored at 10°C for 3 months until the experiment was conducted.

Assessment of seed lot fractions

The seeds within each seed lot were thoroughly mixed and working samples were taken. The un-graded seed lots were studied using X-ray to estimate the proportion of various seed lot fractions: filled, shrivelled, insect-damaged and empty seeds. The seeds were X-rayed at 0.35 mA current in 15 kV for 2 min (Faxitron, Model: 43855A). The developed X-ray sheets were examined under light, and seeds were carefully grouped into filled, shrivelled, insect-damaged and empty seeds. Seeds with visible embryo and megagametophyte were counted as filled seeds while partially filled and non-filled seeds were counted as shrivelled and empty seeds, respectively. Irrespective of the above classes, seeds with exit holes made by emerging larvae or adult insects were counted as insect-damaged seeds. For each seed lot, 100 seeds were examined.

Sorting with petroleum flotation

The un-graded seed lots were separated into floaters and sinkers using petroleum ether (specific density = 0.64; boiling point 40–60°C). The petroleum ether solution was taken in a separating funnel, and a known quantity of seeds were added and stirred to facilitate the separation process. The floating and sunken fractions were separately collected after few minutes when no more exchange between floating and sunken seeds was observed. The quantity of each fraction was determined to compute the proportion of floating and sunken fraction and tested for germinability. The seeds were rinsed thoroughly with tap water before sowing them on germination papers in Petri dishes that were kept in germination room maintained at 25 ± 2°C, 95 ± 2% relative humidity and a photoperiod of 12/12 h light/dark. Initiation of germination was noticed after 4 days and final count was taken after 21 days. For both un-graded and sorted seeds, four replications each with 100 seeds from each seed lot were used.

Analysis of morphological characters

Based on the percentage germination of floating and sunken seeds, seed lots were classified into three groups: those with low germination in the sunken fraction (seed lots 3 and 5), those with more than 10% germination in the floating fraction (seed lots 2 and 9), and those with low germination in the floating fraction and high germination in the sunken fractions (all seed lots except those in the above two groups). To understand the causes of low germination in sunken fractions of seed lots 3 and 5 and more than 10% germination in floating fractions of seed lots 2 and 9, we examined seed morphological characters of filled and empty seeds from these seed lots and compared with the rest of the seed lots where the separation of empty and filled seeds was more distinct. The floating and sunken seeds from each group were first X-rayed and sorted into filled and empty seeds. For each seed fraction, the wing surface area, density, weight, volume and wing quotient of empty and filled seeds were studied. The image of 200 filled and empty seeds each from floating and sunken fractions was grabbed into image analyzing software (Leica Quantimet called QWin 500) using a CCD camera, and average wing surface area (mm^2) was calculated. Seed density was calculated as the ratio of seed weight to volume. Seed weight and volume were estimated in four replications each with 50 seeds. Volume was estimated by water displacement method and single seed average value was expressed in μl. Seeds were weighed using electronic balance at 0.00001 mg accuracy and the average seed weight was expressed in μg. Wing quotient was computed as the ratio of wing surface area (mm^2) to seed weight (μg).

Statistical analysis

The proportion of different seed lot fractions identified by X-ray analysis were computed for each seed lot and pair-wise *t*-test were performed to compare the proportion of filled and non-filled (shrivelled, empty and insect-damaged seeds) seeds. The separation efficiency, defined as the quantity of floating and sunken seeds obtained after petroleum flotation to the total seed samples used, was computed for each seed lot. The percentage germination of un-graded, floating and sunken seeds of each seed lot was computed and compared using pair-wise *t*-test. The percentage data sets were arcsine transformed to meet the normality assumption (Zar 1996). A linear regression analysis was performed to examine the relationship between 1,000-seed weight and germination of un-graded seed lots.

Results

The X-ray analysis revealed that the average proportion of filled and non-filled (shrivelled, empty and insect-damaged seeds) seeds was the same ($t_{[0.05,\ 18]} = -0.067$, $P = 0.947$); i.e., 50% each. There was a marked variation in seed fractions between seed lots, ranging from 28–84% for filled seeds, 11–54% for empty seeds, 1–47% for shrivelled seeds and 0–8% for insect-damaged seeds (Table 1). Among non-filled seeds, the proportion of empty seeds was the highest followed by shrivelled seeds while the proportion of insect-damaged seeds was very low. Of a total of 19 seed lots examined in this study, eight seed lots had more than 50% filled seeds, five seed lots had more than 30% shrivelled seeds, five seed lots had more than 50% empty seeds and 10 seed lots had no infestation or insect damage. The mean proportion of floating and sunken seeds after petroleum flotation was nearly similar to the mean proportion of filled and non-filled seeds identified by X-ray (Table 1). Wide variation in separation efficiency of sunken (23–87%) and floating (13–77%) seeds was observed among seed lots. The separation efficiency of sunken seeds was more than 70% in four seed lots while five seed lots had a relatively low separation efficiency of sunken seeds.

The germination of un-graded seed lots varied between 22% and 90% with a mean germination of 48% (Table 2). Germination was more than 80% in three seed lots while 13 seed lots had less than 50% germination. This inter-seed lot variability in germination of un-graded seed lots was partly related to the variation in seed weight among seed lots ($R^2 = 0.487$; SE = 15%; Figure 1). Petroleum flotation resulted in significantly higher germination in the sunken fraction than in both floating fraction ($t_{[0.05,\ 18]} = 35.53$, P 0.0001) and un-graded seed lots ($t_{[0.05,\ 18]} = 9.054$, P 0.0001). The germination of floating seeds was also significantly lower than the un-graded seed lots ($t_{[0.05,\ 18]} = -9.68$, P 0.0001). As a whole, grading of seed lots using petroleum flotation yielded 42% improvement in germination of the sunken fraction compared with un-graded seed lots

Table 1 The proportion (%) of different seed lot fractions identified by X-ray analysis, and the separation efficiency of sunken and floating seeds (%) after petroleum flotation

Descriptive statistics	Filled seeds	Shrivelled seeds	Infested seeds	Empty seeds	Separation efficiency	
					Sunken	Floating
Minimum	28	1	0	11	23	13
Maximum	84	47	8	54	87	77
Mean	50	17	1	32	53	47
SD*	17	14	2	15	18	18

* SD = standard deviation

Table 2 Percentage germination of *C. equisetifolia* seeds in un-graded seed lots as well as in floating and sunken fractions after petroleum flotation

Descriptive statistics	Un-graded seed lots	Floating fraction	Sunken fraction
Mean minimum	22	1	59
Mean maximum	90	16	100
Grand mean	48	4	90
Standard error	21	4	10

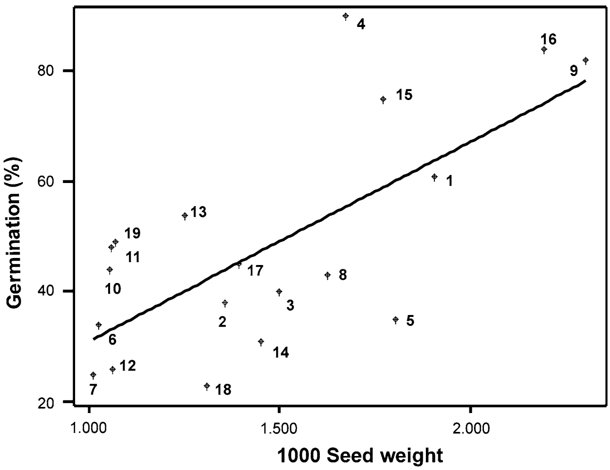

Fig. 1 Relationship between 1,000 seed weight and percentage germination of un-graded seed lots of *C. equisetifolia* (n = 19 seed lots)

while only 4% of germinable seeds were discarded as floaters (Table 2). A marked variation in germination of sunken and floating seeds was observed among seed lots. The germination of sunken seeds was more than 90% in 12 seed lots, 80–90% in two seed lots and relatively low in two other seed lots (seed lots 3 and 5 with 69 and 59% germination, respectively). The germination of floating seeds was less than 4% in 16 seed lots, 7% in one seed lot and more than 10% in two seed lots (seed lots 2 and 9 with 13 and 16 % germination, respectively).

The sunken fraction of seed lots 3 and 5 were found to possess 36% empty seeds with a seed density of 0.652, which tended to be higher than the density of empty seeds (0.470) in the floating fraction (Table 3A). The wing surface area, seed volume and wing quotient of empty seeds in the sunken fraction were relatively lower than empty seeds in the floating fraction while the weight of empty seeds was relatively higher in the sunken than floating fraction (Table 3A). In seed lots 2 and 9, the floating fraction was observed to have 19% filled seeds with a seed density of 0.560, which was comparably lower than the density of filled seeds (0.701) in the sunken fraction (Table 3B). Filled seeds in the floating fraction had higher wing surface area, seed volume and wing quotient than filled seeds in the sunken fraction while the weight of filled seeds was nearly the same for both fractions (Table 3B). The rest of the seed lots showed unambiguous separation of empty and filled seeds as floaters and sinkers, respectively. There was an apparent difference in seed weight, seed density and wing quotient between floaters and sinkers of these seed lots, i.e., the first

Table 3 Morphological characteristics of filled and empty seeds in sunken and floating fractions of different groups

Morphological characters	Empty seeds		Filled seeds	
	Sunken	Floating	Sunken	Floating
(A) Group 1 (seed lots 3 and 5)				
Wing surface area (mm^2)	0.108	0.139	0.175	–*
Seed weight (µg)	1.427	1.173	2.750	–
Seed volume (µl)	2.188	2.496	3.793	–
Seed density (µg/µl)	0.652	0.470	0.725	–
Wing quotient (mm^2/µg)	0.076	0.118	0.063	–
(B) Group 2 (seed lots 2 and 9)				
Wing surface area (mm^2)	–*	0.094	0.090	0.152
Seed weight (µg)	–	0.827	1.325	1.312
Seed volume (µl)	–	1.969	1.890	2.342
Seed density (µg/µl)	–	0.420	0.701	0.560
Wing quotient (mm^2/µg)	–	0.114	0.068	0.116
(C) Group 3 (the rest of the seed lots)				
Wing surface area (mm^2)	–*	0.107	0.110	–*
Seed weight (µg)	–	0.904	1.585	–
Seed volume (µl)	–	1.823	2.198	–
Seed density (µg/µl)	–	0.496	0.721	–
Wing quotient (mm^2/µg)	–	0.118	0.069	–

* Morphological characters were not quantified due to negligibly low number of seeds in each fraction

two seed characters being higher for the sunken fraction while the wing quotient being higher for the floating fraction (Table 3C). As a whole, the result suggested that the efficacy of petroleum flotation was largely influenced by the wing surface area, wing quotient and seed density of filled and empty seeds.

Discussion

The study revealed that seed lots of *C. equisetifolia* are composed of a large quantity of empty and shrivelled seeds with substantial inter-seed lot variability (Table 1). *C. equisetifolia* is a wind-pollinated species with a random mating system and fertilization takes place more than 1 month after pollination, a phenomenon uncommon in angiosperms (Sogo et al. 2004). This prolonged period between pollination and fertilization with five distinct steps of pollen-tube growth and selection of male and female gametophytes may have an impact on production of quality seeds. In addition, environmental factors during seed development, such as day length, temperature, light quality, soil nutrients and water availability, have strong influence on seed filling, thereby accounted for production of shrivelled seeds (Wulff 1995; Gutterman 2000). Reproductive output (the total quantity of reproduction, such as number of flowers and fruits/seeds) also varies among individual mother trees, primarily due to differences in total size and changes in resource allocation patters (Bazzaz et al. 2000), which could be responsible for the occurrence of ill-filled seeds. Insect infestation is another factor that poses a serious problem in seed production.

Seeds partially consumed by larvae were usually less vigorous while seeds that were severely attacked were completely empty of their contents and, hence, failed to germinate (El Atta 1993; Bates et al. 2000, 2001; Tigabu and Odén 2004). However, the effect of insect infestation on seed production of *C. equisetifolia* appears to be less important as evidenced from the substantially low proportion of insect-infested seeds (Table 1).

Apparently, the cause of low germination performance of *C. equisetifolia* seed lots is the occurrence of a large quantity of empty, shrivelled and insect-damaged seeds. The average germination of un-graded seed lots recorded in the present study is consistent with earlier studies that also reported less than 50% germination (Jerlin and Srimathi 1997; Anandalakshmi et al. 2001; Umarani and Vanangamudi 2002). The variation in germination of un-graded seed lots is partly related to the variation in seed weight among seed lots as shown by fairly good positive relationship between these two characters (Fig. 1), which is consistent with previous studies where an increase in germination and seedling vigour of *C. equisetifolia* with increasing seed weight has been observed (Umarani et al. 1997; Mahadevan et al. 1999; Umarani and Vanangamudi 2002). The most interesting part of this finding is that seed weight alone is not a good indicator of germination performance, rather the quantity of unproductive seeds that most affects germinability of seed lots. Maternal factors, such as position of the seed in the fruit/tree and the age of the mother plant during seed maturation, have also an influence on seed germination (Wulff 1995; Gutterman 2000), which partly explains the observed variation in germination between individual seed lots as well as the quantity of shrivelled seeds.

Removal of non-germinable seeds using petroleum flotation resulted in significantly higher germination in the sunken fraction (Table 3). For most seed lots, the percentage germination of sunken seeds was more than 90% indicating that petroleum flotation is more appropriate and efficient to upgrade the germinability of *C. equisetifolia* seed lots than grading using sieves, blowers and specific gravity separator (Maideen et al. 1990; Umarani et al. 1997; Umarani and Vanangamudi 2002) or selection of heavy cones by size grading followed by density grading of seeds (Vinaya Rai 1990). The proportion of germinable seeds discarded as floaters is remarkably low. This is an additional advantage of the petroleum flotation technique because high loss of viable seeds into the discarded fraction as floaters may not be economically feasible from seed supplier's point of view and may have an impact on maintaining the genetic diversity of seed lots. Given the relative availability of petroleum ether, its safety for handling and technical simplicity, the petroleum flotation technique can be applied on a large scale to upgrade germinability of *C. equisetifolia* seed lots, thereby reducing the cost of raising and planting seedlings in nurseries and in the field.

Although petroleum flotation worked well to separate filled and empty seeds of *C. equisetifolia*, the separation efficiency was relatively low for few seed lots as shown by 36% empty seeds in the sunken fractions of seed lots 3 and 5 and 19% filled seeds in the floating fractions of seed lots 2 and 9. The relatively low separation efficiency in these seed lots could be related to their wing size, which in turn is expected to influence the specific density and buoyant force. The higher the wing surface area, the lower the specific density but the higher the buoyant force and vice versa. The empty seeds in the sunken fraction of seed lots 3 and 5 possess tiny wings with a wing quotient of 0.076 but were heavier and denser than empty seeds in the floating fractions, which resulted in a low buoyant force that made them sink. On the contrary, filled seeds in the floating fraction of seed lots 2 and 9 possess relatively bigger wing surface area with wing quotient of 0.116, resulting in high buoyant force that made them float. Wing size has been implicated as a major limitation for successfully separation of filled and dead-filled/empty seeds of *Cupressus lusitanica* by

IDS technique using water as flotation medium (Bergsten and Sundberg 1990). As seeds of *C. equisetifolia* are winged samara (Turnbull and Martensz 1981) and de-winging is difficult to achieve due to the fragile nature of seeds, the best alternative would be to collect a large quantity of seeds from as many parents as possible. This will average out the wing size variability and increase the quantity of filled seeds obtained after petroleum flotation.

The fact that 50% of the seed lots are composed of unproductive seeds accentuates the need for developing methods for enhancing the production of filled seeds in seed orchards or seed production areas. For example, cultural treatments such as nitrogen fertilization (Graham 1986), root pruning (Eysteinsson and Greenwood 1990; Philipson 1995), spacing or stand density (Shearer and Schmidt 1987) as well as heat and drought treatments (Philipson 1995) have been shown to improve floral induction and subsequent seed production in other species (e.g., *Larix* species). In addition, some success in production of cones and filled seeds has been achieved using girdling alone or in combination with application of gibberellins (Bonnet-Masimbert et al. 1998). To our knowledge, such works have not been done on Casuarina. Therefore, further research is required to determine the best treatment that enhances the production of filled seeds of *C. equisetifolia*.

Conclusions

The study provides evidence that the low germination performance of *C. equisetifolia* seed lots is due to the occurrence of a large quantity of empty and shrivelled seeds as revealed by X-ray analysis. Such unproductive seeds can effectively be removed using petroleum flotation technique. Wing surface area, seed density, and wing quotient largely influence the separation efficiency of petroleum flotation.

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