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Improving seedling production for *Vitex doniana*

S. N'DANIKOU[1,2], E.G. ACHIGAN-DAKO[1], D.A. TCHOKPONHOUÉ[1], F. ASSOGBA KOMLAN[3], R.S. VODOUHÈ[2] AND A. AHANCHÉDÉ[1]

[1] Horticulture and Genetics Unit, Faculty of Agronomic Sciences, University of Abomey-Calavi, BP 2549 Abomey-Calavi, Republic of Benin (E-mail: ndanikou@gmail.com)
[2] Bioversity International, West and Central Africa office, 08 BP 0932, Cotonou, Republic of Benin
[3] National Agricultural Institute of Research of Benin (INRAB), BP 884 Cotonou, Republic of Benin

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Abstract

Uniform germination is one of the important agronomic requirements for successful domestication of wild-harvested economic plants. In this study we investigated the effects of storage and hydration-dehydration regimes on *Vitex doniana* seed viability, germination speed and seedling growth and vigour. These effects were tested in a factorial combination of four storage durations (0, 2, 4 and 8 months) and five hydration-dehydration regimes including the control, alternating one hour soaking in tap water with eight hours sun-drying for 3, 7, 14 and 21 days. After two months storage at room temperature, germination percentage was not significantly different from initial germination; seed viability and germination decreased with storage beyond two months. For the first time, germination in *V. doniana* reached 83%, for seeds stored for two months and then submitted to 21 days of hydration-dehydration cycles. Two months storage combined with hydration-dehydration regimes for 14 or 21 days improved germination speed and seedling growth and vigour. We recommend that fresh seeds be stored for a maximum of two months and stratified by consecutive hydration-dehydration cycles for 14 or 21 days to obtain a homogenous seedling cohort. These results represent an important step towards domesticating *V. doniana* in West Africa.

Introduction

Uniform germination of seeds is a critical determinant for successful domestication of plant species (Kupzow, 1980). However, seed dormancy remains a bottleneck to the propagation of many forest species of economic importance, since about 70% of all major taxonomic groups of seed plants have dormant seeds (Baskin and Baskin, 2003). Seed represents the planting material that best conserves the gene pool in species or varieties, from one planting generation to another. In this respect, how to handle seed dormancy in forest species to produce homogenous seedling cohorts for commercial plantations remains an important issue for many wild-harvested economic plants. The current study investigated how to quickly overcome physical dormancy in seeds of *Vitex doniana* Sweet for commercial seedling production. The species was selected based on its importance in supporting the livelihoods of rural and urban dwellers in West Africa, with applications in food and medicine (Kristensen and Lykke, 2003; Okigbo, 2003; Ky, 2008; Achigan-

Dako *et al.*, 2010; Ajenifujah-Solebo and Aina, 2011). It is among the top most important wild edible plants in southern Benin and also appears among the most threatened species (N'Danikou *et al.*, 2011).

Vitex doniana seeds present a combination of physical (PY) and physiological dormancy (PD), based on classification by Baskin and Baskin (2004) and Silveira (2013). However, little is known about dormancy breaking requirements and no reliable technique is available yet. Imbibition tests revealed that *V. doniana* seeds are physically dormant (N'Danikou *et al.,* 2014) but different treatments tested so far resulted in germination rates below 60% after six months (Mapongmetsem, 2006; Ky, 2008; Ahoton *et al.,* 2011). Moreover, germination is spread over a long period (1-12 months) triggering various growth speeds in seedlings.

In seeds combining physiological and physical dormancy, there have been indications of beneficial effects of seed storage and hydration-dehydration of seeds to break water-impermeable layers and weaken the effects of germination inhibitors (Baskin and Baskin, 2003; Kucera *et al.,* 2005). In a recent study, we hypothesised a beneficial effect of hydration-dehydration on germination and seedling growth for *V. doniana* (N'Danikou *et al.,* 2014); a beneficial effect has been demonstrated for seeds of teak (*Tectona grandis* L.f.) which also belongs to the Verbenaceae family (Chacko *et al.,* 1997). In this study, we tested this hypothesis, combining seed storage and hydration-dehydration treatments, before sowing seeds and evaluating germination and seedling growth and vigour.

Material and methods

Experimental design

Seeds used in the experiment were bought from Ikpinle market, Adja-Ouere district in southern Benin. According to vendors these seeds were collected in natural habitats 2-3 days before they were brought to the market. For the purpose of the experiment, seeds were screened to remove impurities and immature seeds to obtain a homogenous seed batch. The seed batch was divided into four lots of 250 seeds each. Seeds of lot S_1 were sown fresh with no storage, while lots S_2, S_3 and S_4 were stored for 2, 4 and 8 months respectively, at room temperature (24-28°C). Seeds were put on fabric and maintained open for the whole storage period. After storage (and for lot S_1), we applied five hydration-dehydration regimes, with T_0 being the control (no hydration-dehydration) and T_1, T_2, T_3 and T_4 treated over 3, 7, 14, and 21 days respectively (table 1). Treated seeds were soaked in tap water for one hour and sun-dried for eight hours during the day, then stored at room temperature overnight and rehydrated the following day and so on, for 3, 7, 14 or 21 cycles, each cycle lasting one day (24 hours). The experiment was carried out from September 2012 to August 2013 at the National Agricultural Research Institute of Benin (INRAB)'s research station at Agonkanmey, Abomey-Calavi Benin. The experimental design was a complete randomised block consisting of a factorial combination of the four storage durations and the five different hydration-dehydration regimes, with five replicates of 10 seeds each per treatment. Each seed was directly sown in one polystyrene nursery bag (approximately 0.64 L capacity) filled with ferralitic soil.

Table 1. Treatments used to break dormancy in seeds of *Vitex doniana*.

Dormancy treatments	Duration of seed storage			
	Control (S1)	Two months (S2)	Four months (S3)	Eight months (S4)
	Combinations (treatments)			
T0: Control, no dormancy treatment	S1T0	S2T0	S3T0	S4T0
T1: three days hydration-dehydration regime	S1T1	S2T1	S3T1	S4T1
T2: seven days hydration-dehydration regime	S1T2	S2T2	S3T2	S4T2
T3: fourteen days hydration-dehydration regime	S1T3	S2T3	S3T3	S4T3
T4: twenty one days hydration-dehydration regime	S1T4	S2T4	S3T4	S4T4

Seeds were sown 50 mm deep in the soil with the hilum oriented upwards. Nursery pots were watered daily. After germination, seedlings were raised in ambient conditions under a shelter and watered daily.

Data collection

Data collection followed N'Danikou *et al.* (2014). Germination was recorded daily for 12 months from the day of sowing to the end of the experiment, while seedling growth data (e.g. height, number of leaves and basal stem diameter) were measured weekly. Growth parameters were recorded from two weeks after germination.

A seed viability test was carried out on non-germinated seeds, using the imbibed seed crush test (Borza *et al.*, 2007).

Data analysis

Germination percentage was calculated as a proportion of viable seeds.

Germination speed was assessed by calculating a) the time to first germination (TFG), b) the mean germination time (MGT), and c) the time necessary to reach the threshold of 25% germination (T_{25}) which was attained in all healthy seed lots. MGT is calculated as follows:

$$\mathrm{MGT}_p = \sum_{i=1}^{k} t_i \Big/ \sum_{i=1}^{k} \mathrm{NG}p_i$$

where p is the treatment, t_i is the time taken by each seed in replicate i of treatment p to germinate, and $\mathrm{NG}p_i$ the number of germinated seeds in replicate i of treatment p, k is the number of replicates in treatment p.

The germination data were analysed using a generalised linear model with binomial and quasi-binomial (to account for over-dispersion) error structures to test the effects of the different dormancy breaking treatments on germination. Effects of the treatments on

the time to first germination, the mean germination time and on the time to reach 25% germination (T_{25}) were compared using a generalised linear model with quasi-poisson error structure. Mixed-effect model with Akaike Information Criterion (AIC) was used to analyse the effects of fixed factors on seedling growth (stem diameter, height and number of leaves). The Student-Newman-Keuls and Kruskal-Wallis tests were used as appropriate to compare mean values of germination speed (MGT, TFG and T_{25}) in different treatments. Correlation test and simple regression were used to analyse relationships between growth parameters. Statistical analyses were performed in R statistical software (version R.2.15.3, 2013).

Results

Effects of storage and hydration-dehydration regimes on seed viability and germination
Seeds stored for more than two months showed significant declines in viability and germination percentage ($P < 0.001$; table 2). Viability was 82.8% for fresh seeds (S_1) and 84.8% after two months storage (S_2). The slight difference between S_1 and S_2 was not significant ($P = 0.57$). After two months storage, seeds rapidly lost their viability, dropping to 18.0% after four months storage (S_3) and 0.8% after eight months storage (S_4).

Six months after sowing, germination had reached 72.2% for S_1 seeds and 65.6% for S_2 seeds. Germination was very erratic for S_3 seeds and no germination was obtained for S_4 seeds. Due to the very low viability for S_3 and S_4 seeds, these seed lots were not included in further analysis of the effects of hydration-dehydration treatments on seed germination.

Final germination at twelve months were not significantly different from that recorded at six months (180 days) after sowing ($P = 0.60$). The highest germination after six months (83.1%) was recorded in seeds stored for two months (S_2) with 21 cycles of hydration-dehydration (T_4). After twelve months, germination for these seeds was just 87.1%.

Alternating hydration-dehydration for several cycles had a significant effect on germination percentage ($P < 0.001$). Germination increased with the number of hydration-dehydration cycles. The generalised linear model revealed a significant ($P < 0.001$) interaction effect of storage and the treatment applied, with treatments S_2T_4 (82.4% germination) and S_2T_3 (83.1%) being the best treatments for removing seed dormancy. Storage for long durations decreased germination, while longer treatments with multiple hydration-dehydration cycles improved seed germination.

Effects of storage and hydration-dehydration regimes on germination speed
Storage and hydration-dehydration regime significantly improved germination speed ($P < 0.01$) (table 3). Mean germination time (MGT) was lower in seeds stored for two months (S_2) compared with seeds sown fresh (S_1). The MGT of all the treatments in seed lot S_2 was less than 90 days, while it was above 90 days in almost all the treatments in seed lot in S_1. First germination in S_2 lots occurred 15 days after sowing (mean 20 days). In fresh seeds (S_1), first germination occurred 31 days after sowing (mean 50 days).

Table 2. Viability and germination following different treatments on *Vitex doniana* seeds. Viability was determined at the end of the experiments (after 12 months).

Treatments[α]	Viability (%)	Germination after 12 months (%)[¥]
S1T0	86.0	68.7
S1T1	80.0	71.7
S1T2	76.0	83.8
S1T3	80.0	73.8
S1T4	92.0	87.1
S2T0	84.0	38.1
S2T1	84.0	59.1
S2T2	80.0	76.5
S2T3	82.0	82.4
S2T4	94.0	87.1
S3T0	40.0	NA*
S3T1	12.0	NA
S3T2	12.0	NA
S3T3	12.0	NA
S3T4	14.0	NA
S4T0	2.0	NA
S4T1	0.0	NA
S4T2	0.0	NA
S4T3	2.0	NA
S4T4	0.0	NA

[α]Each treatment comprised 50 seeds sown as five lots of 10 seeds each. See table 1 for the definition of each treatment; [¥]Germination percentage calculated as the proportion of viable seeds; *NA = not applicable, most seeds were dead.

Table 3. Time to 25% germination, time to first germination and mean germination time of *Vitex doniana* seeds submitted to different storage periods and hydration-dehydration treatments.

Duration of hydration-dehydration	Time to 25% germination (T_{25}) (days)		Time to first germination (TFG) (days)		Mean germination time (MGT) (days)	
	S1	S2	S1	S2	S1	S2
0 days (T0)	123.2 ± 33.2^{a}	95.3 ± 11.6^{abc}	96.4 ± 22.0^{a}	68.0 ± 21.5^{bc}	124.5 ± 29.0^{a}	85.9 ± 23.5^{b}
3 days (T1)	118.8 ± 8.8^{a}	66.2 ± 14.0^{cd}	99.0 ± 14.6^{a}	55.2 ± 8.3^{c}	128.5 ± 22.4^{a}	76.1 ± 26.3^{b}
7 days (T2)	110.0 ± 8.6^{ab}	45.4 ± 22.7^{de}	83.4 ± 6.6^{ab}	27.4 ± 14.1^{b}	115.8 ± 24.4^{a}	76.7 ± 43.4^{b}
14 days (T3)	84.2 ± 19.6^{bc}	36.2 ± 12.3^{e}	55.6 ± 20.4^{c}	20.2 ± 3.3^{d}	92.6 ± 30.2^{b}	56.3 ± 35.7^{c}
21 days (T4)	78.8 ± 20.7^{c}	31.6 ± 8.3^{e}	49.8 ± 15.7^{c}	24.6 ± 8.1^{d}	87.2 ± 26.4^{b}	51.0 ± 29.1^{c}

*For the same parameter and in both columns together (S1 and S2), numbers that are followed by the same superscript letters are not statistically different at 0.05 probability level; S1 (no storage); S2 (two months storage).

In addition, the storage duration and the number of hydration-dehydration cycles, both had significant effects on T_{25} ($P < 0.01$). T_{25} was reached earlier in seeds consecutively imbibed and dehydrated over longer periods (i.e. more cycles), within 32 days for S_2 compared with 79 days for S_1 (table 3). About 75 days after the first germination, the highest germination percentage was obtained in S_2T_4 (79%).

Moreover, a significant interaction effect of the storage and seed dormancy treatment (hydration-dehydration regime) on TFG was noted ($P < 0.05$), but not on MGT ($P > 0.05$). Two months storage and 14 or 21 cycles of hydration-dehydration were optimal. In addition, the interaction effect on T_{25} was significant ($P < 0.01$).

Effects of treatments on seedling vigour and growth

Seedling stem diameter, height and leaf number

Seedling growth was monitored for four months (120 days). In both seed lots (S_1 and S_2), the dormancy breaking treatments improved seedling growth (figure 1). Seedlings regenerated from seeds that were submitted to hydration-dehydration treatments had greater diameter than those from untreated seeds. This difference was more prominent in seeds stored for two months (S_2). During the four months of monitoring in S_2, seedlings regenerated from treatments T_3 and T_4 (stem diameters of 5.6 ± 3.3 and 6.0 ± 3.3 mm, respectively) grew better than seedlings regenerated from untreated seeds (diameter 2.72 ± 4.41 mm). The mixed effects models analysis with temporal pseudoreplication indicated that storage duration and number of hydration-dehydration cycles had highly significant interaction effect on seedling stem diameter ($P < 0.001$). The highest values for seedling growth were recorded for seeds stored for two months (S_2) and consecutively hydrated and dehydrated for 14 (T_3) and 21 days or cycles (T_4) before sowing.

After four months the seedlings obtained from treated seeds grew to greater heights than those that germinated from untreated seeds (figure 1). The mixed effects model analysis indicated that both storage and seed dormancy treatment improved seedling height ($P < 0.05$).

The green and tender leaves are the most appreciated edible part of the species. Thus we assessed biomass yield and found that after four months, seedlings from S_1 seeds have produced fewer than 15 leaves (figure 1). Seedlings obtained from S_2T_3 and S_2T_4 seeds produced an average of eight pairs of leaves. Seedlings regenerated from treated seeds produced more biomass than those from untreated seeds. The mixed effects model analysis indicated a highly significant interaction effect of both storage and hydration-dehydration regimes on subsequent seedling biomass production ($P < 0.01$).

Correlation between growth parameters

The correlation matrix indicated strong and significant correlations between the growth parameters ($r \geq 0.87$; $P < 0.001$). The correlation was stronger respectively between height and diameter, and diameter and number of leaves ($r > 0.9$), than between height and number of leaves (0.87). However, the correlation between the total number of leaves and the number of compound leaves was not strong. In fact, close observations revealed that seedlings produced five to twenty pairs of simple leaves before compound leaves appeared. Overall, the first compound leaves appeared 42 days after germination.

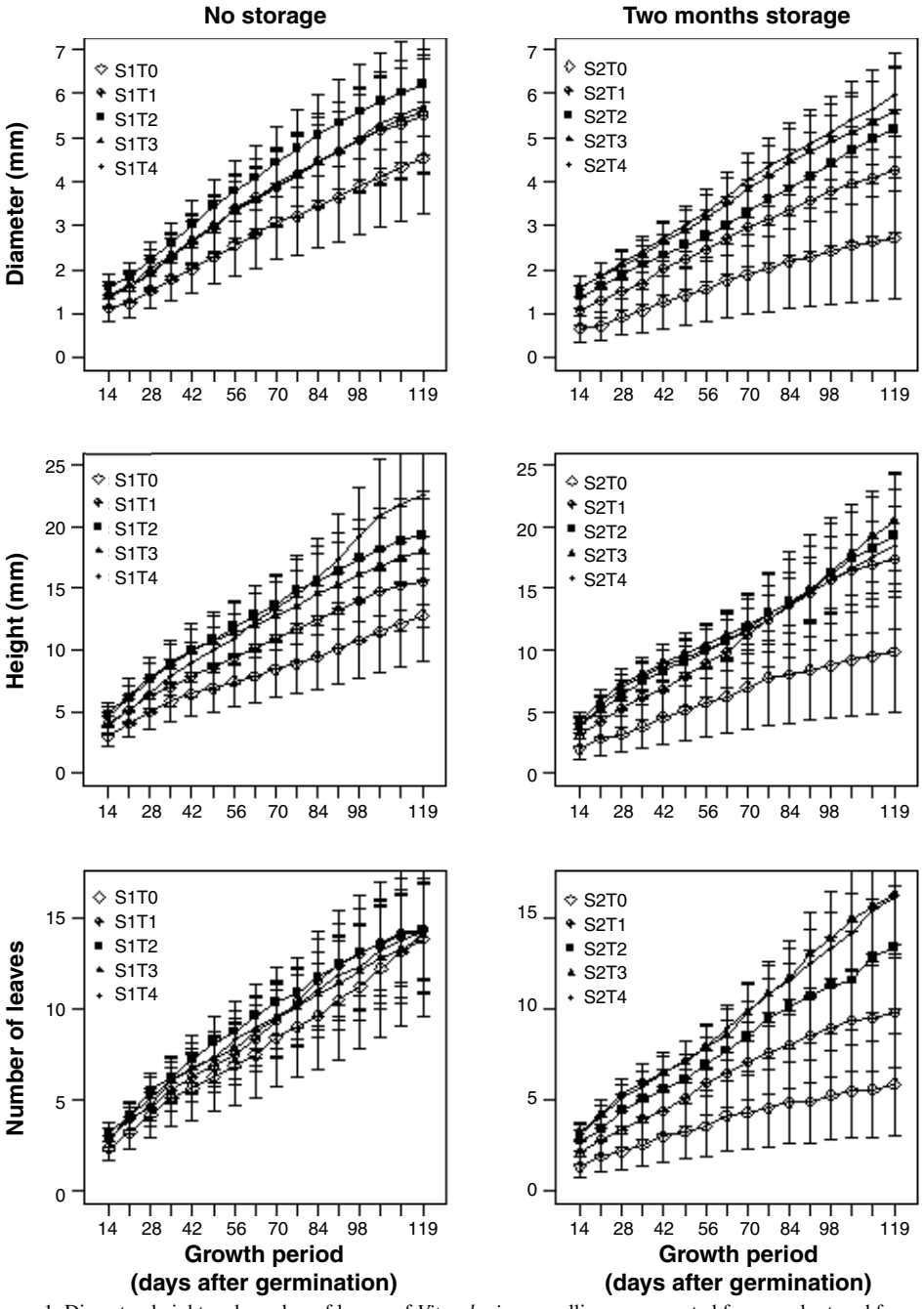

Figure 1. Diameter, height and number of leaves of *Vitex doniana* seedlings regenerated from seeds stored for zero and two months and submitted to five different dormancy breaking regimes. See table 1 for the definition of each treatment.

Discussion

The stratification technique applied in this study to break seed dormancy in *Vitex doniana* has been used since 1925 (Kandya and Kandya, 1990) and been successful in breaking the hard seed coat and increasing water uptake for seeds of *Tectona grandis* (Ngulube, 1986; Yadav, 1992; Chacko *et al.*, 1997). *V. doniana* seeds that were hydrated-dehydrated for several cycles took less time to germinate compared with untreated seeds. This beneficial effect was also reported for *Rumex crispus* L. seeds (Vincent and Cavers, 1978). In *V. doniana*, one hydration-dehydration cycle alone is not sufficient, but there should be multiple cycles. Alternating hydration with dehydration involves a physical action that can be associated with scarification. This action results in rupture of covering layers, caused by the successive increase and decrease in seed volume (water gain and loss, respectively). Rupture of the seed coat allows water intake by the different seed covering layers (e.g. testa and endosperm) and seed embryo expansion (Kucera *et al.*, 2005). Uptake and loss of water result in loss of dormancy, perhaps due to reduction in germination-inhibiting hormones such as ABA in addition to the physical effect on the covering layers. By imbibing water, the seed embryo becomes biochemically active, synthesising RNA, DNA and proteins (Vincent and Cavers, 1978). This leads to the emergence of the radicle which is considered as the completion of the germination process (Kucera *et al.*, 2005).

Knowledge of the best seed storage conditions and the optimum storage duration is a prerequisite for successful domestication / cultivation and *in situ* management of wild-harvested species (José *et al.*, 2011). In the current study, 83% germination was obtained for two months-stored seeds that received dormancy release treatments. This is a great improvement for this important and useful tropical tree, considering the low germination results reported to date (Mapongmetsem *et al.*, 2005; Ky, 2008; Ahoton *et al.*, 2011; N'Danikou *et al.*, 2014). Seeds stored beyond two months lost their viability and germinated poorly. This negative effect of long term storage on seed viability was also reported on plantation trees with similar seed texture such as *Tectona grandis* (Ngulube, 1986), *Celtis australis* L. (Singh *et al.*, 2004), and *Casuarina* spp. (Omran *et al.*, 1989). However, we found that the interactive effect of short-term storage and consecutive hydration-dehydration cycles was beneficial. Based on these findings, we recommend that freshly collected mature seeds of *V. doniana* be stored for two months and then consecutively soaked (for one hour) and sun-dried (for eight hours) for 14 or 21 cycles, before sowing.

Having achieved successful germination for this species, to further enhance horticultural prospects, future research on the sexual propagation of the species should aim to achieve even higher and faster germination (i.e. > 80% germination in less than 30 days). The current study aimed to remove physical dormancy in seeds. The fact that 12 to 62% of ungerminated seeds were still alive in healthy seed lots (S_1 and S_2) after 12 months of experiment might indicate physiological as well as or instead of mechanical dormancy in the species. This is more remarkable with the poor germination after 12 months (less than 40%) in seeds stored for two months and which received no dormancy treatment (S_2T_0), compared with the control in fresh seeds (about 70% in S_1T_0). Thus, current results call for further examination of the seed's (external and internal) morphology, and determining

the trend in the ratio of important germination inhibitors (e.g. ABA and phenols) over the germination promoters (e.g. gibberellins and ethylene).

These results are important for the establishment of private nurseries and development of seedling enterprises. However, to build a sustainable production system for *V. doniana*, there is need for policy development that encourages users to engage in cultivation of the species. This would give advantages, including the introduction of new commodities for urban and peri-urban agriculture and markets, enhancement of livelihoods for the poor, and sustainable use of biodiversity and the environment (several mammal and bee species forage on *V. doniana*). To achieve this, further investigations are required on the agronomic requirements (e.g. water, fertilisers, pest and disease management, harvesting techniques) and the socioeconomic determinants for its domestication and subsequent cultivation.

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