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In Vitro Regeneration via Somatic Embryogenesis of *Schizozygia Coffeoides Baill* (Mpelepele)

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Abstract: *Schizozygia coffeoides* (Mpelepele) is an endangered medicinal shrub with substantial antifungal and antibacterial properties. The sustainable utilization of this shrub is hindered by its limited regeneration potential since there exists no protocol for *In vitro* regeneration of not only this plant but the entire Apocynaceae family. The aim of this study was to develop an appropriate sterilization and regeneration protocol for *In vitro* regeneration of *S. coffeoides* using leaf discs, with the specific aims of determining the best NaOCL concentrations for explant sterilization as well as the appropriate plant growth hormone combinations for development of embryogenic calli, shoot development and rooting. Sterilization was optimized using NaOCl at the concentrations of 5.2mM, 7.8mM, 10.4mM and 13mM at exposure times of 10, 15, 20 and 25 minutes. The different concentrations of NaOCl had a significant effect on the survival of explants to sterilization (P=<0.0001), with the lower concentrations (5.2mM and 7.8mM) showing high levels of explant contamination and high concentration (13mM) leading to explant scotching. Similarly, the time explants were subjected to the sterilization substances was also found to significantly affect their ability to survive the sterilization process (P=0.0027). The best time and NaOCl concentration interaction for the sterilization of leaf explants was found out to be 10.4mM for 20 minutes. This time-NaOCl concentration interaction was found out to significantly produce a higher number of clean surviving explants than all other interactions (P=<0.0001). The best callus formation frequency of 68% was observed in MS media supplemented with 2.0mg/lBAP+0.8mg/lKin+0.4mg/l NAA+0.5mg/lTDZ, compared to a 39% callus formation frequency observed in media supplemented with 1.5mg/lBAP+0.8mg/lKin+0.4mg/lNAA+0.5mg/lTDZ and a 13% callus formation frequency observed in media supplemented with 1mg/lBAP+0.4mg/lKin+0.2mg/lNAA+0.05mg/lTDZ. Although somatic embryos formed in all media types, only media supplemented with 2.0mg/lBAP+0.8mg/lKin+0.4mg/lNAA+0.5mg/lTDZ formed most somatic embryos which survived to maturity and formed shoots, as most calli and embryos forming in other PGR supplementations died with subsequent subcultures. Roots only formed in shoots cultured in media supplemented with 1.0 BAP+0.5 IBA. The successful development of this regeneration protocol is expected to greatly contribute to mass production and conservation of this important shrub.

Keywords: Sterilization, Plant Growth Regulators, Callus Induction, Somatic Embryo

1. Introduction

Africa is endowed with a rich wealth of medicinal plants which have a high valuable source of natural products for therapeutic properties. According to the world health organization, it is estimated that 80% of people worldwide rely on plant based medicine for primary healthcare [1]. Despite the abundance of plant source in Africa, these plants are constantly exploited for new drug development and validation of traditional medicine. Therefore, many of these plant species are in danger of extinction as they are subjected to extensive wild crafting and unsustainable techniques of harvesting [2].

Schizozygia coffaeoides (Mpelepele) is an endangered

medicinal shrub plant that is rich in two known alkaloids; schizozygine and isoschizogaline as well as a new indoline alkaloid 6, 7-dehydro-19β-hydroxyschizozygine. The crude extracts of this shrub have shown substantial antibacterial and antifungal properties which could be attribute to these substances [3]. The shrub is found throughout temperate regions in forests of altitudes 0-1500M above sea and distributed among East African countries, Angola, Zaire and the Comoro's Island. In Kenya, It is predominately found in the Shimba hills and Kilifi coastal regions covering an area of 19,250 Heactre [4]. Like many other Apocynaceous plants, *S. coffaeoides* is a rich source of Alkaloids from which it derives its medicinal properties. In the Comoro's Island the leaves and the fruits are used to coagulate the rubber of other plants. Decoctions of the stem bark is given to nursing mother while the very bitter leaves and bark are popular remedies against fatigue and applied to supporting and fostering wounds [5]. In Kenya, the shrub is used for the treatment of skin related problems, gastro-intestinal diseases, sexually transmitted diseases, Malaria, snake bites and eye infections[3, 4].

Due to its continued use for medicinal purposes, the sustainable development of *S. Coffeoides* will depend on developing an efficient regeneration protocol that will enhance the conservation and sustainable use of this endangered plant. Modern biotechnology offers a viable means and an important tool for mass propagation and genetic improvement of not only *S. Coffeoides* but also other medicinal plant in this family. This began with the discovery that plant cells can be regenerated *in vitro* through a process called somatic embryogenesis [6]. The capacity for somatic embryogenesis is a remarkable property of any plant cell. It is the developmental pathway by which somatic cells develop into structures that resemble zygotic embryos (bipolar and without vascular connection to the parental tissue) through an orderly series of characteristic embryological stages without fusion of gametes [7]. The success of somatic embryogenesis largely relies on the selection of a suitable plant part which is to be used as the starting material for the experiment [8]. There are no documented studies on the regeneration of *S. Coffeoides* which happens to be the only species in this family. As a result, there is need to develop a suitable regeneration protocol for this important plant. This study therefore sought to establish an efficient sterilization and regeneration protocol from the leaf explants of *S. coffaeoides.*

2. Materials and Methods

2.1. Plant Materials

Mpelepele cuttings were obtained from Shimba hills in the coastal region of Kenya. The cuttings were kept in a cool box and transported to the Institute for Biotechnology research (IBR) tissue culture laboratory, Jomo Kenyatta University of Agriculture and Technology (JKUAT). The cuttings were transplanted in potting bags containing well mixed forest soil, sand and manure in the ratio of 2: 1: 1 and kept in the IBR green house. They were watered four times a week using a spraying can. Well adapted plants were used as mother plants in subsequent experiments. The research work was carried out at plant tissue culture laboratory in IBR at JKUAT.

2.2. Media and Culture Condition

MS 1962 [6], basal medium was used in all experiments. It was supplemented with 3% (w/v) sucrose, 0.28% (w/v) gelrite and plant growth regulators (PGRs) as required and pH adjusted to 5.8 using 0.1N HCL and or 0.1N NaOH. The media was then dispensed in 200ml culture jam jars (30ml per jar) and autoclaved at 121°C at 15 (psi) above atmospheric pressure for 15min. The sterilized media was kept at room temperature for one day before use. The chemicals used were of analytical grade and experiments were carried under sterile conditions in the laminar flow hood. Cultures were grown at 25±2°C under a 16 hour light and 8 hour dark period in growth chambers illuminated by 40W Philips® white fluorescent tubes. The intensity of light was between 2500 and 3000μmol^{-2} s^{-1}.

2.3. Explant Preparation and Sterilization

Preparation and sterilization of explants was done according to Ndakidemi *et al*. [2013] with some adjustments. The 3rd and 4th leaves were collected from 8 months mother plants in the greenhouse. The explants were placed in glass beakers and thoroughly cleaned by keeping them under running tap water for 30 minutes. They were then placed in new glass beakers containing 25ml of distilled water with 1ml of detergent and 0.25ml of the antibacterial savlon® and kept for 10 minutes. This was followed by rinsing the leaves thrice with sterile distilled water. The explants were kept in distilled water containing a fungicide (ridomil) and 0.1ml of tween 20® for 1 hour. They were there after rinsed thrice with autoclaved distilled water and transferred to the lamina flow hood. To study the effects of sodium hypochlorite (NaOCl) and time of exposure on these explants, the explants were subjected to NaOCl at concentrations of 5.2 mM, 7.8 mM, 10.4 mM and 13 mM as well as exposure times of 10, 15, 20 and 25 minutes then rinsed thrice using sterile distilled water. The explants were trimmed to 5mm then inoculated on full MS media without hormones and kept in the growth chamber. Cultured explant were monitored daily for 14 days to check bacterial and fungal contamination as well as those that were scorched. The total number of explant contaminated, survived and scorched was recorded.

2.4. Callus Induction

For callus induction explants were placed on MS medium supplemented with various cytokinins (6-benzlademine (BAP), Kinetin (Kin) and Thidizuron (TDZ)) in combination with an auxins (naphthalene acetic acid (NAA)) at three different concentrations. Callus formed per treatment were routinely sub-cultured (every two week) in fresh medium with the respective PGR concentration. The cultures were

observed constantly for any morphological response; colour, texture and shape of the callus using eye and microscopic observation. The callus formation frequency per treatment was recorded as the number of calli forming as a percentage of the cultured explants. Each treatment contained at least 12 explants and replicated 3 times.

2.5. Embryo Formation, Shooting and Rooting

Callus formed were sub-cultured on full MS medium supplemented with BAP, Kin, NAA and TDZ at varying concentrations (1.0mg/l+0.4mg/l+0.2mg/l+0.05mg/l), (1.5mg/l+0.6mg/l+0.3mg/l+0.1mg/l) and (2.0mg/l+0.8mg/l+0.4mg/l+0.5mg/l) respectively until somatic embryos were formed. Further sub-culturing was done on the same media for embryo maturation. Cultures were monitored weekly for shoot proliferation. The number of shoots forming per calli was observed and recorded for each media type. Elongated axillary shoots were transferred to a rooting MS medium supplemented with BAP and IBA. All the experiments were replicated 3 times and each contain at least 4 explant. The treatments were observed weekly for signs of rooting on each shoots.

2.6. Experimental Design and Statistical Analysis

The experiments were set in a completely randomized design (CRD) with 3 replications. Data was keyed into excel spreadsheet and analyzed using statistical analysis system (SAS). The mean number of contaminated explant by bacterial and fungi as well as those that died as a result of scorching were determined. The best suitable concentration and time of exposure was determined. Data were further subjected to analysis of variance to detect differences among treatment means by separation using multiples range test. The effect of variation and interaction of the various plant regulators used and time taken on induction of callus, shoots and root were compared by Turkeys test at $P \geq 0.05$.

3. Results

3.1. The Effect of NaOCl and Exposure Time on the Sterilization of Explants

In this study sterilization of *S. coffaeoides* leaf explants was achieved by using a single sterilant; sodium hypochlorite (NaOCl). It was observed that exposing leaf explants to different concentrations of this sterilant and at different times resulted in differences in sterilization levels of the explants based on the level of bacterial contamination as well as that of fungi observed growing in cultures. Irrespective of the exposure time, Analysis of variance revealed that varying the levels of NaOCl concentration resulted in significant differences on the levels of bacterial contamination observed in culture (P=<0.0001) as well as the% number of clean surviving explants (P=<0.0001) but showed no significant difference in the fungal contamination observed in culture (P= 0.5844). At low NaOCl concentrations of 5.2 mM and 7.8 mM, it was observed that the levels of bacterial contamination were high (51.67% and 43.33%) compared to the low contamination levels (3.33% and 18.33%) observed at 10.4 mM and 13 mM (Figure 1). Although 13.0 mM NaOCl showed lower bacterial and fungal contamination, actually, it was observed that these NaOCl levels (13 mM) lead to most of the explants being scotched hence died. Therefore, NaOCl levels of 10.4 mM were observed to be ideal for explant sterilization since they produced relatively low levels of bacterial and fungal contamination (3.33% and 15.00%) and the highest levels of un-scotched clean explants which were regenerable (78%).

Analysis of variance also revealed that irrespective of the NaOCl levels, the time of exposure did not have a significant effect on bacterial contamination (P= 0.2033) as well as fungal contamination (P= 0.9520) but significantly affected the number of clean surviving explants that could regenerate (P=0.0027). Indeed, it was observed that the ability of the explant to survive and be regenerable in culture was inversely proportional to the exposure time (Figure 1). Lower exposure times of 10 and 15 minutes led to higher explant survival (53.33% and 48.33%) rates compared to higher exposure times of 20 and 25 minutes, which led to decreased explant survival rates (40% and 13.33%).

The interaction of NaOCl concentration and time of exposure on bacterial and fungal contamination as well as explant survival produced interesting results. It was observed through analysis of variance that the interaction of these two parameters did not have a significant difference on bacterial contamination (P=0.0045) as well as fungal contamination (P=0.1138), but had a significant effect on the number of surviving regenerable explants in culture (P=<0.0001). The best interaction level for these two parameters was observed to be at 10.4 mM NaOCl concentration for 20 minutes, since at this interaction level there was relatively low bacterial and fungal contamination and the level of explant survival was optimum (Table 1).

Table 1. *Effect of NaOCl and time of exposure interaction on explant sterilization.*

% NaOcl	Time of exposure (Min.)	% Bacterial contamination	% Fungal contamination	% surviving clean explants
5.2 mM	10	53.33±13.33^{a}	6.67±6.67^{a}	26.67±17.64^{bcd}
5.2 mM	15	53.33±24.04^{a}	13.33±6.67^{a}	26.67±17.64^{bcd}
5.2 mM	20	40.00±11.55^{a}	33.33±24.04^{a}	26.67±13.33^{bcd}
5.2 mM	25	60.00±11.55^{a}	13.33±6.67^{a}	20.00±11.55^{cd}
7.8 mM	10	26.67±26.67^{a}	0.00±0.00^{a}	53.33±26.67^{abc}
7.8 mM	15	33.33±17.64^{a}	20.00±11.55^{a}	53.33±13.33^{abc}
7.8 mM	20	46.67±6.67^{a}	13.33±13.33^{a}	33.33±17.64^{abdc}
7.8 mM	25	66.67±6.67^{a}	20.00±11.55^{a}	6.67±6.67^{cd}
10.4 mM	10	0.00±0.00^{a}	6.67±6.67^{a}	63.33±6.67^{ab}

% NaOcl	Time of exposure (Min.)	% Bacterial contamination	% Fungal contamination	% surviving clean explants
10.4 mM	15	0.00±0.00[a]	20.00±11.55[a]	70.00±11.55[a]
10.4 mM	20	3.33±3.33[a]	6.67±6.67[a]	73.33±6.67[abc]
10.4 mM	25	13.33±13.33[a]	33.33±6.67[a]	46.67±13.33[abcd]
13.0 mM	10	13.33±13.33[a]	46.67±6.67[a]	20.00±11.55[cd]
13.0 mM	15	6.67±6.67[a]	40.00±20.00[a]	13.33±6.67[cd]
13.0 mM	20	33.33±6.67[a]	0.00±0.00[a]	26.67±6.67[bcd]
13.0 mM	25	20.00±11.55[a]	13.33±13.33[a]	0.0±0.00[d]

Values with the same letter in the same column are not significantly different by Tukey's pair-wise comparison ($P \geq 0.05$).

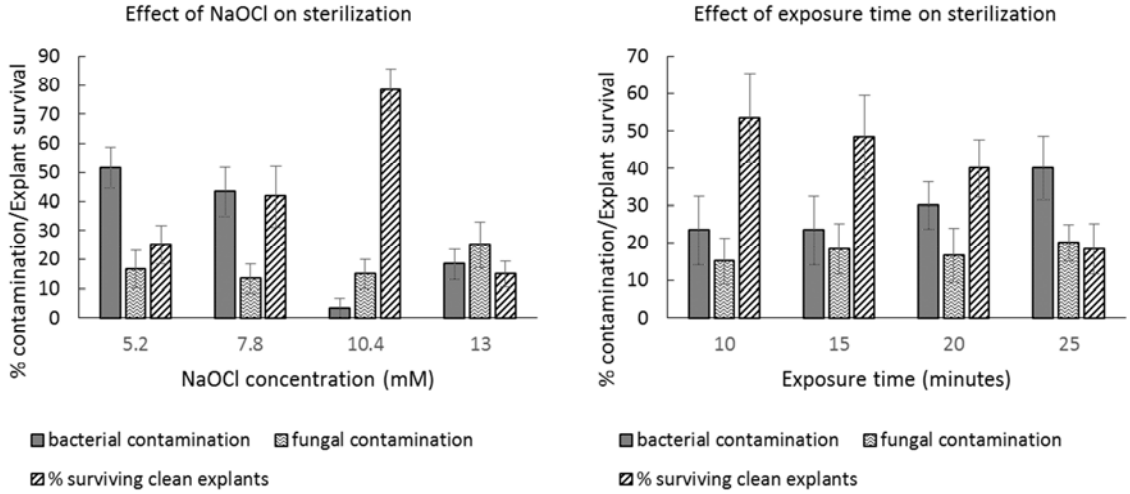

Figure 1. *The effects of NaOCl concentration and exposure times on sterilization.*

3.2. Callus Induction and Multiplication

The effect of plant growth regulators BAP, Kin, NAA and TDZ at different combinations to form both embryogenic and non embryogenic calli was assessed. When leaf explants were cultured in media containing these hormones, it was observed that callus started developing about 9-13 days of culture from the edge of the leaf and extended into the interior in the responding leaves (Figure 2a). However, depending on the hormone combination used, the appearance of these calli differed. Cream yellow compact calli were observed on 2.0mg/lBAP+0.8mg/lKin+0.4mg/NAA+0.5mg/lTDZ (high) hormone combination which started forming after 13 days of culture (Figure 2b). This hormone combination showed the highest callus formation frequency (68%), although it resulted in a mixture of both embryogenic and non embryogenic calli. At 1.5mg/lBAP+0.8mg/lKin+0.4mg/lNAA+0.5mg/lTDZ and 1mg/lBAP+0.4mg/lKin+0.2mg/lNAA+0.05mg/lTDZ, soft friable white loosely packaged calli were observed and started forming earlier after 9 days of culture (Figure 2c). The 1.5mg/lBAP+0.8mg/lKin+0.4mg/lNAA+0.5mg/lTDZ hormone combination had a callus formation frequency of 39% while the 1mg/lBAP+0.4mg/lKin+0.2mg/lNAA+0.05mg/lTDZ hormone combination had a callus formation frequency of 13%. Although these two low hormone combinations formed reasonable embryogenic calli, these soft friable calli could not survive beyond two subcultures and turned brown and died after the second subculturing. Interestingly, it was observed that when the white friable calli from the low hormone combinations were transferred to the high hormone combination, they survived multiple subcultures. Data analysis revealed that there was a significant difference in callus formation using the three hormone combinations (P=<0.001), with most calli forming at the 2.0mg/lBAP+0.8mg/lKin+0.4mg/NAA+0.5mg/lTDZ combination and the least calli forming at 1mg/lBAP+0.4mg/lKin+0.2mg/lNAA+0.05mg/lTDZ combination (Table 2).

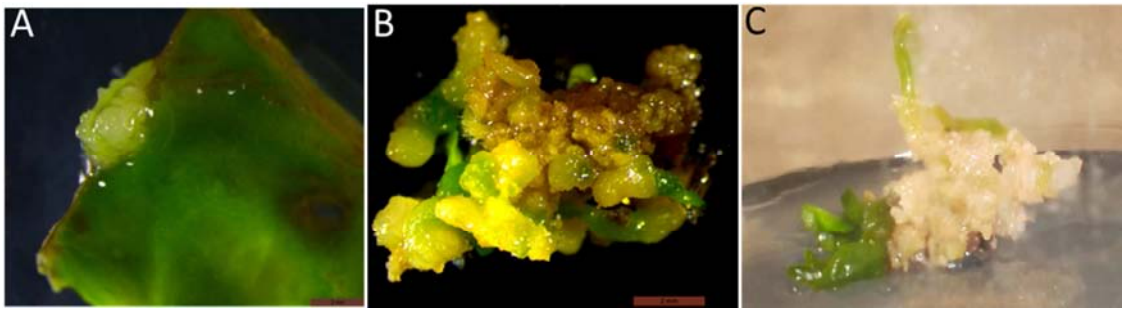

Figure 2. *Embryogenic calli appearance at different hormone combinations. (A) The beginning of the callus formation process at the margin of the leaf. (B) Hard compact cream yellow callus forming at high hormone combination. (C) Soft white callus forming at low hormone combination medium.*

3.3. Embryo Formation, Shooting and Rooting

Two weeks after callus formation in all the three media types, live calli were sub-cultured to fresh media containing the same concentration of growth hormones as in callus induction and allowed to develop somatic embryos. It was observed that although somatic embryos formed in all the three media types, most embryos forming in media supplemented with 1.5mg/lBAP + 0.8mg/lKin + 0.4mg/lNAA + 0.5mg/lTDZ and 1mg/lBAP + 0.4mg/lKin + 0.2mg/lNAA + 0.05mg/lTDZ did not grow to maturity since with subsequent sub-culturing, these embryos died as the callus turned brown. However, medium supplemented with 2.0mg/lBAP + 0.8mg/lKin + 0.4mg/lNAA + 0.5mg/lTDZ was noted to be effective on which a maximum number of embryo were formed. These observed somatic embryos contained all the stages of embryogenesis i. e. globular, heart, torpedo and cotyledonary (Figure 3i) and were light green at the early formation stages and turned dark green at maturity. These results confirmed that the requirement of PGRs at different concentration has effect on the stages of the embryo development. Also somatic embryos with nodular structure forming shoots and roots on callus clumps were observed (figure 3ii).

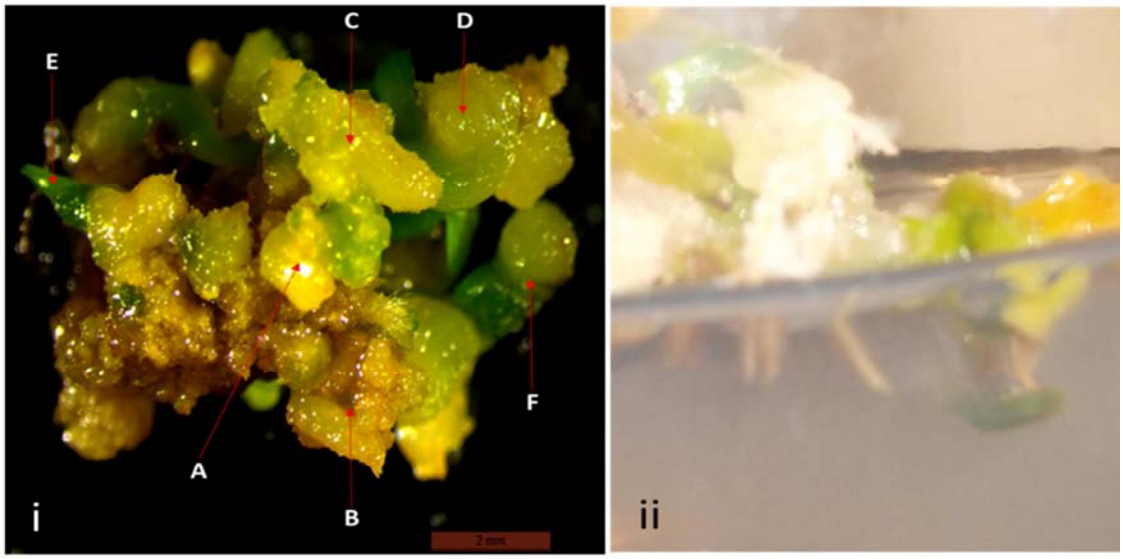

Figure 3. *(i) Somatic embryo development. (A) The globular stage (B) The heart stage (C) The torpedo stage (D) The cotyledonary stage (E) Mature somatic embryo (F) Young somatic embryo (ii) Roots and shoots forming from callus.*

Table 2. *The effect of different growth regulators on explant callus formation and shooting.*

BAP+Kin+NAA+TDZ	Callus	shoots
1.0+0.4+0.2+0.05	13±4.5[a]	2±0.0[a]
1.5+0.6+0.3+0.1	39±8.2[b]	9±2.8[a]
2.0+0.8+0.4+0.2	68±8.4[c]	29±6.6[b]

Values with the same letter in the same column are not significantly different by Tukey's pair-wise comparison (P ≥ 0.05).

Once somatic embryos matured, they were transferred to fresh media for development of shoots. Somatic embryos were considered mature as soon as the shoot (epicotyl) formed and elongated with presence of a radicle structure which is a precursor to root induction. The effect of different media on shoot induction and multiplication was evaluated by counting the number of emerging shoots after four weeks of culture. Shoot primordia were observed within 21 days of transfer to new media. Statistical analysis revealed that there was a significant difference in the number of shoots in the three media types (P=0.011), with most shoots forming in MS medium with 2.0mg//BAP + 0.8mg/lKin + 0.4mg/lNAA + 0.5mg/lTDZ compared to other treatments (Table 2). Actually, as PGRs levels increased, the number of shoots decreased significantly. At 1mg/lBAP + 0.4mg/lKin + 0.2mg/lNAA + 0.05mg/lTDZ, the mean number of shoots were 2, while at 1.5mg/lBAP + 0.8mg/lKin + 0.4mg/lNAA + 0.5mg/lTDZ the mean number of shoots was 9 and a mean of 29 emerging shoots observed at 2.0mg//BAP + 0.8mg/lKin + 0.4mg/lNAA + 0.5mg/lTDZ.

The role of organic supplement on root induction was examined after optimizing the synergistic effect of the cytokinin BAP and an auxin IBA. After 8 weeks of culture under light condition, root primordia were observed only in one treatment (Table 3). Statistical analysis revealed that there was a significant difference in the number of roots emerging from the shoots (P=<0.003). A mean of one root per shoot was observed in the treatment supplemented with 1.0 BAP+0.5 IBA extending from the shoots (Figure 4). The other treatments supplemented with 0.5 BAP+0.25 IBA and 1.5 BAP+0.75 IBA did not form any roots at all.

Table 3. *The effect of BAP and IBA on root formation.*

BAP+IBA	Roots
0.5+0.25	0.00[a]
1.0+0.5	1.00[b]
1.5+0.75	0.00[a]

Values with the same letter in the same column are not significantly different by Tukey's pair-wise comparison (P ≥ 0.05).

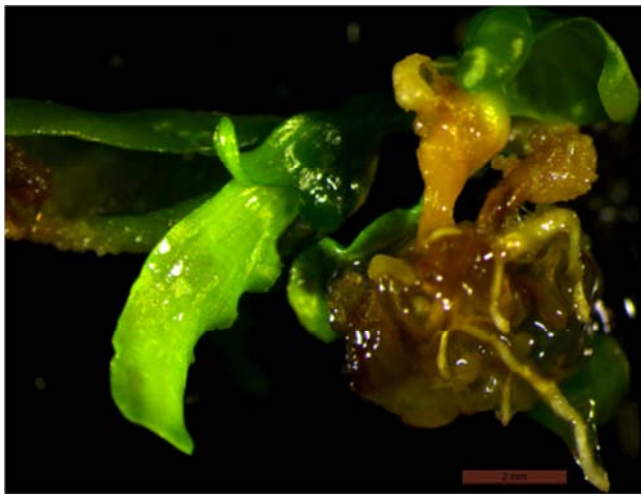

Figure 4. *Roots forming on regenerated shoots in media supplemented with 1.0 BAP+0.5 IBA.*

4. Discussion

The first step in any plant tissue culture process is to get explants that are free from microbial contamination. Toward this aim, many strerilants are used, most of which are commercial bleaches. One popular sterilization agent amongst many used to disinfect explants is Jik® bleach, used at time intervals of between 20-45minutes. From this study, it was observed that optimum sterilization using this commercial bleach was achieved at a concentration of 10.4 mM for 20 minutes and that higher concentrations of 13 mM and longer exposure times of 25minutes caused scorching to the leaf. This adverse tissue damage observed on higher concentrations of NaOCl could be is due to the fact that the sterilant is toxic to plant tissues [10]. Other studies have reported efficient sterilization of explants using NaOCl. For example, Tiwari *et al*. (1998) reported an efficient sterilization of different explants of Brahmi (*Bacopa monniera*) using NaOCl. Although its use alone could not achieve efficient sterilization of explants, its supplementation with a systemic fungicide and an antibiotic enabled it achieve efficient sterilization of these explants. In agreement with our findings, supplementing NaOCl with the systemic fungicide redomil® ensured efficient sterilization compared to explant sterilization using NaOCl only. Ndakidemi *et al*. (2013), working on *B. huillensis* leaf explants reported efficient sterilization using NaOCl concentrations of 1.5% v/v as the optimum concentration for 10 minutes supplemented with tween 20 and the antifungal cefotaxime. Actually, almost all sterilization protocols reported using NaOCl involve additional use of either an antifungal or antibacterial or in most cases both.

Somatic embryogenesis has been achieved successfully in several woody plants from leaf explants [9, 12, 13]. Somatic embryos arise either directly on somatic explants or on intermediate callus in which cells somehow acquire competence. In this study increased levels of PGRs resulted in the expression of somatic embryogenesis with creamy yellow compact calli which started forming at the cut edges of the explants. This observation is in agreement with that of Hatanaka et al. (1991) who observed that embryogenic calli started forming at the cut edges of *Coffea canephora* explants. This phenomenon could be due to the fact that the cut surfaces are probable sites for rapid uptake of mineral and hormones resulting in the high percentage of calli formation. The results confirm that the requirement of PGRs at different concentrations has a significant effect on callus induction and proliferation as well as development of somatic embryos.

5. Conclusion

Conservation and sustainability of *Schizozygia coffeoides* is imperative for future use. This study revealed the best sterilization protocol and the regeneration via somatic embryogenesis showing the derivered explant as a good somatic embryogenesis potential since callus initiation was noticed as creamy white (pre-embryogenic masses or pre-globular pro embryos) which are loosely packed and can be used for initiating cell suspension cultures. Additionally, the presence of cytokinins BAP in combination with TDZ was efficient for shoot multiplication and proliferation. The explant derived from the young leaf shows good somatic embryogenesis potential which is a major criteria in somatic embryogenesis alongside several factors including the genotype carbon source type and concentration of exogenous growth regulators. The somatic embryogenesis process developed in this study could be utilized for *In vitro* culture and transformation studies, as well as secondary metabolites production experiments and cell suspension cultures to improve the plant.

References

[1] WHO. 2005. National policy on traditional medicine and regulation of herbal medicines Report of a WHO global survey. Geneva.

[2] Atanasov AG, Waltenberger B, Pferschy-Wenzig EM, Linder T, Wawrosch C, Uhrin P, Temml V, Wang L, Schwaiger S, Heiss EH, Rollinger JM, Schuster D, Breuss JM, Bochkov V, Mihovilovic MD, Kopp B, Bauer R, Dirsch VM, and Stuppner H. 2015. Discovery and resupply of pharmacologically active plant-derived natural products: A review. Biotechnol Adv 33: 1582–1614.

[3] Magadula JJ, and Erasto P. 2009. Bioactive natural products derived from the East African flora. R Soc Chemestry 26: 1535–1554.

[4] Kariba RM, Houghton PJ, and Yenesew A. 2002. Antimicrobial activities of a new schizozygane indoline alkaloid from Schizozygia coffaeoides and the revised structure of isoschizogaline. J Nat Prod 65: 566–569.

[5] Maurice D, and Henri J. 1971. Contribution à l'inventaire des plantes médicinales de la côte Est de Madagascar. ORSTOm 8: 35–47.

[6] Murashige T, and Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant.

[7] Jiménez VM. 2001. Regulation of in vitro somatic embryogenesis with emphasis on to the role of endogenous hormones. Rev Bras Fisiol Veg 13: 196–223.

[8] Singh P, Singh A, Shukla AK, Singh L, Pande V, and Nailwal TK. 2009. Somatic embryogenesis and in vitro regeneration of an endangered medicinal plant sarpgandha (rauvolfia serpentina l.). Life Sci J 6: 57–62.

[9] Ndakidemi CF, Mneney E, and Ndakidemi PA. 2013. Development of Sanitation Protocol for Leaf Explants of B. huillensis for in Vitro Culture. Am J Plant Sci 4: 2425–2430.

[10] Çölgeçen H, Koca U, and Toker G. 2011. Influence of different sterilization methods on callus initiation and production of pigmented callus in Arnebia densiflora Ledeb. Tubitak 35: 513–520.

[11] Tiwari V, Singh BD, and Tiwari KN. 1998. Shoot regeneration and somatic embryogenesis from different explants of Brahmi [Bacopa monniera (L.) Wettst.]. Plant Cell Rep 17: 538–543.

[12] Wang HC, Chen JT, and Chang WC. 2006. Somatic embryogenesis and plant regeneration from leaf, root and stem-derived callus cultures of Areca catechu. Biol Plant 50: 279–282.

[13] Othmani A, Bayoudh C, Drira N, Marrakchi M, and Trifi M. 2009. somatIC embryoGenesIs and plant reGeneratIon In Gymnema sylvestre. Plant Cell Tissue Organ Cult 38: 71–79.

[14] Hatanaka T, Arakawa O, Yasuda T, Uchida N, and Yamaguchi T. 1991. Effect of plant growth regulators on somatic embryogenesis in leaf cultures of Coffea canephora. Plant Cell Rep 10: 179–182.