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# Procedure for *in vitro* seed sterilization, germination and aseptic seedling establishment of *Zehneria capillacea* (Schumach) C. jeffrey

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## Abstract

The most important step in plant tissue culture technique is standardizing a protocol for explant sterilization using phytotoxic sterilants. In this study, two sterilants, sodium hypochlorite (household bleach) and mercuric chloride were evaluated to determine the most suitable concentration and exposure time for explant sterilization of *Zehneria capillacea* seeds, an undomesticated diploid herbaceous edible cucurbit that is rarely distributed in the Niger Delta region of Nigeria. 0.1% mercuric chloride for 1minute and 20% sodium hypochlorite for 15 minutes achieved an efficient (95%) seedling establishment. The study also evaluated the effect of aseptic seed coat removal on *in vitro* seedling establishment. Seeds with intact coat had 0% germination six weeks after initiation into Murashige and Skoog (MS) medium while all the decorticated seeds showed 100% germination with cotyledonary growth and roots one week after initiation, indicating that the seed coat is a major barrier to the *in vitro* germination of this species. It was also observed that seed orientation during culture initiation influenced seedling vigor and growth. Three weeks after culture initiation, the aseptic seedlings generated from decorticated seeds were observed to have grown 4-5 cm in height with formation of tendrils, apical and axillary nodes, which could be used as explant source for various *in vitro* culture studies such as callus induction, shoot multiplication, *in vitro* polyploidization and production of secondary metabolites.

**Keywords:** Aseptic Seedling; *In vitro* Germination; Seed Coat; Seed Orientation, Sterilization; *Zehneria capillacea*

## 1. Introduction

*Zehneria capillacea* (Shumach) C. Jeffrey is a diploid herbaceous climber that belongs to the family Cucurbitaceae [1]. In Nigeria, the family is represented by 41 species in 21 genera, comprising both cultivated and wild species and is of tremendous economic importance as vegetables, oil and medicinal plants [2]. Tissue culture of many cucurbits have been reported by various authors such as *Cucurbita maxima*, *Benincasa hispida* [3], *Cucumis melo* var.*flexuosus*, *Momordica dioica* [4,5], *Telfairia occidentalis* [6], *Lagenaria siceraria, Zehneria scabra* [7,8] and *Coccinia indica* [9]. Despite the enormous data on *in vitro* studies and utilization of cultivated cucurbits, knowledge on uses of wild cucurbits found in Africa is limited [10]. The wild species of plants are gene reservoirs for tackling biotic and abiotic stresses and for improving the quality of cultivated species. [8, 11] reported the first antimicrobial activities and *in vitro* culture of *Zehneria scabra*.

The establishment of a micropropagation protocol is necessary to begin application of biotechnological tools, for example, *in vitro* polyploidization [12, 13]. The sterilizing agents used for optimization of *in vitro* sterilization protocol are ethanol, sodium hypochlorite (NaOCl), calcium hypochlorite, mercuric chloride ($HgCl_2$) and hydrogen peroxide. Due to the phytotoxicity of these sterilants, the concentration and duration of exposure have to be determined for different

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explants. [14] Treated shoot tips of *Gypsophilia paniculata* with 22% sodium hypochlorite for 20 minutes. [15] treated shoot tips of *Luffa cylindrica* with 0.05% mercuric chloride for 2.5 minutes. Nodal segments of *Zehneria scabra* were surface sterilized with 0.1% $HgCl_2$ for 3 minutes [8]. There is no available data on the conservation status and *in vitro* studies of *Zehneria capillacea* which grows wild in Nigeria. The objective of this study is to determine the most effective sterilization procedure for *Zehneria capillacea* seeds using mercuric chloride and commercial bleach and to evaluate the effect of seed coat removal on establishment of aseptic seedlings.

## 2. Material and methods

### 2.1. Plant Material and Culture Medium

Mature seeds of *Zehneria capillacea* collected from the wild in Choba (Lat. 4°54'06.11"N and Long. 6°55'19.52"E) and Idi Ose (Lat. 7°29'59.57"N and Long. 3°54'47.35"E) were used as explants for optimizing sterilization procedure and seedling establishment. The media formulation described by [16] referred as MS medium was used as the culture medium for the *in vitro* germination and establishment of aseptic seedlings. The full strength MS basal medium was supplemented with 3% (w/v) sucrose and 0.2% (w/v) gelrite without plant growth regulators. The pH was adjusted to 5.7 with 1 N NaOH and 1 N HCl prior to the addition of the gelrite. The medium was distributed in aliquots of 15mls into culture vessels and autoclaved at 121°C and 1.06kg/cm2 for 15 minutes.

### 2.2. Seed sterilization and initiation

Seeds of *Zehneria capillacea* were subjected to germination and float test to determine the viability. Viable seeds were immersed in 70% ethanol for I minute followed by 0.1%(w/v) mercuric chloride solution for 1min and in 20%, 40%, 60%, 80% and 100% sodium hypochlorite for 15 minutes. One drop of tween 20 was added as a wetting agent. The seeds were rinsed five times in sterile distilled water to remove all traces of the sterilant and soaked in sterile distilled water for 6 hours to soften the coat. The seed coat of sterilized seeds were aseptically removed or left intact (control) before initiation into the culture medium. A total of sixty seeds were cultured per treatment (2 seeds per culture vessel) in a completely randomized design with four replications. All cultures were incubated at 25 ± $1^0$C under 16h/8h photoperiod using cool white fluorescent light. Data obtained was subjected to analysis of variance to test the significance of the treatment difference.

## 3. Results

**Table 1a** Germination Percentage of *Zehneria capillacea* seeds sterilized with different Concentrations of Sodium hypochlorite (NaOCl) for 15 minutes and 0.1% mercuric Chloride ($HgCl_2$) for 1 minute from a CRD experiment with 4 replications and 6 treatments.

| Treatment | Germination Percentage | | | | Treatment Total | Treatment Mean |
|---|---|---|---|---|---|---|
| **NaOCl (15 min)** | | | | | | |
| 20% | 95 | 96 | 98 | 94 | 383 | 95.75 |
| 40% | 96 | 96 | 94 | 96 | 382 | 95.5 |
| 60% | 97 | 94 | 95 | 95 | 381 | 95.25 |
| 80% | 93 | 96 | 98 | 95 | 382 | 95.5 |
| 100% | 98 | 96 | 93 | 96 | 383 | 95.75 |
| **$HgCl_2$ (1 min)** | | | | | | |
| 0.10% | 95 | 94 | 96 | 96 | 381 | 95.25 |
| Grand total | | | | | 2,292 | |
| Grand mean | | | | | | 95.5 |

The effect of different concentrations and treatment duration of sodium hypochlorite and mercuric chloride on *in vitro* germination of *Zehneria capillacea* is shown on table 1a. Seed germination commenced on the $2^{nd}$ day after initiation irrespective of the sterilizing agent and maximum percentage germination was obtained by the $7^{th}$ day. Seeds exposed

to 0.1% $HgCl_2$ for 1 min had 95% germination which was not significantly different from seeds exposed to the various concentrations of NaOCl (20, 40, 60, 80 and 100%) for 15 min (Table 1b). There was no contamination and phytotoxic effect recorded for the entire treatment regime (Table 3). Since seed germination was not significantly affected by NaOCl concentration, 20% for 15 minutes was utilized for subsequent experiments.

**Table 1b** Analysis of Variance of Germination Percentage data

| Source of Variation | Degree of Freedom | Sum of Squares | Mean Square | Computed F[a] | Tabular F | |
|---|---|---|---|---|---|---|
| | | | | | 5% | 1% |
| Treatment | 5 | 1 | 0.2 | 0.08[ns] | 2.77 | 4.25 |
| Experimental error | 18 | 45 | 2.5 | | | |
| Total | 23 | 46 | | | | |

cv = 1.65%; [a]ns = non-significant

**Table 2** Effect of two sterilants (NaOCl and HgCl2) on *in vitro* germination of *Zehneria capillacea* decorticated and intact seeds

| Treatment | Germination Percentage | | Contamination (%) |
|---|---|---|---|
| | Decorticated[a] | Intact | |
| **NaOCl (15 min)** | | | |
| 20% | 96.25±0.82 | 0 | 0 |
| 40% | 96.00±0.71 | 0 | 0 |
| 60% | 96.88±0.68 | 0 | 0 |
| 80% | 96.88±0.70 | 0 | 0 |
| 100% | 96.75±0.75 | 0 | 0 |
| **$HgCl_2$ (1 min)** | | | |
| 0.10% | 95.88±0.71 | 0 | 0 |

[a]ns = non-significant (P>0.05)

The results indicate that the seed coat is a major barrier for *in vitro* seed germination (Table 2). All the seeds with intact seed coat (fig, 1a) did not germinate six weeks after initiation (Fig. 1b) while decorticated seeds started germinating two days after initiation (Fig. 1d). One week after initiation, germinated seeds showed cotyledonary growth and long hypocotyl area having roots (Fig. 1e). After three weeks, seedlings were observed to have grown 4-5 cm in height with formation of tendrils, axillary nodes and apical buds (Fig.1f and 1g).

It was also observed that seed (explant) orientation during inoculation significantly affected seedling growth ($P < 0.05$) as shown in Table 3. All the seedling traits scored for seeds that were inoculated with radicle on the medium were significantly different from the seeds inoculated with plumule on the medium except the root length which was the same for both (Table 3). Seeds inoculated with plumule downwards in the medium had inverted seedlings which were stunted and vitrified (Fig 1h) with a mean seedling height of 4.20 ± 0.13cm and 2.25 ± 0.10 cm number of nodes (Table 3). The seeds inoculated with radicle on the medium had normal upright seedlings with a mean height of 6.89 ± 0.20cm and 4.95 ± 0.18cm number of nodes. There were no phenolic exudates observed in both.

**Table 3** Effect of explant orientation on *in vitro* seedling development of *Z. capillacea* in MS medium. (Traits were scored 21 days after culture initiation except hypocotyl length).

| Trait | Radicle downwards | Plumule downwards |
|---|---|---|
| *Hypocotyl length (cm) | 1.43 ± 0.02 | 0.78 ± 0.02 |
| Seedling height (cm) | 6.89 ± 0.23 | 4.20 ± 0.13 |
| Number of nodes | 4.95 ± 0.18 | 2.25 ± 0.10 |
| Internode length (cm) | 1.56 ± 0.04 | 0.43 ± 0.14 |
| Number of leaves | 4.95 ± 0.20 | 2.25 ± 0.10 |
| Petiole length (cm) | 0.65 ± 0.02 | 0.32 ± 0.03 |
| Root length (cm) | 10.68 ± 0.28 | 10.52 ± 0.22 |
| Tendrils | present | absent |
| Vitrification | absent | present |
| Phenolic exudates | absent | absent |
| Seedling vigour | vigorous | stunted |
| Contamination | none | none |

*scored 7 days after inoculation

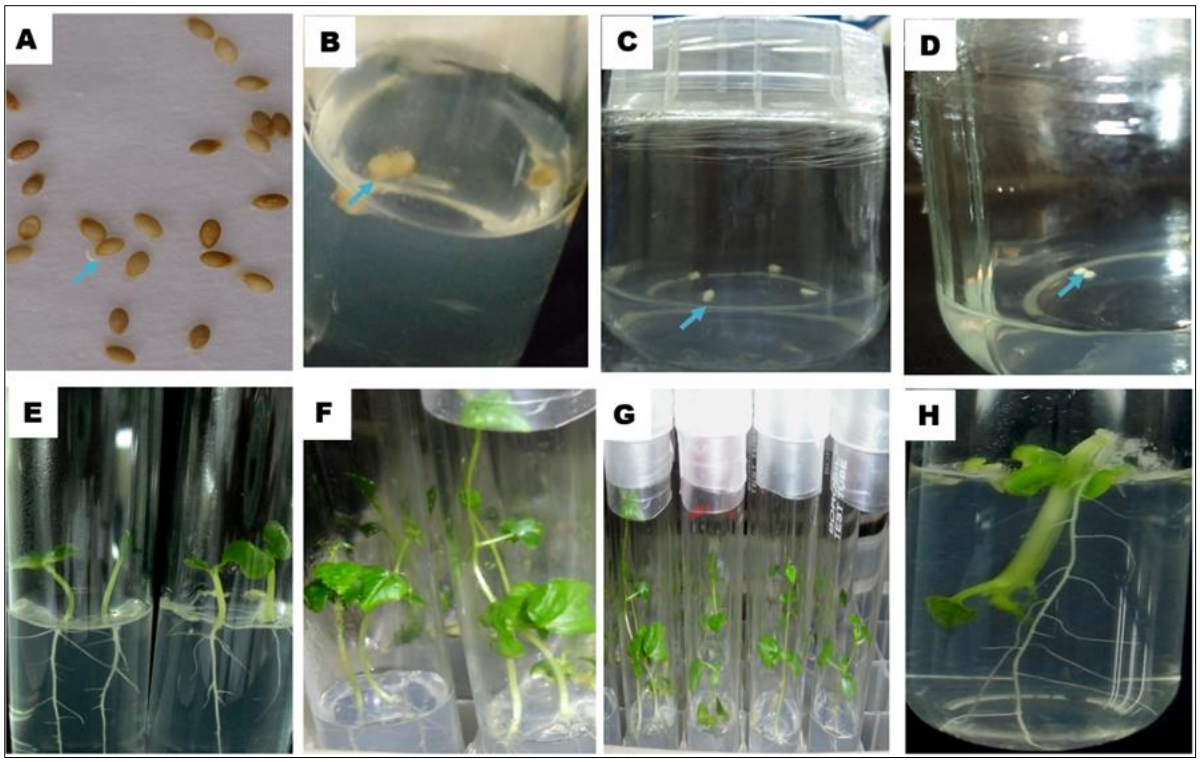


**Figure 1** *in vitro* seed germination and seedling development of *Zehneria capillacea* on MS medium without plant growth regulators. A) Sterilized intact seeds before initiation; B) Intact seeds six weeks after initiation, C) Decorticated seeds one day after initiation; D) Decorticated seeds two days after intiation, arrow shows open cotyledons; E) 7 days old seedlings from de-corticated seeds; F and G) 21 days old seedlings; H) 21 days old inverted seedling

## 4. Discussion

Optimization of explants sterilization protocol is the most important step for a successful *in vitro* culture establishment [17]. Tomato seeds exposed to 0.1%$HgCl_2$ for 10 min completely inhibited germination [18] while groundnut seeds exposed to 0.1% $HgCl_2$ for 8 min was found to be more effective as sterilizing agent (82 – 100% explants survival) compared to NaOCl [19]. Ginger rhizomes and nodal segments of *Zehneria scabra* soaked in 0.1%$HgCl_2$ solution for 5min and 3 min respectively was effective in sterilizing the explants without inhibiting shoot formation [20, 8]. Bamboo seeds sterilized with 20% NaOCl for 20 min gave maximum germination by the $12^{th}$ day of germination [21]. Observations in the present report reveals that exposure time affects seed germination using 0.1%$HgCl_2$ while the different concentrations (20 -100%) of NaOCl for 15 min. gave the same germination percentage.

In the current study, the two sterilizing agents (0.1%$HgCl_2$ for 1 min and NaOCl for 15 min) gave maximum percentage germination. Removal of seed coat after sterilization is also recommended as the seed coat was observed to completely inhibit *in vitro* germination. Soaking the seeds for 6 hours after sterilization softens the seed coat for easy removal. Seedlings generated using this sterilization procedure could be utilized as source of explants to generate aseptic seedlings which could be used for various *in vitro* studies such as callus initiation shoot multiplication, polyploidy induction and conservation of this edible wild cucurbit.

A major limitation to aseptic culture establishment is microbial contaminants which may be introduced into the culture medium from explants and affect the potential of plant regeneration [1]. To obtain sterile cultures, the explants (seeds) used in this study were dipped in 70% ethanol for 1 min followed by treatment with various concentrations of sodium hypochlorite (NaOCl) for 15 min and 0.1% (w/v) aqueous mercuric chloride ($HgCl_2$) for 1 min. There was no contamination recorded using the two sterilizing agents as indicated in table 1. All the explants had statistically similar germination percentage (96%) indicating efficient sterilization without damage to the plant tissue. The results obtained in this study are in conformity with earlier reports of utilizing NaOCl and $HgCl_2$ for explant sterilization of cucurbits.

In this study, sterile seeds were obtained using 20 -100% NaOCl for 15mins and 0.1% $HgCl_2$ for I min. The seeds were able to withstand higher concentration of the sodium hypochlorite. However, 20% NaOCl for 15 min is recommended for seed sterilization because it is cheaper, easily available in Nigeria and less toxic compared to $HgCl_2$ which may not be appropriately disposed if disposal facilities for mercury-containing waste products are not available in plant tissue culture laboratories. Sterilized seeds were decorticated prior to initiation to enhance germination.

## 5. Conclusion

*In vitro* aseptic seedling establishment was accomplished by washing seeds under running water for five minutes, dipping seeds in 70% ethanol for one minute followed by immersion in 20% - 100% NaOCl for fifteen minutes and rinsing five times with sterile distilled water under aseptic conditions. This was compared to sterilization with 0.1% $HgCl_2$ for 1 minute. The sterile seeds were decorticated and initiated on MS basal medium solidified with 0.2% gelrite without plant growth regulators. The different concentrations of the NaOCl and 0.1% $HgCl_2$ were effective in eliminating surface contaminants on the seeds and did not affect the germination. The cotyledons of decorticated seeds opened a day or two after initiation and produced aseptic seedlings which provided explants for optimization of hormonal requirements for *in vitro* multiplication and rooting. The seeds with intact seed coat did not germinate after six weeks of initiation indicating that the seed coat is a major barrier to *in vitro* seed germination while the seed orientation in the medium had significant influence on seedling growth. Based on these findings, it can be concluded that seeds of *Z. capillacea* should be aseptically decorticated after sterilization before initiation into hormone free MS medium for *in vitro* germination and seedling establishment. In addition, seeds should be initiated vertically with the radicle in contact with the medium and the plumule facing upwards.

## Compliance with ethical standards

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*Disclosure of conflict of interest*

No potential conflict of interest reported by the authors.

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