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Development of seed germination and *in vitro* propagation protocols for *Vitex doniana*

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ABSTRACT

Propagation of *Vitex doniana* by seeds has been limited by low yield and a prolonged germination period which, along with over-harvesting, has led to a decline in its natural population. The present study aimed to improve seed germination and to establish *in vitro* organogenesis protocols to conserve superior genotypes. The effects of seed pre-treatments and environmental conditions on seed germination were investigated through conventional germination methods. Removal of the seed coat followed by germination on filter paper resulted in the highest percentage germination (87 %). The best germination temperature range was 25 °C-35 °C (80–87 %). A direct organogenesis protocol was established when *in vitro* nodal explants were cultured for eight weeks on modified Woody Plant Medium (WPM) supplemented with 0.3 mg/L 6-benzylaminopurine (BAP), resulting in 4 shoots/explant. Shoots were rooted on the modified WPM containing 0.5 mg/L indole-3-acetic acid (IAA), resulting in 80 % rooting and 4 roots/shoot, after four weeks. Successful acclimatisation was achieved, with 93 % survival in the greenhouse. For somatic embryogenesis, pro-embryos that formed from pairing 1 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) with 1 mg/L picloram or each tested auxin with 0.5 mg/L BAP failed to develop into somatic embryos on different maturation media. For time and cost savings and high yield, the present study found that germination be performed using de-coated seeds on filter paper at 25–35 °C followed by transfer to soil upon germination. The present study reports the first direct organogenesis protocol for *V. doniana*, allowing for clonal propagation of high-value genotypes.

1. Introduction

Vitex doniana Sweet (Verbenaceae) is a tree species of economic importance, and occurs in the savannah and open woodland vegetation types of western, eastern, and southern regions of Africa (Dalziel and Hutchison, 1955; Orwa et al., 2009). The pulp from the fruit is used to make jam (Alabo, 1999), local wine (Okigbo et al., 1998), and food sweetener (Egbekun et al., 1996). The leaf, stem bark, and fruit of the plant are utilised in traditional medicine as antibacterial, analgesic, antiviral, diuretic, and antidepressant (Jean et al., 2019). The wood is used to produce furniture, agricultural tools, and charcoal (Orwa et al., 2009). Hence, the tree faces over-exploitation, which, coupled with its relatively poor seed germinability has placed pressure on wild populations in north-western Nigeria (Danjuma and Abubakar, 2017), Burkina Faso (Souleymane et al., 2009) and Benin (Oumorou et al., 2010).

Conservation of the species can be achieved through sexual or asexual methods, the route depending on the objective of the propagation. Although propagation by seeds is the preferred method to conserve and maintain genetic diversity (Pence, 2011), the seeds of *V. doniana* are characterised by a hard-woody coat, imposing physical dormancy (Orwa et al., 2009), necessitating pre-sowing treatment. N'Danikou et al. (2015) reported 83 % seed germination after six months of sowing, when seeds of *V. doniana* were soaked in water for one hour followed by sun-drying for eight hours daily for 21 days, while Salisu and Jiya (2016) obtained 26 % germination when intact seeds were soaked for 24 h in hot water at 60 °C until the temperature dropped to ambient level. Hence, the effect of pre-germination treatments and other physiological and environmental factors require investigation and elucidation for this species, for both conventional and *in vitro* propagation, where germination conditions can be closely controlled.

Asexual propagation, primarily through cuttings can also be used to identify and vegetatively proliferate superior genotypes for commercial purposes. The challenge in *V. doniana* (Sanoussi et al., 2012), as with most vegetative propagation protocols, lies in the induction of adventitious roots from cuttings (de Assis et al., 2004; Pinto et al., 2013; Mazri et al., 2022). Determining the physiological requirements for adventitious rooting is often achieved through *in vitro* experiments, which also represents a useful vehicle for germplasm storage (Engelmann, 2011; Pence et al., 2020), along with the

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opportunity to propagate via a range of explant material (George and Debergh, 2008; Coelho et al., 2020).

In vitro or micropropagation can be achieved through organogenesis or somatic embryogenesis, either directly from the explants or indirectly through an intervening callus phase (George and Debergh, 2008: Fehér, 2019). Direct organogenesis involves shoot initiation and multiplication from a pre-existing meristem, followed by root induction under the correct combinations of plant growth regulators (PGRs) and culture conditions (George, 2008; Duclercq et al., 2011). In contrast, somatic embryogenesis is the process through which somatic cells are induced to become embryogenic, which then 'germinate' and develop into complete plants (Ammirato, 1987; Emons, 1994; Heringer et al., 2018). These propagation options should be explored for species that are recalcitrant and yet vulnerable in the wild. There is only one report on *in vitro* regeneration of *V. doniana* via somatic embryogenesis and none on organogenesis. Dadjo et al. (2015) induced cotyledonary-stage somatic embryos from leaf explants derived from 2–4 year old field grown seedlings using half-strength MS (Murashige and Skoog, 1962) salts supplemented with 8.45 mg/L AgNO3 and 30.6 mg/L tryptophan, each combined with 0.11 mg/L N-phenyl-N-1,2,3-thiadiazol-5-ylurea (TDZ). However, the somatic embryos did not convert to plantlets in that study. As the conversion of somatic embryos to plantlets can be affected by the availability of PGRs in the preceding stages, the correct type and quantity of PGR, particularly auxin and other media components that will promote somatic embryogenesis in this species, remain to be investigated. Therefore, the first aim of this study was to understand the physical and physiological barriers to seed germination by investigating the effects of pre-sowing seed treatments, watering frequencies, light conditions and temperature on seed germination. Also, the conditions for *in vitro* seed germination was investigated to establish an alternative route to conventional seed germination. The second aim was to understand the physiological requirements for direct organogenesis and indirect somatic embryogenesis in *V. doniana*, which will be useful in conserving *V. doniana* through mass propagation and preservation of superior genotypes.

2. Material and methods

2.1. Source of seeds

Ripe fruits of *V. doniana* were purchased every September (2017–2022) from Yar'kutungu market, Katsina, Nigeria (13° 0 15.85 N; 7°36 19.15 E). Each time, fruits were purchased from three different sellers and combined to form a composite sample in order to get adequate genetic variety of the natural population. The identity of the plant specimen was confirmed at the herbarium of the botany department, Ahmadu Bello University Zaria, Nigeria (ABU0959). The pulp of ripe fruits was removed to extract the seeds, which were then washed with tap water, dried outside in the sun, and stored at ambient temperature (21–25 °C) for 16 days (including transportation time) before use. The seeds were transported under ambient temperature to the University of KwaZulu-Natal, Durban, South Africa, for the laboratory work. Germination was performed both in the greenhouse and under *in vitro* conditions, as explained in the subsequent sections. A flowchart of the experiment design is presented in Fig. 1.

2.2. Seed germination

2.2.1. Effect of seed pre-treatments on germination

Various seed pre-treatments were tested for seed germination. These were: (1) manual cracking of seed coats with a hammer followed by soaking in tap water for one hour (hereafter referred to as the seed with cracked coat); (2) removal of the seed coat (hereafter referred to as de-coated seeds); (3) soaking de-coated seeds in distilled water containing 0.5 mg/L indole-3-butyric acid (IBA) for 0.5 h (4) soaking seeds in tap water for four days on an orbital shaker (New Brunswick Scientific, USA) at a low speed (83 rotations per minute) in a growth room with a 16-h light (37 μmol m^{-}2 s^{-}1)/ 8-h dark photoperiod, at 25 and 21 °C, respectively; (5) soaking seeds in hot tap water at 60 °C; (6) soaking seeds in hot tap water at 100 °C, where (for treatments 5 and 6) they stayed until the temperature dropped to the ambient level, for four days on an orbital shaker in the same condition as the preceding treatment; and (7) the control (untreated seeds).

Following the seed pre-treatments, 10 seeds from each treatment were sown in 23 cm (length) x 15 cm (diameter) x 6 cm (depth) seedling trays (Plasgrow, South Africa) containing potting mix (Grovida, South Africa). Seedling trays were kept in a greenhouse (uncontrolled environment) at the University of KwaZulu-Natal Westville Campus (29° 52 S; 30°59 E; 25 °C day/ 18 °C night) and watered three times (120 ml per cycle) a day for three minutes each by an automatic water sprinkler using municipal water. A seed was considered to have germinated when the seedling emerged from the soil. The number of seedlings that emerged from the soil was recorded every two days for 63 days. The study was repeated three times for each treatment.

2.2.2. Effect of the watering frequency on germination and seedling growth

Seeds with cracked coats were sown in 23 cm (length) x 15 cm (diameter) x 6 cm (depth) seedling trays containing potting mix and then placed in the greenhouse. Seeds were watered to field capacity (well-watered) once a day for one of the treatments and once on alternate days for the other treatment. A total of 15 seeds and four replicates were used for each treatment. Germination percentages were recorded following five weeks of sowing seeds in soil.

2.2.3. Effect of auxin on germination in vitro

De-coated seeds were decontaminated in a laminar flow hood with 70 % (v/v) ethanol (ILLOVO, South Africa) for one minute, followed by 2.5 % (v/v) sodium hypochlorite (NaOCl)

(Reckitt Benckiser, South Africa) for 20 min. After each disinfection treatment, the seeds were rinsed three times with distilled water for less than one minute per rinse. Afterwards, seeds were placed on 10 ml germination media containing half-strength Murashige and Skoog (1962) (MS) (Sigma-Aldrich, USA), 10 g/L sucrose, and 8 g/L agar (Sigma-Aldrich, USA), in 9 cm (height) x 2 cm (diameter) glass culture tubes (one seed per tube) for four weeks. Indole-3-butyric acid at 0.5 mg/L was added to the germination medium, while the medium without the auxin served as the control. Thereafter, seedlings were subcultured onto 50 ml of the fresh media without IBA in 9.6 cm (height) x 7.6 cm (diameter) Magenta^{TM} (Bioworld, USA) culture vessels (one seedling per Magenta box) for a further four weeks. Cultures were maintained under a 16-h light (37 μmol m^{-2} s^{-1})/ 8-h dark photoperiod in a growth room, at 25 and 21 °C, respectively. The pH of media was adjusted to 5.6–5.8 before autoclaving (HL-341, Laboratory Supplies, South Africa) at 121 °C and 1 kPa for 20 minutes. Fifteen seeds and three replicates for each seed type.

2.2.4. Effects of light and temperature on germination

De-coated seeds were disinfected, as mentioned in Section 2.2.3. Afterwards, 15 seeds were transferred onto sterile filter paper on a 9 cm Petri dish (Labotec, South Africa) moistened with 2.5 ml of sterile distilled water that contained 0.5 mg/L of IBA (as the auxin was supplemented to the germination medium in Section 2.2.3). For the light experiment, three Petri dishes containing 15 seeds each were placed in a growth room in 16-h light (37 μmol m^{-2} s^{-1})/ 8-h dark photoperiod at 25 and 21 °C, respectively. Another set of three Petri dishes were maintained in dark conditions in a growth room at ambient temperature (21 °C). For the temperature experiment, the Petri dishes were incubated in plant growth chambers (GC-300TLH, JEIO

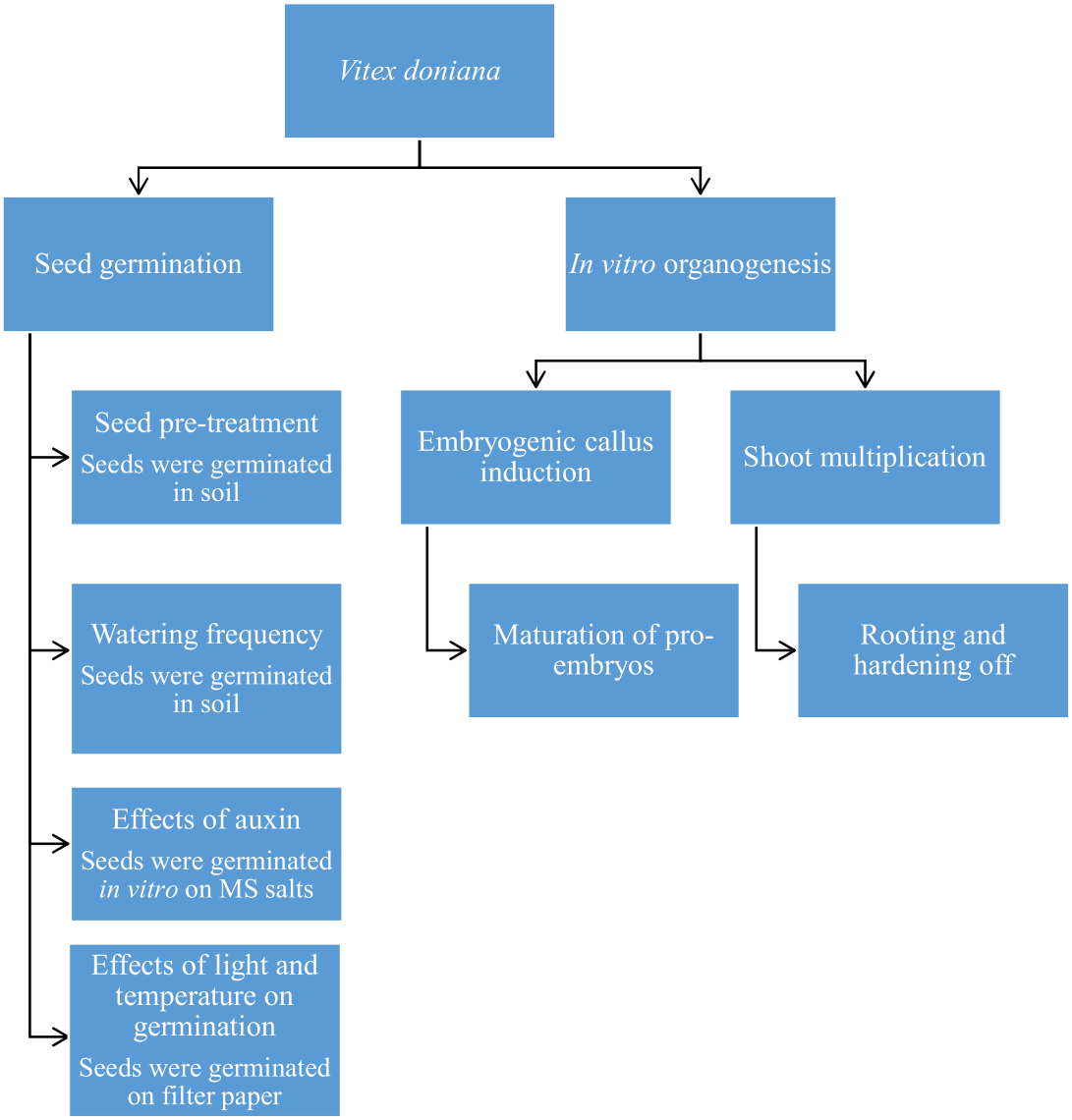

Fig. 1. A flowchart illustrating the experiment design.

TECH, Korea) at 15, 25, 35, and 40 °C and in a growth room at 25/21 °C (16-h/ 8-h) for 14 days. The filter paper was re-moistened every four days with 1–2 ml of sterile distilled water that contained 0.5 mg/L IBA. A seed was considered germinated by the protrusion of a radicle ≥ 1 cm long (Bewley, 1997). There were four replicates per treatment.

2.3. In vitro *organogenesis*

Eight-week-old *in vitro*-germinated seedlings (each having four nodes) were used for the *in vitro* organogenesis studies. The *in vitro* seedlings were produced as described in Section 2.2.3, using the germination medium that contained 0.5 mg/L IBA at the initiation stage.

Unless otherwise stated, the pH of media was adjusted to 5.6–5.8 before autoclaving at 121 °C and 1 kPa for 20 min. Cultures were maintained in a growth room in 16-h light (37 μmol m^{-2} s^{-1})/ 8-h dark photoperiod, at 25 and 21 °C, respectively.

2.3.1. *Shoot induction and multiplication*

Shoots from the *in vitro*-germinated seedlings were cut into nodal sections with a size 24 surgical blade (Healthease®, South Africa) and then placed in 9 cm (height) x 2 cm (diameter) culture tubes (one node per tube) containing 10 ml of media for shoot induction and multiplication. The media contained Woody Plant Medium (WPM; Lloyd and McCown, 1980) (HIMEDIA, India), 30 g/L sucrose, 8 g/L agar, 0.15 g/L each of calcium chloride ($CaCl_2$) (BDH Chemicals Ltd., England) and magnesium sulphate heptahydrate ($MgSO_4{\cdot}7H_2O$) (Merck Laboratory Supplies, South Africa), 0.2 g/L potassium dihydrogen phosphate (KH_2PO_4) (Merck Laboratory Supplies, South Africa), 0.1 mg/L D-biotin (Sisco Research Laboratory, India), 0.1 mg/L calcium pantothenate (Sigma-Aldrich, USA), 0.025 mg/L cobalt chloride hexahydrate ($CoCl_2{\cdot}6H_2O$) (ACE Chemicals, South Africa) and 0.3 mg/L each of BAP, or 6-γ-γ-dimethylallyaminopurine (2iP), or 6-furfuryl-aminopurine (kinetin). Medium without PGR served as the control. There were 25 nodal explants and three replicates for a treatment.

2.3.2. *Rooting and hardening off*

Shoots (approximately 1.5–2 cm) from *in vitro* cultures maintained for eight weeks, were transferred to root induction media in culture tubes (one shoot per tube) containing 10 ml WPM, 30 g/L sucrose, 8 g/L agar, 0.15 g/L each of $CaCl_2$ and $MgSO_4{\cdot}7H_2O$, 0.2 g/L KH_2PO_4, 0.1 mg/L D-biotin, 0.1 mg/L calcium pantothenate, 0.025 mg/L $CoCl_2{\cdot}6H_2O$, and 0.5 mg/L indole-3-acetic acid (IAA). The medium

that was devoid of auxin served as the control. Twenty shoots and three replicates were used for this study.

Rooted plantlets (≥ 3 cm shoot height) were taken out of the culture media and rinsed in distilled water to remove remnants of agar from the roots. The plants were transferred into 9 cm (height) x 5 cm (diameter) culture bottles containing sterile potting mix (nutrient-rich substrate), and watered with ¼-strength MS salts and vitamins. The bottles were placed in a growth room in 16-h light (37 μmol m^{-2} s^{-1})/ 8-h dark photoperiod, at 25 and 21 °C, respectively. After the first week, the humidity was gradually reduced by loosening the caps of the bottles for another week. In the third week, the caps were removed entirely from the bottles, and the plants were transferred into 10 cm (diameter) x 8.3 cm (depth) pots and kept in the greenhouse and watered three times a day for three minutes each by an automatic water sprinkler using municipal water. The plants were maintained for eight weeks, after which the percentage survival was recorded.

2.3.3. Embryogenic callus induction

Leaf explants from *in vitro*-germinated seedlings were cut across the midrib into 1 cm (height) x 1.5 cm (length) pieces using a size 24 carbon steel surgical blade. Four pieces of explants were placed in 9 cm Petri dishes on 26 ml callus induction medium (CIM). The CIM contained MS salts with vitamins, 30 g/L sucrose, 10 g/L agar, and PGRs. For the initial experiment, explants were maintained for eight weeks without subculture on CIM containing: (1) 1 mg/L 2, 4-D (2) 1 mg/L 2,4-D with 0.5 mg/L BAP; (3) 1 mg/L 4-amino-3, 5, 6-trichloropicolinic acid (picloram) (4) 1 mg/L picloram with 0.5 mg/L BAP and (5) 1 mg/L 2,4-D with 1 mg/L picloram. In a related experiment, and in order to investigate the effect of exposure of explants to PGR analogues on embryo maturation, explants were cultured on CIM for six instead of eight weeks as was performed for the initial experiment. The CIM contained (1) 1 mg/L 2,4-D combined with 1 mg/L picloram (2) 1 mg/L 2,4-D combined with 0.5 mg/L BAP and (3) 1 mg/L picloram combined with 0.5 mg/L BAP. Cultures were maintained in a growth room in the dark at 25 °C day/ 21 °C night.

2.3.4. Maturation of proembryos

Embryogenic calli were placed in 9 cm Petri dishes (four calli per Petri dish) with maturation media that included MS salts and vitamins, 30 g/L sucrose, and 10 g/L agar. Four maturation media were tested: (1) MS without PGR; (2) MS supplemented with 0.1 mg/L BAP; (3) MS with 2 mg/L abscisic acid (ABA); and (4) MS with a combination of 0.1 mg/L BAP and 2 mg/L ABA. Calli from the initial experiment were transferred onto the first maturation medium (MS without PGR), whereas those from the subsequent experiment were transferred onto the four maturation media. Cultures were maintained for eight weeks in the light.

2.3.5. Microscopy

After six or eight weeks of callus initiation, morphology of calli was observed using a stereomicroscope (NIKON AZ100, Japan). The proportion of pro-embryos at each stage of embryo development was recorded based on estimation within the microscopic field of view.

2.4. Statistical analyses

Statistical analyses were performed with Statistical Package for the Social Sciences (SPSS) software, version 25 (IBM Incorporation, USA). Prior to statistical analysis, arcsine transformation was applied to the percentage data. A normality test was performed for all data types using a one-sample Kolmogorov-Smirnov (K-S) test ($p < 0.05$). Afterwards, data were subjected to the one-way analysis of variance (ANOVA) or paired samples *t*-test where appropriate. When the ANOVA indicated a significant difference, a Tukey's Honest Significant Difference (HSD) test was used to compare the means at a 95 % confidence interval. The means were assigned different letters to indicate significant differences among treatments.

3. Results

3.1. Seed germination

In an effort to improve seed germination, this study investigated the effects of seed pre-treatments, watering frequencies, PGR, and light and temperature on seed germination.

3.1.1. Effect of seed pre-treatments on germination

The percentage of germination of *V. doniana* seeds was observed every two days for 63 days. The results showed that the seed coat was a hindrance to water uptake (Table 1). During the first 21 days of the experiment, only de-coated seeds and those seeds where the coats were cracked and soaked in water for one hour, germinated (20 %) (Table 1). At day 42, both untreated (control) and four-day-soaked seeds started to germinate (Table 1). The final germination percentage (FGP), after 63 days, was significantly higher in seeds with cracked coats (60 %) than in the control (6.6 %) and other treatments (6.6–33.3 %) (Table 1).

Having established the above, the seed pre-treatment of cracking seed coats and then soaking them for an hour was used to investigate the effect of watering frequency on seed germination. There was no significant difference in the final percentage germination of seeds watered daily or on alternate days (Table 2).

3.1.2. Effect of auxin on germination in vitro

In an effort to establish an alternative, more reliable and more efficient germination protocol for seeds of *V. doniana*, the *in vitro* route was presently investigated. As it had been demonstrated that the seed coat was a hindrance to water uptake (Table 1), seeds with cracked coats and de-coated seeds were placed on half-strength MS salts for the *in vitro* germination experiment. Seeds with cracked coats did not germinate even after being left in culture for 10 weeks (result not shown), while de-coated seeds germinated with the FGP

Table 1
The effect of seed pre-treatments on the percentage germination of *V. doniana* seeds sown in soil and maintained in the greenhouse, after 63 days. Mean ± standard deviation followed by different letters within the same column are significantly different (one-way ANOVA, post hoc Tukey test, p ≤ 0.05, n = 10).

	Germination (%)		
Treatment	Day 21	Day 42	Day 63
Control (untreated seeds)	0 ± 0.0	3.3 ± 5.7^{b}	6.6 ± 11.5^{b}
Seeds cracked with a hammer, followed by soaking in tap water for 1 h.	20 ± 0.0^{a}	53.3 ± 11.5^{a}	60 ± 10.0^{a}
De-coated seeds.	20 ± 10^{a}	20 ± 10^{b}	23.3 ± 5.7^{b}
De-coated seeds were soaked in distilled water containing 0.5 mg/L IBA for 0.5 h.	20 ± 10.0^{a}	20 ± 10.0^{b}	20 ± 10.0^{b}
Intact seeds were soaked in tap water at 60 °C and shaken on an orbital shaker for 24 h.	0 ± 0.0	0 ± 0.0	33.3 ± 28.8^{b}
Intact seeds were soaked in tap water at 100 °C and shaken on an orbital shaker for 24 h.	0 ± 0.0	0 ± 0.0	6.6 ± 5.7^{b}
Intact seeds were soaked in water and shaken on an orbital shaker for 4 days.	0 ± 0.0	3.3 ± 5.7^{b}	13.3 ± 15.2^{b}

Table 2
Effect of watering regime on seed germination and seedling growth, five weeks after sowing. Mean ± standard deviation (paired samples *t*-test, $p > 0.05$, n = 15).

Watering regime	Germination (%)
Daily	73.3 ± 17.2[a]
Alternate days	66.6 ± 10.9[a]

Table 3
The effect of IBA on germination of de-coated seeds *in vitro*, after 14 days. Mean ± standard deviation followed by different letters within the same column denote significant differences (paired samples *t*-test, $p \leq 0.05$, n = 15).

IBA (mg/L)	Germinating seeds without radicle (%)	Germinating seeds with radicle (%)
0	60 ± 13.3[b]	40 ± 6.6[b]
0.5	86 ± 5.4[a]	66 ± 12.1[a]

Fig. 2. *In vitro* germination of de-coated seeds on half-strength MS salts: A) absence of radicle protrusion in seeds germinated on medium without 0.5 mg/L IBA; B) Radicle protrusion in seeds germinated on medium with 0.5 mg/L IBA.

(66 %) achieved following 14 days in culture (Table 3). However, in some seeds, only cotyledons emerged without the radicle protrusion (Table 3, Fig. 2). Although the protrusion of the radicle is considered a sign of germination, this set of seeds was referred to as 'germinating seeds without radicles' to distinguish them from seeds whose radicles had protruded. Radicle protrusion was significantly increased when 0.5 mg/L IBA was added to the germination medium (Table 3). Consequently, 0.5 mg/L IBA was always supplemented to the medium for all germination experiments not undertaken in the greenhouse.

Following these findings, fully de-coated seeds were used to investigate the effects of light and temperature on seed germination. In both experiments, seeds were germinated on filter paper, as it is more resistant to high temperature compared to gel media.

3.1.3. *Effects of light and temperature*

The FGP was not significantly different between light (75 %) and dark (80 %) conditions whereas temperature significantly affected seed germination (Table 4). Only seeds incubated at 25 and 35 °C and at ambient temperatures of 25/21 °C (16 h/ 8 h) germinated following 14 days of incubation in a given temperature, having FGPs of 80, 87, and 80 %, respectively (Table 4).

3.2. In vitro *organogenesis*

3.2.1. *Shoot induction and multiplication*

The main objective of the shoot induction and multiplication stage was to develop an *in vitro* organogenesis protocol for clonal propagation in order to preserve superior genotypes and to achieve mass proliferation to sustain populations that are otherwise difficult to conserve. In a preliminary experiment, a purple colouration was observed in the growing shoots, which was hypothesised to be an indication of phosphorous deficiency (results not shown), as high amounts of sulphur in the WPM can interfere with phosphorus uptake (Grifoni et al., 2015). Hence, 0.2 g/L KH_2PO_4 was supplemented to the shoot induction and multiplication media, which subsequently resulted in healthier looking, greener shoots.

Shoot multiplication was significantly promoted in the presence of BAP (4 shoots/explant) compared with other cytokinins (2iP, and kinetin) or the control (Table 5). There was callus formation at the basal cut end of the nodal explants, which was significantly higher in the control than in the PGR treatments (Table 5). The percentages survival and bud break did not significantly differ among treatments (Table 5).

3.2.2. *Root induction and hardening off*

The best shoot multiplication treatment (0.3 mg/L BAP) was used to produce sufficient numbers of shoots for the rooting experiment. Shoots rooted in both media with and without 0.5 mg/L IAA. However, percentage rooting in the auxin-free medium was significantly lower (60 %) than in the medium with 0.5 mg/L IAA (80 %) (Table 6). In contrast, the number of roots per shoot was not significantly different between the treatment (4) and the control (3) (Table 6). Rooted plantlets acclimatised successfully with 93 % survival. Different stages involved in the protocol development are shown in Fig. 3 A - G.

3.2.3. *Embryogenic callus induction*

Leaf explants started forming calli after three weeks on different induction media. Combination of PGRs (auxin-auxin or auxin-cytokinin) significantly increased the percentage of callus formation (Table 7). The combination of PGRs also influenced the formation of pro-embryos as no structures were observed under a stereomicroscope in calli formed from explants treated with a single auxin (Fig. 4 A - D). At eight weeks, only the calli from the treatments where pro-embryos were observed were transferred to PGR-free medium (maturation medium) for further development. At this stage, pro-embryos

Table 4
Effect of temperature on germination of de-coated seeds in Petri dishes on filter paper moistened with distilled water containing 0.5 mg/L IBA, after 14 days. Mean ± standard deviation followed by different letters are significantly different amongst treatments (one-way ANOVA, post hoc Tukey test, $p \leq 0.05$, n = 15).

	Germination (%)	
Temperature (°C)	Day 7	Day 14
15	0 ± 0.0	0 ± 0.0
25	80 ± 10.0[a]	80 ± 10.0[a]
35	87 ± 5.4[a]	87 ± 5.4[a]
40	0 ± 0.0	0 ± 0.0
25/21- 16-h light/8-h dark	80 ± 8.1[a]	80 ± 8.1[a]

Table 5
The effect of different types of cytokinins on the shoot multiplication of *in vitro* nodal explants sourced from *in vitro*-germinated seedlings, after eight weeks. Mean ± standard deviation followed by different letters within the same column denote significant differences (one-way ANOVA, post hoc Tukey test, $p \leq 0.05$, n = 25).

Cytokinin (mg/L)	Survival (%)	Explants with callus (%)	Bud break (%)	Number of shoots/explant
0	72 ± 43.8^{a}	90 ± 22.3^{a}	72 ± 22.1^{a}	2 ± 0.5^{b}
0.3 BAP	84 ± 16.7^{a}	60 ± 14.1^{b}	85.7 ± 35.85^{a}	4 ± 0.2^{a}
0.3 2iP	96 ± 8.9^{a}	44 ± 16.7^{b}	89.5 ± 22.3^{a}	2 ± 0.4^{b}
0.3 kinetin	76 ± 43.3^{a}	8 ± 17.8^{c}	89 ± 20.9^{a}	2 ± 0.2^{b}

Table 6
Rooting (%) and the number of roots of shoots multiplied *in vitro*, followed by root induction with and without 0.5 mg/L IAA, after 4 weeks. Mean ± standard deviation within columns followed by different letters denote significant differences (paired samples *t*-test, $p \leq 0.05$, n = 20).

IAA (mg/L)	Rooting (%)	Number of roots/shoot
0	60 ± 52.9^{b}	3 ± 0.0^{a}
0.5	80 ± 34.6^{a}	4 ± 0.5^{a}

were mostly at the globular - heart - stages of development (Fig. 4 B and D).

3.2.4. Maturation of pro-embryos

Initially, calli which had been on CIM for eight weeks were transferred to the PGR-free medium for pro-embryo development. However, pro-embryos did not develop further after eight weeks on this medium. Consequently, the callus induction time was reduced from eight weeks to six weeks as prolonged exposure of explants to exogenous auxins can hinder pro-embryo development (*e.g.*, Parrott et al., 1988; Bonneau et al., 1994; Filippov et al., 2006; She et al., 2013). This was investigated using three different combinations of PGRs in the CIM. The media were: (1) 1 mg/L 2, 4-D with 1 mg/L picloram; (2) 1 mg/L 2, 4-D with 0.5 mg/L BAP; and (3) 1 mg/L picloram with 0.5 mg/L BAP. Only leaf explants cultured on CIM containing auxin-cytokinin combinations formed pro-embryos at six weeks (Fig. 5 A - C). After six weeks on the CIM, only the calli from the auxin-cytokinin treatments were transferred to different maturation media: (1) PGR-free medium; (2) MS with 0.1 mg/L BAP; (3) MS with 2 mg/L ABA; and (4) MS with a combination of 0.1 mg/L BAP and 2 mg/L ABA, for pro-embryo development. The calli were maintained on the maturation media for eight weeks.

Pro-embryos did not develop into somatic embryos when the exposure time of explants to exogenous PGRs was reduced from eight to six weeks. Similarly, the PGRs added to the maturation media did not promote pro-embryo development.

4. Discussion

4.1. Seed germination

The results for seed pre-treatments indicated that *V. doniana* seeds exhibit physical dormancy caused by the hard seed coat. Manual cracking of the hard seed coat followed by an-hour of soaking in tap water resulted in the highest FGP compared to the control and de-coated seeds (Table 1). It is known that seed coats can impose physical dormancy by limiting or preventing the seed from absorbing water to start the germination process (Baskin and Baskin, 2000; Finch–Savage and Leubner–Metzger, 2006; Zhang et al., 2019). In other *Vitex* species such as *V. agnus-castus* (Belhadj et al., 1996) and *V. glabrata* (Hossain et al., 2018), acid scarification and sand paper scraping of the hard seed coat significantly improved germination, respectively. The low FGP in de-coated seeds when compared to seeds with cracked coats was likely caused by damage to the seed from soil abrasion as seeds subjected to the same treatment had high FGPs when germinated on filter paper (Table 3). Nevertheless, results from the present study offer a significant improvement for the germination of *V. doniana* seeds compared with the literature thus far (*e.g.* N'Danikou et al., 2015). Presently, FGPs were achieved at 63 days of sowing seed in soil and at 14 days on filter paper or on MS salts

Fig. 3. *In vitro* propagation of *V. doniana* through direct organogenesis; A) shoot multiplication on WPM without cytokinin (control); B) shoot multiplication on WPM with 0.3 mg/L BAP; C) shoot multiplication on WPM with 0.3 mg/L kinetin; D) shoot multiplication on WPM with 0.3 mg/L 2iP; E) root induction on WPM without auxin (control); F) root induction on WPM with 0.5 mg/L IAA; G) two-month-old acclimatised plants.

Table 7
The effect of PGRs on embryogenic callus induction from *in vitro* leaf explants. Explants were cultured in callus induction media for 8 weeks without subculture. Mean ± standard deviation followed by different letters within the same column denote significant differences (one-way ANOVA, post hoc Tukey test, $p \leq 0.05$, n = 10).

PGR (mg/L)				
2,4-D	Picloram	BAP	Explant with callus (%)	Proportion of pro-embryos per plate
0.5	0.5	0	100 ± 0.0[a]	0.1
1	1	0	97.5 ± 7.90[a]	0.6
1	0	0	52.5 ± 32.16[b]	0.0
0	1	0	40 ± 35.74[b]	0.0
1	0	0.5	97.5 ± 7.90[a]	0.8
0	1	0.5	92.5 ± 16.87[a]	0.6
0.5	0.5	0.5	100 ± 0.0[a]	0.5

compared to six months reported by N'Danikou et al. (2015). Burying seeds in soil for prolonged periods can lead to soil-borne fungi infecting the seeds, completely preventing germination (Khan et al., 1976; Mycock and Berjak, 1992; Finch-Savage, 2015). More details of seed germination on filter paper and on MS are discussed later.

Seed deterioration was commonly observed during seed coat removal. Ageing is the primary cause of this deterioration by causing oxidative damage to membranes, lipids, proteins, DNA and RNA, resulting in the loss of seed vigour and viability (Khan et al., 1976;De-Vitis et al., 2020, Klaedtke et al., 2022). This ageing process is influenced by the storage conditions, mainly temperature and moisture (Ebone et al., 2019). In the present study, seeds were kept at ambient temperature (21–25 °C), which can be ideal for ageing *V. doniana* seed. Nevertheless, visually healthy seeds were observed up to seven months of storage after extraction from the new fruit. However, as time in storage increased, the frequency of identifying and selecting visually healthy seeds gradually decreased. There is, therefore, a need to investigate the optimal storage conditions for *V. doniana* seed.

Given that seeds with cracked coats resulted in the highest percentage germination in soil, seeds subjected to this priming treatment were used to investigate the effect of the watering frequency on seed germination. It is well established that variability in the soil moisture can influence seed germination and seedling development (Lamichhane et al., 2018; Maeda et al., 2021). Presently, the FGP between the watering frequencies were not significantly different (Table 2). The fact that *V. doniana* naturally grows in areas with 750–2000 mm mean annual rainfall (Orwa et al., 2009) may account for these results. De-coated seeds were used for germination on MS or filter paper to avoid the impact of the seed coats on water uptake. It was necessary to also investigate the effect of auxin on seed germination, as in some germinating seeds only cotyledons emerged without radicle protrusion (Fig. 2 A and B). The addition of 0.5 mg/L IBA to the germination medium (MS salts) significantly increased percentage germination (Table 3). Auxin is known to promote ethylene production (Machakova et al., 2008), and ethylene promotes germination by making seeds less sensitive to endogenous ABA, a germination inhibitor (Baskin and Baskin, 2004). Hence, the result indicate that apart from the physical dormancy imposed by the seed coat in this species, *V. doniana* seeds may also exhibit physiological dormancy imposed by PGRs. Further study is therefore required to understand the interactions of endogenous PGRs on germination in *V. doniana.* Seeds of *Arabidopsis thaliana* showed a similar type of germination response in seeds that were not treated with gibberellic acid (GA), resulting in the emergence of cotyledons without radicles (Hauvermale and Steber, 2020). Gibberellic acid (GA) is widely used to overcome physiological dormancy (Baskin and Baskin, 2004).

The seeds of *V. doniana* were non-photoblastic, as their germination was light-insensitive (Fig. 4). The regulatory effect (promotion or inhibition) of light on seed germination differs from species to species (Wartidiningsih and Geneve, 1994; Wang et al., 2009; El-Keblawy et al., 2014; Bu et al., 2017; Zhang et al., 2021; Chen et al., 2022). The present result is consistent with the result reported for *Verbena officinalis,* which is also a member of Verbenaceae that light

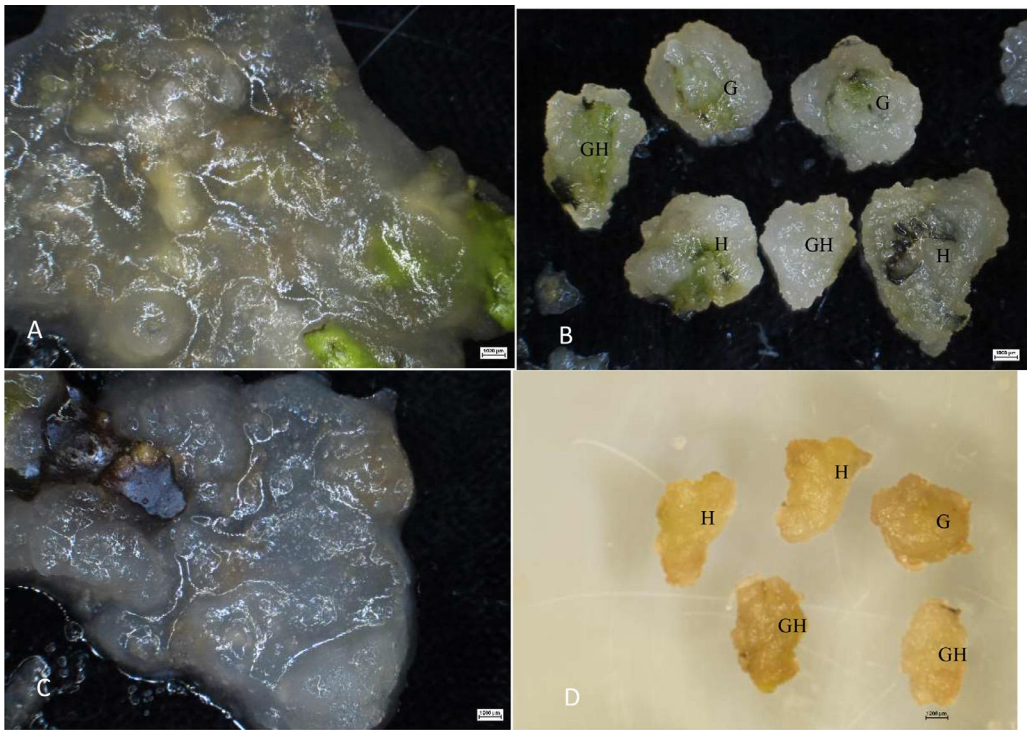

Fig. 4. Pro-embryo formation in calli produced from leaf explants after eight weeks on CIM: A) absence of pro-embryos in calli formed on 1 mg/L 2, 4- D; B) pro-embryos in calli formed on 1 mg/L 2,4-D combined with 0.5 mg/L BAP; C) absence of pro-embryos in calli formed on 1 mg/L picloram; D) pro-embryos in calli formed on 1 mg/L picloram combined with 0.5 mg/L BAP. Stages of pro-embryo development are: G = globular; GH = globular-heart; and H = heart. Bar = 1000 μm.

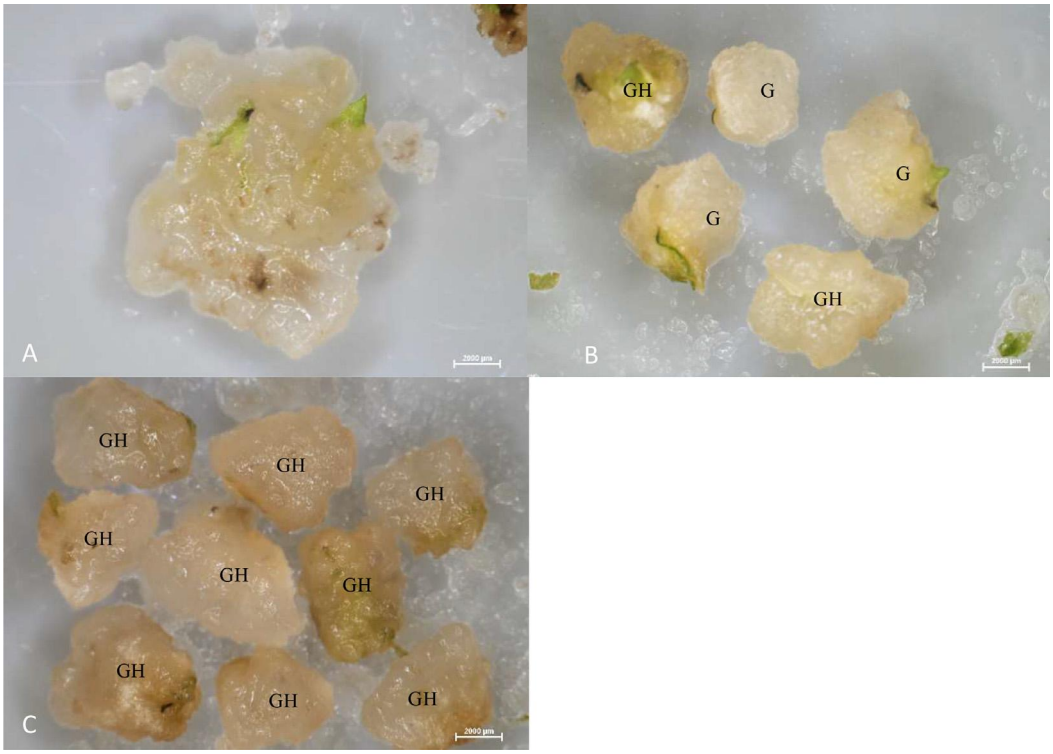

Fig. 5. Pro-embryos (G = globular; and GH = globular-heart) produced through callus induction when leaf explants were cultured for six weeks on: A) media with a combination of 1 mg/L 2, 4-D and 1 mg/L picloram, with no pro-embryos; (B) media with a combination of 1 mg/L 2, 4-D and 0.5 mg/L BAP; and C) media with a combination of 1 mg/L picloram and 0.5 mg/L BAP. Bar = 1000 μm.

conditions had no significant effect on seed germination (Toscano et al., 2018).

The germination of *V. doniana* seeds are, however, highly influenced by temperature. High FGPs were obtained when seeds were incubated at 25 and 35 °C and 25/21 °C (16 h/ 8 h), while no seed germinated at the low temperature of 15 °C or at the higher temperature of 40 °C (Table 4.3). Low temperatures affect seed germination by arresting metabolic activities (Hills and van Staden, 2003), whereas high temperatures can denature the suite of proteins necessary for germination (Yan and Chen, 2020). It appears that the optimal temperatures for seed germination among members of Verbenaceae are similar. For example, the optimal temperature for seed germination of *Lippia multiflora* is 25 - 35 °C (Mattana et al., 2017), while that of *Caryopteris incana* is 20 - 25 °C (Shi et al., 2022). In Nigeria, the fruiting season of *V. doniana* is from August to September, during which the minimum and maximum temperatures are around 20 and 30 °C, respectively (Amadi et al., 2014).

Seed germination on both media containing MS salts (Table 3) or on filter paper (Table 4) resulted in high FGPs, indicating that *V. doniana* seed does not require an external supply of nutrients to germinate. Although the seed embryo utilises the storage reserves to germinate, there are species such as members of *Cypripedium* that require an external supply of nutrients for a reasonable percentage of seed germination to be achieved (Zeng et al., 2014). Species belonging to this genus have zygotic embryos with varied, but sufficient nutrient requirements to germinate (Zeng et al., 2014).

4.2. Direct organogenesis

As different cytokinins can act differently in promoting morphogenesis (van Staden et al., 2008), three types were tested in this study at 0.3 mg/L - BAP, 2iP, and kinetin, for shoot induction and multiplication. Of these, BAP produced the highest number of shoots per explant (Table 5). This morphogenic response may be attributed to the relative stability and persistence of BAP in tissues over those of kinetin and 2iP, as well as different transport and metabolic pathways (van Staden et al., 2008). The natural cytokinins, 2iP and zeatin, are rapidly degraded by cytokinin oxidase, contributing to their relatively reduced efficacy over time, compared with more stable cytokinins (van Staden et al., 2008). Although kinetin is a metabolically stable cytokinin, it is relatively weaker (degrades more easily) than BAP (van Staden et al., 2008). It has been proposed that some synthetic cytokinins can be degraded by an enzyme different from the cytokinin oxidase in some plant species (Mok and Mok, 2001). As the application of exogenous PGRs does not substitute the role of endogenous ones in regulating morphogenesis (Machakova et al., 2008), the ability of low concentration of BAP to promote shoot multiplication as well as the fact that shoots multiplied in PGR-free medium (albeit at a comparatively lower rate) indicated that *V. doniana* contains sufficient endogenous cytokinins to induce shoot organogenesis.

Similar to *V. doniana*, the effectiveness of BAP in multiple shoot formation has also been reported in other *Vitex* species, including *Vitex negundo* (7.4 shoots per explant; Ahmad and Anis, 2011) and *V. trifolia* (4.4 shoots per explant; Ahmed and Anis, 2014). Furthermore, BAP is widely used for the micropropagation of a number of commercially-important tree species (*e.g.*, Mittal et al., 1989; Barakat and El-Lakany, 1992; Bueno et al., 2003; Anis et al., 2010; Watt, 2014; Vitambas et al., 2020).

In the present study, *in vitro* organogenesis required media supplements to counter discoloration brought about by nutrient deficiency. A few purple shoots were formed, irrespective of the PGR treatment, which was likely caused by the high sulphur to phosphorus ratio in the woody plant medium (4.9:1). The development of purple leaves caused by phosphorus deficiency has been reported in some plants such as *Solanum lycopersicum* (Ulrychova and Sosnova, 1970) and *Lupinus luteus* (Wiwart et al., 2009). It is well known that high concentration of sulphur reduces the uptake of phosphorus by

plants (Grifoni et al., 2015). Phosphorus is involved in carbon dioxide fixation and photosynthetic phosphorylation (de Bang et al., 2021). Deficiency of the element upregulates the biosynthesis and subsequent accumulation of anthocyanins, resulting in the development of purple shoots or leaves (de Bang et al., 2021). In the present study, the formation of purple shoots was prevented when the multiplication medium (WPM) was supplemented with 0.2 g/L KH_2PO_4, or when MS was used (results not shown) as the culture medium. MS contains almost equal ratio of sulphur to phosphorus (1.3:1) compared to WPM (4.9:1). The findings indicated that *V. doniana* needs phosphorus and sulphur in approximately equal ratios for normal shoot development. The correct ratio of sulphur and phosphorus can be provided to the culture media by adjusting their ratio in WPM in favour of phosphorus or using MS salts as an alternative to the modified WPM.

During organogenesis, it was observed that bud break and callus development at the basal cut of the explant started occurring concurrently after 10 days of culture initiation. However, the percentage explants with callus at eight weeks of culture was significantly higher in the control than in treatments with cytokinin (Table 5). Given that endogenous auxin is transported downward in a polar direction, its accumulation at the base of the explant can result in callus formation (Marks and Simpson, 1994; Machakova et al., 2008). However, cytokinin affects differential auxin distribution by disrupting the polar transportation (Pernisova et al., 2009), which was attributed to the low percentage of callus development at the basal cut ends of explants in treatments with cytokinin compared to those in the control (without cytokinin). In some cases, this antagonistic effect of cytokinin on auxin transport can adversely affect the rooting ability of *in vitro* shoots as reported by Nakhooda et al. (2012). Those authors reported that the omission of cytokinin or the replacement of kinetin with a less stable cytokinin (trans-zeatin) significantly improved the rooting percentage in *Eucalyptus grandis* x *E. nitens* shoots (Nakhooda et al., 2012). Nevertheless, in the present study, the cytokinin supplied during the multiplication stage did not inhibit adventitious root induction, indicating that the combinations and concentrations selected were close to optimum for this species. Furthermore, while additional exogenous auxin in the form of 0.5 mg/L IAA did promote adventitious rooting, *in vitro* shoots were able to induce roots under endogenous auxins alone, once the exogenous cytokinins were excluded from the medium. Similarly, for other species of *Vitex* (*V. negundo*), Ahmad and Anis (2011) reported the best rooting to have occurred in the presence of 0.2 mg/L of the naturally-occurring auxins IBA or IAA, compared with NAA.

4.3. Somatic embryogenesis

In order to understand the PGR requirement for somatic embryogenesis in *V. doniana*, auxins (1 mg/L 2,4-D and 1 mg/L picloram) were tested alone, in combination with each other or with a cytokinin (0.5 mg/L BAP). Only when auxins or an auxin and a cytokinin were combined did pro-embryo formation occur (Fig. 4 A and C). The failure of a single auxin supplied to CIM to promote pro-embryo induction could be attributed to the antagonistic effect of endogenous cytokinins that are already at elevated levels within the *in vitro* explant, as presently derived from the shoot multiplication experiments, on the supplied auxins. An auxin maximum is usually required to promote pro-embryo induction (Evans et al., 1981; Tisserat, 1985; Machakova et al., 2008; De-la-Pena et al., 2015). In the present study, this was achieved by combining exogenous auxins or by pairing an exogenous auxin with an exogenous cytokinin. The exogenous application of cytokinin has been shown to promote the activity of cytokinin oxidase, which, in a feedback loop, results in a rapid degradation of endogenous cytokinin in plant tissues (van Staden et al., 2008). Such an auxin and cytokinin synergism for embryo induction has been reported for some Lamiaceae (previously Verbenaceae), of which *Vitex* belongs. Reported combinations were 0.4 mg/L (2.25 μM) 2,4-D and 0.1 mg/L (0.45 μM) BA for *Rosmarinus officinalis* (Aman and Afrasiab, 2014), 0.5 mg/L 2,4-D and 1 mg/L BAP for *Ocimum basilicum* (Gopi and Ponmurujan, 2006), 0.4 mg/L (1.8 μM) 2,4-D and 4 mg/L (18 μM) kinetin for *Salvia fruticose* (Kintzios et al., 1999), and 0.6 mg/L 2,4-D, 0.1 mg/L picloram and 1 mg/L BA for *Phyla nodiflora* (Ahmed et al., 2011).

Following eight weeks of callus induction on media with paired PGRs (1 mg/L 2,4-D + 1 mg/L picloram, 1 mg/L 2,4-D + 0.5 mg/L BAP and 1 mg/L picloram + 0.5 mg/L BAP), calli were transferred to media containing MS salts without PGRs, for pro-embryo maturation. It is well established that a relatively high level of auxin is needed for pro-embryo induction, but it becomes inhibitory to their subsequent development (Zimmerman, 1993; Machakova et al., 2008; Fehér, 2019). This was observed in the present study, as the pro-embryos did not develop further on the PGR-free medium. The levels of auxins required for callus induction and pro-embryo formation were inhibitory to embryo maturation. Various authors have demonstrated that prolonged explant exposure to exogenous auxin has a detrimental effect on pro-embryo development (Parrott et al., 1988; Bonneau et al., 1994; Filippov et al., 2006; She et al., 2013). This hypothesis was investigated by reducing the culture duration at the callus induction stage from eight to six weeks. The maturation medium was PGR-free, or contained PGRs in the form of 0.1 mg/L BAP or 2 mg/L ABA, added to the maturation media alone or in combination. The addition of BAP to the maturation media was intended to counteract the effect of exogenous auxin persistence in the callus tissues, while ABA is known to promote pro-embryo development by regulating the synthesis and deposition of late embryogenesis abundant (LEA) and storage proteins (Ammirato, 1977; Bell et al., 1993; Sharma et al., 2004; Vahdati et al., 2008; Yang et al., 2020).

Despite shortening the time that explants were exposed to exogenous PGRs or addition of BAP to the maturation media, pro-embryos failed to develop into somatic embryos, indicating that the auxin analogues used in CIM may need to be different, or used in different concentrations, and carefully selected with the aim of allowing pro-embryo development. The regeneration of woody species via somatic embryogenesis remains largely limited by embryo maturation and conversion of somatic embryos into plantlets (Giri et al., 2004; Vieitez et al., 2012; Pais, 2019).

5. Conclusion

The present study demonstrated that seeds of *V. doniana* exhibit physical dormancy imposed by a hard coat. Optimal germination of *V. doniana* seeds can be achieved by germinating de-coated seeds on filter paper or *in vitro* on MS salts at 25–35 °C, which results in high FGPs within for 14 days of germination period. However, for cost-effectiveness, the present study recommends seed germination on filter paper than on MS salts. Following emergence, seedlings should be transplanted into the soil for further growth and development. The overall findings of this study have shed insight on some physiological aspects that determine the propagation of *V. doniana*, which was thus far missing. The findings demonstrated that *V. doniana* contains endogenous PGRs (cytokinin and auxin) at levels that are nearly optimal for adventitious shoot and root induction, as both shoot multiplication and root induction were successfully achieved in the absence of PGRs, or significantly promoted with low concentrations of exogenous cytokinin (0.3 mg/L BAP) and auxin (0.5 mg/L IAA), respectively. To the best of our knowledge, this is the first developed direct organogenesis protocol for *V. doniana*. For somatic embryogenesis, the auxin analogues, 2,4-D and picloram appeared to have persisted in the callus tissues, inhibiting pro-embryo development (Table A). These findings provide a basis upon which future research on *V. doniana*, particularly with regard to somatic embryogenesis can be designed. The information from this study will allow for more

accurate selection of exogenous PGRs that can promote *in vitro* development, regardless of the route. Therefore, a future study could look at identifying and quantifying these endogenous PGRs (cytokinins and auxins) so that the clonal propagation protocols can be better designed with exogenous PGRs that complement endogenous ones for enhanced yield, rather than antagonise them.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Appendix A

Table A
Qualitative estimation of pro-embryos at each stage of embryo development in calli at the induction stage. Pro-embryo estimation within the microscopic field of view; low (+), medium (++), and high (+++); n - 4.

PGR (mg/L)				Proportion of pro-embryos	
2,4-D	Picloram	BAP	Development stage	4 wk.	8 wk.
0.5	0.5	0	G	0.0	0.13
			G/H	0.0	0.0
			H	0.0	0.0
			T	0.0	0.0
			NE	1.0	0.87
1	1	0	G	0.0	0.26
			G/H	0.0	0.26
			H	0.0	0.06
			T	0.0	0.0
			NE	1.0	0.42
1	0	0	G	0.0	0.0
			G/H	0.0	0.0
			H	0.0	0.0
			T	0.0	0.0
			NE	1.0	1.0
0	1	0	G	0.0	0.0
			G/H	0.0	0.0
			H	0.0	0.0
			T	0.0	0.0
			NE	1.0	1.0
1	0	0.5	G	0.06	0.13
			G/H	0.2	0.33
			H	0.0	0.26
			T	0.0	0.06
			NE	0.74	0.22
0	1	0.5	G	0.06	0.0
			G/H	0.33	0.4
			H	0.26	0.2
			T	0.0	0.0
			NE	0.35	0.4
0.5	0.5	0.5	G	0.0	0.13
			G/H	0.26	0.2
			H	0.06	0.06
			T	0.06	0.06
			NE	0.62	0.55

wk. - week, G - globular, G/H - globular-heart, H - heart, T - torpedo, NE - non-embryogenic callus

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