OPTIMIZATION OF CRYOPRESERVATION PROTOCOL FOR *STERCULIA CORDATA* ZYGOTIC EMBRYOS USING TAGUCHI EXPERIMENTS

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NADARAJAN, J., STAINES, H. J., BENSON, E. E., MARZALINA, M., KRISHNAPILLAY, B. & HARDING, K. 2006. Optimization of cryopreservation protocol for *Sterculia cordata* zygotic embryos using Taguchi experiments. *Sterculia cordata* is a tropical forest tree species bearing intermediate to recalcitrant seeds. This study investigated the effects of seed developmental stage, desiccation time and 6-benzyl aminopurine hormone level in recovery media for cryopreservation of *S. cordata* embryos using Taguchi designed experiments. Taguchi experiments, which use Signal to Noise Ratio (SNR) as variables, allowed investigation of all main effects in smaller ($\frac{1}{3}$) replication of the original experiment. Seed developmental stage was the most important factor for *S. cordata* embryo cryopreservation protocol optimization. Taguchi optimization techniques are recommended when germplasm is scarce and the experiment needs to be conducted rapidly.

Keywords: desiccation, seed maturation tropical forest seeds, seed development, moisture content

NADARAJAN, J., STAINES, H. J., BENSON, E. E., MARZALINA, M., KRISHNAPILLAY, B. & HARDING, K. 2006. Pengoptimuman protokol krioawetan bagi embrio zigot *Sterculia cordata* menggunakan kaedah Taguchi. *Sterculia cordata* ialah spesies pokok hutan tropika yang menghasilkan biji benih yang dapat dikategorikan dalam jenis perantaraan hingga rekalsitran. Kajian ini tentang kesan tahap perkembangan biji benih (SDS), masa pengeringan dan paras hormon 6-benzilaminopurin di dalam media pemulihan bagi krioawetan embrio *S. cordata* menggunakan kaedah reka bentuk Taguchi. Kaedah Taguchi yang menggunakan nisbah 'Signal to Noise' (SNR) sebagai pemboleh ubah membolehkan penyiasatan semua faktor utama dalam perelikaan yang lebih kecil ($\frac{1}{3}$) berbanding perelikaan percubaan asal. Tahap perkembangan biji benih ialah faktor terpenting bagi pengoptimuman protokol krioawetan embrio *S. cordata*. Teknik pengoptimuman Taguchi disyorkan apabila sesuatu plasma germa susah didapati dan uji kaji perlu dilaksanakan dengan cepat.

INTRODUCTION

An important tropical tree species and a member of the Sterculiaceae, *Sterculia cordata* is endemic in Peninsular Malaysia, Borneo, Sumatra and New Guinea. It is distributed throughout South-East Asia (Ng & Low 1982). It produces lightweight softwood used for light interior construction, packing cases, ceilings, veneer and plywood, concrete shuttering and shoe heels. The seeds of *S. cordata* are edible and have a high oil content, which is used for frying, illumination and also in batik works (Lemmens *et al.* 1998). The seeds are dispersed after reaching a moisture content of 29% (fresh weight basis) and usually germinate immediately or soon after falling from the mother tree.

Sterculia cordata seeds, after dispersal, are currently stored at partially dehydrated moisture contents of 12 to 20 °C at the Forest Research Institute Malaysia. However, their viability is maintained only for a few weeks and presently cryopreservation is the only realistic choice for long-term storage. Selecting an appropriate seed maturation stage is extremely important in the development of cryopreservation protocols especially for those seeds within an intermediate–recalcitrant category.

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Based on their post-harvest storage behaviour, *S. cordata* seeds could be classified as intermediate or recalcitrant (Roberts 1973, Ellis *et al.* 1990). Definition of intermediate seed storage behaviour is based on the response of longevity to storage environment (Ouedraogo *et al.* 1996). For intermediate seeds, seed longevity benefits to a considerable extent from desiccation and/or reduction in temperature. However, longevity is decreased by further reduction in temperature below about 10 °C and/or by further reduction in moisture content below apparent optimum values in equilibrium with about 40–50% relative humidity. It is often observed that seeds may be damaged immediately by desiccation to relatively low moisture contents (7 to 12%) depending on species (Ellis *et al.* 1990). The critical moisture contents of intermediate seeds, below which more rapid loss in viability occurs during hermetic storage, vary considerably with species, degree of maturity, method of seed extraction and post harvest handling.

Sterculia cordata seeds have large fleshy cotyledons and hence are also prone to fungal infection and attacks by pests, which further reduce viability during storage. Therefore, under these conditions, long-term storage of these seeds is not currently possible and cryopreservation offers the most promising solution. Cryopreservation is the process by which living tissues are conserved in liquid nitrogen (LN) at -196 °C. Successful cryoconservation depends upon the application of cryoprotective treatments without which living cells undergo lethal injury. A wide variety of plant species have now been cryopreserved although special consideration is required for tropical tree genetic resources (Normah *et al.* 1996, Engelmann & Takagi 2000, Benson 2004).

This paper reports, for the first time, cryopreservation of *S. cordata* embryos. Excised embryos were used due to larger size (2 cm height and 1.5 cm diameter; Figure 1) and high shedding moisture content of seeds (29% on fresh weight basis). Different seed developmental stages (SDS) were studied for cryopreservation. Seed developmental stage is a very important criterion in cryopreservation especially for non-orthodox seeds (i.e. intermediate and recalcitrant categories). This is because complex physiological changes take place as the seeds mature including changes in moisture content (Walters 1999). Moisture content is an important factor in

Figure 1   *Sterculia cordata* seedpods and seeds

cryopreservation and determining the critical moisture content is a basic requirement in many seed cryopreservation studies.

MATERIALS AND METHODS

Seed collection

Seedpods at developmental stage one (SDS 1), two (SDS 2) and three (SDS 3) were collected from mother trees at the Forest Research Institute Malaysia (FRIM). SDS 3 were collected at their optimum maturity when they shed from mother trees. SDS 2 and SDS 1 were collected at one and two weeks respectively before collecting seeds at stage three. These intervals were set and fixed based on FRIM's monthly phenological report on the species. Figures 2a and b show the three different developmental stages.

Seed testing and processing

Seed testing was conducted before the seeds were subjected to experimental procedures. These tests include seed moisture content (5×4 seeds), germination (25×4 seeds) and seed weight determination (5×4 seeds) tests. These tests were conducted on freshly collected seeds for three SDS.

Seeds were surface sterilized (10% v/v hypochlorite solution) for 10 min, rinsed once in 30% (v/v) ethanol for 1 min and then rinsed three times in sterile water. The embryos were excised in a laminar airflow cabinet, surface sterilized with 0.3% (w/v) boric acid, rinsed once in 30% (v/v) ethanol for 1 min and rinsed three times in sterile water.
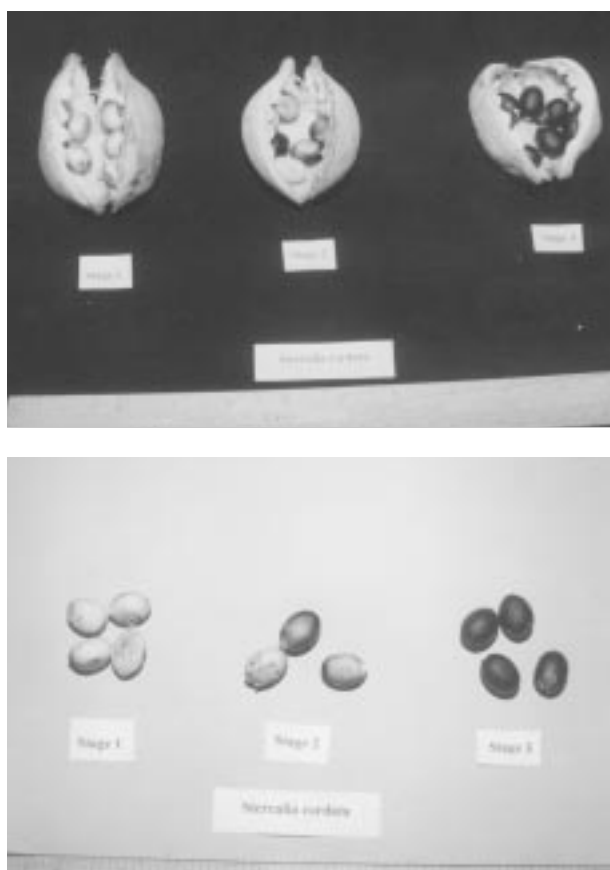
Figure 2   *Sterculia cordata* seedpods (a) and seeds (b) at three different developmental stages

Embryo desiccation

Excised embryos were arranged in a single layer in sterile Petri dishes, placed in the air current of a laminar airflow cabinet, and desiccated for 2, 4 and 6 hours at 23 ± 2 °C and $55 \pm 5\%$ relative humidity. There were 10 embryos in three replicates for each treatment. Embryo moisture contents were determined after desiccation at each developmental stage.

Cryopreservation

After the required desiccation period, embryos were placed in sterile 2 ml cryo-vials and cooled rapidly by direct immersion in liquid nitrogen. They were rapidly warmed by directly plunging and swirling in a water bath at 38 ± 2 °C for 10–15 min.

Germination

Both non-cryopreserved and cryopreserved embryos were germinated in Murashige and Skoog (1962) medium (MS) supplemented with 30 mg l^{-1} sucrose and 5.5 mg l^{-1} plant agar and three different levels of BAP (0.5, 1.0 and 1.5 mg l^{-1}), incubated in a temperature-controlled growth room at 26 °C with 12 hours photoperiod. The development of each seed from sprouting to germination was observed daily for 15 days. Germination day, shoot to root ratio and seedling dry weight before and after cryopreservation were carried out as described in Muthusamy *et al.* (2005).

Data analysis

There were two experiments in this study based on LN storage. Experiment 1 was without LN storage and experiment 2 was with LN storage.

Experiment 1

For the experiment without LN storage, three SDS, three different desiccation times and three levels of BAP in recovery media were investigated as shown below:

(A)   Seed development stage SDS (stages 1, 2 and 3)
(B)   Desiccation time (2, 4 and 6 hours)
(C)   BAP (0.5, 1.0 and 1.5 mg l^{-1})

This is a three cubic (3^3) experiment with three factors at three levels and designed as Taguchi L$_9$ experiment. The response variables chosen for this case study were: germination day, shoot to root ratio and seedling dry weight. These illustrate the three signal to noise ratio (SNR) (smaller is better, nominal value and larger is better respectively) commonly used with Taguchi experimental designs. There were nine treatments and 30 seeds were used per treatment in three replicates with 10 embryos per replicate. Analysis of covariance (ANCOVA) was used for this experiment since two of the factors (desiccation time and BAP concentration) were at consistent intervals. ANCOVA is a technique that sits between analysis of variance and regression analysis (Rutherford 2000). In this

experiment, the data were analysed with desiccation time and BAP concentration as covariates and seed developmental stage as factor with SDS 1 as fixed factor, and SDS 2 and SDS 3 as the compared stages to SDS 1. The significant effects were identified from the ANCOVA table. Regression analysis was carried out to investigate how different is the SDS 2 and SDS 3 compared with SDS 1.

Experiment 2

For the cryopreservation experiment, all three levels of SDS and BAP hormone were tested. However, only two levels of desiccation time were investigated since the embryos desiccated to 6 hours did not show any survival in experiment 1. The factors and their levels are shown below:
(A)   Seed development stage (stages 1, 2 and 3)
(B)   Desiccation time (2 and 4 hours)
(C)   BAP (0.5, 1.0 and 1.5 mg l^{-1})
Taguchi analysis with the appropriate SNR as the response variable was used for this experiment. There were 18 treatments and 30 seeds were used per treatment in three replicates with 10 seeds per replicate. The experiment was analysed as Taguchi L$_{18}$ experiment allowing investigation of all main effects but not any interaction.

RESULTS

There was a decline in seed moisture content and increase in seed dry weight as the seeds matured (Table 1).

The embryos tended to have slightly higher moisture content compared with the seeds (Table 2). The embryos' moisture content also declined gradually as the seed matured.

Experiment 1: Without LN storage

This design consists of only $\frac{1}{3}$ replication (i.e. nine treatment combinations) of the original experiment. For this fractional replicate it was possible to investigate all the main effects but not any interaction.

Germination before cryopreservation

Overall germination percentage at SDS 1 after 2 hours' desiccation was approximately 40% and it declined to about 16 and 0% after 4 and 6 hours' desiccation respectively (Table 3). This shows that the immature embryos were extremely sensitive to desiccation. Although germination percentage increased for SDS 2, the embryos still showed desiccation sensitivity. The germination was higher (93.3%) for SDS 3 at 2 hours' desiccation but it declined rapidly to about 80 and 60% after 4 and 6 hours' desiccation respectively. Overall, SDS 3 was the optimum stage whereby *S. cordata* embryos showed greatest germination (Table 3).

Germination day

The Taguchi analysis showed that both SDS 2 and SDS 3 had significantly different effects from SDS 1 (p = 0.000). SDS 2 increased the SNR values for germination day by the average of 1.72 (1.4 days earlier than SDS 1) and SDS 3 increased the SNR value for germination day by the average of 4.71 (3.3 days earlier than SDS 1) compared with SDS 1, provided other conditions are fixed. Both BAP and desiccation time did not have any significant effect on the germination day.

Shoot to root ratio

None of the main effects had significant effect on the shoot to root ratio SNR.

Dry weight

Both SDS 2 (p=0.004) and SDS 3 (p=0.000) had significantly different effects from SDS 1. SDS 2 increased the SNR values for dry weight by the average of 2.23 (0.16 g increase in dry weight) and SDS 3 increased the SNR value for dry weight by the average of 5.27 (0.32 g increase in dry weight) compared with SDS 1, provided other conditions are fixed. Both BAP and desiccation time did not have any significant effect on the dry weight.

Experiment 2: Post-cryopreservation germination

The overall viability as percentage germination was 0% for SDS 1, 50% for SDS 2 and 70% for SDS 3, regardless of desiccation time and BAP level (Figure 3).

Seed developmental stage (SDS) showed a

Table 1   *Sterculia cordata* seed moisture content, dry weight and germination percentage at three different developmental stages

Parameter	SDS 1 (Mean ± SD)	SDS 2 (Mean ± SD)	SDS 3 (Mean ± SD)
Moisture content (%)	34.33 ± 0.58	30.00 ± 1.0	29.33 ± 0.58
Dry weight (g)	3.66 ± 0.12	3.97 ± 0.13	4.44 ± 0.08
Germination (%)	40.00 ± 0.10	86.00 ± 5.29	100.00 ± 0

SD = standard deviation

Table 2   Moisture contents of *Sterculia cordata* embryos at shedding and after 2, 4 and 6 hours of desiccation

Treatment	SDS 1 (Mean ± SD)	SDS 2 (Mean ± SD)	SDS 3 (Mean ± SD)
Shedding / 0 hour	37.61 ± 0.68	33.66 ± 0.78	30.85 ± 0.69
2 hours desiccation	31.89 ± 0.63	29.06 ± 0.66	24.54 ± 0.41
4 hours desiccation	25.61 ± 0.60	22.78 ± 0.53	19.28 ± 0.61
6 hours desiccation	21.77 ± 1.28	18.67 ± 0.86	16.49 ± 0.70

SD = standard deviation

Table 3   Taguchi L_9 experimental design, the selected treatment combinations and their mean germination percentage

Seed development stage	Drying time (hours)	BAP (mg l^{-1})	Mean germination (%) (Mean ± SD)
1	2	0.5	40.0 ± 0
1	4	1.0	16.7 ± 05.8
1	6	1.5	0 ± 0
2	6	0.5	46.7 ± 17.3
2	4	1.0	60.0 ± 2
2	2	1.5	76.7 ± 05.8
3	6	0.5	60.0 ± 0
3	4	1.0	80.0 ± 10
3	2	1.5	93.3 ± 05.8

SD = standard deviation

significant effect on germination day (p = 0.000) of the embryos and seedling dry weight (p = 0.000). Embryos at SDS 3 germinated earlier and had greater dry weight compared with SDS 2. All embryos from SDS 1 were dead after cryopreservation for both desiccation treatments.

DISCUSSION

Selecting an appropriate seed maturation stage is extremely important in the development of cryopreservation protocols especially for those seeds of an intermediate–recalcitrant category. Whilst orthodox seeds undertake maturation drying, which programmes the switch from a developmental mode to a germinative mode (Kermode 1995), many recalcitrant seeds especially in the tropics do not undergo maturation drying and therefore do not experience reduced cellular

metabolism and a clear physiological termination of seed development (Pammenter & Berjak 1999).

For many tropical tree seeds, development is followed by germination without interruption during fruit dehiscence and dispersal. It has been reported that those seeds, which do not undertake maturation drying, are usually large, with very well developed embryos. For example, *Aesculus hippocastanum* (Tompsett & Pritchard 1993) and *Hevea brasiliensis* (Chin *et al.* 1981) seeds. All these seeds increase in dry weight until fruit dehiscence, with slight or no loss in fresh weight However, a decrease in water content is characteristic of several seeds that do not undertake maturation drying (Greggains *et al.* 2000). For these, embryo growth may continue (increasing in dry weight) after dehiscence in the absence of enough water to promote germination. However, in the tropical forest, with very
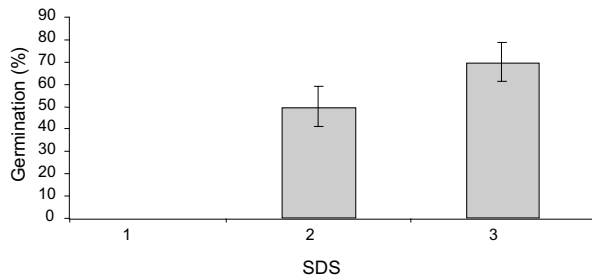
Figure 3 Percentage germination of *Sterculia cordata* embryos after freezing in liquid nitrogen at three different maturity stages

high rainfalls and humidity levels, the seeds shed from mother trees on humid soils and therefore continue hydrating and subsequently germinate.

Characterization of *Sterculia cordata* seeds

Table 1 shows that moisture content declines drastically as the seed matures from SDS 1 to SDS 2 but than stabilises at SDS 2. This observation was different from those reported for orthodox seeds where seed moisture content continues to decline until the seed is fully matured and ready to germinate (Bailly *et al.* 2001).

In this study, the seed dry weight continued to increase until the end of maturity. During maturation, reserve material accumulation occurs to such a degree that dry weight increases in spite of the already initiated decrease in water content. Aberlence-Bertossi *et al.* (2003) reported similar observation in oil palm (a recalcitrant seed producing species), whereby embryos underwent dehydration up to 120 days after pollination. However their moisture content remained high at maturity and their dry weight during maturation increased between 80 and 140 days after pollination and was then stable to the point of maturation.

In *S. cordata*, the percentage germination was relatively low (40%) for seeds harvested at SDS 1 (Table 1). However, it increased to 86% as the seeds matured to SDS 2 and to 100% at SDS 3. These data indicate that the seeds are naturally preparing for germination as they mature and optimum germination is attained at the end of maturation process.

Effects of maturation and desiccation

Embryos at SDS 3 germinated most rapidly followed by those at SDS 2 and SDS 1. This supports the supposition that seeds at SDS 3 are ready to germinate once placed in the appropriate medium. Sprouting is triggered immediately. It also explains why seeds in SDS 2 took longer to germinate compared with those in SDS 3 and seeds in SDS 1 took the longest time to germinate. SDS significantly affected the dry weight of the seedling. Seedling showed greater dry weight at SDS 3 followed by SDS 2 and SDS 1. This explains that as seeds mature, they tend to have greater dry matter content compared with immature seeds. Those seeds with greater dry weight have larger stored reserves that can be made available to the germinating embryo and therefore produced seedlings with greater dry weight.

Percentage germination before cryopreservation showed that SDS 1 embryos were extremely sensitive to desiccation and their germination cannot therefore be improved by desiccation. All embryos were dead after 6 hours' desiccation. Percentage germination increased when seed moisture content declined naturally as the seeds matured (Table 1). However, when this outcome was compared with experimentally induced desiccation, germination of *S. cordata* embryos was not improved. It was reported that bean (*Phaseolus vulgaris*) seed tolerance to drying developed fully only at the end of seed maturation, approximately 50 days after anthesis (Bailly *et al.* 2001). Bailly *et al.* (2001) also confirmed that drying of very young seeds, which were not at all tolerant to desiccation, induced strong deterioration of cell membranes as indicated by high electrolyte leakage. Embryos from all SDSs also showed a decline in germination as they were desiccated for longer periods. Similar observations were reported for castor bean (Kermode 1990) and *P. vulgaris* seeds (Dasgupta *et al.* 1982). However, Nedeva and Nikilova (1999) reported an increase in germination with desiccated premature *Triticum aestivum* (wheat) seeds.

Post-cryopreservation recovery

The freezing tolerance of *S. cordata* seeds was identified by their embryos' ability to germinate after immersion in liquid nitrogen. Post-cryopreservation germination of developing seeds increased abruptly as the seeds matured and when their moisture content was lower (Figure 3).

The embryos survived liquid nitrogen storage with a percentage germination of 70% at SDS 3 and 50% at SDS 2 but not at SDS 1 (Figure 3). SDS 3 was found to be optimum for cryopreservation of *S. cordata* embryos. The reduced germination at SDS 2 indicated that tissues were damaged at water contents much higher than those in seeds at SDS 3. The percentage germination continued to increase with seed maturation as the moisture content decreased and dry weight increased.

Increase in post-cryopreservation germination may also be due to other seed physiology factors, which may have reduced freezable water content, thereby, allowing seed survival. During maturation, when seeds have initiated the desiccation process, some solutes were evidently still accumulating, as demonstrated by the increase in dry weight (Table 1). These results may indicate that the reduction in seed water content may not completely explain the greater acquisition of freezing tolerance for embryos at SDS 3 as compared with those at SDS 2.

It was observed that freezing tolerance increased sharply at a later stage of seed development when there is an accumulation in dry weight. This phenomenon may be related to the increase of the so-called late embryogenesis accumulated (LEA) proteins, which are supposedly involved in the maturation and desiccation tolerance acquisition (Blackman *et al.* 1991), as well as other solutes such as oligosaccharides (Castillo *et al.* 1990). The raffinose family of oligosaccharides has been reported as endogenous cryoprotectants, which accumulate during the cold hardening of Puma rye (Koster & Lynch 1992). Moreover, Koster (1991) found that raffinose is a crucial oligosaccharide for the prevention of sucrose crystallization in drying embryo axis, promoting glass formation, a phenomenon that is prerequisite for the survival of tissues to freezing at ultra-low temperatures.

Experimental design and data analysis

Both experiments 1 and 2 were designed as Taguchi experiments. One major difference in the Taguchi approach compared with factorial experiment is that of experimental design (Staines *et al.* 1999). Taguchi uses orthogonal arrays, a particular set of fractional replicates of factorial designs (Nadarajan 2005). This, in conjunction with the associated set of linear graphs, permits quickly designed experiments that allow given main effects and specified interactions to be investigated (Muthusamy *et al.* 2005). Fractional replicates consist of a carefully chosen subset or fraction of full experiment (Wu & Hamada 2000). For Taguchi analysis, the optimal treatment combination is that which maximises SNR (Staines *et al.* 1999, Muthusamy *et al.* 2005). The SNR depends on whether the response variable is to be as small as possible, close to a nominal value or as large as possible.

Muthusamy *et al.* (2005) showed that same conclusions as factorial experiments could be obtained with Taguchi experiments. Furthermore, they claimed that a more consistent conclusion could be obtained using Taguchi method evidenced by the distribution of raw data. These properties make Taguchi experimental design a favourable alternative to traditional factorial designs. Full experiments are rarely necessary in practice for large number of factors, especially as significant three or more factor interactions are not regarded as important (Wu & Hamada 2000). In experiment 1, only $\frac{1}{3}$ fraction of the original experiment was used. This fractional replication allows for investigation of all main effects but not any 2-factor interactions due to limited degrees of freedom. However, this $\frac{1}{3}$ fractional experiment is useful if only main effects are to be investigated or to carry out screening or preliminary experiments or when there is limited number of samples to run a big experiment (Babiak *et al.* 2000).

For the (2×3^2) cryopreservation experiment, no smaller fractional replication is possible. However, if another level is added for the desiccation time factor, 3^3 experiment (similar to experiment 1) could have been designed and there is an advantage to conduct $\frac{1}{3}$ replication of the original experiment to investigate all the main effects.

CONCLUSIONS

The seed developmental stage was the most important factor for cryopreservation of *S. cordata* embryos whereby embryos at SDS 3 showed greatest germination. The capacity of desiccated immature embryos to germinate post-cryopreservation did not depend on the degree of desiccation reached by artificial drying but only on their age at harvest time. Taguchi optimization techniques are recommended when germplasm is scarce and the experiment needs to be conducted rapidly or when smaller scale experiments are preferred for preliminary screening tests.

ACKNOWLEDGEMENTS

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