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Rapid germination and development of *Acacia sieberiana* DC *in vitro*

Hussien M. DAFFALLA[1]*, Kauther S. ALI[2], Magdoleen G. OSMAN[1], Yousif O. YAHIYA[2]

[1]*National Centre for Research, Commission for Biotechnology and Genetic Engineering, Department of Plant Cell and Tissue Culture, Mohamed Nageeb St., Khartoum 2404, Sudan; hdaffalla@yahoo.com (*corresponding author); magdogamar@yahoo.com*
[2]*University of Bahri, College of Applied and Industrial Sciences, Department of Biotechnology, Khartoum North, Sudan; kauthersirelkhatim@gmail.com; yousifomeryahya111@gmail.com*

Abstract

Acacia sieberiana is an ecological and economical important forest tree. The plant has poor germination due to dormancy; therefore, *in vitro* culture can provide a fast supply of plantlets and can be a basis for further biotechnology research. The effect of sodium hypochlorite, Murashige and Skoog (MS) medium, sucrose and cytokinin were investigated. The results indicated that all the treatments induced significant ($P < 0.05$) effects on germination and seedling development depending on their concentrations. Germinates free of contamination were established successfully with all the concentrations of sodium hypochlorite, however, the germination was reduced as the concentration increased. A higher germination rate of 98.3% was attained with full MS strength and 3% sucrose concentration. The highest germination percentage obtained was 100% with 2 mg/L thidiazuron. The maximum shoot length (13.5 cm) and seedling dry weight (0.49 g) were recorded on full MS medium while the maximum root length (13.4 cm) was obtained on ½ MS strength. However, when full MS medium augmented with 5% sucrose enhanced shoot length and dry weight to (14.2 cm) and (0.5 g), respectively. While the highest root length (10.2 cm) was obtained by 3% provided in full MS medium. Application of cytokinins improved the number of node and dry weight of seedlings to 6.9 nodes with 4 mg/L thidiazuron and 0.54 g by 2 mg/L benzyladenine. The present study describes the establishment of *in vitro* seed germination and seedling growth system for *A. sieberiana* for the first time.

Keywords: *Acacia sieberiana*; cytokinin, dormancy; MS strength; *in vitro* seed culture; seedling; sucrose level

Introduction

Acacia sieberiana DC var. *sieberiana* referred to in English as whitethorn, umbrella thorn, paperbark thorn, or flat-topped thorn and in Sudan commonly known as *Kuok* or *Kuaka*. It is a multipurpose leguminous tree belonging to the subfamily Mimosaceae within Fabaceae family. The tree is widely distributed in Eastern, Western, and Southern Africa through the savannah, wooded grasslands to the semi-arid regions under 500 mm/year rainfall. It is known to grow along the banks of seasonal and permanent rivers, slopes of hills, coastal

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plain, or low grounds (Kordofani and Ingrouille, 1991). *A. sieberiana* is a deciduous indehiscent species, erect large tree 5-20 m high, with flat or rounded crown (El Amin, 1990).

A. sieberiana has been utilized for wood and non-wood products to provide various purposes. The tree is very important for social forestry and agroforestry programs due to its rapid growth and nitrogen fixation capability (Pahla *et al.*, 2014). Gums collected from the tree are edible and used as chewing gum, in making ink, for cosmetics, and also as astringent and emulsifiers (Salisu *et al.*, 2017). The pods, shoots and leaves are highly nutritious with low insoluble proanthocyanidins and high soluble phenolics (Mugunga and Sahinkuye, 2020) favorable to feed livestock. The woods are used in making furniture, hut-building, fencing, tool handles and mortars as they are termite resistant (Sulieman *et al.*, 2012; Salisu *et al.*, 2017). On the other hand, different parts of *A. sieberiana* which are rich in tannins are used traditionally in decoction/infusion forms for the treatment of general ailments such as inflammations, fever, tiredness, and to improve lactation after childbirth in addition to many other ailments as reported by several authors (Christiana *et al.*, 2012; Salisu *et al.*, 2017; Tchatchedre *et al.*, 2019).

Indeed, almost all species of *Acacia* are regenerated in natural habitats only from seed. During seeding season, *Acacia* species habitually produced substantial quantities of seeds. However, as a survival strategy, large quantities of those produced seeds are accumulated viable but dormant in the soil. This inherited seed dormancy resulted in impermeability of the seed testa to water and gases which obstruct embryo expansion (Miransari and Smith, 2014). The hard seed-coat protects the embryo from harsh environments and prevents out-season emergence so upholds seed durability in postponing for the finest chance for seedling establishment. *A. sieberiana* and *A. nilotica* have the thickest seed coat among the acacias of Sudan (Warrag and Eltigani, 2005). Such seeds fail to germinate when conditions are appropriate for germination. In natural stands, the conditions necessary to allow seeds to break dormancy and germinate can be highly variable even among seed genotypes of the same species (Luna *et al.*, 2014). Infestation by bruchid beetles and veld fire were two environmental factors that were reported affecting *Acacia* seeds viability and germination. For example, *Acacia* seeds stored in soil bank reported to decline by 92% after one natural fire in South Africa (Sabiiti and Wein, 1987), however, this percentage depends on fire intensity, maximum temperature (Kanz, 2001) and duration of exposure (Mucunguzi and Oryem-Origa, 1996). Otherwise, the fire was reported to improve *A. sieberiana* seedlings emerging (Sabiiti and Wein, 1987). On the other hand, *A. sieberiana* trees produced an enormous quantity of seed estimated at 980 seeds/m^2, yet bruchid larvae reported to infest 50-96% of these seeds (Mugunga and Sahinkuye, 2020; Sabiiti and Wein, 1987). Nonetheless, Mucunguzi (1995a) found that 88.4-98.8% of infested *A. sieberiana* seeds collections were viable. Furthermore, it was observed that bruchid infestation accelerated germination of *A. sieberiana* seed, which was not pretreated, to 17.1% (Mucunguzi, 1995b). In a study on germination of *A. sieberiana* seeds under field conditions, results proved that seedlings emergence and survival was only 16.3% after about 32 weeks of planting (Kanz, 2001). Also, in another study curried in Zambia, the seedling emergence rate of *A. sieberiana* was 65.2% after 14 weeks of sowing while seedling mortality reached 48.3% of 1-year-old total emerged plants (Chidumayo, 2008). Even more, in a study of natural regeneration of *A. sieberiana*, the result of inventory shown after several weeks only one seedling was observed within over 310 ha of the study area (Mugunga and Sahinkuye, 2020). From all the above-studied literature, the positive effects of fire and bruchid infestation on the natural regeneration of *A. sieberiana* seed is not predicted and depends on ambiguous environmental conditions such as water availability and grazing. Correspondingly, Warrag and Eltigani (2005) specified that as the clean seeds produced the highest germination percentages deny the common concept that the natural germination is due to damaged and infected seeds. As well, Mugunga and Sahinkuye (2020) concluded the adverse outcome of treating *A. sieberiana* seeds with dry heat reject the hypothesis of the positive effect of fire on germination or regeneration of the species. In addition to germination and emergence difficulties, the tree also has been greatly threated in its natural habitat due to overexploitation. That, in a study assessing changes in the vegetation of indigenous

plant species in Gadarif region, Sudan, *A. sieberiana* was considered almost disappeared from the study area (Sulieman *et al.*, 2012). In the same study, the author stated the species abundance is strictly related to their uses as those utilized for live fences, firewood and forage, including *A. sieberiana*, are the most declined.

In vitro seed culture techniques can be a valuable alternative for mass propagation and conservation of forest trees ensuring incessant supplies of the saplings for afforestation schedules. Intact seeds are ideal for establishing the tissue culture of forest trees because they can be cheaply and easily collected, sored, sterilized, and then used to produce a large number of juvenile plants free of contaminations. Also, seeds are representative of entire genomics pool of the population target for conservation (Alves *et al.,* 2006). Generally, even after pretreatment seed was sown *ex vitro* might germinate slowly and non-uniformly resulting in prolonged times to obtain considerable populations seedlings and which would be of irregular sizes and ages. In contrast, *in vitro* induction of seed germination will increase the probability of germination with the rapid and uniform development of seedlings. Seed explants can be used for induction of direct multiple shoots proliferation by directly culturing on a cytokinin-augmented medium.

In vitro germination of seeds as well as micropropagation of *A. sieberiana* has never been reported according to our knowledge. Successful experiments were reported on *in vitro* culture of various *Acacia* species (more than 20 genera) as reviewed by Gantait *et al.* (2016). Therefore, the development of a protocol for seed sterilization and germination of this multipurpose plant is indispensable.

Materials and Methods

Collection and preparation of seed explant

Mature dried pods of *A. sieberiana* were picked from trees that naturally grew in Eldamazein area, Blue Nile state, Sudan. The obtained pods were opened to extract seeds which were then cleaned and kept in airtight bottles at 25 °C in a storage room until use. Before culturing, the seeds were pretreated, to facilitate softening of the hard seed coat, to break mechanical seed dormancy. The seeds were soaked in concentrated sulphuric acid for 60 min with regular shaking and then washed thoroughly under running water for 5 min to remove all residues of sulphuric acid and kept in distilled water. The pretreated seeds were surface-sterilized under laminar flow firstly with ethanol (70%, v/v) for 10 sec, followed by 3-4 times washes in autoclaved distilled water. Then, commercial sodium hypochlorite (Clorox 0.5% free chlorine) solution was applied with aid of 2-3 drops of tween 20 and shaking for 10 min. The seeds were rinsed 4-5 times with sterile distilled water to remove residues of disinfectant. Disinfected seeds were sequentially placed on soft tissue in sterilized Petri dishes to absorb liquid on the surface of the seeds.

Media and culture conditions

Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) with 30 g/L sucrose was utilized in all experiments. The pH was adjusted to 5.8 with the NaOH. The media were augmented with 7 g/L type I agar melted in a microwave and dispensed as 15 mL per tube. The solidified media with culture tubes were autoclaved at 121 (1.12 kgf/cm^2) for 15 min. For the germination experiment, one seed was inoculated per a glass culture tube (20 × 3 cm). Culture tubes were wrapped with plastic sheets (to exclude light) until germination. All cultures were kept in an incubation room at 23 ± 2 °C under a 16 h photoperiod with a light intensity of 60 μmol/m/s provided by cool-white fluorescent lamps.

Experimental procedures

A series of experiments were carried to study the responses of *A. sieberiana* to the *in vitro* conditions. The effects of sodium hypochlorite, MS medium, sucrose and cytokinin on seed germination and seedling development were investigated. A variable range of concentrations (5, 10, 15, or 100%) of sodium hypochlorite

were applied to examine the effects of sodium hypochlorite on seed germination and seedling development. To optimize *in vitro* germination and plantlets development of *A. sieberiana*, different strengths of MS basal medium (mineral salts and vitamins) were prepared. The tested strengths include full MS (1 MS), half MS (½ MS), quarter MS (1/4 MS) and zero MS (0 MS) which is media free of MS salts and consisted of distilled water only. All media were provided with standard MS sugar and fixed values of pH and agar as specified above. Different concentrations of sucrose (1, 2, 3, 4, and 5%) were used to examine effects on seed germination and seedling growth. The different concentrations were supplemented in the best MS strength defined in the previous experiment test. To study the germination behavior and seedling development of *A. sieberiana* in response to cytokinins treatment various concentrations (1, 2, 3, 4, and 5 mg/L) of BA and TDZ were studied following the general germination protocol.

Seed germination and seedling growth parameters

The protrusion of ≈ 2.0 mm radicle through seed coat was considered physiological germination. Germinated seeds were counted starting from the third day and recorded daily until no further germination was recorded (for 12 days). By end of the experiments, germinability (G), mean time to germination (MGT), mean germination rate (MR), and coefficient of variation of the germination time (CVt) were calculated. Germinability (G) is represented by the percentage of germination in the experimental conditions. The total numbers of the germinated seeds for each treatment were summed up to determine the cumulative germination. Mean germination time (MGT) was calculated as follows:

$$MGT = \sum (n \times g)/N$$

Where: n= number of seeds germination per day; g= number of days needed for germination; N= total number of germinated seeds.

Mean germination rate (MR) is calculated as the reciprocal of the mean germination time:

$$MR = 1/MGT$$

Coefficient of variation of the germination time (CVt) is calculated by the expression:

$$\mathrm{CV}t = \frac{St}{MGT}100$$

Where: St: standard deviation of the germination time.

All seeds germinated on the germination experiments were kept on the same media, then the cultures used for seedling growth experiments. Observations on seedling growth including shoot length, root length, number of nodes, and dry weight were recorded after three weeks of sowing.

Data collection and statistical analysis

The data were collected from nine replicates for four treatments (NaOCl 4 levels, MS 4 levels, sucrose 5 levels, 2 cytokinins 5 levels) within each of the two independent experiments (germination and seedling growth). Statistical analysis was performed using one-way analysis of variance procedure (ANOVA) on the excel computer program. Duncan's multiple range test (DMRT) was used to perform means separation at a significance level of $P \leq 0.05$.

Results and Discussion

Seed germination

Seed germination involves morphological and physiological changes on the embryo appeared as expansion and elongation activities after seed absorbing water, thereafter, radicle penetrate out of the seed coat (Miransari and Smith, 2014). The germination process is characterized by three parameters that can be measured; i.e., percentage, rate, and uniformity (Hartmann *et al.*, 2002); apprising the dynamics of the germination process.

Effect of sodium hypochlorite on seed germination

In the current study, the effects of different concentrations of sodium hypochlorite solution were investigated on the germination of *A. sieberiana* (Table 1). All the concentrations of NaOCl produced free-contamination cultures, but with significant ($P \leq 0.05$) variation in the germination percentages (Table 1). Maximum germination (98.3 %) was obtained in *A. sieberiana* seeds treated with the lower concentration (5%) of NaOCl while germination reduced significantly ($P \leq 0.05$) with increasing NaOCl concentrations (Table 1). This suggested that, due to the increase in permeability of seed coat after pretreatment and in NaOCl-enriched environs, amount of NaOCl might reach embryo affecting the germinability. Sodium hypochlorite is the most used detergent for surface sterilization of different types of explants in various plant species. Household bleach (Clorox) is an equilibrium mixture composed mainly of 5.25% NaOCl and 4% NaCl in addition to sodium salts as stabilizer and buffer; soluted in water with 11.4 (Chun *et al.*, 1997). The reaction of NaOCl and water in the bleach solution yield two products NaOH and HOCl which is the active principle in chlorine disinfection. Chlorine compounds are extremely reactive elements and potent oxidizers cable to exterminate microbes by oxidizing biological molecules such as proteins and nucleic acids. However, this property also makes it highly reactive with amino acids, nucleic acids, amines, and amides metabolism in tissues of sterilized seeds (Abdul-Baki, 1974). Seemingly, a portion of sodium hypochlorite persists in the sterilized tissues even after several rinsing of seeds with water and the toxic effects undergo during germination. However, Abdul-Baki (1974) stated that the residual hypochlorite can be removed by soaking seeds in 0.01 N HCl for 10 min without reducing germinability. The concentration of NaOCl and sterilization period together have adverse effects on viability and regeneration frequency of explant (Yildiz and Er, 2002). In the present study, this harmful effect noticeably increases with increasing concentration of NaOCl appeared as negative effects on germination percentage and rate (Table 1). A similar negative effect of high concentrations of NaOCl on seed germinability was reported on *Linum usitatissimum* (Yildiz and Er, 2002) and *Melissa officinalis* (Kiani *et al.*, 2017).

Table 1. Effect of sodium hypochlorite in different concentrations (5-100%) on seed germination of *A. sieberiana* after 12 days of culture

NaOCl (%)*	G (%)	MGT (day)	MR (day^{-1})	CVt (%)
5	98.32^{a} (± 1.7)	2.7^{a} (± 0.02)	0.37^{a} (± 0.0)	25.2^{a} (± 0.48)
10	75.0^{b} (± 2.9)	2.9^{a} (± 0.1)	0.35^{a} (± 0.01)	28.8^{a} (± 3.78)
15	73.33^{b} (± 1.7)	2.8^{a} (± 0.0)	0.36^{a} (± 0.0)	33.09^{a} (± 0.52)
100	49.17^{c} (± 0.8)	2.8^{a} (± 0.17)	0.36^{a} (± 0.02)	30.95^{a} (± 1.29)

Values represent means (± standard errors). G: germinability, MGT: mean time to germination, MR: mean germination rate, CVt: coefficient of variation of the germination time. * Seed surface sterilization time is 10 min.

Table (1) showed the influence of sodium hypochlorite on the germination speed which reflected MGT values. The shortest time for germination (2.7 days) was observed with the concentration 5%, however, no significant ($P \leq 0.05$) differences were observed between all NaOCl concentrations (Table 1). Regarding MR of the germination, the highest value (0.37) was observed in 5% sodium hypochlorite and the lowest value was (0.35) on the concentration 10%. Also, 5% sodium hypochlorite recorded the lower CVt value (25.2%) explains the germination uniformity in relation to the mean germination time. Whereas sodium hypochlorite at 15% showed a higher CVt value (33.1 %), which indicates more irregular germination when compared to the other concentrations. On the other hand, seeds of *A. sieberiana* did not present significant differences in the CVt.

Effect of MS medium on seed germination

The results of this study showed that *in vitro* seed germination of *A. sieberiana* was affected by the strength of the MS basal medium (Table 2). Full MS basal medium was found significantly ($P \leq 0.05$) the best

for the seed germination with final germination 98.3% followed by the 0 MS medium (water agar medium) with 84.8%. This is in agreement with Bodede *et al.* (2018) that *A. nigrescens* seeds gave higher germination in full MS compared to ½ MS and plain agar media. On contrary, full MS was lower than ½ MS in percentage germination of *A. nilotica* seed (Dhabhai and Batra, 2010). A similar better effect of full MS medium on germination of seed compared to reduced strength or water agar media was also reported on other species such as *Drypetes roxburghii* (Murthy and Reddy, 2014) and *Begonia Malabarica* (Murugan *et al.*, 2016). This is contrary to that reported on *Melissa officinalis* (Kiani *et al.* 2017), *Pterocarpus marsupium* (Mishra *et al.*, 2013) and *Tectona grandis* (Mishra *et al.,* 2018) where ½ strength produced maximum seed germination compared to ¼ and full MS strengths media. The environment surrounding the seed including various parameters such as salinity, acidity, temperature and light, have a splendid influence on the germination process by affecting hormonal balance within the seed (Miransari and Smith, 2014). In the present study, these parameters are supposed to be equally provided for *A. sieberiana* seeds in all treatments except salinity which varies according to the MS strength. Nitrate (NO_3^-) play important role in seed germination because it is a source of N which is a germination enhancer by adjusting the K^+/Na^+ ratio and increasing ATP production and seed respiration [(Miransari and Smith, 2014). The level of NO_3^- is high in MS basal medium beside another source of nitrogen provided by ammonium (NH_4^+). Therefore, full MS strength contains a high concentration of nitrogen. The improved germination percentage of seeds cultured on the water agar medium might be due to the lack of salts nutrients which encourage seeds to germinate faster as indicated with the lower MGT value obtained (2.5 days), higher MR value (0.4), and low CVt. Previous reports on germination of pre-treated seeds of *A. sieberiana* recorded an average time of germination of 2.0 days and rate of germination 1.8 (seeds/day) on filter paper moist with distilled water (Mucunguzi, 1995b). Similarly, seeds of *Grewia tenax* needed fewer days to germinate in plain agar medium in comparison to other MS strengths (Daffalla *et al.*, 2016). The mineral demand during the process of germination is probably related to the amount of reserves in the seed, genotypes, seed age, size and growth factors during germination of seeds *in vitro*.

Table 2. Effect of MS strengths* on seed germination of *A. sieberiana* after 12 days of culture

MS strengths	G (%)	MGT (day)	MR (day^{-1})	CVt (%)
0	84.87^{ab} (± 1.7)	2.51^{b} (± 0.06)	0.40^{a} (± 0.03)	21.19^{a} (± 4.1)
¼	71.97^{b} (± 1.1)	2.86^{b} (± 0.16)	0.36^{ab} (± 0.03)	36.91^{a} (± 4.9)
½	73.36^{b} (± 0.6)	3.45^{a} (± 0.1)	0.29^{b} (± 0.01)	36.15^{a} (± 4.3)
1	98.33^{a} (± 1.7)	2.73^{b} (± 0.15)	0.37^{a} (± 0.0)	29.59^{a} (± 1.4)

Values represent means (± standard errors). G: germinability, MGT: mean time to germination, MR: mean germination rate, CVt: coefficient of variation of the germination time. MS: Murashige and Skoog basal medium. :* sucrose applied at 3%

Effect of sucrose on seed germination

The effect of various concentrations of sucrose on seeds germination was presented in Table 3. The significantly ($P \leq 0.05$) maximum seed germination recorded was 98.3% obtained on MS medium supplemented with 30 g sucrose. Similarly, the highest seed germination percentage induction with 30 g sucrose was reported on *Drypetes roxburghii* (Murthy and Reddy 2014), *Prunus armeniaca* seeds (Yildirim *et al.*, 2007) and *Eustoma grandiflorum* (Roni *et al.*, 2017). The sucrose at 20 g showed a lower MGT value (2.5 days) combined with a higher MR value (0.4) indicated a fast germination rate among sucrose concentrations. Although both these values were not significantly different from other concentrations. Seeds of *A. sieberiana* presented greater homogeneity (significantly lower value of CVt; 39.4%) with 10 g sucrose.

Table 3. Effect of sucrose* in different concentrations (1-5%) on seed germination of *A. sieberiana* after 12 days of culture

Sucrose (g/L)	G (%)	MGT (day)	MR (day^{-1})	CVt (%)
10	76.64^{b} (± 1.8)	2.69^{a} (± 3.3)	0.38^{a} (± 0.02)	39.39^{c} (± 1.5)
20	81.73^{b} (± 1.4)	2.51^{a} (± 3.3)	0.40^{a} (± 0.03)	54.72^{a} (± 0.4)
30	98.25^{a} (± 1.8)	2.66^{a} (± 3.3)	0.38^{a} (± 0.03)	44.55^{bc} (± 2.9)
40	66.62^{c} (± 2.9)	3.38^{a} (± 3.3)	0.30^{a} (± 0.0)	43.78^{bc} (± 1.4)
50	58.35^{c} (± 0.9)	2.83^{a} (± 3.3)	0.36^{a} (± 0.03)	48.54^{b} (± 1.8)

Values represent means (± standard errors). G: germinability, MGT: mean time to germination, MR: mean germination rate, CVt: coefficient of variation of the germination time. *Supplemented in full strength MS medium

Effect of cytokinins on seed germination

A. sieberiana seeds showed comparatively better (not significant) germination in MS medium supplemented with 2 mg/L TDZ with 100% seed germination compared to 98.3% achieved on cytokines-free MS medium (Table 4). These values were significantly ($P \leq 0.05$) different from the other TDZ concentrations and all BA concentrations. On the other hand, all BA concentrations showed a decrease in seed germination of *A. sieberiana* compared to the control. Similarly, BA reduced the percentage of seed germination of *Albizia lebbeck* (Perveen *et al.*, 2013). In general seed germination was decreased with increasing cytokinins concentration (Table 4). However, TDZ achieved the lowest time for germination with a lower MGT value (2.1 days) and a higher MR value (0.5) indicated faster seed germination. The lower CVt value recorded was (0.0%) with 5 mg/L TDZ because all germination at this concentration was recorded at the time (MGT = 3.0 days). An alike better effect of cytokinins in decreasing days required to germination was reported in *Grewia tenax* (Daffalla *et al.*, 2016). Cytokinins are plant hormones, regulating a range of plant activities including seed germination. They are active in all stages of germination and can regulate different functions related to the physiology of the embryo such as development and differentiation (Miransari and Smith, 2014).

Table 4. Effect of BA and TDZ in different concentrations (0-5 mg/L) on seed germination of *A. sieberiana* after 12 days of culture

PGRs (mg/L)		G (%)	MGT (day)	MR (day^{-1})	CVt (%)
Control		98.29^{a} (± 0.0)	2.70^{a} (± 0.0)	0.38^{ab} (± 0.0)	33.12^{ab} (± 5.9)
BA	1	81.55^{b} (± 3.1)	2.76^{a} (± 0.4)	0.38^{ab} (± 0.1)	59.54^{a} (± 6.2)
	2	59.52^{c} (± 1.3)	2.90^{a} (± 0.4)	0.36^{ab} (± 0.1)	62.14^{a} (± 9.6)
	3	58.33^{c} (± 3.6)	3.22^{a} (± 0.3)	0.32^{b} (± 0.0)	52.29^{a} (± 9.9)
	4	54.17^{c} (± 1.5)	3.26^{a} (±0.3)	0.31^{b} (± 0.0)	46.88^{a} (± 5.9)
	5	24.52^{d} (± 2.5)	2.67^{a} (± 1.5)	0.18^{b} (± 0.1)	31.43^{ab} (± 6.4)
TDZ	1	80.95^{b} (± 2.7)	2.13^{a} (± 0.5)	0.52^{a} (± 0.1)	45.16^{a} (± 3.6)
	2	100^{a} (± 0.0)	2.43^{a} (± 0.2)	0.42^{ab} (± 0.0)	44.04^{a} (± 6.0)
	3	80.95^{b} (±2.5)	2.43^{a} (± 0.4)	0.43^{ab} (± 0.1)	49.33^{a} (± 4.2)
	4	76.19^{b} (± 2.3)	2.26^{a} (± 0.3)	0.46^{ab} (± 0.1)	53.04^{a} (± 3.6)
	5	19.05^{d} (± 0.8)	3.0^{a} (± 0.0)	0.33^{ab} (± 0.0)	0.0^{b} (± 0.0)

Values represent means (± standard errors). G: germinability, MGT: mean time to germination, MR: mean germination rate, CVt: coefficient of variation of the germination time. BA: benzyl adenine, TDZ: thidiazuron.

In the present study, *A. sieberiana* seeds were pretreated with concentrated sulfuric acid for 60 min. According to the Tree Seeds Treatments Guide prepared by the National Tree Seed Center, Sudan (NTSC, 1998), *A. sieberiana* seeds pretreated with concentrated sulfuric acid for 90 min gave 85% germination and untreated seeds gave 3% germination. Pretreatment of *A. sieberiana* seeds with sulfuric acid for 60 min has been reported by several authors with variable success such as 88.4% (Mucunguzi, 1995b), 76% (Mucunguzi and Oryem-Origa, 1996), 78% (Kanz, 2001), and 80% (Pahla *et al.*, 2014). However, in contrast, Salisu *et al.* (2017)

stated that *A. sieberiana* seeds treated with sulfuric acid for 60 min did not germinate and reducing the acid concentration to 50% resulted in 20%, and interestingly untreated seeds gave 90% germination. On the other hand, other methods of seed pretreatment have been applied on *A. sieberiana* seeds such as hot water which resulted in lower germination to 53% (Pahla *et al*., 2014) and 60% (Tybirk, 1991). Besides, hot wire scarification gave 90% (Mugunga and Sahinkuye, 2020) and the maximum germination reported was 94% using mechanical scarification (Matekaire and Maroyi, 2009). In light of altogether previous reviews on *ex vitro* germination of *A. sieberiana*, the current result indicated that *in vitro* culture of seed improved the germination rate capable uniformity and homogeneity of seedlings in a short period of culture. That, in the present study, 98.3% germination was achieved using MS devoid of growth regulators and 100% when MS augmented with TDZ after 12 days of culture. The higher germination percentage (94%) reported by Matekaire and Maroyi (2009), above mentioned, can be attributed to the fact that seeds in the study were surface sterilized in sodium hypochlorite and incubated under optimum temperature ≤27 °C, and when temperature raised to 30 °C germination percentage decreased to 80%. Also, Mugunga and Sahinkuye (2020) stated that although a higher percentage of germination was got by *A. sieberiana* at glasshouse the germination was highly irregular and it took up to 16 weeks. Improving seed germination under *in vitro* culture compared to that *ex vitro* was also reported in other species. Only 50% of *Prunus armeniaca* seeds was germinated in glasshouse after two months of sowing, while 75% of the seeds were germinated *in vitro* after 14 days of culture (Yildirim *et al*., 2007).

In vitro seed germination of other *Acacia* was previously reported on *A. nilotica* (90%) (Dhabhai and Batra, 2010), and *A. raddiana* (83.3%) and *A. nilotica* (96.7%) treated with sulphuric acid (Aziz *et al.* 2001), treated with hot water, and 100% on *A. nigrescens* with mechanical scarification (Bodede *et al*., 2018). Also, Attia *et al.* (2017) studied *in vitro* germination of 8 *Acacia* species seeds pretreated with boiling water found maximum germination was 73.7% by *A. ehrenbergiana.*

Effect of MS medium on seedling growth

The results on the effects of MS medium strengths on seedling growth of *A. sieberiana* after 3 weeks of culture were presented in Table 5. The strengths of MS medium showed a variable effect on shoot length of *A. sieberiana* with the highest length obtained was (13.5 cm) on full MS medium, however, the value was not significant from other strengths. In contrast, Altayeb 92004) reported that ½ strength MS medium gave the higher shoot length of *A. seyal.* Also, a finer effect of ½ MS medium on shoot length was reported on *Pterocarpus marsupium* (Mishra *et al.,* 2013). The significantly ($P \leq 0.05$) maximum root length (13.4 cm) was obtained on ½ strength MS medium. Likewise, a half-strength MS medium was found effective in improving the root growth of *Begonia malabarica* (Murugan *et al.,* 2016) and *Pterocarpus marsupium* (Mishra *et al.,* 2013). Half strength MS medium has a low osmotic potential favor for fine-tuning of seedlings during the germination and root development stages (Roni *et al*., 2017). The control treatment (distilled water augmented with sucrose and solidified with agar) produced an average root length of 4.6 cm. Matekaire and Maroyi (2009) found that a radicle length of 2.4 cm for *A. sieberiana* seeds incubated in moist filter paper. The highest value on DW of the seedling (0.49 g) was recorded on full MS which is not significant from the value (0.38 g) of ½ MS. Although 0 strength MS medium produced the second value of shoot length (12.6 cm) it produced the lower values on both root length and seedling DW. The presence of large quantities of stored carbon, mineral elements and hormones in cotyledons may be responsible for the occurrence of germination in the hormones-free medium that also supports the growth and development of *A. sieberiana* seedlings. Further, the 0 strength MS medium resulted in early initiation of seed germination (lower MGT 2.5 days, Table 2) and seedlings grew fast, therefore, have long shoots.

Table 5. Effects of MS strengths* on seedling growth of *A. sieberiana* after three weeks of culture

MS strength	Shoot length (cm)	Root length (cm)	Seedling DW (g)
0	12.59^{a} (± 0.30)	4.59^{b} (± 0.38)	0.1679^{b} (± 0.02)
¼	11.73^{a} (± 0.41)	6.36^{b} (± 1.54)	0.258^{b} (± 0.04)
½	11.78^{a} (± 1.08)	13.43^{a} (± 1.38)	0.3775^{a} (± 0.05)
1	13.51^{a} (± 0.70)	10.23^{ab} (± 1.13)	0.4892^{a} (± 0.03)

Values are mean (± standard errors). Mean values within the column followed by the different letter are significantly different at the 0.05% probability level using Duncan's new multiple range test. DW: dry weight. * Sucrose applied at 3%.

Effect of sucrose on seedling growth

The current study revealed that increasing the concentration of sucrose in MS medium boosted the shoot length of *A. sieberiana* seedlings (Table 6). The significantly highest value on shoot length (14.2 cm) was obtained on 50 g/L. Improving shoot length with rising sucrose concentration was also reported on *Givotia rottleriformis* (Rambabu *et al.*, 2006). In the contrast, 30 g/L produced the highest shoot length on *A. seyal* (Altayeb, 2004). On the other hand, in the present study, using the concentration 30 g/L appear optimum for the root growth of *A. sieberiana* as the mean length recorded was (10.2 cm), however, it was not significantly different from other sucrose concentrations. Similarly, 30 g/L induced the maximum root length for *Begonia malabarica* (Murugan *et al.* 2016) and *Givotia rottleriformis* (Rambabu *et al.*, 2006). Our findings showed full MS medium with 50/L g sucrose has a better effect in terms of biomass allocation with the significantly higher DW value obtained was 0.51 g. Murugan *et al.* (2016) have been reported a similar result in *Begonia malabarica*. The presence of exogenous sucrose in the medium as the carbohydrate source led to control nutrient mobilization, hypocotyl elongation, cotyledon greening and expansion, and later shoot development and root growth (Roni *et al.*, 2017). Sucrose supplemented in the medium can trigger the carbon source and fix the CO2 at a high level inside the air-tight culture vessel during the photoperiod. The CO2-enriched conditions in a culture vessel enhance the photosynthesis of *in vitro* plantlets resulting in a high morphogenesis and growth rate of shoots and roots (Shin *et al.*, 2013).

Table 6. Effects of sucrose concentrations* on seedling growth of *A. sieberiana* after three weeks of culture

Sucrose (g/L)	Shoot length (cm)	Root length (cm)	Seedling DW (g)
10	8.73^{b} (± 1.53)	6.83^{a} (± 1.4)	0.4744^{b} (± 0.009)
20	10.2^{ab} (± 0.8)	9.03^{a} (± 1.72)	0.4773^{b} (± 0.005)
30	13.47^{a} (± 1.11)	10.19^{a} (± 0.93)	0.4968^{ab} (± 0.004)
40	13.49^{a} (± 0.85)	9.46^{a} (± 1.23)	0.4969^{ab} (± 0.01)
50	14.16^{a} (± 0.65)	8.03^{a} (± 1.2)	0.5131^{a} (± 0.012)

Values are mean ± standard errors. Mean values within the column followed by the different letter are significantly different at the 0.05% probability level using Duncan's new multiple range test. DW: dry weight. *Supplemented in full strength MS medium.

Effect of cytokinins on seedling growth

Generally, cytokinins decreased shoot and root lengths of seedlings (Table 7). MS medium free of cytokinins produced significantly the maximum shoot and root lengths (13.5 cm) and (10.3 cm), respectively. BA at 1.0 mg/L produced the significantly highest shoot length (10.3 cm) among all BA concentrations and overall TDZ treatments. This is in agreement with the result on *Grewia tenax* where cytokinins suppressed elongation of shoot (Daffalla *et al.*, 2016). Root length of *A. sieberiana* was declined remarkably with the application of cytokinins (Table 7) with only 1.9 cm length achieved by 1.0 mg/L TDZ. A parallel negative effect of cytokinins on the root growth of *in vitro* raised seedlings was also reported on *Drypetes roxburghii* (Murthy and Reddy, 2014) and *Prunus armeniaca* (Yildirim *et al.*, 2007). In the present study, augmenting the

germination medium with cytokinins disturb the development of the root system as secondary roots declined by TDZ (Figure 1A) and were absent in the BA medium (Figure 1B) compared to the control (Figure 1A), where a long taproot developed with a large number of secondary roots including branched tertiary roots.

Table 7. Effects of BA and TDZ in different concentrations (1-5 mg/L) on seedling growth of *A. sieberiana* after 3 weeks of culture

PGRs (mg/L)		Shoot length (cm)	Root length (cm)	Number of nods	Seedling DW (g)
Control		13.51^{a} (± 0.7)	10.27^{a} (± 1.38)	2.57^{b} (± 0.48)	0.484^{a} (± 0.04)
BA	1	10.27^{b} (± 1.34)	1.8^{b} (± 0.29)	2.86^{b} (± 0.94)	0.477^{a} (± 0.01)
	2	8.16^{bc} (± 1.08)	1.73^{b} (± 0.34)	4.71^{ab} (± 0.71)	0.539^{a} (± 0.01)
	3	7.73^{bc} (± 0.52)	1.36^{b} (± 0.07)	4.29^{b} (± 0.71)	0.514^{a} (± 0.01)
	4	7.6^{bc} (± 0.95)	1.34^{b} (± 0.25)	4.14^{b} (± 0.99)	0.513^{a} (± 0.00)
	5	6.8^{c} (± 0.78)	1.04^{b} (± 0.6)	3.29^{b} (± 0.68)	0.504^{a} (± 0.03)
TDZ	1	6.97^{c} (± 0.82)	1.89^{b} (± 0.25)	4.43^{b} (± 0.48)	0.482^{a} (± 0.01)
	2	5.49^{cd} (± 0.32)	1.84^{b} (± 0.1)	5.14^{ab} (± 0.86)	0.504^{a} (± 0.02)
	3	5.41^{cd} (± 0.63)	1.81^{b} (± 0.08)	5.86^{ab} (± 1.01)	0.510^{a} (± 0.03)
	4	5.24^{cd} (± 0.47)	1.8^{b} (± 0.11)	6.86^{a} (± 0.4)	0.505^{a} (± 0.01)
	5	3.69^{d} (± 0.93)	0.69^{b} (± 0.2)	2.71^{b} (± 0.81)	0.204^{b} (± 0.06)

Values are mean (± standard errors). Mean values within the column followed by the different letter are significantly different at the 0.05% probability level using Duncan's new multiple range test. DW: dry weight.

Figure 1. Effects of cytokinins on seedling development of *A. sieberiana* after 21 days of *in vitro* culture
A) Seedling on TDZ (1.0 mg/L) medium developed few numbers of short secondary roots, B) seedling on BA (1.0 mg/L) medium showed no secondary roots, C) Seedling on cytokinin-free MS media (control) showed development of primary root with the formation of secondary roots.

The present investigation also highlighted different morphogenic responses of the developed seedlings. The addition of various levels of cytokinins has stimulated the formation of nodes on the emergent seedlings. The significantly maximum number of nodes obtained was 6.9 nodes on MS medium supplemented with 4 mg/L TDZ compared to 4.7 nodes with 2.0 mg/L BA and 2.6 nodes on the control treatment. On the opposite, the application of cytokinins reduced the number of nodes in *Grewia tenax* shoots (Daffalla *et al.*, 2016). Seedlings with a high number of nodes were very useful for micropropagation of a plant as each node produced auxiliary bud can utilized for multiple shoot induction. During this study, the highest DW of (0.54 g) was observed on seedling grew MS medium supplemented with 2.0 mg/L BA while (0.51 g) was obtained on 3.0 mg/L TDZ and (0.48 g) on the control. Cytokinins can affect the activities of meristemic cells in roots and

shoots. The cytokinins can regulate different functions related to the development and physiology of seedlings such as hypocotyls and shoot growth, root growth, nutrient uptake, and handling stress (Miransari and Smith, 2014).

Conclusions

The overall results of the present investigation can be concluded to define optimal conditions for *in vitro* germination and plantlets development of *Acacia sieberiana*. The best percentage of germination was recorded by seeds treated with 5% of sodium hypochlorite, and germination declined with increased concentration. Although water agar medium recorded the fast germination rate and gave a reasonable high germination percentage, full MS was the best since the maximum germination percentage was obtained. Also, water distilled water (0 MS) medium, which is characterized with low cost, can be used to maintain the growth of *Acacia sieberiana* seedlings *in vitro* for three weeks or more suitable for commercialization purposes. That was proved by the outcome of using 3% sucrose which gave the highest germination. In addition, TDZ improved germination rate and percentage over the full MS medium. Regarding seedling development, full MS improved shoot length and dry weight of seedling, and half MS enhanced root growth. Sucrose at 3% favors root length while at 5% improved shoot length and seedling dry weight. Using cytokinins (BA, TDZ) supplemented in MS in high concentrations generally decreased the shoot and root lengths of seedlings, except TDZ at 4 mg/L which improved the number of nodes. Thus, this protocol can be used as a base for mass production and genetic improvement aiming at conservation of the species and for afforestation programs.

Authors' Contributions

Data analysis and editing the manuscript: HMD, Supervision: KSA, Conceptualization: MGO, Laboratory work and writing original draft: YOY. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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