



OPEN Direct somatic embryogenesis induction in *Aspilia Africana* (Pers.) C. D. Adams, and assessment of genetic homogeneity and physiology of regenerants

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Aspilia africana (Pers.) C. D. Adams is a valuable medicinal plant, and the expanding therapeutic use of the plant due to explosion of human population is causing depletion of its wild population, thus requiring propagation. This study established an effective method for direct somatic embryogenesis in *A. africana* using leaf explants. We evaluated the effects of exogenous plant growth regulators (PGRs) and some molecules on induction, development, and maturation of somatic embryos. Murashige and Skoog (MS) medium supplemented with 1.0 mg/L benzylaminopurine (BAP) and 3.472×10^{-2} mg/L adenosine 5-monophosphate (AMP) optimally induced direct somatic embryogenesis in *A. africana* leaf explants (100% response and 9.50 ± 0.29 somatic embryos per explant). Differentiation and maturation of somatic embryos was enhanced under osmotic stress induced by using 9 g/L gelrite in MS medium augmented with 0.5 mg/L abscisic acid (ABA) and 6.634×10^{-2} mg/L nicotinamide adenine dinucleotide (NAD) (6.27 ± 0.36 globular, 3.40 ± 0.35 heart, 2.60 ± 0.51 torpedo, and 4.73 ± 0.41 cotyledonary). Half strength MS medium containing 0.5 mg/L gibberellin (GA) and 0.1 mg/L naphthaleneacetic acid (NAA) optimally supported germination ($31 \pm 1.73\%$) of cotyledonary somatic embryos. Pre-germination treatment of cold (stratification at 4°C) further stimulated somatic embryo germination (60.00%) and their conversion into plantlets (26.67%). After five weeks of acclimatization, the survival rate of somatic embryo derived *A. africana* plants was 75.00%. Histological observations and flow cytometric analysis confirmed different somatic embryo stages and stability in genome size of regenerated plants, respectively. Moreover, chlorophyll contents and photosynthetic rates were similar between zygotic and somatic embryo derived plants. This method could be employed in mass clonal regeneration, conservation, synthetic seed production, cryopreservation, and genetic improvement of *A. africana*. Additionally, the system would provide suitable model for investigating molecular, biochemical, and physiological events, which occur at the induction and development of embryogenesis in *A. africana*.

Keywords *Aspilia Africana*, Chlorophyll contents, Genetic homogeneity, Growth regulators, Photosynthetic rates, Somatic embryogenesis

Aspilia africana (Pers.) C. D. Adams popularly known as wild sunflower is a valuable medicinal plant which has been used for many years to treat different diseases across various African communities¹. The plant belongs to family Asteraceae and it is distributed across tropical African countries including Nigeria, Ethiopia, Uganda, Central African Republic, Congo, and Senegal². The diseases that *A. africana* has been used to treat traditionally include wounds, malaria, measles, osteoporosis, gonorrhea, gastrointestinal disorders such as stomach ache, diabetes mellitus, tuberculosis, and rheumatic pains^{3,4}. Accordingly, pharmacological studies have shown that phytochemicals of *A. africana* either singly or in combination exhibited various bioactivities namely

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wound healing, anti-inflammatory, antimicrobial, antidiabetic, gastroprotective, anticancer, antioxidant, and antimalarial effects⁵. Apart from its important medicinal uses, *A. africana* is also utilized as forage for livestock, such as chicken, goats, sheep, cattle, and rabbits, and it is reported to be one of the most browsed plants by these animals in Africa^{1,6}. The expanding therapeutic and other uses due to explosion of human population, climate change, and environmental pollution are causing depletion of wild *A. africana* population⁶ thus requiring propagation of this valuable plant. However, conventional plant propagation is associated with many challenges including low viability and occurrence of seed borne diseases⁷. Indeed, Okello et al.⁸ reported very low percent seed germination (between 11.67% and 15.67%) of *A. africana* in various soil types.

Micropropagation serves as a solution to these challenges. Regarding *A. africana* *in vitro* propagation, direct and indirect *in vitro* regeneration methods via organogenesis have been established^{9,10}. As an alternative production technique, somatic embryogenesis is a powerful biotechnological tool for clonal regeneration, germplasm conservation, synthetic seed production, cryopreservation, and genetic improvement^{11,12}. Somatic embryogenesis occurs if a somatic cell dedifferentiates to a totipotent embryonic stem cell, which can become an embryo *in vitro*¹³. Direct somatic embryogenesis and indirect somatic embryogenesis are the two ways of inducing somatic embryogenesis¹⁴. During direct somatic embryogenesis, somatic embryos are induced directly from explants under specific conditions without an intermediate callus phase, while indirect somatic embryogenesis happens through an intermediate callus phase^{11,12}. Somatic and zygotic embryos undergo comparable developmental stages of globular, heart, torpedo, and cotyledonary in dicots such as *A. africana*, or globular, scutellar, and coleoptilar in monocots^{11,15}.

In plant tissue cultures, regeneration through somatic embryogenesis could present several advantages over organogenesis, including the attainability of single cell origin and possible automation of the massive production of embryos in bioreactors and *ex vitro* planting as synthetic seeds¹⁶. Additionally, the bipolar nature of embryos enables direct growth into plantlets, eliminating need for the rooting stage required for plant regeneration through organogenesis, thereby making regeneration via somatic embryogenesis quicker compared to organogenesis¹¹. Further, single epidermal cell origins for embryos could prevent chimeras, allowing preference of somatic embryogenesis for plant transformation¹⁷.

Somatic embryogenesis has been induced in various species of plants namely *Digitalis lanata* Ehrh, *Camellia oleifera* Abel, and *Castanea mollissima* Blume among others^{18–20}. However, to the best of our knowledge, reports on somatic embryogenesis in *A. africana* are not available in literature to date. In this view therefore, this study aimed to establish an efficient and scalable method for direct somatic embryogenesis in *A. africana* using leaf explants. We evaluated the effects of exogenous PGRs, phytochrome- α (PSK), AMP, and NAD on induction, development, and maturation of somatic embryos. The ploidy level of regenerated plantlets was measured using flow cytometry to confirm their genetic stability. Furthermore, SPAD and the FluorPen FP110 series were used to test and compare chlorophyll pigment content and photosynthetic rates, respectively, of somatic and zygotic embryo derived plants. The proposed method could be useful for mass clonal regeneration, conservation, synthetic seed production, cryopreservation, and genetic improvement of *A. africana*. Additionally, this system would provide suitable model for investigating molecular, biochemical, and physiological events, which occur at the induction and development of embryogenesis in *A. africana*.

Materials and methods

Plant material and Preparation of explants

With permission from local authorities, *A. africana* seeds (mature and dry) were collected and provided by Natural Chemotherapeutics Research Institute (NCRI) from about 50 healthy plants growing at Pece, Gulu, Uganda in East Africa. On arrival at Korea Institute of Oriental Medicine (KIOM), Republic of Korea, the seeds were stored in a room at temperature 25 ± 1 °C until the time of experiment. A voucher specimen (number KYM-KIOM-2021-1) was deposited by Dr. Sungyu Yang at the Korean Herbarium of Standard Herbal Resources (Index Herbarium code: KIOM) at KIOM, Herbal Medicine Resources Research Center, Republic of Korea. To raise seedlings for explant sources in aseptic conditions, seeds of *A. africana* were germinated *in vitro* by adopting the method of Okello et al.¹ with slight modification. Briefly, the seeds were initially washed for 2 min under running tap water and taken to a laminar flow cabinet. Next, the seeds were again washed using autoclaved double-distilled water, surface sterilized for 1 min with 70% (v/v) ethanol, and rinsed thrice with autoclaved double-distilled water. This was followed by surface sterilizing with 2% (v/v) sodium hypochlorite for 3 min and rinsing thrice using autoclaved double-distilled water. The seeds were allowed to dry between sterile filter papers and afterwards inoculated on MS medium with vitamins augmented with gibberellin (GA; 0.5 mg/L) and transferred to culture room. After growth for four weeks, the *in vitro* germinated seedlings were cultured in MS medium containing vitamins, supplemented with BAP (1.0 mg/L)². For constant supply of plant materials, shoots of seedlings raised *in vitro* were sub-cultured in the same medium after every four weeks. Leaves obtained from *in vitro* shoots served as explants.

Embryo initiation and proliferation

Effects of PGRs on somatic embryogenesis of A. africana

Effects of PGRs on somatic embryogenesis in *A. africana* was evaluated in two phased experiments using leaf explants of size approximately 1 cm x 1 cm (obtained from 4 weeks old healthy plantlets growing *in vitro*) (Fig. 1A). In the first experiment, effects of four different cytokinins, BAP, thidiazuron (TDZ), kinetin (Kn), isopentenyl adenine (2iP), at varying concentrations (0.1, 0.5, 1.0, 2.0, and 3.0 mg/L) were evaluated separately. MS medium containing vitamins was supplemented with the cytokinins (BAP, TDZ, Kn, 2iP), 30 g/L sucrose, and 3 g/L gelrite; pH of the medium was adjusted to 5.7–5.8 and autoclaved for 20 min at 121 °C, then poured in crystal-grade polystyrene petri dishes (100 × 20 mm) to 50 mL level. Leaf explants were placed (adaxial surface in contact with medium) on the solidified medium and each petri dish contained three explants with eight

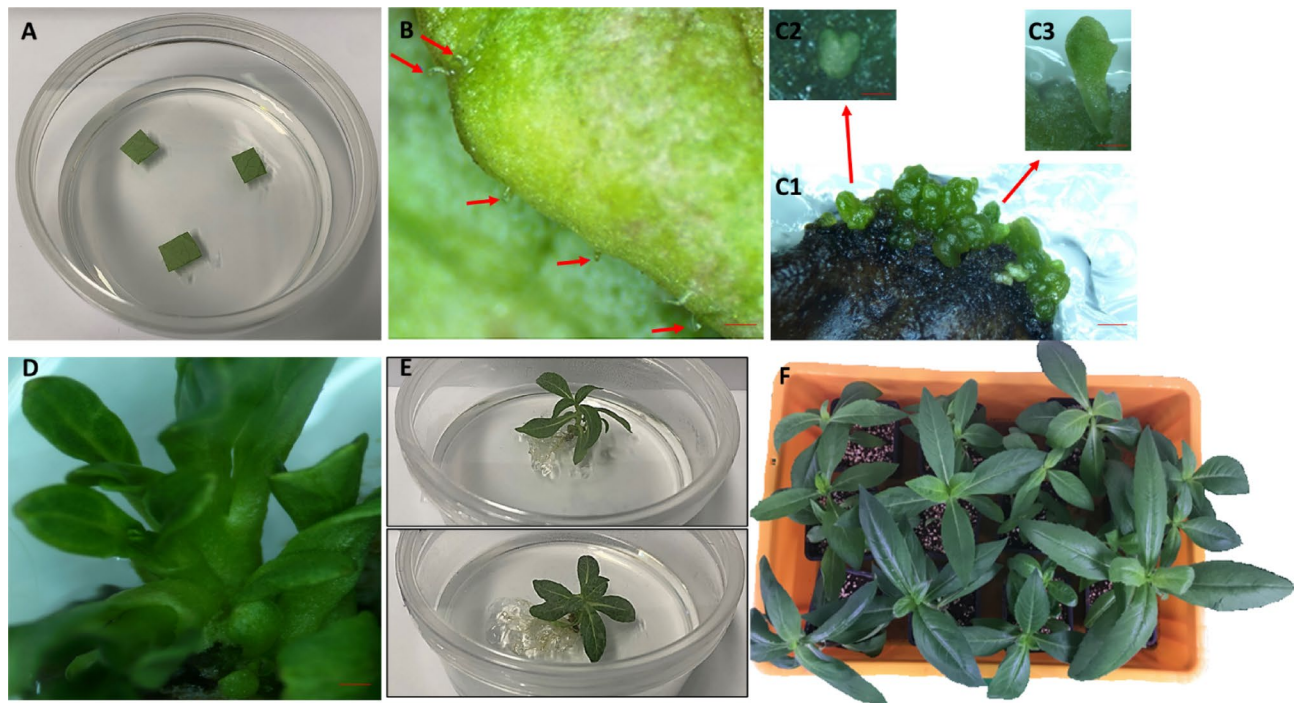


Fig. 1. Direct somatic embryogenesis in *A. africana* from leaf explants. (A) Leaf explants on induction medium. (B) Induction and development of globular shape embryos. (C1) development of heart and torpedo embryos. (C2) heart shape embryo. (C3) Torpedo shape embryo. (D) Proliferated cotyledonary embryos. (E) Well developed plantlets. (F) Plantlets growing in containers having horticultural soil mixed with perlite (2:1). Bars: (b–d) = 1 mm.

replicates (total 24 explants per treatment). All cultures were incubated at 16 h photoperiod ($33.73 \mu\text{mol}/(\text{m}^2/\text{s})$ light intensity) and 80% r.h. Unless otherwise mentioned, culture conditions and other medium components in all subsequent evaluations were identical to those described at this induction stage. After four weeks of culture, frequency (%) of explants that developed somatic embryos and the number of somatic embryos per explant were captured (Fig. 1B). The cytokinin that showed optimal result (1.0 mg/L BAP) was used in the next experiment.

In the second experiment, leaf explants were cultured in MS medium containing 1.0 mg/L BAP combined separately with three different auxins, indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), and NAA at concentrations of 0.1, 0.5, 1.0, 2.0, and 3.0 mg/L. Similarly, each petri dish contained three explants and this was replicated eight times (total 24 explants per treatment). Frequency (%) of explants that developed somatic embryos and the number of somatic embryos per explant were registered after four weeks of culture (Fig. 1B). Treatment with optimal result (still 1.0 mg/L BAP alone) was employed in further experiment.

Effects of combining PGR with PSK, AMP, and NAD on somatic embryogenesis of A. africana

Leaf explants were cultured in MS medium containing 1.0 mg/L BAP fortified independently with PSK, AMP, and NAD at various concentrations, PSK (8.449×10^{-4} , 8.449×10^{-3} , 8.449×10^{-2} , and 8.449×10^{-1} mg/L), AMP (3.472×10^{-4} , 3.472×10^{-3} , 3.472×10^{-2} , and 3.472×10^{-1} mg/L), and NAD (6.634×10^{-4} , 6.634×10^{-3} , 6.634×10^{-2} , and 6.634×10^{-1} mg/L). Each petri dish consisted of three explants with eight replicates (total 24 explants per treatment). Notably, globular embryos developed earlier on the surface of leaf explants in media having both PGR and any of PSK, AMP, and NAD (twenty-four days), in contrast with medium devoid of PSK, AMP, and NAD (four weeks or 28 days). After twenty-four days of culture, frequency (%) of explants that developed somatic embryos and the number of somatic embryos per explant were captured (Fig. 1B). Leaf explants with embryos at twenty-four days of culture were used to evaluate differentiation and maturation in subsequent experiment.

Embryo differentiation and maturation

Effects of ABA on A. africana somatic embryo differentiation and maturation

Leaf explants with embryos at twenty-four days of culture from initiation medium were randomly transferred to MS medium augmented with ABA at different concentrations of 0.1, 0.5, 1.0, 2.0, 3.0, and 5.0 mg/L for differentiation and maturation of somatic embryos. Accordingly, ABA was added when medium temperature was about 60°C after autoclaving. MS medium devoid of ABA served as control. Each petri dish contained a single explant with 15 replicates (a total of 15 explants per treatment). After ten days of culture in maturation medium, number of somatic embryos per leaf explant at various stages viz. globular, heart, torpedo, and cotyledonary was

counted and registered (Fig. 1C1, C2, C3, and D). Treatment with best result, in this case, 0.5 mg/L ABA was used in the following evaluation.

Effects of combination of ABA with PSK, AMP, and NAD on A. africana somatic embryo differentiation and maturation

To observe influence of combining ABA with PSK, AMP, and NAD on differentiation and maturation of somatic embryos, leaf explants with embryos at twenty-four days of culture from initiation medium were randomly shifted to MS medium having 0.5 mg/L ABA supplemented separately with PSK (8.449×10^{-4} , 8.449×10^{-3} , 8.449×10^{-2} , and 8.449×10^{-1} mg/L), AMP (3.472×10^{-4} , 3.472×10^{-3} , 3.472×10^{-2} , and 3.472×10^{-1} mg/L), and NAD (6.634×10^{-4} , 6.634×10^{-3} , 6.634×10^{-2} , and 6.634×10^{-1} mg/L). MS medium fortified with 0.5 mg/L ABA alone served as control. Every petri dish had a single explant with 15 replicates (total 15 explants per treatment). Number of somatic embryos per leaf explant at various stages (globular, heart, torpedo, and cotyledonary) was counted and recorded, after ten days of culture in maturation medium (Fig. 1C1, C2, C3, and D). Treatment that showed best result (0.5 mg/L ABA + 6.634×10^{-2} mg/L NAD) was used in the next assessment.

Effects of osmotic stress, ABA, and NAD on A. africana somatic embryo differentiation and maturation

To examine the role of ABA and NAD coupled with osmotic stress in maturation of *A. africana* somatic embryos, MS medium fortified with 0.5 mg/L ABA and 6.634×10^{-2} mg/L NAD was combined with either 3 g/L or 9 g/L gelrite (total two treatments). Leaf explants with embryos at twenty-four days of culture from initiation medium were randomly transferred to respective culture medium. Each petri dish contained a single explant with 15 replicates (total 15 explants per treatment). After ten days of culture in maturation medium, number of somatic embryos per leaf explant at various stages (globular, heart, torpedo, and cotyledonary) were counted and noted.

Embryo pre-germination treatments, germination and conversion into plantlets, and acclimatization

Suitable medium for embryo germination was determined by culturing cotyledonary stage somatic embryos on embryo germination media of full/half/quarter strength semi solid MS fortified with 0.5 mg/L GA and 0.1 mg/L NAA. After three weeks, germination frequency (percent of germination) was determined as the percent of somatic embryos showing emergence of shoot out of total somatic embryos inoculated. The best medium for germination (Half strength MS medium fortified with 0.5 mg/L GA and 0.1 mg/L NAA) was used in further evaluation.

Regarding pre-germination treatments, mature somatic embryos (cotyledonary and torpedo stages) were obtained from maturation medium and subjected to two conditions (1) stratification treatment at 4°C for 5 days while on germination medium and (2) direct placement of embryos onto germination medium and kept in culture room (this served as control).

Embryos from stratification treatment were kept in the dark for 5 days before being shifted to light in a 16-h photoperiod to germinate. After 3 weeks of growth, the germinated embryos were shifted to same germination medium for additional 4 weeks. Germination frequency (percent of germination) as the percent of somatic embryos showing emergence of shoot out of total somatic embryos inoculated, and percent of plantlets as percent of germinated somatic embryos with shoot and root out of total germinated somatic embryos with shoot inoculated, were determined after 3 weeks and 7 weeks of growth, respectively.

Upon sufficient growth (Fig. 1E), the plantlets were removed from culture containers, medium adhered to the roots washed with water, and planted in sterile horticultural soil mixed with perlite (2:1) contained in plastic containers placed in a greenhouse (Fig. 1F). Plantlets were covered with a transparent polythene bag which were removed after fourteen days. The plantlets were watered two times a week, and rate of survival was recorded after seven weeks of growth.

Morphological and histological observations

Observation of embryos at various stages of development were performed under a stereo-zoom binocular microscope (SMZ745T, China) and a digital camera (Nikon, China) was used to photograph images (Fig. 1B, C1, C2, C3, and D).

Histological analysis of *A. africana* somatic embryos was performed at various developmental stages (globular, heart, torpedo, and cotyledonary) at 21–40 days after inoculation following the method of Okello et al.¹⁰ with slight modification. Briefly, fresh leaf explant tissues with somatic embryos at various stages of development were immediately fixed in 70% ethanol for at least 24 h. After, the tissues were sequentially dehydrated in ethanol 50, 70, 80, 90, 95, 98, and 100% (for 30 min each) at 25 ± 1 °C. The dehydrated tissues were successively cleared in ethanol-xylene mixtures at different ratios (ethanol : xylene) 3:1, 1:1, and 1:3, and twice in xylene (for 30 min each) at 25 ± 1 °C. Further clearing was done in xylene-paraffin mixtures at different ratios (xylene : paraffin) 2:1, 1:2, and twice in paraffin (for 1 h in each) at 60 ± 1 °C. Then, the tissues were embedded in paraffin wax overnight and 12-µm sections were sliced using a microtome. Tissue sections were dewaxed by sequentially dipping for 5 min in the following: xylene (twice), ethanol-xylene mixture (1:1), ethanol 100% (twice), 95%, 70%, and 50%. Next, the sections were stained by successively dipping in the following: safranin 1% (1 h), water, ethanol 50%, 70%, 95% (3 min each), fast-green (3 min), ethanol 100% (twice and 1 min each time), and xylene (thrice and 15 min each time). After mounting with Balsam medium, images of stained tissues were scanned and observed and captured using digital slide scanner (3DHitech Panoramic Desk II DW; 3DHitech Kft., Budapest, Hungary) and CaseViewer software (version 2.4.0; 3DHitech Kft., Budapest, Hungary), respectively.

Genetic stability assessment by flow cytometry

Flow cytometry was used to determine the genetic stability of direct somatic embryo *A. africana* plants following the method of Choi et al.²¹ with slight modifications. With a known 2 C DNA content of 2.50 pg, the leaves of *Glycine max* cv. Polanka served as an internal standard. Briefly, randomly picked leaf tissues (approximately 0.5 cm² from somatic embryo *A. africana* plants and zygotic embryo *A. africana* plants were independently co-chopped together with leaf tissue of internal standard in a plastic petri dish containing 500 µL of nuclei extraction buffer (CyStain Ultraviolet Precise P Nuclei Extraction Buffer; Sysmex Partec, Germany). Prior to filtering the contents through 50 µm mesh (CellTrics, Sysmex Partec, Germany) into tubes, they were incubated for 30–90 s. To each filtrate, staining solution (2000 µL) consisting of staining buffer, propidium iodide, and RNase A was added, and the mixtures were incubated for 60 min at 25 °C while protecting from light. Measurement of the fluorescence intensity of isolated nuclei was performed using a flow cytometer (BD FACSCalibur, USA). The ploidy of somatic embryo *A. africana* and zygotic embryo *A. africana* plants was determined through comparing their relative fluorescence intensity with that of the internal standard, based on the formula below:

$$2 \text{ C DNA of } A. africana = 2.50 \text{ pg} \frac{\text{Intensity of test}}{\text{Intensity of standard}}.$$

Where *intensity* is the fluorescence intensity of isolated nuclei.

Chlorophyll content measurement

The leaf chlorophyll content of somatic embryo *A. africana* plants (five weeks after acclimatization) and zygotic embryo *A. africana* (about 2 months old) growing in the same greenhouse was measured and compared using SPAD chlorophyll meter (SPAD-502 Plus, Konica Minolta, Inc., Japan). Before clumping the SPAD chlorophyll meter onto *A. africana* leaves to get readings of chlorophyll content, it was calibrated. Per plant, six leaves were chosen randomly from lower-stem region (a pair), mid-stem region (a pair), and apical region (a pair) and registered chlorophyll content values from four points of each leaf (Fig. 2A and B). Average chlorophyll content value of the six leaves represented chlorophyll content of an individual plant. Chlorophyll contents of same plants (10 somatic embryo *A. africana* plants and 10 zygotic embryo *A. africana* plants) were recorded weekly across seven weeks (Fig. 2C).

Chlorophyll fluorescence measurement

FluorPen FP110 (Drásov 470, 664 24 Drásov, Czech Republic) was employed to capture chlorophyll fluorescence of the plants. Prior to recording chlorophyll fluorescence, plant leaves were dark-adapted with leaf clip gaskets for about 1 h. Chlorophyll fluorescence measurements were taken weekly for seven weeks from six randomly selected leaves per zygotic embryo *A. africana* plant (total 10 plants) and somatic embryo *A. africana* plants (total 10 plants). To get Fv/Fm values, more than 4,000 µmol m⁻² s⁻¹ saturating flash was used as per the FluorPen FP110 OJIP protocol. In the dark-adapted state, minimal chlorophyll fluorescence intensity (Fo) was measured when photosystem II (PSII) reaction center was open, maximal chlorophyll fluorescence intensity (Fm) was measured during application of saturation light pulse, and variable chlorophyll fluorescence (Fv) was recorded when minimal non-photochemical processes occurred.

Statistical analysis

Completely randomized design was applied to the experiments performed. Embryogenesis initiation and proliferation stage consisted of three experiments, and during all, each treatment petri dish contained three explants with eight replicates (total 24 explants per treatment). In the first experiment, effects of four different cytokinins (BAP, TDZ, Kn, and 2iP) at five varying concentrations (total 21 treatments) were evaluated. In the second experiment, 1.0 mg/L BAP was combined separately with three different auxins (IAA, IBA, and NAA) at five varying concentrations (total 16 treatments). In the third experiment, 1.0 mg/L BAP was fortified independently with three molecules (PSK, AMP, and NAD) at four various concentrations (total 13 treatments).

The embryo differentiation and maturation stage comprised three experiments, and during all, each treatment petri dish contained a single explant with 15 replicates (total 15 explants per treatment). During the first experiment, effect of ABA at six different concentrations (total 7 treatments) was investigated. In the

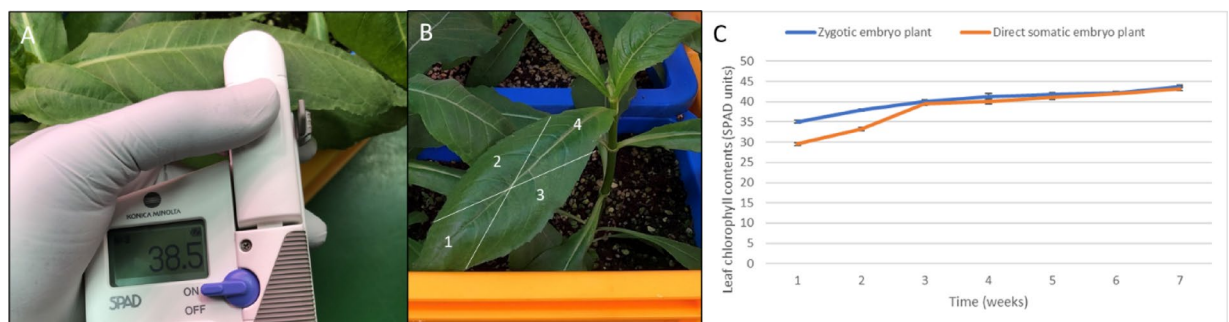


Fig. 2. Chlorophyll content measurement of zygotic embryo plant leaves and direct somatic embryo plant leaves of *A. africana*. (A) Measuring chlorophyll content of leaf using SPAD meter (B) Specific points on a leaf taken to represent the average chlorophyll content of an individual leaf. (C) Chlorophyll contents of zygotic embryo *A. africana* plants compared with that of the direct somatic embryo *A. africana* plants across 7 weeks.

second experiment, 0.5 mg/L ABA was combined with three molecules (PSK, AMP, and NAD) at four various concentrations (total 13 treatments). In the third experiment, 0.5 mg/L ABA + 6.634×10^{-2} mg/L NAD was combined with either 3 g/L or 9 g/L gelrite (total 2 treatments).

During embryo germination and conversion into plantlets stage, two experiments were conducted, and during both experiments, each treatment had 100 embryos. In the first experiment, embryo germination was evaluated in MS medium at three different strengths (full/half/quarter) supplemented with 0.5 mg/L GA and 0.1 mg/L NAA (total 4 treatments). The second experiment involved two embryo germination treatments of stratification at 4° C before transfer to culture room and direct placement in culture room. Unless otherwise stated, all experimental data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc tests using GraphPad Prism v 10.1.1. Means were considered significantly different at $P < 0.05$.

Results

Embryo initiation and proliferation

Effects of PGRs on somatic embryogenesis of A. africana

Except Kn, all cytokinins induced somatic embryo formation from leaf explants, although with varying degree of response (Fig. 3A and B). Initially, the number of somatic embryos increased with increasing concentration of cytokinins before decreasing at high concentrations (Fig. 3A). Accordingly, swelling and formation of embryogenic clumps on leaf explants occurred in the first and second week, respectively. After four weeks, globular embryos developed on the surface of leaf explants. In the initial test, among cytokinins, MS medium augmented with 1.0 mg/L BAP showed the best results with 5.92 ± 0.29 somatic embryos per explant and 100% response (Fig. 3A and B). These values of BAP (1.0 mg/L) did not markedly differ from those of TDZ (2.0 mg/L) (Fig. 3A and B). The lowest values were observed in MS medium devoid of cytokinins and those supplemented with Kn at all concentrations, which did not form somatic embryos (i.e., 0.0 somatic embryos and 0.0% frequency of explants that developed somatic embryos) (Fig. 3A and B).

In the successive experiment, excluding MS medium fortified with 1.0 mg/L BAP + 1.0 mg/L IBA, combination of BAP (1.0 mg/L) with different auxins resulted in decreased formation of somatic embryos in all concentrations (Fig. 4A and B). MS medium supplemented with 1.0 mg/L BAP + 1.0 mg/L IBA generated the highest number of somatic embryos per explant (6.33 ± 1.35) and this did not differ significantly ($p < 0.05$) from those of MS medium supplemented with BAP (1.0 mg/L), BAP (1.0 mg/L) + IBA (2.0 mg/L), BAP (1.0 mg/L) + IBA (3.0 mg/L), and BAP (1.0 mg/L) + IAA (3.0 mg/L) (Fig. 4A and B). Without significant ($p < 0.05$) variation from the values of MS medium fortified with BAP (1.0 mg/L) + IBA (2.0 mg/L), BAP (1.0 mg/L) + NAA (3.0 mg/L), BAP (1.0 mg/L) + IAA (3.0 mg/L), and BAP (1.0 mg/L) + IBA (0.5 mg/L), the highest percentage of explants that developed somatic embryos ($91.67\% \pm 5.46\%$) was recorded in MS medium fortified with BAP (1.0 mg/L), BAP (1.0 mg/L) + IBA (1.0 mg/L), and BAP (1.0 mg/L) + IBA (3.0 mg/L) treatments (Fig. 4A and B). However, the lowest number of somatic embryos formed (0.0) and frequency of explants that developed somatic embryos (0.0%) were registered in MS medium augmented with BAP 1.0 (mg/L) + NAA (0.1 mg/L). 1.0 mg/L BAP alone with the second highest number of somatic embryos (5.75 ± 0.48) and no marked difference from that of first, 1.0 mg/L BAP + 1.0 mg/L IBA) and joint highest percentage response of explants ($91.67\% \pm 5.46\%$) was taken as the optimal PGR treatment for inducing direct somatic embryogenesis, since some calluses were observed in 1.0 mg/L BAP + 1.0 mg/L IBA treatment. Therefore, 1.0 mg/L BAP was used for further evaluation.

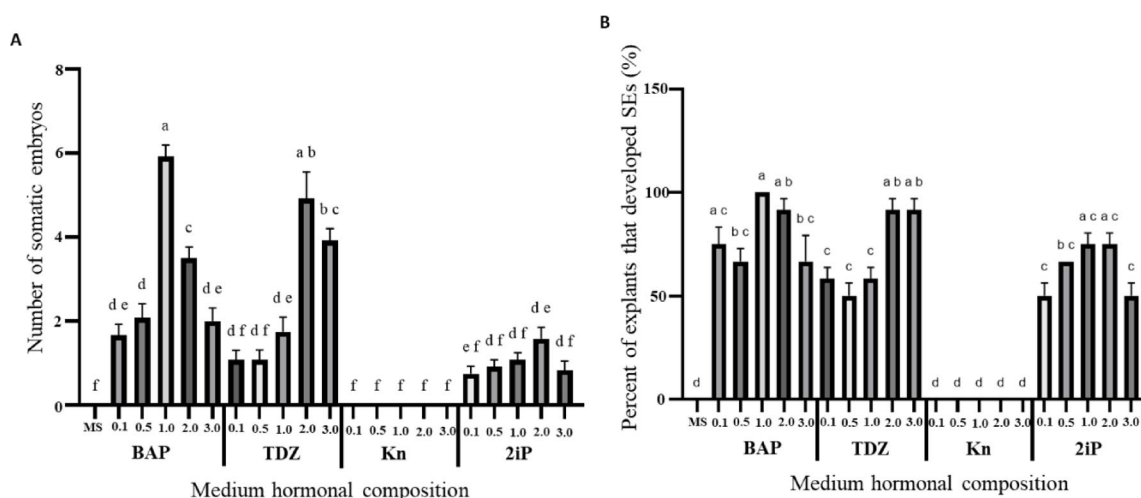


Fig. 3. Effects of Cytokinins at different concentrations on number of somatic embryos formed and percent of explants that developed somatic embryos via direct somatic embryogenesis from leaf explants of *A. africana*. (A) Number of somatic embryos formed. (B) Percent of explants that developed somatic embryos. The medium used was MS, and all concentrations are in mg/L. Values represent the Mean $\pm$ SE of 24 explants. Same letters are not significantly different by Tukey's test and $p \leq 0.05$.

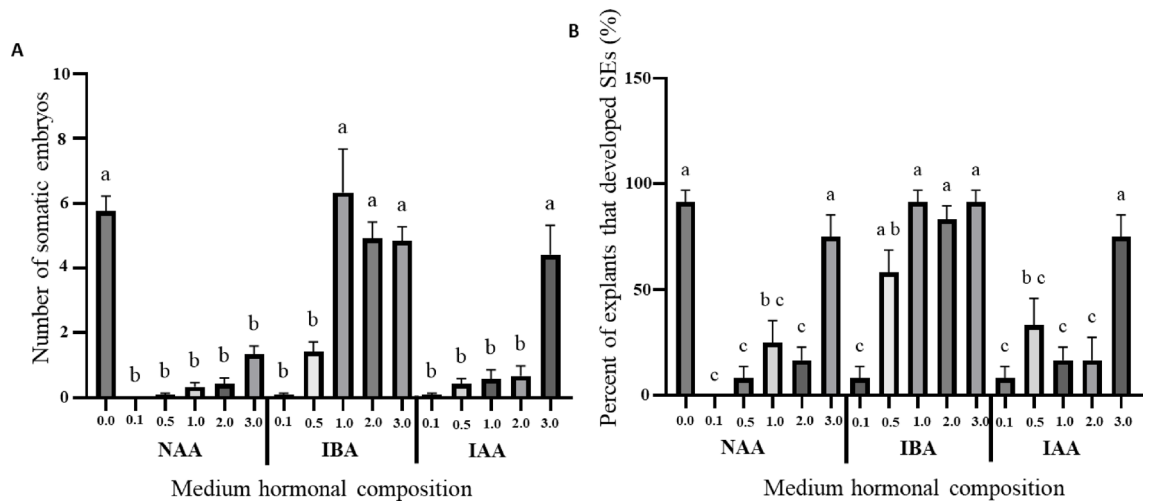


Fig. 4. Effects of PGR combination at different concentrations on number of somatic embryos formed and percent of explants that developed somatic embryos via direct somatic embryogenesis from leaf explants of *A. africana*. (A) Number of somatic embryos formed. (B) Percent of explants that developed somatic embryos. All media (MS) contained 1.0 BAP, and all concentrations are in mg/L. Values represent the Mean $\pm$ SE of 24 explants. Same letters are not significantly different by Tukey's test and $p \leq 0.05$.

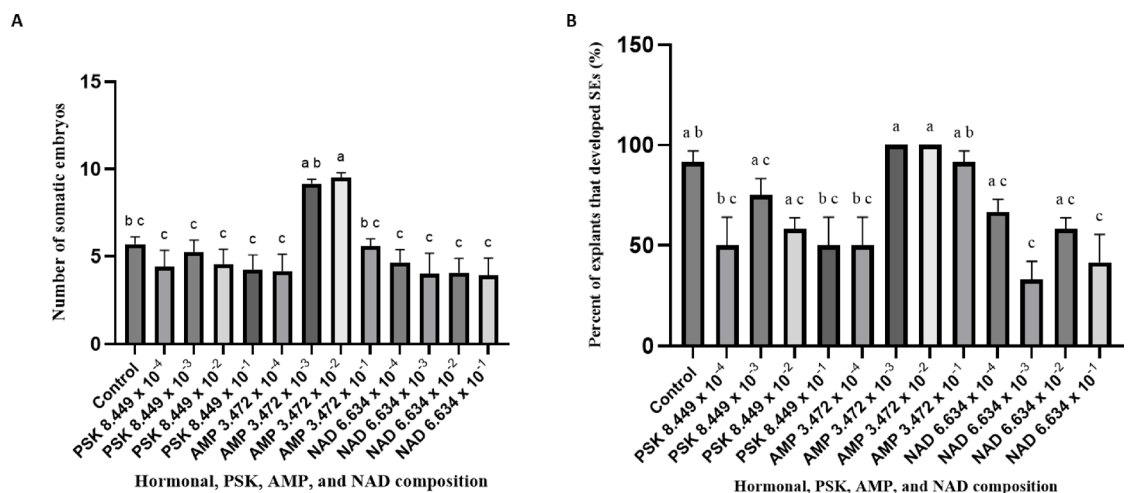


Fig. 5. Effects of combination of Cytokinin and PSK, AMP, and NAD at various concentrations on number of somatic embryos formed and percent of explants that developed somatic embryos via direct somatic embryogenesis from leaf explants of *A. africana*. (A) Number of somatic embryos formed. (B) Percent of explants that developed somatic embryos. All media (MS) contained 1.0 BAP, and all concentrations are in mg/L. Values represent the Mean $\pm$ SE of 24 explants. Same letters are not significantly different by Tukey's test and $p \leq 0.05$.

Effects of combining PGR with PSK, AMP, and NAD on somatic embryogenesis of *A. africana*

Combination of 1.0 mg/L BAP with PSK, AMP, and NAD, except AMP (3.472×10^{-3} , 3.472×10^{-2} , and 3.472×10^{-1} mg/L) reduced both number of somatic embryos and frequency of embryogenesis (Fig. 5A and B). MS medium fortified with BAP (1.0 mg/L) plus AMP (3.472×10^{-4} mg/L) yielded the highest mean somatic embryo number (9.50 ± 0.29), and this was markedly higher ($p < 0.05$) than the mean somatic embryo number of other treatments (apart from 1.0 mg/L BAP + 3.472×10^{-3} mg/L AMP) (Fig. 5A). Lowest mean somatic embryo number of 4.0 ± 1.19 was recorded in MS medium augmented with BAP (1.0 mg/L) plus NAD (6.634×10^{-3} mg/L) (Fig. 5A). Similarly, MS augmented with BAP (1.0 mg/L) combined with AMP (3.472×10^{-2} , 3.472×10^{-3} mg/L) produced maximum percentage of explants that developed somatic embryos (100%), while BAP (1.0 mg/L) combined with NAD (6.634×10^{-3} mg/L) showed the lowest percentage of explants that developed somatic embryos (33.33%) (Fig. 5B). As stated above, globular embryos developed earlier on the surface of leaf explants in media containing both PGR and any of PSK, AMP, and NAD (twenty-four days) compared to medium devoid of PSK, AMP,

and NAD (four weeks or twenty-eight days). Thus, 1.0 mg/L BAP + 3.472×10^{-2} mg/L AMP optimally induced somatic embryogenesis in *A. africana* from leaf explants compared to other treatments in the present study.

Embryo differentiation and maturation

Effects of ABA on A. africana somatic embryo differentiation and maturation

Differentiation and development of various somatic embryo stages of globular, heart, torpedo, and cotyledonary occurred when leaf explants with embryos at twenty-four days of culture from initiation medium were transferred to MS medium augmented with ABA at different concentrations (0.1, 0.5, 1.0, 2.0, 3.0, and 5.0 mg/L) (Fig. 6A, B, C, and D). Maximum mean cotyledonary stage embryo number (1.67 ± 0.16) was registered in MS medium augmented with 0.5 mg/L ABA, and this markedly differed ($p < 0.05$) from that in all other treatments (Fig. 6D). Based on this result, 0.5 mg/L ABA was employed in further assessments.

Effects of combination of ABA with PSK, AMP, and NAD on A. africana somatic embryo differentiation and maturation

ABA (0.5 mg/L) combined with PSK, AMP, and NAD at concentrations PSK (8.449×10^{-4} , 8.449×10^{-3} , 8.449×10^{-2} , and 8.449×10^{-1} mg/L), AMP (3.472×10^{-4} , 3.472×10^{-3} , 3.472×10^{-2} , and 3.472×10^{-1} mg/L), and NAD (6.634×10^{-4} , 6.634×10^{-3} , 6.634×10^{-2} , and 6.634×10^{-1} mg/L) differently influenced differentiation and development of somatic embryos at various stages (globular, heart, torpedo, and cotyledonary) on leaf explants containing embryos transferred from initiation medium (Fig. 7A, B, C, and D). The initial globular, heart, torpedo, and cotyledonary embryo stages formed at 23, 25, 26, and 28 days, respectively. MS medium fortified with 0.5 mg/L ABA + 6.634×10^{-2} mg/L NAD showed the highest number of cotyledonary stage somatic embryos per explant (3.33 ± 0.33), which was markedly different ($p < 0.05$) from control (0.5 mg/L ABA alone) and most other treatments (Fig. 7D). The lowest mean cotyledonary stage somatic embryo number of 0.80 ± 0.22 was observed in MS medium fortified with 0.5 mg/L ABA + 8.449×10^{-1} mg/L PSK (Fig. 7D).

Effects of osmotic stress, ABA, and NAD on A. africana somatic embryo differentiation and maturation

Osmotic stress showed increased differentiation and development of various stages of somatic embryos on leaf explants having embryos transferred from initiation medium (Table 1). Maturation of somatic embryos was markedly higher ($p < 0.05$) in osmotic medium (MS + ABA 0.5 mg/L + NAD 6.634×10^{-2} mg/L + Gelrite 9 g/L) with 4.73 ± 0.41 number of cotyledonary embryos per explant than in non-osmotic medium (MS + ABA 0.5

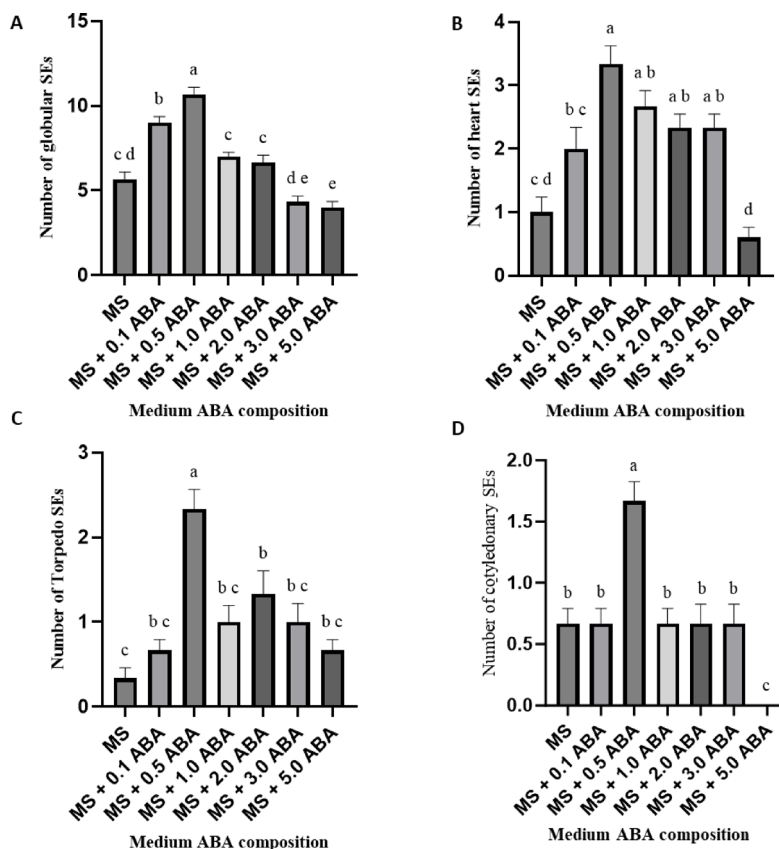


Fig. 6. Effects of different concentrations of abscisic acid (ABA) on differentiation and maturation of somatic embryos from leaf explants of *A. africana*. (A) Globular shape embryos. (B) Heart shape embryos. (C) Torpedo shape embryos. (D) Cotyledonary shape embryos. Same letters are not significantly different by Tukey's test and $p \leq 0.05$.

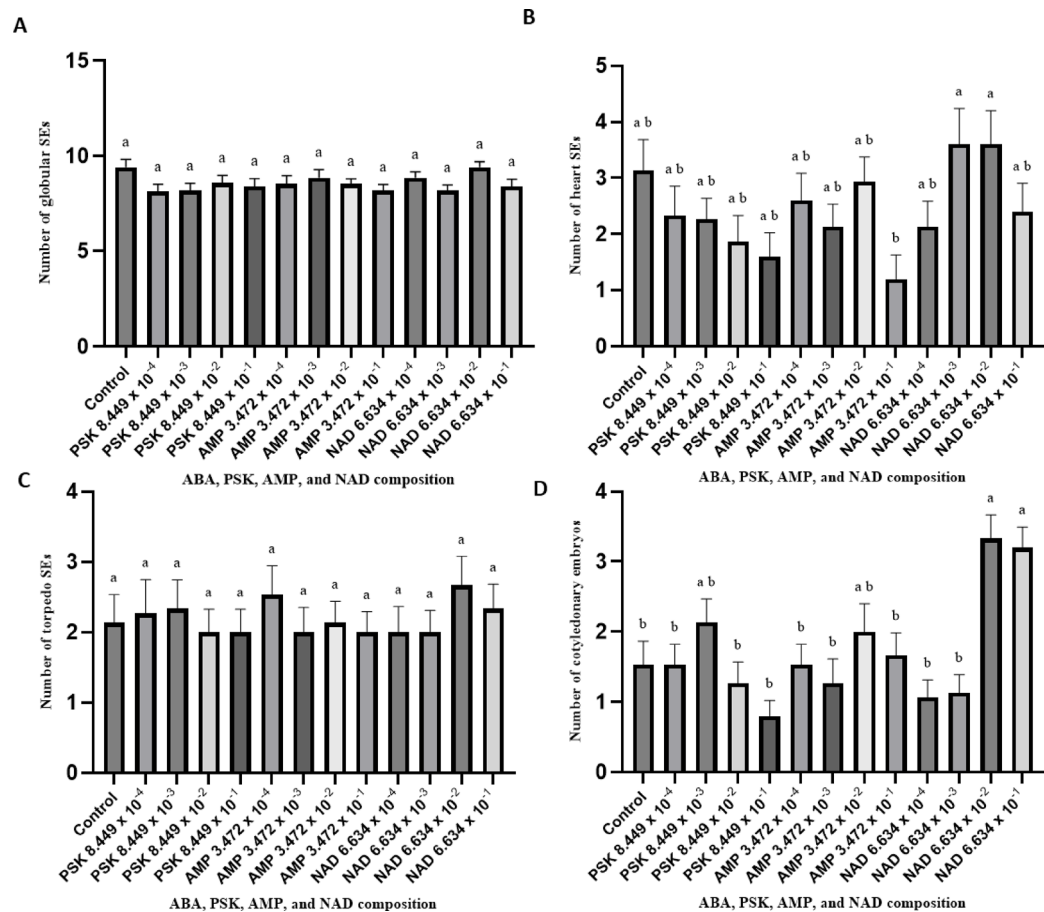


Fig. 7. Effects of combination of abscisic acid (ABA) and PSK, AMP, and NAD at various concentrations on differentiation and maturation of somatic embryos from leaf explants of *A. africana*. (A) Globular shape embryos. (B) Heart shape embryos. (C) Torpedo shape embryos. (D) Cotyledonary shape embryos. All media (MS) contained 0.5 ABA, and all concentrations are in mg/L. Same letters are not significantly different by Tukey's test and $p \leq 0.05$.

Treatment	Different stages of somatic embryogenesis			
	Globular	Heart	Torpedo	Cotyledonary
Non-osmotic medium MS + ABA 0.5 + NAD 6.634 × 10 ⁻² + Gelrite 3 g/L	5.47 ± 0.26 a	3.13 ± 0.43 a	2.07 ± 0.41 a	3.20 ± 0.35 b
Osmotic medium MS + ABA 0.5 + NAD 6.634 × 10 ⁻² + Gelrite 9 g/L	6.27 ± 0.36 a	3.40 ± 0.35 a	2.60 ± 0.51 a	4.73 ± 0.41 a

Table 1. Effect of osmotic stress on differentiation and maturation of somatic embryos in *A. africana*.

mg/L + NAD 6.634 × 10⁻² mg/L + Gelrite 3 g/L), which yielded 3.20 ± 0.35 number of cotyledonary embryos per explant (Table 1).

Embryo pre-germination treatments, germination and conversion into plantlets, and acclimatization

In addition to reducing the strength of MS medium, its supplementation with 0.5 mg/L GA and 0.1 mg/L NAA increased germination of cotyledonary stage somatic embryos (Fig. 8). The cotyledonary somatic embryos elongated and developed shoot after three weeks in germination media. Half strength MS medium fortified with 0.5 mg/L GA and 0.1 mg/L NAA produced the highest germination percent (31 ± 1.73%), and this was markedly ($p < 0.05$) different from the rest of the treatments, except in quarter strength MS medium augmented with 0.5 mg/L GA and 0.1 mg/L NAA (Fig. 8). The lowest percent of germination (14 ± 1.15%) was observed in PGR free MS medium.

Generally, stratification at 4° C treatment influenced the germination of somatic embryos (Fig. 9 and Table 2). Stratification at 4° C resulted into better embryo germination (60.00%) and conversion to plantlets (26.67%) compared to direct culture in medium (Table 2).

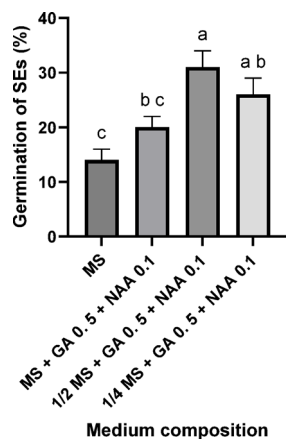


Fig. 8. Germination of somatic embryos in different medium compositions. Same letters are not significantly different by Tukey's test and $p \leq 0.05$.

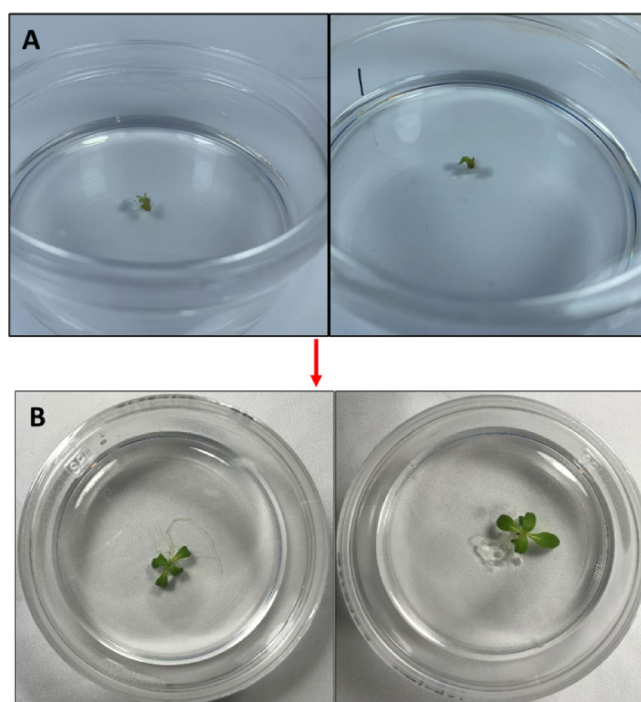


Fig. 9. Effect of stratification at 4° C on germination of *A. africana* somatic embryos and their conversion to plantlets. (A) Cotyledonary somatic embryos cultured in germination medium before stratification at 4° C. (B) Somatic embryo derived plantlets growing in germination medium two weeks after stratification at 4° C.

Treatment	Germination of SEs (%)	Percent of SE plantlets (%)
Direct culture in medium	30.77 b	12.50 b
Stratification-4° C	60.00 a	26.67 a

Table 2. Effect of stratification at 4° C on germination of somatic embryos and their conversion to plantlets.

After five weeks of acclimatization, the survival rate of somatic embryo derived *A. africana* plants was 75.00% (Table 3). The plantlets were morphologically similar to zygotic embryo derived *A. africana* plants.

Number of plantlets planted ex vitro	Number of plantlets that survived 5 weeks after acclimatization to ex vitro condition	Percent (%) of plantlets that survived 5 weeks acclimatization to ex vitro condition
100	75	75

Table 3. Number of somatic embryo derived plantlets planted *ex vitro* and their survival rate after acclimatization.

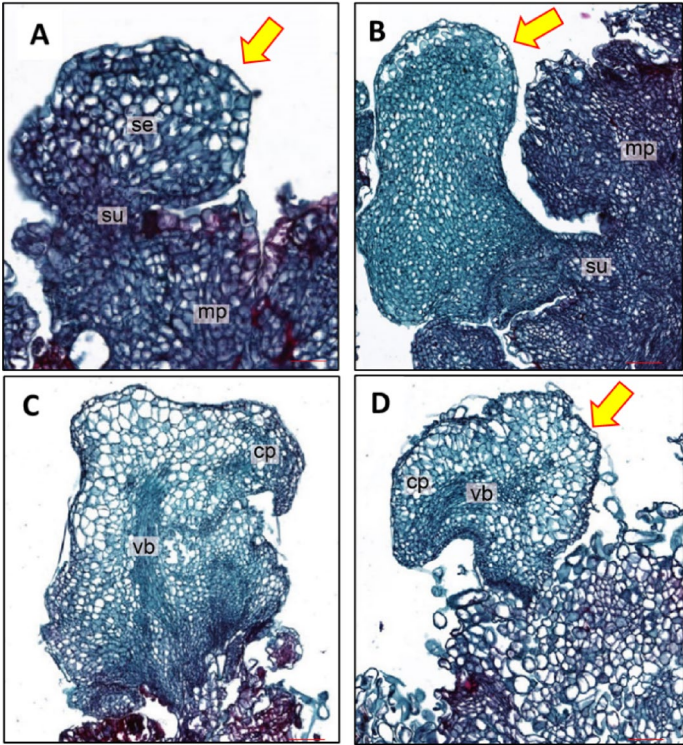


Fig. 10. Histological observation of direct somatic embryogenesis in *A. africana* from leaf explants. **(A)** Histological section of globular somatic embryo. **(B)** Histological section of heart stage somatic embryo. **(C)** Histological section of torpedo stage somatic embryo. **(D)** Histological section of cotyledonary somatic embryo. cp.: cotyledon primordia, mp: mother plant, se: somatic embryo, su: suspensor, vb: vascular bundle. Bars: **(b, d)** = 0.100 mm, **(a, c)** = 0.200 mm.

Morphological and histological observations

Histological analysis showed the regeneration pathway of somatic embryogenesis in *A. africana*. Longitudinal sections of embryonic structures revealed that accelerated cell divisions begin from the leaf explant surfaces and cut edges (Fig. 10A). Within 2 weeks of culture, formation of cell aggregates occurred, developing into globular embryos (Fig. 10A). The globular somatic embryos majorly comprised small compact meristematic cells, having dense cytoplasm, notable nuclei, and a layer of protoderm on their surface (Fig. 10A). From globular embryos, subsequent stages of embryos developed asynchronously, including heart, torpedo, and cotyledonary stages, after 4 weeks of culture (Fig. 10B, C, D). It was observed that the cotyledonary stage embryos had a closed vascular system, thus no vascular bundle connection with mother tissue (Fig. 10D).

Genetic stability assessment by flow cytometry

As per flow cytometry histograms, the ploidy levels of somatic and zygotic embryo *A. africana* plants were identical (Fig. 11A and B). The 2 C DNA contents of zygotic and somatic embryo *A. africana* plants were 6.33 pg and 6.25 pg, respectively (Fig. 11A and B). The 2 C DNA contents of zygotic (6.33 pg) and somatic (6.25 pg) embryo *A. africana* plants corresponded to the genome size (2 C) of 6,190 Mbp and 6,113 Mbp, respectively (where 1 pg is ~ 978 Mbp, Doležel et al.²²).

Chlorophyll content measurement

Initially (in the first week), the chlorophyll contents of zygotic embryo *A. africana* plants (35.06 ± 0.30) were comparatively higher than that of somatic embryo *A. africana* plants (29.5 ± 0.35) (Fig. 2C). During the first two weeks, increase in chlorophyll contents of somatic embryo *A. africana* plants was relatively higher than

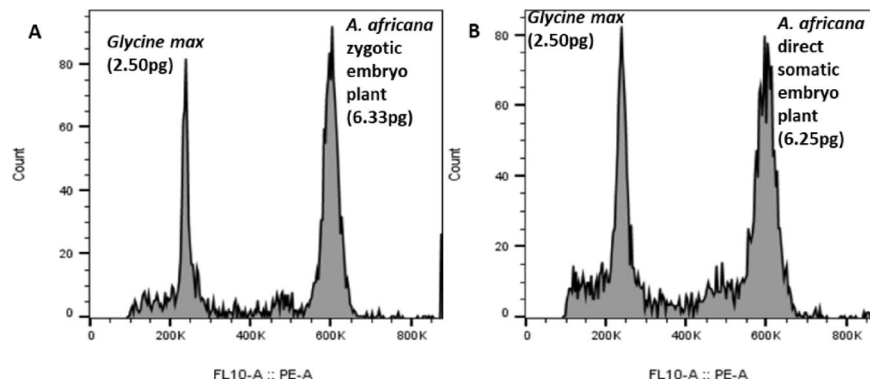


Fig. 11. Flow cytometry histograms of fluorescent nuclei isolated from *A. africana*. (A) 2 C-DNA content from zygotic embryo *A. africana* plants. (B) 2 C-DNA content from direct somatic embryo *A. africana* plant.

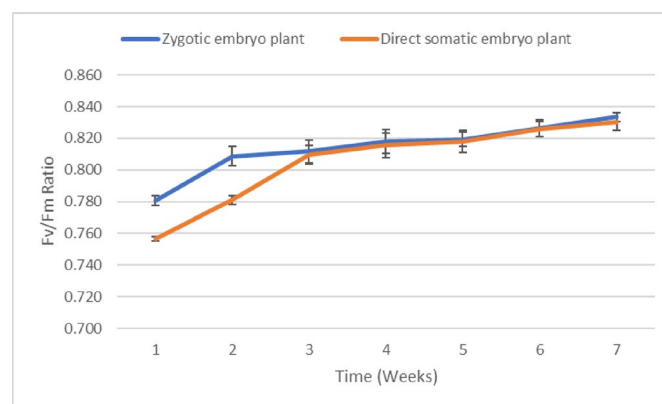


Fig. 12. Comparison of mean Fv/Fm ratio of zygotic embryo *A. africana* plants compared with that of the direct somatic embryo *A. africana* plants across 7 weeks.

that in zygotic embryo *A. africana* plants, and this led to comparable contents by the third week (Fig. 2C). From week three to seven, minimal increase in chlorophyll contents was registered for both zygotic embryo *A. africana* plants (from 40.01 ± 0.54 to 43.75 ± 0.35) and somatic embryo *A. africana* plants (from 39.55 ± 0.39 to 43.26 ± 0.46) (Fig. 2C). Notably, from third week onwards, there was no significant difference in chlorophyll contents of zygotic embryo *A. africana* plants and somatic embryo *A. africana* plants (Fig. 2C).

Chlorophyll fluorescence measurement

Measurement of the Fv/Fm values in dark-adapted state, which is an indicator of potential maximum quantum efficiency of PSII, revealed the photosynthetic rates of zygotic embryo *A. africana* plants and somatic embryo *A. africana* plants. Across seven weeks, the mean Fv/Fm values of zygotic embryo *A. africana* plants and somatic embryo *A. africana* plants ranged from 0.78 ± 0.01 to 0.83 ± 0.01 and from 0.76 ± 0.0 to 0.83 ± 0.02 , respectively (Fig. 12). Accordingly, Fv/Fm values of zygotic embryo *A. africana* plants and somatic embryo *A. africana* plants across the last four weeks were similar (Fig. 12).

Discussion

Generally, somatic embryogenesis is induced when plant tissues are exposed to the right stimulus, in most cases PGRs^{23,24}. PGR requirement for optimal somatic embryogenesis induction varies among plant species and even genotypes^{18,20} thus, types, concentrations, combinations, and ratios of exogenous cytokinins and/or auxins play a crucial role. Importance of cytokinins and auxins in triggering embryogenic response could possibly be attributed to their involvement in cell cycle regulation and cell division²⁵.

Among cytokinins used in this study, 1.0 mg/L BAP optimally induced somatic embryogenesis from *A. africana* leaf explants (number of somatic embryos per explant and percentage response of explants being 5.75 ± 0.48 and $91.67\% \pm 5.46\%$, respectively). In most species, auxins have been used for inducing somatic embryogenesis. However, in the present study, cytokinins effectively induced direct somatic embryogenesis. Noteworthy is that combination of cytokinins and auxins did not show synergistic effects on initiation and proliferation of somatic embryos. Superiority of BAP in inducing somatic embryogenesis could probability be due to its metabolic stability in in vitro cultures and ability to initiate cell division^{26,27}. This finding is in

agreement with other reports that also recorded highest number of somatic embryos in media containing BAP, including in *Curcuma longa* L.²⁸ and *Metabriggsia ovalifolia* W. T. Wang²⁹.

Exogenous metabolites, small molecules, and peptides such as PSK, AMP, and NAD not only similarly regulate plant growth like cytokinins and auxins but also regulate other processes including reproduction, bioenergetic, biotic and abiotic responses, essential compounds for nucleic acid synthesis, and other components of cell metabolism^{30,31}. Some researchers have used these metabolites, molecules, and peptides including PSK, AMP, and NAD to supplement culture media and promote somatic embryogenesis in different plant species^{32–35}.

In this study, for induction and proliferation of somatic embryos, supplementation of MS medium containing 1.0 mg/L BAP with 3.472×10^{-2} mg/L AMP generated the biggest number of somatic embryos per explant (9.50 ± 0.29) and percent of explants that developed somatic embryos (100%), coupled with earlier formation of embryos than control. The observed result could possibly be because adenosine, a component of AMP is reported to be a major substrate for purine salvage, which is highly associated with growth and development of embryos³⁰. Additionally, during early phase of somatic embryogenesis, active utilization of purines may be necessary to enhance endogenous nucleotide pool, needed to maintain rapid proliferation and cell division of embryogenic tissue in the presence of BAP or/and 2, 4-D³⁰. This finding suggests that AMP is potentially a major metabolite that confers embryogenic competence during *A. africana* somatic embryogenesis. Similarly, Lee et al.³⁴ alluded to the enhancement of somatic embryo development in *Peucedanum japonicum* Thunb. by AMP.

Maturation is a critical stage of somatic embryogenesis since accumulation of storage materials occurs at this point^{36,37}. In many plant species, presence of exogenous ABA at various concentrations has been reported to promote maturation of somatic embryos^{38,39}. In the current study, maximum number of cotyledonary stage embryos (1.67 ± 0.16) was registered in 0.5 mg/L ABA. Ability of ABA to enhance embryo maturation may potentially be attributed to its capacity to induce key metabolic changes that facilitate storage of reserves, thus increasing contents of fatty acids and storage proteins in somatic embryos^{40,41}. Furthermore, ABA has been reported to improve quality of somatic embryos through increasing their desiccation tolerance and prevention of precocious germination⁴². Our result implies that exogenous application of ABA at low concentration (0.5 mg/L) favours maturation of *A. africana* somatic embryos. Consistent with the present finding, exogenous ABA favoured maturation of somatic embryos in other species such as *Pinus elliottii* Engelm.⁴³, *Eucalyptus camaldulensis* Dehnh⁴⁴, and *Copiapoa tenuissima* Ritt. forma *monstruosa*³⁶.

Further supplementation of maturation medium (MS containing 0.5 mg/L ABA) with 6.634×10^{-2} mg/L NAD yielded the highest number of cotyledonary embryos per explant (3.33 ± 0.33). Effectiveness of NAD in improving maturation of *A. africana* somatic embryos could likely be explained by the fact that it is a core component of reduced nicotinamide adenine dinucleotide (phosphate)-oxidised nicotinamide adenine dinucleotide (phosphate) [NAD(P)+/NAD(P)H], an important redox couple in plant cells⁴⁵. Accordingly, somatic embryogenesis is known to be influenced by redox environment of the culture medium, and oxidized environment favours completion of embryogenesis through actions such as improving functionality of shoot apical meristems (SAMs)⁴⁶. Relatedly, Kintzios et al.³³ also showed that exogenous application of nicotinic acid, a key constituent of NAD+ and NADP+, strongly supported embryogenesis in *Rosa hybrids* L. leaf explants.

Another important factor that is reported to influence maturation of somatic embryos is osmotic water potential of the culture medium⁴⁷. Indeed, in our study, maturation of somatic embryos was enhanced in osmotic stress medium (4.73 ± 0.41 cotyledonary embryos per explant) compared to non-osmotic medium (3.20 ± 0.35 cotyledonary embryos per explant). The increased level of maturation of somatic embryos in osmotic stress medium is likely because this condition allows proper development of root apical meristem (RAM) and SAM⁴⁷. This implies that somatic embryos matured under osmotic stress potentially have high conversion rate to plantlets due to well-developed RAM and SAM⁴⁸. This could explain successful development of plantlets from *A. africana* somatic embryos obtained from leaf explants recorded in this study. Similarly, Valencia-Lozano et al.⁴⁷ recorded more efficiency in maturation and conversion to plantlets of somatic embryos of *Coffea arabica* var. Typica under osmotic stress and PGR presence in medium.

Accordingly, media strength markedly influenced germination of *A. africana* somatic embryos with highest percent of germination observed in half strength MS medium ($31 \pm 1.73\%$). The reason may be that variation in strength of culture medium is also known to directly affect osmotic potential⁴⁹. Indeed, half strength MS medium appreciably supported germination of somatic embryos in other species such as *Hypoxis hemerocallidea* Fisch., C.A. Mey. & Avé-Lall⁴⁹ and *Silybum marianum* L.⁵⁰. Additionally, augmentation of germination medium with 0.5 mg/L GA and 0.1 mg/L NAA optimally supported germination of somatic embryos in *A. africana*. This supplementation was based on previous findings of Okello et al.¹ and Okello et al.⁸ who revealed that 1.44×10^{-3} M (0.5 mg/L) GA and 0.1 mg/L NAA strongly enhanced in vitro seed germination and growth of *A. africana*, respectively. Positive effects of GA on somatic embryo germination have been recorded in various plant species^{28,51}.

Cold treatment has commonly been applied to break dormancy and improve seed germination^{52,53}. Indeed, in this study, subjecting the embryos to cold treatment at 4° C improved germination and conversion to plantlets, and this may be because cold mimics stratification⁵⁴. Our finding is consistent with that of Fernández-Guijarro et al.⁵² and Deng & Cornu⁵⁵ who reported enhanced somatic embryo germination after cold treatment in *Quercus suber* L and *Juglans regia* L, respectively.

Due to the fact that in vitro regenerated plants can sometimes show abnormal anatomies, morphologies, and physiologies, their acclimatization is important because it facilitates survival of the plantlets *ex vitro*²⁶. In our study, the survival rate of somatic embryo derived *A. africana* plantlets was 75.00% after acclimatization, suggesting appropriate adaptation of the plantlets to greenhouse condition. Several other workers also registered more than 70.00% survival rate of somatic embryo plantlets after acclimatization including in *Viola canescens* Wall. Ex. Roxb⁵⁶ *Tolunnia* Louise Elmore ‘Elsa’⁵⁷ and *Hemides musindicus*⁵⁸.

Histological observation is a key tool in proving the morphological differentiation and structure development of somatic embryos²⁰. Isolation of vascular bundle from the mother tissue has been used to distinguish somatic embryogenesis from organogenesis²⁰ and in this study, cotyledonary stage embryos had a closed vascular system, indicating reliability of somatic embryogenesis. Histological analysis also revealed that embryonic cells aggregation directly formed somatic embryos, confirming direct somatic embryogenesis in *A. africana*. Anatomical evidences of somatic embryogenesis exhibited sequential cellular differentiation and stages of development in *A. africana*, similar to zygotic embryogenesis (in dicots), characterized by developmental stages of globular, torpedo, and cotyledonary stages¹¹.

Flow cytometry has become a primary method used for measuring content of plant nuclear DNA such as genome size and its related parameters like ploidy level⁵⁹. The wide application of this technique is attributed to associated merits, including being rapid, convenience of sample preparation, and ability to accurately detect small differences in DNA content⁶⁰. Several researchers have employed flow cytometry in determining stability of plant genome size/ploidy level, following in vitro regeneration as tissue culture might lead to somaclonal variation^{18,61,62}. During somatic embryogenesis, somaclonal variation is reported to be influenced by many factors, including PGRs such as BAP, light conditions, wounding, and it is most common when callus phase is involved⁶³. For example, Catalano et al.⁶³ revealed that alteration of ploidy levels (from diploid to tetraploid) in 9.3% of somatic embryo derived *Vitis vinifera* occurred when pistil explants were cultured in medium containing BAP (4.4 μ M) and beta-naphthoxyacetic acid (10 μ M). In this study, there was no marked variation between the genome size/ploidy level of zygotic embryo derived *A. africana* plants (6.33 pg/6,190 Mbp) and somatic embryo derived *A. africana* plants (6.25 pg/6,113 Mbp), meaning genetic integrity of the regenerated plants was maintained. This result corresponds to that reported for *Juglans regia*⁶⁴ *Cucumis melo* L.⁶⁵ and *Brassica juncea* L.⁶².

Chlorophyll is a key photosynthetic pigment that affects photosynthetic capacity, health, and growth of plants²⁶. SPAD meter has been commonly used for measuring chlorophyll content because it is non-destructive, quick, and its result is consistent with those of other destructive chlorophyll measuring methods^{66,67}. The first week of measurement in the current study revealed that chlorophyll contents in zygotic embryo *A. africana* plants (35.06 ± 0.30) were higher than those in somatic embryo *A. africana* plants (29.5 ± 0.35). This may probably be due to indisposed chloroplasts of in vitro raised plants, decreased phytochemical activity of leaves during initial periods of acclimatization, characteristic lower chlorophyll content in younger leaves compared to older leaves as somatic embryo *A. africana* plants were younger^{26,68}. Previously, lower chlorophyll contents in in vitro regenerated plants were reported for some plants, for instance *Tecoma stans* L.⁶⁹ and *Apios americana* Medik.²⁶. In the initial two weeks, there was relatively higher rate of increase in chlorophyll contents of somatic embryo *A. africana* plants compared to that in zygotic embryo *A. africana* plants, resulting into comparable chlorophyll contents by the third week. The observed higher rates of chlorophyll content increase in somatic embryo *A. africana* plants could likely be attributed to increased: supply of nutrients such as magnesium and potassium (necessary for chlorophyll synthesis) from soil, phytochemical activity, and age of leaves². From week three to seven, with minimal increase in chlorophyll contents, there was no significant difference in chlorophyll contents of zygotic embryo *A. africana* plants and somatic embryo *A. africana* plants. The minimal increase in chlorophyll contents at this stage might be due to the fact that mature leaves tend to have more stable content of chlorophyll than young leaves⁶⁷. Lack of significant difference in chlorophyll contents of zygotic embryo *A. africana* plants and somatic embryo *A. africana* plants revealed similarity of photosynthetic rates in both plant groups across the last four weeks. Okello et al.² reported comparable trend for in vitro *A. africana* regenerated via organogenesis and mother plants.

The quantum yield efficiency of PSII (Fv/Fm) is a sensitive chlorophyll parameter that is commonly used to show photosynthetic performance of plants⁷⁰. In this study, the initially lower Fv/Fm values (2 weeks) expressed in somatic embryo *A. africana* plants may possibly be attributed to poorly differentiated and immature photosynthetic tissues²⁶. Fv/Fm values increased in somatic embryo *A. africana* plants and became similar to those of zygotic embryo *A. africana* plants in the third week; this could be because as acclimatization progresses, further development of tissues and leaf mesophyll differentiation occur, making the plants more adapted for photosynthesis⁷¹. Fv/Fm values of zygotic embryo *A. africana* plants and somatic embryo *A. africana* plants across the last four weeks were similar, suggesting similar rate of photosynthesis between them. Moreover, the Fv/Fm values of both plant groups were within range for plants growing under no stress (0.75 to 0.85)⁷² meaning their growth conditions were favourable and devoid of stress. This finding is consistent with that of Okello et al.² who observed related Fv/Fm values between in vitro *A. africana* regenerated via organogenesis and donor plants. In other species, similar Fv/Fm values were also recorded and these include *Prunus africana* (Hook f.) Kalkman⁶⁷ *Paphiopedilum insigne* (Wall. ex Lindl.) Pfitzer⁷³ and *Guadua chacoensis* (Rojas) Londoño & P. M. Peterson⁷⁴.

Conclusions

For the first time, micropropagation method via direct somatic embryogenesis in *A. africana* from leaf explants was established. MS medium supplemented with 1.0 mg/L BAP and 3.472×10^{-2} mg/L AMP optimally induced direct somatic embryogenesis in leaf explants (100% response and 9.50 ± 0.29 somatic embryos per explant). Differentiation and maturation of somatic embryos was enhanced in MS medium augmented with 0.5 mg/L ABA, 6.634×10^{-2} mg/L NAD, and 9 g/L gelrite (6.27 ± 0.36 globular, 3.40 ± 0.35 heart, 2.60 ± 0.51 torpedo, and 4.73 ± 0.41 cotyledonary). Half strength MS medium containing 0.5 mg/L GA and 0.1 mg/L NAA optimally supported germination ($31 \pm 1.73\%$) of cotyledonary somatic embryos. Pre-germination treatment of stratification at 4° C further stimulated somatic embryo germination (60.00%) and their conversion into plantlets (26.67%). Histological observations and flow cytometric analysis confirmed different somatic embryo stages and stability in genome size of regenerated plants, respectively. Moreover, chlorophyll contents and photosynthetic

rates were similar between zygotic and somatic embryo derived plants. However, the major limitations registered in this study are the asynchronous formation of embryos and relatively low conversion of somatic embryos to plantlets (26.67%), requiring improvement. These bottlenecks could be addressed by conducting molecular studies (such as genomics and transcriptomics) to understand molecular basis of somatic embryogenesis in *A. africana*, which would possibly enable identification of molecular markers and manipulation of key factors, leading to improved formation and development of somatic embryos at the same time (synchronously) as well as conversion to plants. Additionally, we also recommend exploring other methods reported to improve conversion of somatic embryos to plantlets in other plant species such as the use of temporary immersion bioreactors in coffee (*Coffea canephora* and *Coffea arabica*)²³. Overall, the established method in this study may be used for mass clonal regeneration, conservation, synthetic seed production, cryopreservation, and genetic improvement of *A. africana*. Additionally, this system could be utilized for investigating molecular, biochemical, and physiological events, that take place during embryogenesis in *A. africana*.

Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions

RG conceptualization, investigation, data curation, formal analysis, and wrote the manuscript. SY histological analysis, flow cytometry analysis, and wrote the manuscript. KH formal analysis, reviewed and edited the manuscript. JM formal analysis, reviewed and edited the manuscript. AH formal analysis, reviewed and edited the manuscript. DO formal analysis, reviewed and edited the manuscript. YB Reviewed and edited the manuscript. YK supervision, reviewed and edited the manuscript, and funding acquisition. All authors read and approved the final manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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