Original article

Regeneration ability and seedling growth in the miracle plant *Synsepalum dulcificum* (Schumach. & Thonn.) Daniell

D.A. Tchokponhoué[1,a], E.G. Achigan-Dako[1], S. N'Danikou[1,2], A.C. Houdégbé[1], C.O. Aboègnonhou Agossou[1], F. Assogba-Komlan[3] and R.S. Vodouhè[2]

[1] Laboratory of Genetics, Horticulture and Seed Sciences, Faculty of Agronomic Sciences, University of Abomey-Calavi, BP 2549, Abomey-Calavi, Republic of Benin

[2] Bioversity International, West and Central Africa Office, 08 BP 0932, Cotonou, Republic of Benin

[3] National Agricultural Research Institute of Benin (INRAB), 01 BP 884, Cotonou, Republic of Benin

Summary

Introduction – **Conservation and management of recalcitrant-seeded species are a major concern, particularly for socio-economically important species such as *Synsepalum dulcificum* in which seed viability and storage behavior are not sufficiently documented.** *Materials and methods* – **In order to improve the seed propagation management of *S. dulcificum*, we investigated the effects of seed provenance, short-term storage, culture medium, and pulp removal on viability, germinability, germination speed, and seedlings growth. A first experiment following a complete randomized block design consisted of two treatments (intact fruit or depulped fruit). In a second experiment we used a split-split-plot design with 16 treatments of a factorial combination of 2 provenances (Houéyogbé and Toffo), 2 culture media (soil and sawdust) and 4 storage times (0, 1, 2, and 7 days).** *Results and discussion* – **The seed viability remained high (85%) on the second day of storage at ambient condition and decreased to 0% when seeds were stored for 7 days. It was affected by the type of culture medium. All factors, except pulp removal, affected germinability and germination speed with percentages ranging from 44 to 99% at two months after sowing. Seedling growth was extremely slow and was affected by the seed provenance, substrate, and storage time.** *Conclusion* – **The time of storage is a major limiting factor for *S. dulcificum* seed viability whereas successive use of sawdust and soil for germination and seedling growth, respectively, is recommended to improve seedling production in the nursery.**

Keywords
Benin, *Synsepalum dulcificum*, seed viability, germination, agroforestry, underutilized species, genetic resource management

Résumé

Aptitude à la régénération et croissance des plantules chez la baie miracle *Synsepalum dulcificum* (Schumach. & Thonn.) Daniell.

Introduction – **La conservation et la gestion des espèces à semences récalcitrantes constituent une préoccupation majeure, en particulier pour les espèces d'importance socio-économique telles que *Synsepalum dulcificum* pour lesquelles la viabilité et le comportement des semences en stockage ne sont pas suffisamment documentés.** *Matériel et méthodes* – **Afin d'améliorer la gestion de la propagation de *S. dulcificum* par semis, nous avons étudié les effets de la provenance des semences, du stockage de courte durée, du substrat de culture et de l'élimination de la pulpe du fruit sur la viabilité, la germination, la vitesse de germination et la croissance des plantules. Un premier essai en blocs aléatoires complets a consisté en deux traitements (fruit intact ou fruits dépulpés). Dans un deuxième essai, nous avons utilisé un modèle en split-split-plot avec 16 traitements en combinaison factorielle de 2 provenances (Houéyogbé et Toffo), 2 substrats (sol et sciure de bois) et 4 durées de stockage (0, 1, 2 et 7 jours).** *Résultats et discussion* – **La**

Significance of this study

What is already known on this subject?

- Little was known on seed regeneration ability and seedling growth in *Synsepalum dulcificum*, an attractive future crop, for which technical knowledge (e.g., seed management) for profitable and large scale cultivation is yet to be determined.

What are the new findings?

- We demonstrated that seed storage beyond seven days at ambient temperature is detrimental for *S. dulcificum*.
- Seed provenance affects regeneration and is a key factor to consider for the successful reproduction of the species.
- Alternation of use of sawdust and soil improved seed germination and seedling growth respectively in the nursery.

What is the expected impact on horticulture?

- Knowledge and practices to increase the regeneration and seedling growth in *S. dulcificum* is now available. New possibilities rise up to accelerate and scale up the production of miracle berry in West Africa.

[a] Corresponding author: dedeoutchokponhoue@gmail.com.

viabilité des semences s'est montrée élevée (85%) au deuxième jour de stockage à l'air ambiant et a chuté à 0% lorsque les graines ont été stockées pendant 7 jours. Elle a été affectée par le type de substrat. Tous les facteurs, à l'exception de l'élimination de la pulpe, ont eu un effet sur la germination et la vitesse de germination des graines, avec des pourcentages variant de 44 à 99% deux mois après le semis. La croissance des jeunes plants s'est montrée extrêmement lente et a été affectée par la provenance des semences, le substrat et la durée de stockage. *Conclusion* – **La durée de stockage est un facteur limitant déterminant pour la viabilité des graines de *S. dulcificum*, alors que l'utilisation successive de la sciure de bois et du sol pour la germination et la croissance des plantules, respectivement, est recommandée pour améliorer la production des plants en pépinière.**

Mots-clés
Bénin, *Synsepalum dulcificum*, viabilité des semences, germination, système agroforestier, espèces sous-utilisées, gestion des ressources génétiques

Introduction

Recalcitrance often occurred in non-dormant seeds (Baskin and Baskin, 2008) and consequently constrains efficient seed management. Recalcitrance is when seed loses viability in short time (Barbedo and Bilia, 1998) mostly due to loss of water after being shed from the mother plant at high water content. This behavior is observed in a number of worldwide important seed plant species (Baskin and Baskin, 2008) including *Artocarpus heterophyllus* Lamk. (Wesley-Smith *et al*., 2001), *Theobroma cacao* L. (Li and Sun, 1999), and *Trichilia emetica* Vahl (Varghese *et al*., 2009). Environmental conditions deeply affected the level of recalcitrance by interfering with the seed moisture content through temperature, light, hygrometry and others factors (Tweddle *et al*., 2003). Since, there is no desiccation sensitivity standard within genera and even within species (Berjak and Pammenter, 2008; Dussert *et al*., 2000), the investigation of this phenomenon should consequently be species-specific (Erdey *et al*., 2007).

Recalcitrance has also been suspected in *Synsepalum dulcificum* (Schumach. & Thonn.) Daniell but strong evidence in support of the phenomenon is lacking (Chen *et al*., 2012). Locally called "Sisrè" (Fon language) or "Tanmini" (Adja language), *S. dulcificum* is a West African native shrub of the Sapotaceae family which produces a red fruit called "miracle berry", used as sweetener (Burkill, 2000). The sweetening property is attributed to a glycoprotein called ''miraculin'' (Milhet and Costes, 1984). Since 1927, *S. dulcificum* fruit has been used as a substitute to sugar in Benin (Juhé-Beaulaton, 1995). It is also a highly valued nutraceutical species whose leaves are used to treat diabetes and enuresis, bark for prostatitis, its root used in cough and tuberculosis treatment, and the almond used against anemia and stomach ache (Yang *et al*., 2005; Oumorou *et al*., 2010). In Taiwan, *S. dulcificum* has many applications in medical cosmetology and food supplementation (Wang *et al*., 2011). Its antioxidant activities have also been demonstrated (Inglett and Chen, 2011). The miracle berry is rich in anthocyanin and was reported to be beneficial for food industries**.** For instance, the anthocyanin offers a good stability to carbonated beverages (Buckmire and Francis, 1978). The miracle berry was also rich in vitamin C, Iron, Zinc, Copper and leucine (Njoku *et al*., 2015), and can contribute to improve the nutritional profile of local populations. In Benin, *S. dulcificum* is an important source of income and the fruit sale annually generates up to 6,300 XOF tree^{-1} (Houeto, 2015). At the international level, the pure powder of the miracle berry costs 3,000 $ kg^{-1} (http://miraclefruitfarm.com/shop/commercial-sample-of-miracle-fruit-powder-50-g#). Additional scientific evidences were recently highlighted on the ability of the species to substitute sugar, particularly in sour beverages (Rodrigues *et al*., 2016). A complete review on the current knowledge and breeding perspectives in the species can be found in Achigan-Dako *et al.* (2015). Despite these benefits, almost no active cultivation practices were observed at farmers' level; the most common exploitation strategy being the harvest of naturally regenerated or spared individuals during land clearing whereas large-scale exploitations are needed to optimize the species benefit.

During the last decades numerous scientific works have stressed the taste-modifying and the antioxidant properties, and the nutritional importance of the miracle plant (Chen *et al*., 2010; Kurihara and Terasaki, 1982). However, current conservation status and horticultural practices remained poorly documented. The successful tissue culture technique developed in the species using embryo (Ogunsola and Ilori, 2008) is not accessible to producers yet. Unfortunately, to the best of our knowledge, there is limited scientific work on the seed-based regeneration ability of the species (Yang *et al*., 2005; Adansi and Hollowey, 1977; Oumorou *et al*., 2010) though high germination rates (90%) were achieved (Yang *et al*., 2005). Also, none of those studies assessed seed viability in the species. Milhet and Costes (1984) reported that the threshold for seed storage is three days; however, evidence of this statement is lacking. Furthermore, information on the seedling growth was also scanty.

The objective of this work was to assess and improve seedling production through: i) determining seed viability; ii) defining suitable fruit conditions at sowing (intact or depulped fruits); and iii) investigating the influence of seed provenance, seed storage, and culture media on the regeneration ability and the seedling growth under nursery conditions. We hypothesized that: i) using depulped fruits would accelerate their germination; ii) germination rates and seedling growth of *S. dulcificum* would be affected by seed provenance and sowing media, and that iii) at ambient conditions seed storage duration would affect seed viability in *S. dulcificum*.

Materials and methods

Plant materials and experimental design

The intact fruit of *Synsepalum dulcificum* (Figure 1A) used for this stage of the experiment is a drupe with a sweet-acid pulp covered with a red skin (Burkill, 2000) whereas the seed (Figure 1B) exhibits a hard, shiny and oily testa with a large embryo that fills the cavity (Ayensu, 1972). Germination ability and growth pattern in *S. dulcificum* were assessed in two complementary experiments.

Fruits used in a first experiment were collected on a 3-m tall tree located in a cultivated farm of the municipality of Abomey-Calavi (6°27'0"N, 2°21'E) and they were treated per progeny. The site of Abomey-Calavi is characterized by a ferralitic soil and a subequatorial climate with an annual rainfall

Figure 1. *Synsepalum dulcificum* plant materials used in the study. (A) Intact fruits; (B) Seeds (depulped fruits).

ranging from 1,000 to 1,400 mm and a mean temperature from 20 to 33 °C. The first experiment was carried out at the International Institute of Tropical Agriculture (IITA) station in Abomey-Calavi, Benin. The experimental design was a complete randomized block design with two treatments representing two different states of fruit at sowing: intact fruits (T0) or depulped fruits (T1). Each treatment was made up of 7 replicates of 10 seeds.

Seeds used in a second experiment were harvested from two different locations, on a single 3.5-m tall tree per location and treated per progeny. The first lot was collected in a cultivated farm of the municipality of Houéyogbé (*Hy*) (6°30'N, 1°34'E) in Benin whereas the second batch was harvested from Toffo municipality (*Tf*) (6°92'N, 2°27'E) in a home garden. With a mean annual rainfall of 936 mm and a mean temperature ranging from 24.0 to 33.7 °C, the municipality of Houéyogbé is characterized by a subequatorial climate and ferralitic and hydromorphic soils. The municipality of Toffo is characterized by a subequatorial climate, an annual rainfall ranging from 800 mm to 1,400 mm. Soils in that municipality include vertisols, hydromorphic and ferralitic soils. Temperature varied from 27 to 30 °C. We hand-picked mature and ripe fruits from the two localities and stored them at ambient temperature (25 °C) for four durations (0, 1, 2 and 7 days). The fruits were subsequently depulped and then immediately sown in two different media: soil (*Sa*) and sawdust (*Sw*). The soil medium had a pH of 7.24 and the sawdust medium had a pH of 6.86. The soil was of sandy-clay type and weakly ferralitic and was sterilized by heating while the sawdust was not. This trial was carried out at the research station of the National Agricultural Research Institute of Benin (INRAB) in Abomey-Calavi. The experiment design used was a split-split-plot design combining three factors: seed provenance (*Hy* and *Tf*), the culture medium (*Sa* and *Sw*), and seed storage duration (0, 1, 2, and 7 days) giving a total of sixteen treatments (Table 1). There were five blocks of 20 seeds per treatment and seeds were horizontally sown in a black polystyrene nursery bag (754.2 mL).

In the first experiment, we used a total of 140 seeds whereas in the second experiment, we used 1,600 seeds. Both trials were carried out from November 2013 to June 2014 and during this period, nursery bags in each trial were watered twice a day using a 15-L watering cane.

Data collection

Germination was recorded daily for 60 consecutive days. A seed was deemed germinated and recorded as such when seed hypocotyl was visible above the culture medium. Growth parameters such as basal stem diameter, height, and number of leaves were measured fortnightly during five months. Recording of growth parameters started two weeks after germination. At the 60th day after sowing, we performed an imbibed seed crush test (Borza *et al*., 2007) on non-germinated seed to determine numbers of viable and dead seeds per treatment. In *S. dulcificum*, the embryo of dead seeds was yellowish or brown, and collapsed after a slight pressure and there was an odor of decay, while the embryo of viable seeds was greenish or white and firm when pressed.

Germination rate is important for seedling production in the nursery. It determines the seedling cohort homogeneity, a crucial parameter in production and plantation management and also for marketing. In this study, we assessed germination rate using three parameters: the time to first germination (TFG), the mean germination time (MGT) and the time to 40% germination (T_{40}).

Table 1. Factors combination in the second experiment on *Synsepalum dulcificum*. Fruits collected in Houéyogbé (Hy) or Toffo (Tf), stored (for 1, 2 or 7 days) or not (0 day), were sown in soil (Sa) or in sawdust (Sw) substrate.

Provenances	Substrates	Storage duration (in days)			
		0	1	2	7
Houéyogbé (Hy)	Soil	D0HySa	D1HySa	D2HySa	D7HySa
	Sawdust	D0HySw	D1HySw	D2HySw	D7HySw
Toffo (Tf)	Soil	D0TfSa	D1TfSa	D2TfSa	D7TfSa
	Sawdust	D0TfSw	D1TfSw	D2TfSw	D7TfSw

Data analysis

We estimated seed lot viability and germination by calculating viability percentage (Vp) and germination percentage (G_p) as:

$$V_p = \left(N_{gp} + N_{vp}\right) \times 100/N$$

and

$$G_p = (N_{gp} \times 100)/(N_{gp} + N_{vp})$$

where N_{vp} is the number of seeds which were still alive in a treatment p after the imbibed seed crush test, N_{gp} the number of seeds that germinated in the treatment *p* and *N* the total number of seeds sown in the treatment (100 seeds for each treatment). The germination speed was assessed by computing a) the time to first germination (TFG), b) the time to 40% germination (T_{40}), and c) the mean germination time (MGT) (N'Danikou *et al.*, 2014) as:

$$MGT_p = \sum_{i=1}^{k} n_{gpi} \times ti / \sum_{i=1}^{k} n_{gpi}$$

where *p* is the treatment, t_i is the duration since germination experiment started up to date *i,* and n_{gpi} the total number of seeds in treatment *p* that have germinated at date *i*.

We calculated T_{40} because 40% was the minimum percentage observed in each treatment at the end of the experiment.

To test the effects of fruit depulping and the interactions with geographical origin, culture medium and storage time on seed germination, we used the generalized linear model (glm) with binomial and quasibinomial error structure (to account for over-dispersion). We used the glm with Poisson and quasi-Poisson error structure (where necessary) to test the effects of different factors on germination rate. To account for temporal pseudo-replication due to repeated measures of growth parameters, we used a mixed effect model fit by maximum likelihood to test the effects of pulp removal and the interactions among fixed factors (geographical origin, the culture medium and the storage length) on seedling diameter and height growth. Generalized linear mixed models fit by maximum likelihood were used to test the effects of the same factors on leaf production. Models were compared using Akaike Information Criterion (AIC) and ANOVA. We also tested the correlation between growth parameters. Data were analyzed using "gregmisc", "lme4", and "nlme" R packages (R Development Core Team, 2013).

Results and discussion

In the first experiment, the pulp removal affected nor the germination rates neither the growth parameters (data not shown). The results below are solely related to the second experiment.

Seed viability

The viability of *S. dulcificum* ranged from 0 to 100% (Table 2). The effect of seed provenance was not significant ($P = 0.362$). In contrast, the effects of culture medium and storage on seed viability were highly significant ($P < 0.001$) with a higher rate for sawdust (98.66 ± 2.24%) compared to soil (94.83 ± 6.63%). The viability rates two months after sowing were 95.16% ± 6.5 and 98.33% ± 3.03 respectively for Houéyogbé and Toffo whereas the rates were 97.25 ± 3.43, 96 ± 8.05, and 97 ± 2.99% respectively for ''no storage'', ''one day storage'', and ''two days storage''. All seeds stored

Table 2. Viability and germination of *Synsepalum dulcificum* seeds (second experiment). Values indicate means ± standard deviation of five replications ($n = 20$ seeds). Details of treatments are given in Table 1.

Treatments	Viability (%)	Germination (%)
D0HySa	95.55 ± 4.18 a	75.25 ± 9.37d
D1HySa	85.00 ± 10.00b	44.14 ± 9.34e
D2HySa	95.47 ± 0.00a	82.10 ± 2.88d
D7HySa	00.00 ± 0.00 c	NA
D0HySw	97.47 ± 2.74a	97.94 ± 2.81b
D1HySw	99.00 ± 2.24a	90.89 ± 4.22c
D2HySw	98.52 ± 2.24a	95.89 ± 5.62b
D7HySw	00.00 ± 0.00 c	NA
D0TfSa	97.47 ± 4.47a	92.61 ± 8.02c
D1TfSa	100.00 ± 0.00a	99.00 ± 2.23a
D2TfSa	95.47 ± 4.18a	96.83 ± 2.89b
D7TfSa	00.00 ± 0.00 c	NA
D0TfSw	98.52 ± 2.24a	96.00 ± 4.18b
D1TfSw	100 ± 0.00a	96.00 ± 4.18b
D2TfSw	98.47 ± 2.74a	96.84 ± 4.7b
D7TfSw	0.00 ± 0.00 c	NA
P-value	0.000***	

Mean values followed by the same letter within a column are not statistically different.
NA = not calculated (all seeds died).
***: significant at α = 0.001.

for seven days at ambient conditions died. This finding corroborates with those of Milhet and Costes (1984) who found that storage beyond three days reduced the germinability of the species. Similar negative effect of storage duration on seed viability was also observed in many other species *e.g.*, *Nephelium lappaceum* L. "(Cavalcante *et al*., 2008), *Magnolia ovata* Spreng. (José *et al*., 2011), *Annona squamosa* L. and *A. cherimola* × *A. squamosa* (Pereira *et al*., 2014), though with various extents. The fact that provenance did not affect seed viability highlights the adaptation potential of the species to a new environment. This is valuable information while selecting genotypes for breeding purposes. Furthermore, the sawdust promoted better seed aeration and hydration, conserving seed viability. The same trend here observed with *S. dulcificum* had also been reported with *Zostera marina* L. in which the sediment type was reported to influence the viability rate (Jarvis and Moore, 2014).

Effect of treatments on seed germination percentages

Although germination in *S. dulcificum* was reported to be difficult (Milhet and Costes, 1984; Ogunsola and Ilori, 2008), for the first time a record of 99% germination was reached two months after sowing. Indeed, there was a highly significant effect of treatments on the seed germination percentages (Table 2). Higher germination percentages were observed for fruits collected in Toffo, stored for 24 h and sown in soil culture medium (99.00 ± 2.24%), and for fruits collected in Houéyogbé and directly sown in sawdust culture medium (97.94 ± 2.81%), while lower germination percentages were obtained when seeds were collected in Houèyogbé, stored one day before sowing (44.14 ± 9.34%) or directly sown in soil medium (75.25 ± 9.37%). Previous studies related to the propagation of the species reported a maximum germination of 90% (Yang *et al*., 2005). Germination percentages in

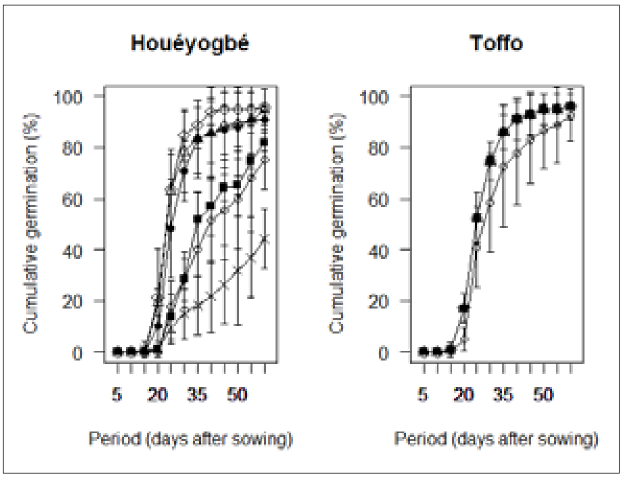

FIGURE 2. Cumulative germination at two months after sowing of *Synsepalum dulcificum* seeds collected from the municipalities of Houéyogbé and Toffo, stored for various durations and sown on different media: ○ D0Sa, △ D0Sw, × D1Sa, ● D1Sw, ■ D2Sa, ◇ D2Sw. D0: No seed storage; D1: Seeds stored 24 h; D2: Seeds stored 48 h; Sa: Soil medium, Sw: Sawdust medium. Refer to Table 1 for details on treatments. Each point corresponds to the mean ± standard deviation of five replications (*n* = 20 seeds).

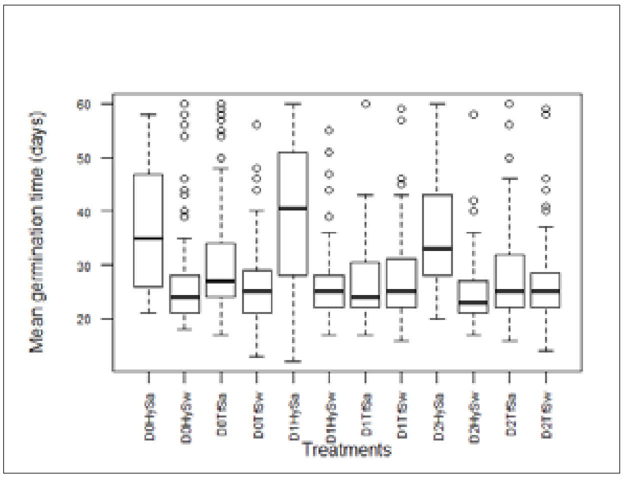

FIGURE 3. Mean germination times in seeds of *Synsepalum dulcificum* collected from the municipalities of Houéyogbé and Toffo, stored for various durations and sown on different culture media. Refer to Table 1 for the details on treatments.

TABLE 3. Time to first germination (TFG) and time to 40% germination (T_{40}) of *Synsepalum dulcificum* seeds (second experiment). Values indicate means ± standard deviation of five replications (*n* = 20 seeds). Details of treatments are given in Table 1.

Treatments	Germination parameters (in days)	
	TFG	T_{40}
D0HySa	22.00 ± 1.41 ^{a}	36.20 ± 2.94 ^{b}
D1HySa	20.80 ± 5.06 ^{a}	54.00 ± 8.74 ^{c}
D2HySa	22.00 ± 2.34 ^{a}	33.00 ± 2.00 ^{b}
D0HySw	18.60 ± 0.54 ^{a}	23.00 ± 1.00 ^{a}
D1HySw	18.40 ± 1.51 ^{a}	23.80 ± 1.92 ^{a}
D2HySw	18.60 ± 1.51 ^{a}	23.00 ± 1.58 ^{a}
D0TfSa	20.00 ± 2.12 ^{a}	25.40 ± 2.07 ^{b}
D1TfSa	18.20 ± 1.30 ^{a}	22.80 ± 1.09 ^{a}
D2TfSa	18.20 ± 1.78 ^{a}	23.80 ± 1.30 ^{a}
D0TfSw	16.80 ± 2.16 ^{a}	23.60 ± 1.34 ^{a}
D1TfSw	17.80 ± 1.09 ^{a}	23.80 ± 1.30 ^{a}
D2TfSw	17.00 ± 2.00 ^{a}	24.20 ± 2.19 ^{a}
P-value	0.06^{ns}	0.000***

Mean values followed by the same letter within a column are not statistically different.
^{ns} : not significant; *** : significant at α = 0.001.

Synsepalum dulcificum were comparable to previous reports on *Vitellaria paradoxa* (Gaertn.) Hepper (Iroko *et al.*, 2013), another desiccation sensitive species of Sapotaceae (Daws *et al.*, 2006); however, the germination percentage was higher than that reported for *Chrysophyllum albidum* (G. Don) (Aduradola *et al.*, 2005), and *Zapota manilkara* (Van Royen) (Jacq) (Gill) (Buitrago-Rueda *et al.*, 2004), both from the Sapotaceae family as well.

There was a very highly significant effect of seed provenance, culture medium and storage duration on the germination percentage ($P < 0.001$). Germination was also highly affected by two ways interactions of [seed provenance × culture medium] and [seed provenance × storage length] ($P < 0.001$). The best germination percentages were obtained with planting material from Toffo, sown in the sawdust culture medium, and stored for 0 to 2 days (Table 2). Germination success was reported to be under the control of numerous factors including storage conditions (Moncaleano-Escandon *et al.*, 2013), seed origin (Jarvis and Moore, 2014; O'Farrill *et al.*, 2011), medium type, state of fruit at sowing (Mukonyi *et al.*, 2011) and other environmental factors. The favorable effect of the sawdust culture medium could be associated to its both physical and chemical characteristics. On one hand, sawdust was a light medium that promoted an easy protrusion of hypocotyl in contrast to the soil that was denser. Additionally, the sawdust was slightly acidic and fulfills one of the conditions required for good germination in *S. dulcificum* (Milhet and Costes, 1984). The effect of seed provenance observed in this experiment calls for further investigation. Probably additional data on environmental conditions and stands management scheme may help explain the source of variation. Plant management schemes were reported to highly influence the disturbance level and this latter affects the plant reproduction ability, physiology, growth and survival (Snyder and Williams, 2003; Gaoue *et al.*, 2014). Because the mother plant which seeds were sampled in Houéyobé was in an open field, disturbance (*e.g.*, fire, excessive fruit and bark harvesting) might have reduced its regeneration abilities. Nonetheless, more ecological data, as well as morphological and molecular data are required to fully explain the observed variations. The negative effects of storage were observed mostly on seeds stored for 7 days. Moreover, it is worth mentioning that the difference observed among the four storage durations was prominent in seeds stored for 24 h. The seeds stored for 0 h and 48 h exhibited similar germination percentages. This suggests that *S. dulcificum* seeds can be stored up to 48 h without any detrimental reduction in germination. A follow-up experiment would be useful to determine the storage duration threshold, and to investigate the most suitable conditions for extended storage of these seeds. *Synsepalum dulcificum* seeds can probably be stored for several days if storage temperature and seed moisture content can be set to an optimum.

Germination started at 15th day after sowing, and rapidly increased between the 20th and the 45th day before it stabilized around the 55th day after sowing (Figure 2).

Effect of treatments on seed germination rates

The seed provenance and the culture medium showed highly significant ($P < 0.001$) effects on the mean germination time (MGT) (Figure 3). Seeds collected from Toffo germinated more quickly (27.6 ± 8.5 days) than those from Houéyogbé (30.5 ± 11.2 days). Likewise, sawdust ensured a more rapid germination (26.5 ± 7.9 days) comparatively to soil (31.9 ± 11.2 days). The interaction between the seed provenance and the culture medium had also a very significant effect on the MGT ($P < 0.001$). Although the effect of storage was not significant on MGT ($P = 0.1$), the glm analysis revealed a highly significant effect of the [seed provenance × storage duration] two-way interaction ($P < 0.001$) and a very significant effect of the [seed provenance × culture medium × storage duration] three-way interaction ($P < 0.0001$) on the MGT.

The treatments that improved MGT included D2HySw (fruits collected in Houéyogbé, stored for 48 h and sown in sawdust culture medium), D2TfSw (fruits collected in Toffo, stored for 48 h and sown in sawdust culture medium), D0HySw (fruits collected in Houéyogbé, not stored and sown in sawdust culture medium), D0TfSw (fruits collected in Toffo, not stored and sown in sawdust culture medium), and D1TfSa (fruits collected in Toffo, stored for 24 h and sown in soil culture medium).

The mean time to first germination (TFG) ranged from 16.8 ± 2.2 to 22.0 ± 1.4 days (Table 3). Only the culture medium had significant effect on TFG ($P = 0.03$); seed provenance and storage did not affect this parameter ($P = 0.69$). In sawdust, first germination occurred after 17.9 ± 1.6 days while in soil; this was after 20.2 ± 2.9 days.

The seed provenance, storage time and germination culture medium had also very significant ($P < 0.01$) effects on the time to 40% seed germination (T_{40}). Germination was more homogenous with seeds collected from Toffo (23.9 ± 1.7 days) compared to those from Houéyogbé (32.2 ± 11.8 days). T_{40} was lower for seeds sown on sawdust (23.56 ± 1.52 days) compared to those sown on the soil (32.5 ± 11.5 days). There were also significant interaction effects of seed provenance and culture medium ($P < 0.001$) and of seed provenance and storage duration ($P < 0.05$) on T_{40}. The highest T_{40} (54.0 ± 8.7 days) was observed with D1HySa (fruits collected in Houéyogbé, stored for 24 h and sown in soil culture medium) (Table 3).

These findings suggest the culture medium to be an important driver to increase germination homogeneity in *S. dulcificum*. In fact, sawdust gave the lowest MGT, TFG and T_{40}. Similar findings were reported by Jarvis and Moore (2014) who revealed a determinant effect of sediment on the mean time to germinate of *Zostera marina* L. Despite the quite encouraging MGT value obtained in *S. dulcificum,* that duration could be still shortened as in *Tamarindus indica* L. (13 days) (Fandohan *et al*., 2010). In addition to the medium, seed provenance also plays a role in the germination speed (Fandohan *et al*., 2010).

An overall trend in *S. dulcificum* was that it presented a homogenous germination. While 20% germination only was reached after one month in *Vitex doniana* Sweet (N'Danikou *et al*., 2014), 40% germination in *S. dulcificum* was reached in less than a month. Nonetheless, the two species exhibit different storage behavior, *V. doniana* presents orthodox and dormant seeds. The cumulative germination curve revealed that, whatever the seed provenance and the culture medium, germination peaked between 20 and 25 days after sowing, corroborating the observation of Oumorou *et al.* (2010) with a peak between the 23rd and the 28th day after sowing.

Effect of treatments on seedling growth

After five months monitoring the highest values for diameter, height and number of leaves were 4.8 mm, 8 cm and 22 leaves, respectively, indicating that *S. dulcificum* is a slow-growing species. However, seed provenance and culture medium could enhance the species growth as reported for *Cordia africana* Lam. in Ethiopia (Loha *et al*., 2006) and for *Santalum album* L. in China (Liu *et al*., 2009).

The effects of seed provenance, culture medium and storage time on seedling height were highly significant ($P < 0.001$). Overall, the seeds collected from Toffo grew better than those from Houéyogbé (Figure 4). Treatments with soil ensured the best seedling growth compared with sawdust medium. Treatments with higher mean height included D2TfSa-fruits collected in Toffo, stored for 48 h and sown in soil culture medium (6.69 ± 1.55 cm), D1TfSa-fruits collected in Toffo, stored for 24 h and sown in soil culture medium (6.52 ± 1.35 cm), and D0TfSa-fruits collected in Toffo, not stored and sown in soil culture medium (5.76 ± 2.06 cm). Furthermore, the mixed-effect model analysis revealed a three-way interaction between seed provenance, culture medium, and storage duration on seedling height ($P < 0.001$).

Seedling stem diameter growth was influenced by the provenance and the storage time ($P < 0.05$). There was also a highly significant three-way interaction effect on the seedling diameter ($P < 0.001$). Best treatments included D2TfSa-fruits collected in Toffo, stored for 48 h and sown in soil culture medium (2.21 ± 0.57 mm) and D1TfSa-fruits collected in Toffo, stored for 24 h and sown in soil culture medium (2.20 ± 0.36 mm) (Figure 5).

Provenance, culture medium, and storage duration affected the leaf production ($P < 0.01$). The highest number of leaves was obtained with seeds collected from Toffo, sown in soil, and stored for 48 h (Figure 6). Treatment D2TfSa (fruits collected in Toffo, stored for 48 h and sown in soil culture medium) produced the maximum leaf number (10.46 ± 4.14 leaves) ($P < 0.001$).

In contrast to the germination trial where sawdust was the best medium, we noticed that seedlings growth was better promoted in soil. This incongruence between germination and growth medium was already reported in *S. dulcificum* by Yang *et al.* (2005) and in *Aloe turkanensis* Christian by Mukonyi *et al.* (2011). The soil was a more stable medium compared to sawdust and ensured a better root fixation and development which resulted in better seedlings growth (Burdett *et al*., 1983). Furthermore, sawdust is an immature medium in which mineralization is in progress. During the experiment the early development stage of seedlings in sawdust had coincided with the decomposition process in that medium. This resulted in weakened seedlings; some of them were even burnt. As good nursery practice, seedlings germinated in sawdust should be transplanted on to another medium (*e.g.*, soil) after two weeks.

Relationships between seedling growth parameters

We found a linear relationship between leaf number, stem diameter and height of seedlings. There were strong and highly significant correlations between leaf number and stem diameter (r = 0.79, $P < 0.001$) and between leaf number and seedling height (r = 0.71, $P < 0.001$). The adjusted determinant ($R^2_{adjusted}$) indicated that 64.7% of variation in the number of leaves produced at nursery stage is explained by the seedling diameter and height. The multiple linear regression equation reads:

leaf number = -7.05 + 5.54 stem diameter + 0.851 height

Implications and prospects for conservation and production

The rapid loss of viability after a few days storage at ambient conditions exposed the species as highly recalcitrant. This calls for urgent genetic resources collection and conservation to set *ex situ* conservation strategies for this species (*e.g.*, field gene banks, cold room storage of seeds, slow growth maintenance of explants, cryopreservation of meristems) for sustainable conservation of the germplasm. Currently, none of these conservation measures exist for the species and germplasms are almost absent in international gene banks (Achigan-Dako *et al.*, 2015). Given the species' recalcitrant seed storage behavior, cryopreservation may be the chief conservation approach to be explored.

Successful germination in *Synsepalum dulcificum* was evidenced in this study. The high germinability observed represents an important achievement for the promotion of the large-scale production of the species. Regeneration methods developed in this study can be expanded in distribution areas to promote the development of nurseries and increase

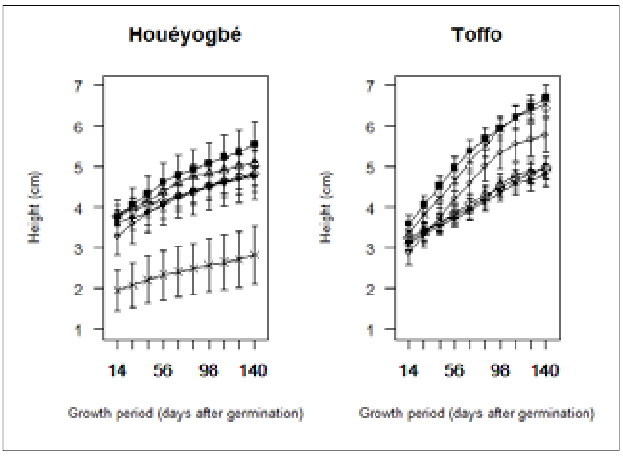

Figure 4. Height growth in seedlings produced by *Synsepalum dulcificum* seeds collected from the municipalities of Houéyogbé and Toffo, stored for various durations and sown on different culture media: ○ D0Sa, △ D0Sw, × D1Sa, ● D1Sw, ■ D2Sa, ◇ D2Sw. D0: No seed storage; D1: Seeds stored 24 h; D2: Seeds stored 48 h; Sa: Soil medium, Sw: Sawdust medium. Refer to Table 1 for the details on treatments. Each point corresponds to the mean ± standard deviation of five replications (*n* = 8–19 seedlings).

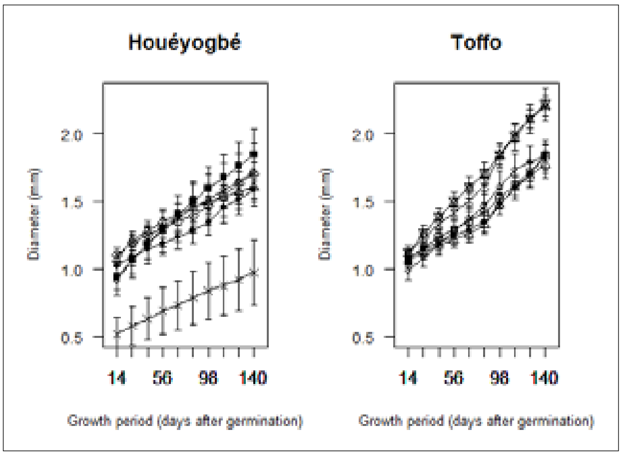

Figure 5. Diameter growth in seedlings produced by *Synsepalum dulcificum* seeds collected from the municipalities of Houéyogbé and Toffo, stored for various durations and sown on different culture media: ○ D0Sa, △ D0Sw, × D1Sa, ● D1Sw, ■ D2Sa, ◇ D2Sw. D0: No seed storage; D1: Seeds stored 24 h; D2: Seeds stored 48 h; Sa: Soil medium, Sw: Sawdust medium. Refer to Table 1 for details on treatments. Each point corresponds to the mean ± standard deviation of five replications (*n* = 8–19 seedlings).

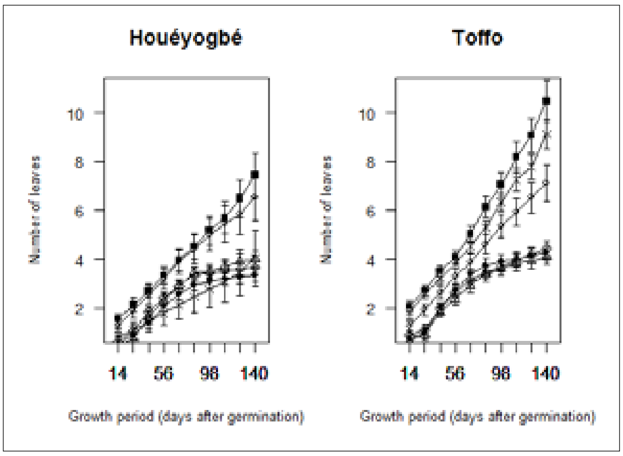

Figure 6. Leaf production in seedlings produced by *Synsepalum dulcificum* seeds collected from the municipalities of Houéyogbé and Toffo, stored for various durations and sown on different culture media: ○ D0Sa, △ D0Sw, × D1Sa, ● D1Sw, ■ D2Sa, ◇ D2Sw. D0: No seed storage; D1: Seeds stored 24 h; D2: Seeds stored 48 h; Sa: Soil medium, Sw: Sawdust medium. Refer to Table 1 for the details on treatments. Each point corresponds to the mean ± standard deviation of five replications (*n* = 8–19 seedlings).

production at the community level. This will also account for *in situ* conservation measures and promotion of the resources in areas where home gardens are present. However, germination and seedling growth parameters were found closely associated to the seed provenance. This suggests an agro-ecological variation in the characteristics of the species that needs to be clarified for the selection of genotypes.

Conclusion

This study explored the effects of seed type (intact fruit or depulped seed), seed provenance, sowing medium and short-term storage duration on the germination and seedling growth of *S. dulcificum*. Factors that improve germination rates and speed include seed provenance and culture medium. The interaction of these factors induced very high germination rate in a short time. While sawdust medium was efficient in promoting germination, soil medium enhanced seedlings growth. This study evidenced that storage of *S. dulcificum* seed at ambient conditions beyond a week would most likely be detrimental. Further study should clarify optimal temperature and moisture content conditions that would help extend the viable storage duration.

In spite of the relatively good effects of both seed provenance and regeneration medium, the seedling growth was still extremely slow. This has an important implication in terms of time to bear the first fruit. Despite the relative ease of reproduction by seed, vegetative propagation should also be explored for quicker fruit production. Moreover, the selection of genotypes to shorten the production cycle should be seriously investigated.

Acknowledgments

This study was fully funded by New Alliance Trust through the Grant No: RGNAT03/14, awarded to the first author.

References

Achigan-Dako, E., Tchokponhoué, D.A., N'Danikou, S., Gebauer, J., and Vodouhè, R.S. (2015). Current knowledge and breeding perspectives for the miracle plant *Synsepalum dulcificum* (Schum. et Thonn.) Daniell. Gen. Res. and Crop Evol. *62*, 465–476. https://doi.org/10.1007/s10722-015-0225-7.

Adansi, M.A., and Hollowey, H.L.O. (1977). Germination of seeds of the sweet or miraculous berry *Synsepalum dulcificum* Daniell. Acta Hortic. *53*, 181–182. https://doi.org/10.17660/ActaHortic.1977.53.24.

Aduradola, A., Adeola, B., and Adedire, M. (2005). Enhancing germination in seeds of African star apple, *Chrysophyllum albidum* (G. Don). Int. J. Food Agric. Environm. *3*, 292–294.

Ayensu, E.S. (1972). Morphology and anatomy of *Synsepalum dulcificum* (Sapotaceae). Botl. J. Linnean Soc. *65*, 179–187. https://doi.org/10.1111/j.1095-8339.1972.tb00932.x.

Barbedo, C., and Bilia, D. (1998). Evolution of research on recalcitrant seeds. Sci. Agri. *55*, 121–125. https://doi.org/10.1590/S0103-90161998000500022.

Baskin, C.C., and Baskin, J.M. (2008). Advances in understanding seed dormancy at the whole-seed level: an ecological, biogeographical and phylogenetic perspective. Acta Bot. Yunnanica *30*, 279–294.

Berjak, P., and Pammenter, N. (2008). From Avicennia to Zizania: seed recalcitrance in perspective. Ann. Bot. (London) *101*, 213–228. https://doi.org/10.1093/aob/mcm168.

Borza, J.K., Westerman, P.R., and Liebman, M. (2007). Comparing estimates of seed viability in three foxtail (*Setaria*) species using the imbibed seed crush test with and without additional tetrazolium testing. Weed Technol. *21*, 518–522. https://doi.org/10.1614/WT-06-110.

Buckmire, R., and Francis, F. (1978). Pigments of miracle fruit, *Synsepalum dulcificum* Schum. as potential food colorants. J. Food Sci. *43*, 908–911. https://doi.org/10.1111/j.1365-2621.1978.tb02453.x.

Buitrago-Rueda, N., Ramírez-Villalobos, M., Gómez-Degraves, A., Rivero-Maldonado, G., and Perozo-Bravo, A. (2004). Effect of seed storage and post seeding light on germination and morphological aspects of young nispero seedlings (*Manilkara zapota* (Van Royen) (Jacq) Gill). Revista de la Facultad de Agronomia, Universidad del Zulia *21*.

Burdett, A., Simpson, D., and Thompson, C. (1983). Root development and plantation establishment success. Plant Soil *71*, 103–110. https://doi.org/10.1007/BF02182645.

Burkill, H.M. (2000). The useful plants of west tropical Africa, Vol. 5. Families S-Z. Cryptogams Addenda (Royal Botanical Gardens).

Cavalcante, Í.H.L., Segeren, A., and Martins, A.B.G. (2008). Emergence and initial growth of rambutan seedlings as a function of seed storage conditions. Fruits *63*, 349–355. https://doi.org/10.1051/fruits:2008031.

Chen, C.Y., Wang, Y.D., and Wang, H.M. (2010). Chemical constituents from the leaves of *Synsepalum dulcificum*. Chem. Nat. Comp. *46*, 495–495. https://doi.org/10.1007/s10600-010-9658-6.

Chen, X.W., Abdullah, T.L., Abdullah, N.A.P., and Hassan, S.A. (2012). Rooting response of miracle fruit (*Synsepalum dulcificum*) softwood cuttings as affected by indole butyric acid. Amer. J. Agricult. Biol. Sci. *7*(4).

Daws, M.I., Garwood, N.C., and Pritchard, H.W. (2006). Prediction of desiccation sensitivity in seeds of woody species: A probabilistic model based on two seed traits and 104 species. Ann. Bot. (London) *97*, 667–674. https://doi.org/10.1093/aob/mcl022.

Dussert, S., Chabrillange, N., Engelmann, F., Anthony, F., Louarn, J., and Hamon, S. (2000). Relationship between seed desiccation sensitivity, seed water content at maturity and climatic characteristics of native environments of nine *Coffea* L. species. Seed Sci. Res. 10, 293–300. https://doi.org/10.1017/S0960258500000337.

Erdey, D., Naidoo, S., Pammenter, N., and Berjak, P. (2007). Drying out in Africa: physical and physiological seed characteristics of selected indigenous plant species. S. Afr. J. Bot. *73*, 501–502. https://doi.org/10.1016/j.sajb.2007.04.049.

Fandohan, B., Assogbadjo, A.E., Kakaï, R.G., and Sinsin, B. (2010). Variation in seed morphometric traits, germination and early seedling growth performances of *Tamarindus indica* L. Int. J. Biol. Chem. Sci. *4*(4).

Gaoue, O.G., Lemes, M.R., Ticktin, T., Sinsin, B., and Eyog Matig, O. (2014). Non timber forest product harvest does not affect the genetic diversity of a tropical tree despite negative effects on population fitness. Biotropica *46*, 756–762. https://doi.org/10.1111/btp.12145.

Houeto, J. (2015). Strategies d'exploitation des pieds reliques et vergers de *Synsepalum dulcificum* (Schumach & Thonn.) Daniell dans la commune de Toffo (Abomey-Calavi, Benin: University of Abomey-Calavi), 64 pp.

Inglett, G.E., and Chen, D. (2011). Contents of phenolics and flavonoids and antioxidant activities in skin, pulp, and seeds of miracle fruit. J. Food Sci. *76*, C479. https://doi.org/10.1111/j.1750-3841.2011.02106.x.

Iroko, O., Asinwa, I., Kareem, A., and Kasim-Ibrahim, F. (2013). Pretreatment effects on seed germination of *Vitellaria paradoxa* (Gaertn) Hepper. Scholarly J. Agric. Sci. *3*, 121–125.

Jarvis, J.C., and Moore, K.A. (2014). Effects of seed source, sediment type, and burial depth on mixed-annual and perennial *Zostera marina* L. seed germination and seedling establishment. Estuaries Coasts 1–15.

José, A.C., Da Silva, E.A.A., Davide, A.C., Melo, A.J.S., and Toorop, P.E. (2011). Effects of drying rate and storage time on *Magnolia ovata* Spreng. seed viability. Seed Sci. Technol. *39*, 425–434. https://doi.org/10.15258/sst.2011.39.2.14.

Juhé-Beaulaton, D. (1995). Les Paysages Végétaux de la Côte des Esclaves du XVII° Siècle à la Veille de la Colonisation: Essai d'Analyse Historique (Paris, France: Université Panthéon-Sorbonne).

Kurihara, Y., and Terasaki, S. (1982). Isolation and chemical properties of multiple active principles from miracle fruit. Biochim. Biophys. Acta *719*, 444–449. https://doi.org/10.1016/0304-4165(82)90232-X.

Li, C., and Sun, W.Q. (1999). Desiccation sensitivity and activities of free radical-scavenging enzymes in recalcitrant *Theobroma cacao* seeds. Seed Sci. Res. *9*, 209–217.

Liu, X.-J., Xu, D.-P., Xie, Z.-S., and Zhang, N.-N. (2009). Effects of different culture media on the growth of Indian sandalwood (*Santalum album* L.) seedlings in Zhanjiang, Guangdong, southern China. Forest Sci. Pract. *11*, 132–138. https://doi.org/10.1007/s11632-009-0023-4.

Loha, A., Tigabu, M., Teketay, D., Lundkvist, K., and Fries, A. (2006). Provenance variation in seed morphometric traits, germination, and seedling growth of *Cordia africana* Lam. New Forests *32*, 71–86. https://doi.org/10.1007/s11056-005-3872-2.

Milhet, Y., and Costes, C. (1984). Some data on sweetener plants biology. Acta Hortic. *144*, 77–84. https://doi.org/10.17660/ActaHortic.1984.144.10.

Moncaleano-Escandon, J., Silva, B.C., Silva, S.R., Granja, J.A., Alves, M.C.J., and Pompelli, M.F. (2013). Germination responses of *Jatropha curcas* L. seeds to storage and aging. Ind. Crop Prod. *44*, 684–690. https://doi.org/10.1016/j.indcrop.2012.08.035.

Mukonyi, K., Oeba, V., Chiteva, R., Lelon, J., and Gathara, M. (2011). Germination and growth performance of *Aloe turkanensis* and *Aloe secundflora* under different substrates. Afr. J. Agric. Res. *6*, 2132–2138.

N'Danikou, S., Achigan-Dako, E.G., Tchokponhoué, D.A., Assogba Komlan, F., Gebauer, J., Vodouhè, R.S., and Ahanchédé, A. (2014). Enhancing germination and seedling growth in *Vitex doniana* Sweet for horticultural prospects and conservation of genetic resources. Fruits *69*, 279–291. https://doi.org/10.1051/fruits/2014017.

Njoku, N.E., Ubbaonu, C.N., Alagbaoso, S.O., Eluchie, C.N., and Umelo, M.C. (2015). Amino acid profile and oxidizable vitamin content of *Synsepalum dulcificum* berry (miracle fruit) pulp. Food Sci. Nutr. *3*, 252–256. https://doi.org/10.1002/fsn3.213.

O'Farrill, G., Chapman, C.A., and Gonzalez, A. (2011). Origin and deposition sites influence seed germination and seedling survival of *Manilkara zapota*: implications for long-distance, animal-mediated seed dispersal. Seed Sci. Res. *21*, 305–313. https://doi.org/10.1017/S0960258511000201.

Ogunsola, K.E., and Ilori, C.O. (2008). In vitro propagation of miracle berry (*Synsepalum dulcificum* Daniell) through embryo and nodal cultures. Afr. J. Biotechnol. *7*, 244–248.

Oumorou, M., Dah-Dovonon, J., Aboh, B.A., Hounsoukaka, M., and Sinsin, B. (2010). Contribution à la conservation de *Synsepalum dulcificum*: régénération et importance socio-économique dans le département de l'Ouémé (Bénin). Ann. Sci. Agron. *14*, 101–120.

Oumorou, M., Dah-Dovonon, J., Aboh, B.A., Hounsoukaka, M., and Sinsin, B. (2010). Contribution à la conservation de *Synsepalum dulcificum*: régénération et importance socio-économique dans le département de l'Ouémé (Bénin). Ann. Sci. Agron. *14*, 101–120.

Pereira, M.C., Crane, J.H., Montas, W., Nietsche, S., and Vendrame, W.A. (2014). Effects of storage length and flowering stage of pollen influence its viability, fruit set and fruit quality in 'Red' and 'Lessard Thai' sugar apple (*Annona squamosa*) and 'Gefner' atemoya (*A. cherimola* × *A. squamosa*). Sci. Hortic. (Amsterdam) *178*, 55–60. https://doi.org/10.1016/j.scienta.2014.08.004.

R Development Core Team (2013). R: A language and environment for statistical computing (Vienna, Austria: R Foundation for Statistical Computing).

Rodrigues, J.F., da Silva Andrade, R., Bastos, S.C., Coelho, S.B., and Pinheiro, A.C.M. (2016). Miracle fruit: An alternative sugar substitute in sour beverages. Appetite *107*, 645–653. https://doi.org/10.1016/j.appet.2016.09.014.

Snyder, K., and Williams, D. (2003). Defoliation alters water uptake by deep and shallow roots of *Prosopis velutina* (Velvet Mesquite). Funct. Ecol. *17*, 363–374. https://doi.org/10.1046/j.1365-2435.2003.00739.x.

Tweddle, J.C., Dickie, J.B., Baskin, C.C., and Baskin, J.M. (2003). Ecological aspects of seed desiccation sensitivity. J. Ecol. *91*, 294–304. https://doi.org/10.1046/j.1365-2745.2003.00760.x.

Varghese, D., Berjak, P., and Pammenter, N. (2009). Cryopreservation of shoot tips of *Trichilia emetica*, a tropical recalcitrant-seeded species. CryoLetters *30*, 280–290.

Wang, H.-M., Choun, Y.-T., Hong, Z.-L., Chen, H.-A., Chang, Y.-C., Yang, W.-L., Chang, H.-C., Ma, I.C.-T., and Chen, C.-Y. (2011). Bioconstituents from stems of *Synsepalum dulcificum* Daniell (Sapotaceae) inhibit human melanoma proliferation, reduce mushroom tyrosinase activity and have antioxidant properties. J. Taiwan Inst. Chem. Eng. *42*, 204–211. https://doi.org/10.1016/j.jtice.2010.05.008.

Wesley-Smith, J., Pammenter, N.W., Berjak, P., and Walters, C. (2001). The effects of two drying rates on the desiccation tolerance of embryonic axes of recalcitrant jackfruit (*Artocarpus heterophyllus* lamk.) seeds. Ann. Bot. (London) *88*, 653–664. https://doi.org/10.1006/anbo.2001.1519.

Yang, G., Chen, W., and Zhang, Z. (2005). Effect of media on seed germination and growth of *Synsepalum dulcificum*. Chin. J. Trop. Agric. *5*, 7.

Received: Jan. 28, 2017
Accepted: Oct. 31, 2017