Optimization of germination, callus induction, and cell suspension culture of African locust beans *Parkia biglobosa* (Jacq.) Benth

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**Abstract**

The present study was carried out to determine the best pre-sowing treatments that can enhance the germination and seedling growth of *Parkia biglobosa* (Jacq.) Also, to establish and long-term maintenance of calli and cell suspension cultures. The result of various pre-sowing treatments showed that seeds soaked in concentrated H$_2$SO$_4$ treatment appeared the highest germination percentage, higher value of plant height, number of leaves, number of branches and stem girth. The MS medium containing 1mg/l 2, 4-D was the best for callus induction of stem explants. The addition of 50 mg/l citric acid to the MS medium was effective for reducing browning of callus than other treatments. However, the viability percent recorded the maximum (87.76%) on the 9th day while the concentration of viable cells per ml reached the higher record (137.5 viable cell/ml) at the 12th and cell viability remains (> 68.39%) throughout 18 days of culture.

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**1. Introduction**

*Parkia biglobosa* is a perennial deciduous tree with a very broad crown that may reach a height of 20 m. The species grows under a wide range of conditions, where annual rainfall ranges from 600 to 1500 mm and the dry season last 5 to 7 months. *Parkia biglobosa* belongs to the family of Fabaceae subfamily Mimosoideae and genus Parkia known as néré in Francophone Africa, and locust bean tree in Anglophone Africa is a multipurpose tree indigenous to sub-Saharan Africa [2]. The genus parkia belongs to the tribe parkieae. It consists of about 35 species with a pantropical distribution [3]. Only 3 species all belonging to the section parkia, occur in continental Africa and the fourth one in Madagascar [4] two of these species are found in Nigeria these are *Parkia bicolor* A. Chev. and *Parkia biglobosa* (jacq.) Benth. *Parkia biglobosa* tree has been used traditionally as food and medicine and high commercial value in the West African region. The plant contains carbohydrates, fats, minerals, vitamins, and tannins and flavonoids. *Parkia biglobosa* possesses antimalarial, antihelminthic, antibacterial, antivenom, antidiabetic and antioxidant properties [5]. Recently, there was a noticeable decrease in the population of *Parkia biglobosa* in natural forests of West Africa due to the small percentage of the seeds produced and germinated in the field leading to the low population of the crop in the savannah. The dormancy of these seeds has been attributed to the presence of endogenous inhibitor as well as the impermeable seed coat [6]. Therefore, extinction studies on seed germination is a preliminary step in its conservation. Different treatment for enhancing the seeds germination of *parkia biglobosa* were performed. Prior treatment of seeds with H$_2$SO$_4$, wet heat and mechanical scarification was found to induce germination of the dormant seeds. These methods could be applied to raise seedlings of the plant for field propagation [7]. However, seedlings of this important plant are rarely seen growing in the wild, so it can consider as a rare plant. The existing trees are aging and fast disappearing [8]. Therefore, it is an urgent need to develop suitable strategies for conservation programs with sustainable exploitation. Recently plant tissue culture techniques and biotechnological tools rapidly succeeded in mass propagation and in vitro conservation of rare or endangered medicinal plants [9–11]. Plant tissue culture refers to growing and multiplying of cells, tissues, and organs of the plant on defined solid or liquid media under the aseptic and controlled environment [12]. The frequency of callus formation in tissue culture is influenced by many factors such as culture medium composition, explants source, genotype, and
environment, etc change to genotype, and environment. Liquid media culture system considered a good supply source of uniform cells in large scale compared to the common callus culture with the long procedures. The cell suspension remains undifferentiated with a short growth cycle, which is grown under tightly controlled environmental conditions, thus increasing reproducibility within and between experiments [15]. Using in vitro culture techniques, the rapid growth of callus and cell suspension culture from which secondary metabolites are to be extracted can be obtained [16].

Few studies have been carried out on Parkia biglobosa to enhance callus induction and tested the factor that affecting callus induction or solvingrowning problems of callus. Also, no cell suspension culture studies have been reported on this multipurpose, endangered woody plant. Therefore, the aim of this study was to determine the best pre-sowing treatment that can enhance the germination percentage and seedling growth of Parkia biglobosa at nursery stages under greenhouse condition. Also, to establish and long-term maintenance of calli and cell suspension cultures in order to be utilized as an efficient tool for various studies in Parkia biglobosa for pharmaceutical and/or industrial future applications.

2. Materials and methods

This study was conducted at the Experimental Laboratories of the Natural Resources Department, Institute of African Research and Studies, Cairo University, and Plant Biotechnology Department, National Research Center, Cairo, Egypt during the period from 2014 to 2017. The Seeds were extracted from matured bud of Parkia biglobosa tree which collected from different locations of Nigeria by Dr. Nasir Hassan Wagini, Department of Biology, Faculty of Natural and Applied Sciences, Umaru Musa Yar’adua University, Katsina, Nigeria.

2.1. Pre-sowing treatments

Pre-sowing was subjected to different treatments, which includes:

- Acid treatment (A): The seeds were randomly selected and soaked in concentrated H₂SO₄ (sulfuric acid) for 5 min. The seeds were washed with distilled water to remove any trace of acid and air-dried before sowing according to Aleiro [7].
- Mechanical treatment (B): The selected seeds were rubbed against the rough surface of the sandpaper until the slight exposure of the cotyledon of the seed according to Okunlola et al. [22].
- Hot water treatment (C): The seeds treated were soaked in 100 °C hot water for about two minutes and air-dried before sowing according to Aleiro [7].
- Cold treatment (D): The seeds were kept in cold water for 24 h at room temperature and air-dried before sowing according to Okunlola et al. [22].
- Control (E): The seeds were sown without any treatment.

The treated seeds and the seeds without any treatment (control) were sown in poly pots filled with a mixture of clay, sand and peat moss (1:1:1), two seeds per pot in four replicates with 10 seeds for each replication. Watering and observation were done on a daily basis for 42 days from the date of sowing and the final germination, the seeds were watered at 3–5 days interval and weeding was done manually by hand pulling.

Data Analysis:

- Germination rate (%): was evaluated according to the following equation [23]:
  \[
  \text{Germination percentage} = \left( \frac{\text{Number of germinated seeds}}{\text{Number of planted seeds}} \right) \times 100
  \]

The seedling vigor index (SVI): was calculated according to [24]:

\[
\text{SVI} = (\text{seedling length (cm)} \times \text{germinated percentage})/100.
\]

Growth parameters: (seedling height (cm), Numbers of leaves, numbers of branches, stem girth (cm).)

2.2. In vitro seed germination under sterilization conditions

Batches of 48 Parkia biglobosa seeds were scarified by (98%) H₂SO₄ for 5 min, then washed with sterile distilled water along with a 0.05% of detergent for 15 min then rinsed with sterile distilled water 5 times. Seeds were surface sterilized with (70%) ethanol alcohol for 3 min and rinsed 5 times with sterile distilled water. Then the seeds were immersed in different concentrations of commercial Clorox solution (contains 5.25% sodium hypochlorite) as 30%, 60% and 90% for different times as 15 or 25 min. Subsequently, seeds were washed five times with sterile distilled water and then sterilized seeds were cultured on sold full strength of MS basal medium containing 3% sucrose, and 0.2% gerlite. The pH of all used media was adjusted to 5.8 using 0.1 N KOH or HCl upon high or low before autoclaving. The media were distributed into 250 ml glass jar, where each jar contained 50 ml and sterilized by autoclaving for 24 min at 121 °C and 1.2 kg/cm². After culturing jars were incubated in illumination light condition at 25 ± 1 °C for 16/8 h day photoperiod. Each treatment considered of 4 replicates (jars) and each replicate contained two seeds. After two weeks of culturing contamination (%) and survival (%) were estimated.

2.3. Callus induction

2.3.1. Effects of different concentrations of plant growth regulators on callus induction from stem root and leaf tissue

For callus induction, twelve types of callus media were used; each medium was supplemented with 3% sucrose, 0.2% gerlite and 2, 4-D (2, 4-Dichlorophenoxyacetic acid) as follow (0.0, 0.5, 1.0, 1.5 mg/l) alone or in combination with kinetin as follow (0.0, 0.5, 0.8 mg/l). Stem, root and leaf segments about (0.5 to 1.0 cm) long were excised from two-weeks-old seedlings and cultured in jars containing 30 ml of callus induction medium. For each medium type consisted of 4 replicates (jars) and each replicate contained 4 explants. Cultures of all different types of callus media were incubated in a growth chamber at 25 ± 1 °C and exposed to 16/8 h day photoperiod-controlled automatically from the white cool light of fluorescent lamps cultured for 8 weeks.

2.3.2. Effects of different types of nutrient media on callus induction from stem tissue

To study the effect of different types of nutrient media on callus induction. Explants (stem) were cultured with the following: MS medium (Murashige and Skoog) medium [25], B5 medium (Gamborg medium) [26], MS salts including B5 vitamins, WPM (Woody Plant Medium) [27]. At a full salt strength of solid medium containing 3% sucrose and 0.2% gerlite, all culture media were supplemented with 1 mg/l 2, 4-D each treatment consisted of 4 replicates. Incubation of all media was carried out as described in the establishment stage of callus as mentioned before.

2.3.3. Maintenance of Parkia biglobosa callus

Sterilized stem segments of the plant were taken from in vitro growing plantlets and cut into pieces (0.5–1.0 cm), then cultured on MS medium containing 3% sucrose, 0.2% gerlite and 2, 4-D supplemented with different concentrations of Ascorbic acid (0.0, 25, 50 mg/l) and citric acid (0.0, 25, 50 mg/l) as antioxidants alone or in combination with 0.5 g activated charcoal. Preparation and incubation of all media were carried out as described in the establish-
ment stage of callus. This experiment consisted of ten treatments, each treatment contains 4 replicates.

2.3.4. Establishment of Parkia biglobosa cell suspension culture

For initiation of suspension culture, 0.5 g of friable callus derived from stem explants was transferred to Erlenmeyer flasks (250 ml) each containing 50 ml liquid MS medium supplemented with 1.0 mg/l 2, 4-D, 3%w/v of sucrose. PH of the medium was adjusted to 5.8 before autoclaving the medium at 120 °C for 20 min. The flasks were agitated at 120 rpm on a gyratory shaker and incubated at 25 °C under continuous low light [28,29]. Cultures were maintained for 18 days. The percentage of viable cells and concentration of viable cells per ml were determined.

2.3.5. Statistical analysis

The layout of the experiment was arranged in a randomized complete blocks design, with 4 blocks (replicates), and the resulted data were subjected to statistical analysis, employing F-test for significance at P ≤ .05 and computing of LSD values to separate means in different statistical groups according to the described method by Gomez and Gomez [30].

3. Results and discussion

3.1. The effect of pre-sowing treatments on germination and seedling growth of (Parkia biglobosa) seeds under greenhouse conditions

3.1.1. Germination indices

3.1.1.1. Germination percentage. Evaluation of germination under different treatments showed substantial variation in germination percentage at 42 days after sowing there was a significant difference (P ≤ .05) in germination percentage between different treatments. However, the data in Table 1 indicated that the maximum germination percentage (100 and 100%) were noticed in seeds treated with concentrated H2SO4 for five minutes, followed by seeds mechanically scarified with sandpaper had germination of (87.5 and 87.5%) without significant difference in first and second seasons, respectively. On the contrary, the minimum percentages (62.5 and 62.5%) had recorded in cold treatment; while seeds treated with hot water had no germination. The differences observed in the germination percentage of seeds subjected to the different treatments imply a significant impact of the various pre-treatments on breaking the seed dormancy.

Dormancy in seeds is usually associated with the factors of the protective covering the seed coat or the enclosed embryo. From the investigation carried out, such treatment as hot water and soaking in cold water, mechanical scarification and application of concentrated H2SO4 were found to induce germination of seeds of Parkia biglobosa.

From the above one can infer that dormancy of the seeds of Parkia biglobosa was probably associated with the seed coat since the treatment that induces germination were those that can effect disruption of the seed coat. A little different between two seasons may be due to climatic conditions in the first and second season but all this differences are insignificant effect. This obtained result goes in line with those findings by Aleiro [7] who revealed that using concentrated H2SO4 achieved the maximum percentage of germination of Parkia biglobosa seed through the short period, which leads to the rupture of the seed coat and enhances the germination rate. Consequently, Ren and Tao [31] reported that the seeds of Calligonum species treated with concentrated H2SO4 showed the highest germination percentage as a result of its desiccant effect on the seed coat allowing easier water uptake and oxygen diffusion.

Agbogid et al. [32] concluded that treatment of the seeds of Dacryodes edulis with concentrated H2SO4 is considered the best method for inducing the germination of the seeds. Also, Ayisire et al. [33] mentioned that using concentrated H2SO4 is necessary for germination of many seed species and recording the highest germinating percentage.

The mechanical scarification by sandpaper had the highest speed of germination, which agrees with the report of Tomlinson et al. [34] who affirmed that mechanical scarification of seed is the most effective way of improving the permeability of the seed coat and overcome seed dormancy. Also, our results are in agreement with Okunola et al. [22] mentioned that the seeds of Parkia biglobosa mechanically scarified by sand paper improved seed germination and seedling growth.

The seeds of Parkia biglobosa treated with hot water showed no germination percentage as a result of its effect on the seed coat that must have ruptured or damaged the seed's embryo. Supportive results were obtained by Okunomo [35] mentioned that the seeds of Parkia bicolor failed to germinate by soaking in hot water due to probably heat shock to the embryo of the seeds. Hence, Abubakar and Mainmanu [8] reported that soaking Parkia biglobosa seeds in hot water for more than 4 s did not improve germination. They reversed the decrease of germination to the effect of heat of the boiling water that leads to the damage of seed embryo.

Our results disagree with findings of Agboola and Adedire [36] and Sabongari [37] who reported that sudden dip of dry seed in boiling water may lead to the rupture of the seed coat allowing water to permeate the tissues causing physiological changes and subsequent germination of the embryo. Also, Nasr et al. [38] found that immersion of Acacia nilotica seeds in boiling water gave significantly higher germination compared with the control.

The seeds of Parkia biglobosa treated with cold water for 24 h increased in germination percentage with time and at 42 days had attained (62.5%) germination. Generally, these results are in accordance with those obtained by Owohobi et al. [39] who found that soaking of Azadirachta indica seeds for 24 h in the cold water, enhancing the rate of seed germination and they mentioned that different species have the variable rate at which seed coat is

| Treatments | Germination (%) | Plant height (cm) | No. of leaves | No. of branches | Stem girth (cm) | Seedling vigor index |
|------------|----------------|------------------|---------------|----------------|----------------|---------------------|
|            | 1st            | 2nd             | 1st           | 2nd           | 1st            | 2nd                | 1st                | 2nd              |
| Control    | 75.00          | 62.50           | 9.25          | 12.00         | 3.75           | 5.50               | 1.75               | 1.75             | 7.63           | 0.30           | 7.63               | 7.88             |
| Acid       | 100.00         | 100.00          | 16.75         | 16.25         | 8.50           | 8.50               | 2.50               | 2.50             | 0.50           | 0.45           | 16.75             | 16.25             |
| Mechanical | 87.50          | 87.50           | 14.50         | 16.00         | 6.50           | 7.75               | 1.75               | 2.25             | 0.40           | 0.38           | 13.50             | 14.75             |
| Hot water  | 0.00           | 0.00            | 0.00          | 0.00          | 0.00           | 0.00               | 0.00               | 0.00             | 0.00           | 0.00           | 0.00               | 0.00               |
| Cold       | 62.50          | 62.50           | 8.50          | 11.50         | 2.00           | 6.00               | 1.50               | 2.00             | 0.20           | 0.30           | 7.00               | 7.75               |
| Mean       | 65.00          | 62.50           | 9.80          | 11.15         | 4.15           | 5.55               | 1.50               | 1.70             | 0.29           | 0.29           | 8.98               | 9.33               |
| LSD 0.05   | 41.60          | 28.99           | 4.08          | 4.08          | 1.27           | 1.27               | 0.54               | 0.54             | 0.07           | 0.07           | 6.68               | 6.68               |

Each treatment was the average of 4 replicates.
permeable to water and gas. Also, Okunlola et al. [22] reported that the seeds of *Parkia biglobosa* treated with cold water for 24 h increased in germination percentage with time and at 35 days had attained with 100% germination. On the contrary, germination percentage decreased with an increase in hours of soaking in cold water from (28% to 24%) at 12 and 24 h, respectively. This confirms to findings of Otegbeye and Momodu [40] that over soaking *Parkia biglobosa* seeds in water may reduce germination through oxygen deficiency. The seeds under control experiment gave similarly high germination but seeds took a longer period to germinate this agreed with Aleiro [7] and Okunlola et al. [22].

### 3.2. Seedling vigor index

Data presented in Table 1 and illustrated in Fig. 1 showed that significant differences at the 5% level among different treatments applied. However, the highest seedling vigor index (16.75) was recorded in seeds treated with concentrated H$_2$SO$_4$ in the first season at 42 days. While; the lowest seedling vigor index (7.0) was recorded in seeds treated with cold water in the first season at 42 days.

The positive effects of acid and mechanical scarification on seed germination may be due to the breaking of seed dormancy, improving seed coat impermeability and increased imbibitions of seeds. These results are in agreement with those obtained by Miranda et al. [41] and Sucande and Clethero [42] who reported that H$_2$SO$_4$ may create or enlarge pores in the seed, enabling water to enter the seed and directly contact the embryo and thus accelerate the germination process. Okunlola et al. [22] found that the mechanical scarification of the seeds of *Parkia biglobosa* may be effective for breaking dormancy and improving the seedling vigor.

![A](https://via.placeholder.com/150)

**A**. Germination of seeds of *Parkia biglobosa* treated with cold water. (B) Germination of seeds of *Parkia biglobosa* treated with concentrated H$_2$SO$_4$.

### 3.3. Growth parameters

**Plant height (cm)**: significant differences (P < .05) were observed among different treatments applied. The results in Table 1 revealed that the taller plants (16.75 and 16.25 cm) were obtained in seeds treated with concentrated H$_2$SO$_4$ and mechanical scarification respectively, in the first and second season at 42 days. While the shortest plants (9.25 and 8.5 cm) were obtained in control and seeds treated with cold water respectively, in the first season at 42 days.

The variation among plant heights can be attributed to the effect of the treatments the seeds were subjected to. The obtained results agreed with the report of Agbogidi et al. [32] who demonstrated that acid pre-treatment of *Dacryodes edulis* has a highly significant effect of improving seed viability and enhances seedling emergence and seedling growth.

From the results, it is evident that seedlings raised from seeds soaked in concentrated H$_2$SO$_4$ had the best plant height, which agrees with the result of El-Juhany et al. [43] who found that treating of *Juniperus procera* seeds with concentrated H$_2$SO$_4$ gave the best plant height.

**A number of leaves**: Overall results showed significant differences at the 5% level among different treatments applied. The data in Table 1 showed that the maximum numbers of leaves (8.5, 8.5) in the first and second season were recorded with concentrated H$_2$SO$_4$ treatment followed by mechanical scarification (6.5 and 7.75) in the first and second season respectively at 42 days of seedling growth. While the minimum number of leaves was obtained with control treatment (3.75) followed by (2.0) with cold water treatment in the first season at 42 days.

The seeds are treated with concentrated H$_2$SO$_4$ or mechanical scarification performed much better than cold and untreated seeds in terms of number of leaves our results are in accordance with those obtained by Mabundza et al. [44] concluded that *Tamarindus indica* L seeds treated with concentrated H$_2$SO$_4$ for 5 min, accelerated germination of the seeds and number of leaves.

**Number of branches**: number of branches were differed significantly (P < .05) between various treatments. A maximum number of branches per plant (2.5 and 2.5) was recorded with the treatment of concentrated H$_2$SO$_4$ in the first and second season followed by mechanical scarification and control respectively at 42 days as shown in (Table 1).

Seeding with the highest number of branches may be as a result of the early germination of the seedlings induced by the methods of dormancy breakage. These results are in agreement with those obtained by Abubakar and Maimuna [8] and Okunlola et al. [22] they concluded that concentrated acid and mechanical scarification treatments were the most successful treatments for enhancing seed germination and the number of branches of *Parkia biglobosa*.

**Stem girth (cm)**: Stem girth of seedlings was significantly (P ≤ .05) influenced by different pre-sowing treatments (Table 1) seedlings originated from seeds treated with H$_2$SO$_4$ 5 min had the highest mean stem girth value of (0.5 and 0.45 cm) followed by mechanical scarification (0.4 and 0.38 cm) while the lowest mean stem girth value of (0.20 and 0.30 cm) was obtained among seedlings under cold treatment, all the above recorded in the first and second season at the end of 42 days.

The seedlings from seed treated with concentrated H$_2$SO$_4$ and cold water treatment differed significantly in terms of stem girth. This can be attributed to the effect of concentrated H$_2$SO$_4$ treatment the seeds were subjected to; seeding with stem girth may be as a result of the early germination of the seedlings induced by this method of dormancy breakage. The general trends of the results obtained agree with that reported by El-Juhany et al. [45] and Olatunji et al. [45] who found that seeds treated with concen-
trated H$_2$SO$_4$ would enhance the germination percentage and gave more stem girth than other seed treatments.

3.4. Conditions of seed germination and sterilization

Sterilization of seeds in the establishment stage is a crucial step in plant tissue culture to find out the best concentration and exposing time of the sterilization agent which lead to minimum contamination and highest survival percentage. *Parkia biglobosa* seeds were treated with different concentrations of sodium hypochlorite solution (5.25%) from 30 to 90% for 15 and 25 min the seed contamination and survival percentages were recorded.

Concerning contamination, overall results showed significant differences at the 5% level among applied treatments. However, data in Table 2 revealed that the treatment of 60% of sodium hypochlorite solution for 25 min showed the best results with 0.0% contamination, while the treatment of 30% of sodium hypochlorite solution for 15 min recorded the highest contamination percentage (87.5%). This means that there is an inverse correlation between contamination percentage and sodium hypochlorite solution concentration and sterilization time.

Concerning survival, there were significant differences among treatments as tabulated in Table 2 the best treatment was 60% of sodium hypochlorite solution for 25 min, which showed the maximum survival percent (100%) in Fig. 2. In contrast, the minimum survival percentage (12.5%) was shown with 30% of sodium hypochlorite solution for 15 min and 90% of sodium hypochlorite solution for 25 min survival percentage was increased using 60% of sodium hypochlorite solution for 15 to 25 min, where it was 37.5% and 100% respectively. However, the survival percentage was decreased from 25% to 12.5% for 90% of sodium hypochlorite solution for 15 min to 90% of sodium hypochlorite solution for 25 min, respectively. The obtained results showed that the best

![Table 2](#)

### Effects of different concentrations of sodium hypochlorite solution (5.25%) at various duration on contamination and survival percentages after 15 days of culturing *Parkia biglobosa* seeds on full MS basal medium at 25 ± 1 °C for 16/8 h day light.

| Treatments | Contamination (%) | Survival (%) |
|------------|-------------------|--------------|
| Clorox% Times (min) | | |
| 30 | 15 | 87.50 | 12.50 |
| 30 | 25 | 50.00 | 25.00 |
| 60 | 15 | 50.00 | 37.50 |
| 60 | 25 | 0.00 | 100.00 |
| 90 | 15 | 37.50 | 25.00 |
| 90 | 25 | 25.00 | 12.50 |
| Mean | | 41.66 | 35.41 |
| LSD 0.05 | | 48.32 | 46.83 |

*Each treatment was the average of 4 replicates and each jar contain 2 seeds.*

![Fig. 2](#)

(A)-Seeds of *Parkia biglobosa* treated with concentrated sulfuric. (B)-Germination of seeds of *Parkia biglobosa* scarified to the concentrated sulfuric during 5 min, the seedling is achieved 15 days after setting in culture: all seeds germinated. (C)-the seedling is achieved 30 days after setting in culture.
disinfectant and highly survival treatments were 60% of sodium hypochlorite solution for 25 min, the similar observation has also been reported that hypochlorite is known to be a very effective killer of bacteria and reducing bacterial populations [46]. Several reports indicated that sodium hypochlorite was found to be more effective disinfectant for sterilizing seeds [17,20,47,48].

3.5. Callus induction

3.5.1. Effects of different concentrations of plant growth regulators on callus induction from stem root and leaf explants

**Percentage of callus production (%):** Effect of MS medium supplemented with different concentrations of 2, 4-D and kinetin on callus induction from the stem, root and leaf explants of *Parkia biglobosa* was investigated as recorded in Table 3 and Fig. 3. The results indicate that the combination of 2, 4-D and kinetin was effective to satisfactorily induce calli from stem explants of *Parkia biglobosa*. The highest callus production was recorded for stem followed by root explants while leaf explants showed no callus production. Stem explants cultured on MS medium supplemented with 1 mg/l 2, 4-D was the best for *Parkia biglobosa* callus production.

This result agrees with the findings of Kumari and Pandey [49] in which they observed that the addition of different concentrations of 2, 4-D from 0.5 to 5 mg/l has a stimulative effect on callus formation of *Carthamus tinctorius*. Also, Ebrahimi and Payan [50] reported similar observations that callus was initiated from cotyledon explants of *Fagonia indica* Burm on MS medium supplemented with 1–5 mg/l 2, 4-D.

Also, these results were in agreement with Amoo and Ayisire [17] who mentioned that callus production was successful in the presence of 2, 4-D alone, highest (100%) of callus induction was observed with MS medium supplemented with 2, 4-D (1.0 and 2.0 mg/l) and suggested that cotyledon explants of *Parkia biglobosa* were auxin-specific. While in the absence of kinetin, the cultures containing corresponding concentrations of 2, 4-D showed higher callus formation. This phenomenon suggests that 2, 4-D played a more important role in callus formation from stem explants compared to kin. However, the combination of 2, 4-D and kinetin were found to produce more calls than kinetin alone. In the treatment recorded in Table 3, one combination containing 1.5 mg/l 2,4-D and 0.8 mg/l kinetin, was proved to be the most efficient in promoting callus development from stem explants with (93.75%) followed by (56.25%) from root explants of *Parkia biglobosa*. While, the lowest amount of callus induction (50%) from stem explants on the medium supplemented with 0.5 mg/l 2, 4-D and 0.5 mg/l 2, 4-D.

| Treatments | Callus (%) | Fresh weight (g) | Dry weight (g) | Dry matter (%) |
|------------|------------|------------------|----------------|---------------|
| 2,4-D (mg/l) | Kin. (mg/l) | Stem | Root | Stem | Root | Stem | Root | Stem | Root |
| 0 | 0 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 0.5 | 0 | 93.75 | 75.00 | 1.53 | 0.66 | 0.17 | 0.06 | 11.27 | 8.74 |
| 1 | 0 | 100.00 | 81.25 | 4.04 | 1.47 | 0.39 | 0.13 | 9.61 | 7.18 |
| 1.5 | 0 | 50.00 | 68.75 | 1.99 | 0.30 | 0.21 | 0.05 | 15.09 | 17.03 |
| 0 | 0.5 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 0.5 | 0.5 | 50.00 | 68.75 | 1.99 | 0.30 | 0.21 | 0.05 | 15.09 | 17.03 |
| 1 | 0.5 | 68.75 | 68.75 | 2.62 | 0.00 | 0.23 | 0.00 | 10.62 | 0.00 |
| 1.5 | 0.5 | 75.00 | 75.00 | 0.87 | 0.00 | 0.10 | 0.00 | 11.67 | 0.00 |
| 0 | 0.8 | 43.75 | 37.50 | 0.88 | 0.32 | 0.16 | 0.01 | 20.79 | 10.91 |
| 0.5 | 0.8 | 75.00 | 62.50 | 1.07 | 0.42 | 0.14 | 0.04 | 14.90 | 8.44 |
| 1 | 0.8 | 75.00 | 43.75 | 1.51 | 0.23 | 0.15 | 0.03 | 10.95 | 17.94 |
| 1.5 | 0.8 | 93.75 | 56.25 | 2.77 | 0.49 | 0.34 | 0.07 | 12.28 | 14.00 |
| Mean | 60.24 | 35.42 | 1.59 | 0.32 | 0.17 | 0.034 | 10.69 | 7.02 |
| LSD 0.05 | 27.50 | 25.81 | 1.12 | 8.99 | 0.09 | 0.10 | 7.20 | 6.84 |

Each treatment was the average of 4 replicates and each jar contains 4 explants.

**Fig. 3.** Effects of growth regulators and explants on callus induction and growth, (A)-callus induction and growth from *Parkia biglobosa* root explants on MS medium supplemented with 2, 4-D (1.0 mg/l) after 8 weeks, (B)- callus induction and growth from *Parkia biglobosa* stem explants on MS medium supplemented with 2, 4-D (1.0 mg/l) after 8 weeks.
kinetin, while root explants showed no callus formation. A similar result was reported by Hou and Jia [51] who observed that 2 mg/l 2, 4-D and 1 mg/l kinetin could induce the high frequency of calli from Aegagrostis lanata. hypocotyle and stem explants. The same trend observed by Varalaxmi et al. [52] who found that calli were induced from cotyledon explants on MS basal medium supplemented with 2, 4-D combined with Kinetin or BAP. Moreover, Osman et al. [53] revealed that the presence of 2, 4-D in the culture media was essentially required to induce callus formation in Barringtonia racemosa L. even though the cytokinin was absent. The effectiveness of 2, 4-D in inducing the formation of calli is attributed to its main characteristic which can stimulate cell division of plant tissues and strongly suppress organogenesis. By looking at the trend of callus formation in this study, an increasing induction percentage was noted associated with an increase in kinetin concentration supplemented together with 2, 4-D hormone at the concentration of 1.0 and 1.5 mg/L. In contrast, Konate et al. [54] studied the effect of adding different concentrations of cytokinins alone and in combination with 2, 4-D was shown to decrease the induction of callus. This may be attributed to an antagonist effect between the cytokinins and the 2, 4-D in callus induction.

3.5.2. Fresh, dry weight (g/jar) and dry matter (%) of produced callus

Overall results showed significant differences at the 5% level among different treatments applied. However, data in Table 3 revealed that the highest fresh and dry weight of callus culture derived from a stem and root explants of Parkia biglobosa plantlets recorded (4.04, 0.39 and 1.47, 0.13 g/jar) respectively after 8 weeks of culturing on MS medium supplemented with 1 mg/l 2, 4-D. When the amount of 2, 4-D increased to 1.5 mg/l in the culture medium the rate of the fresh and dry weight of callus was decreased to (1.99, 0.21 and 0.30, 0.05 g/jar). However, MS medium supplemented with 1 mg/l 2, 4-D + 0.8 mg/l kinetin. Showed the lowest values of fresh and dry weight (1.51, 0.15 and 0.23, 0.03 g/jar), respectively. While the MS basal medium or with 0.5 mg/l kinetin recorded negative results for all used explants. So, the obtained results indicated that stem explants produced the maximum fresh and dry weight of callus when cultured on MS medium supplemented with 1 mg/l 2, 4-D, while the fresh and dry weight of callus derived from root explants recorded the minimum value when cultured on (1 mg/l 2, 4-D + 0.8 mg/l kinetin). Moreover, we can be observed the increasing of the amount of 2, 4-D added to the medium over 1 mg/L would result in reducing the rate of callus formation, these findings are also confirmed by Abdel-Rahman [55] who found that the remarkable increase in average fresh weight of Cissia bicapsularis L. calli was obtained by an increase in MS medium containing 1 mg/l 2, 4-D alone. Also, Al-Ajouni et al. [56] reported that when the amount of 2, 4-D was 1 mg/l in the culture medium of Barely (Hordeum vulgare L.) genotype ‘Mari’, the fresh weight of callus was increased then reduced when the amount of 2, 4-D was 2 mg/l. However, Osman et al. [53] demonstrated that callus culture initiated from endosperm explants of Barringtonia racemosa L. were maintained in WPM media supplemented with 1 mg/l 2, 4-D gave the highest fresh weight of callus. This in contrast to the observation by Mini and Sankaranarayanan [57] who found that MS medium supplemented with 3 mg/l 2, 4-D gave the highest amount of callus fresh weight of Saraca indica culture. Dry matter (%) significantly changed at the 5% level between different treatments correlated to fresh and dry weight of callus, data in Table 3 showed the percentage of dry matter content of callus derived from stem and root explants of Parkia biglobosa which recorded (20.79 and 10.91%), respectively after 8 weeks of culturing on MS medium supplemented with 0.8 mg/l kinetin. However, MS supplemented with 1 mg/l 2, 4-D showed the lowest values of dry matter contents (9.61 and 8.43%), respectively. So, the results concluded that the maximum percentage of dry matter content was recorded with stem explants when the culture in MS medium supplemented with 0.8 mg/l kinetin. Whereas, the minimum value was recorded when cultured on MS medium supplemented with 1 mg/l 2, 4-D with root explants. While the MS basal medium without growth regulators or with 0.5 mg/l kinetin alone recorded negative results for all over the experiment.

3.5.3. Effects of different media types on callus induction from stem tissue

Data in (Table 4) clearly showed that there were significant differences between treatments. In Fig. 4, the best medium for callus other growth parameters was MS medium which recorded (100%) of callus production, 4.04 g/jar fresh weight, and 0.39 g/jar dry weight. MS + B5 medium recorded 81.25% of callus production, 1.19 g/jar fresh weight and 0.16 g/jar dry weight. While B5 medium recorded low values (75%) of callus production, 0.94 g/jar fresh weight, and 0.13 g/jar dry weight. Moreover, the control and WPM showed no callus production during the culture period. The stem explants producing calli in the MS medium fortified with 1 mg/l 2, 4-D was significantly higher than other media types. The obtained results are in agreement with Thangjam and Maibam [58] they reported that the ability of Parkia timoriana callus induction was recorded the heights (100%) in MS medium supplemented with 2, 4-D (1.0 and 2.0 mg/l) than B5 medium supplemented with the same concentration of 2, 4-D. In contrast, Yan et al. [59] found that the highest callus induction from basal plates of Chinese jiaotou (Allium chinense) using MS and B5 media supplemented with 1 mg/l BA and 0.5 mg/l 2, 4-D. The data collected after 8 weeks of culture showed that the highest callus induction (49.3%) was achieved when the B5 medium was used in comparison with 39.7% in MS medium. While Behbahani et al. [60] investigated the factors influencing the callus induction of Barringtonia race-

| Treatments type of medium | Callus (%) | Fresh weight (g) | Dry weight (g) | Dry Matter (%) |
|--------------------------|-----------|-----------------|---------------|---------------|
| Control                  | 0.00      | 0.00            | 0.00          | 0.00          |
| MS                      | 100.00    | 4.04            | 0.39          | 9.61          |
| B5                      | 75.00     | 0.94            | 0.13          | 13.43         |
| MS + B5                 | 81.25     | 1.19            | 0.16          | 13.05         |
| WPM                     | 0.00      | 0.00            | 0.00          | 0.00          |
| Mean                    | 51.25     | 1.23            | 0.14          | 7.22          |
| LSD 0.05                | 31.84     | 0.25            | 0.026         | 1.09          |

* Each treatment was the average of 4 replicates and each jar contains 4 explants.
* Control: (0.7 agar + 3% sucrose+1 mg/l 2, 4-D).
* MS: Murashige and Skoog medium.
* B5: Gamborg medium.
* WPM: Woody plant medium.
They observed that WPM supplemented with 2 mg/l 2, 4-D produced a higher yield for callus induction followed by B5 and MS media. This variation in callus induction using compared media may attribute to the nature of explants tissue and media composition.

3.5.4. Effect of ascorbic and citric acid as antioxidant and charcoal on *Parkia biglobosa* callus maintenance

A blacking or browning of tissues excised from many woody species, the medium in which explants are grown become colored within an hour or two of planting the material as is observed in tissue culture of many tropical and subtropical woody species. The browning and black color developing in callus cell cultures are due to the formation of phenolic exudation. The effect of media composition on callus growth parameters after 8 weeks of culturing on MS media containing different concentrations of ascorbic and citric acid alone or in combination (as antioxidants) with 0.5 mg/l activated charcoal was investigated. There were significant differences among treatments as shown in Table 5 and Fig. 5, the maximum percentage of callus growth parameters and a number of browning were obtained by (50 mg/l citric acid) whereas the minimum rates were obtained with (charcoal + 50 mg/l citric acid). So, it could be concluded that (50 mg/l citric acid) treatment recorded the highest values 100, 3.33 and 0.33 for callus production, fresh and dry weight, respectively. On the other hand, (charcoal + 50 mg/l citric acid) treatment produced the minimum values 25, 1.06 and 0.11 for callus production, fresh and dry weight, respectively and browning color developing in callus cell culture was observed in this treatment. This result describes the effect the plant hormone 2, 4-D plus active charcoal [61,62]. However, there was higher browning on callus cell culture in MS medium devoid antioxidant or charcoal, all the callus which produced with citric or ascorbic acid or without were brown in color and turn black after 3–4 weeks. Therefore, the addition of 50 mg/l of citric acid to the medium was effective in reducing browning of calli of *Parkia biglobosa*. A similar response has been observed in medium supplemented with 50 mg/l of ascorbic acid up to six weeks, the browning color developing in callus cell cultures. The obtained results are in agreement with those concluded by Kumari et al. [63] who reported that to control the browning of *Ricinus communis* callus tissue by adding different concentrations of charcoal, citric acid, and ascorbic acid to prevent the phenolic oxidation. Also, Anthony et al. [64] found that phenolic compounds cause browning problems in woody plants. Using antioxidants could suppress browning of woody plant tissue culture, explants death rates. On the other hand, Bhatt and Dhar [65] concluded that medium supplemented with different concentrations of ascorbic acid, citric acid, and activated charcoal had no improvement effect on the browning of pine callus.

**Table 5**

Effects of MS media supplemented with different concentrations of ascorbic and citric acid as antioxidants alone or in combination with 0.5 g charcoal on calli growth parameters induced from stem of *Parkia biglobosa* after 8 weeks of cultivation under light condition at 25 ± 1 °C

| Treatments | Callus (%) | Fresh weight (g) | Dry weight (g) | Dry Matter (%) |
|------------|------------|------------------|----------------|---------------|
| **Charcoal (gm)** | **Ascorbic (mg/l)** | **Citric (mg/l)** |                |               |
| 0          | 0          | 0                | 62.50          | 2.53          | 0.24          | 9.42          |
| 0.5        | 0          | 0                | 56.25          | 1.56          | 0.17          | 10.53         |
| 0.5        | 25         | 0                | 62.50          | 1.54          | 0.15          | 10.07         |
| 0.5        | 50         | 0                | 56.25          | 1.61          | 0.17          | 10.64         |
| 0.5        | 0          | 25               | 37.50          | 0.97          | 0.11          | 10.81         |
| 0.5        | 0          | 50               | 25.00          | 1.06          | 0.11          | 10.38         |
| 0          | 25         | 0                | 93.75          | 2.35          | 0.22          | 9.40          |
| 0          | 50         | 0                | 100.00         | 3.08          | 0.29          | 9.45          |
| 0          | 0          | 25               | 87.50          | 2.25          | 1.48          | 10.19         |
| 0          | 0          | 50               | 100.00         | 3.33          | 0.33          | 9.74          |
| **Mean**   | 68.13      | 2.03             | 0.33           | 10.05         |
| **LSD<sub>0.05</sub>** | 30.09      | 0.69             | 1.17           | 1.07          |

*Each treatment was the average of 4 replicates and each jar contains 4 explants.*

![Fig. 4. Effect of culture media on callus induction of *Parkia biglobosa* stem explants after 8 weeks. (A)-callus induction from stem explants in MS media including B5 vitamin supplemented with 2,4-D (1.0 mg/l), (B)-callus induction from stem explants in MS media supplemented with 2,4-D (1.0 mg/l), (C)-callus induction from stem explants in B5 media supplemented with 2,4-D (1.0 mg/l), (D)- callus induction from stem explants in woody plant media supplemented with 2,4-D (1.0 mg/l).](image-url)
3.5.5. Establishment of *Parkia biglobosa* cell suspension culture

Three weeks old callus growing on complete MS + 1 mg/L 2, 4-D was used to initiate cell suspensions of *Parkia biglobosa* as shown in Fig. 6. The texture of callus formed at this concentration was friable. The *Parkia biglobosa* cells were grown well in liquid MS media with the same composition of selected callus induction media. The percentage of viable and viable cell numbers per ml of suspensions determined from 28-day-old culture was initially slow. Data showed a lag phase from 0 to 3 days, but as the culture proceeded it showed increasing from day 6 and significantly produced the highest amount of cell number over a period of 9 days an exponential growth phase from 6 to 9 days (log phase). The maximum increase in viable cell number reached on day 12. This means that lag phase from 0 to 3 days and exponential growth phase from 6 to 9 a day and stationary phase from 9 to 12 days.

In our research 2, 4-D has been used for the induction of friable callus for the establishment of cell suspension. The similar result reported by Khafagi [66] who obtained callus for cell suspension establishment of *Peganum harmala* using 0.5 mg/L 2, 4-D. Initially, *Parkia biglobosa* cell suspensions were established in the MS media composition of callus induction (1 mg/L 2, 4-D), 0.5 g of friable callus derived from stem explants were used for inoculation, the age of inoculum cells were 28-days. The obtained results are in accordance with those reported by Soomro and Memon [67] established *Jatropha curcas* suspensions from 28-days-old callus inoculated to media supplied with 0.5 mg/L of 2, 4-D. Also, Mythili et al. [68] and Lee and Chan [69] have established cell suspensions of other species using media with 2, 4-D. We conclude that using 28-days-old callus gave maximum viable cells during 12 days, this means that the age of inoculums influences the establishment of cell suspensions and growth rate. Similar results were obtained by Gonzalez et al. [70] who observed greater cell growth with 28 and 35-day-old-calli. Furthermore, the cell viability remained around 68.39% throughout the experiment period (18 days). Similar observation reported by Mathur and Shekhawat [71] they found that in suspension culture of *Stevia rebaudiana* (Bertoni) still around 75% up to the 18 days of culture. When cell viability remained around 50%, it is considered that the suspension culture establishment has failed [72]. These obtained results in Table 6 showed the maximum viability (%) (87.67%) at the 9th day while...
Table 6
Cell viability in suspension culture of Parkia biglobosa on MS medium supplemented with 1 mg/l 2, 4-D at different time durations.

| Days    | Viable cells/ml | Non-viable cells/ml | Total cells/ml | Viability (%) | Average number of cells/square cm | Concentration of viable cells/ml |
|---------|-----------------|---------------------|----------------|--------------|-----------------------------------|---------------------------------|
| Initial | 12.75           | 8.50                | 21.25          | 59.88        | 3.19                              | 61.75                           |
| 3rd Day | 18.00           | 5.75                | 23.75          | 76.03        | 4.50                              | 90.00                           |
| 6th Day | 22.00           | 5.00                | 27.00          | 82.77        | 5.50                              | 110.00                          |
| 9th Day | 25.25           | 3.75                | 29.00          | 87.76        | 6.31                              | 126.25                          |
| 12th Day| 27.50           | 6.00                | 33.50          | 81.79        | 5.88                              | 137.50                          |
| 15th Day| 23.50           | 7.75                | 31.25          | 74.88        | 5.88                              | 117.50                          |
| 18th Day| 18.75           | 8.75                | 27.50          | 68.39        | 4.69                              | 93.75                           |
| LSD 0.05| 4.84            | 2.89                | 6.01           | 8.05         | 1.21                              | 25.84                           |

the number of viable cells reached the higher record (137.50 viable cells/ml) after 12 days, and the cell viability remained around (68.39%) throughout the 18 days of culture, this confirm that the Parkia biglobosa cell suspension culture growing very well.

4. Conclusion

The result of the study showed that seed scarified in H2SO4 improved seed germination and seedling growth. It is therefore recommended that H2SO4 scarification of the seeds of Parkia biglobosa may be effective for breaking dormancy and have the best seedling performances. This study has revealed an excellent callus production percentage (100%), which we consider it the best callus induction media composition for Parkia biglobosa using stem explants. The addition of citric acid to the callus induction media was very effective to reduce the browning of Parkia biglobosacallus. Parkia biglobosa cell suspension protocol was established for the first time using MS media containing 1 mg/l 2, 4-D and harvest a viable cell after 12 days to be applied to any other experiments.

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