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Efficient *in vitro* Ginger Plant Material Production (*Zingiber officinale* roscoe)

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ABSTRACT

Conventional seed production techniques in *Zingiber officinale* roscoe are not appropriate for addressing problems related to anthropogenic pressure, biotic constraints, and low-rate multiplication by asexual modes. This study evaluated *in vitro* culture conditions for explant disinfection, plantlet proliferation/rooting, and plant acclimatization in ginger. Buds, after treatment following various concentrations of mercuric chloride and immersion durations, are cultured on the basic medium of *Murashige and Skoog* (MS) supplemented with NAA and Cytokinins (BAP / Kinetin / Zeatin) for six weeks. The results indicated a highly significant ($P < 0.001$) and very significant ($P < 0.0001$) difference between disinfection treatments, proliferation, rooting, and organogenesis culture media. Treatments

with $HgCl_2$ (0.05%) for 5 and 10 min on the one hand and with $HgCl_2$ (0.1%) for 10 min of immersion on the other hand were indicated for survival explants. The culture medium with the plant growth regulator combination of 0.5 mg/l (NAA) + 4 mg/l (BAP) was more favorable for shoot proliferation (6.27±0.4), leaves number (9.53±1.3) while the medium with 0.5 mg/l of NAA + 4 mg/l of Kinetin (7.5 cm) better influenced shoot growth. Regarding rhizogenesis, 0.5 mg/l of NAA was more favorable for the length of the roots (7cm) and number of roots formed (20.2). Acclimatization was more successful with weaning under growing room conditions, with a survival rate of 70% after 4 weeks. This study will improve the large-scale production of ginger plantlets and guide research on the production of secondary metabolites.

Keywords: *Zingiber Officinale*, *in vitro* culture, organogenesis proliferation, Mercuric chloride, plant growth regulator.

INTRODUCTION

Ginger (*Zingiber officinale* Roscoe) from the family *Zingiberaceae* is an important plant species whose rhizomes are mainly used as a spice and in traditional medicine for humans (1). Globally, the rhizome is the tissue used in plant vegetative propagation, food storage, and raw materials for pharmaceutical industries (2,3). Ginger is used as a medicinal plant to treat a wide array of illnesses and disorders because of its anti-inflammatory properties. In addition to these properties, ginger has antidiabetic, antiallergic, antiemetic, antihepatotoxic, antinauseant, antioxidant, antiparasitic, antiplatelet, antiseptic, antitussive, cardiovascular, digestive, and hypoglycemic activities (4). Regarding the importance of ginger, it faces high anthropogenic pressure. In contrast, ginger is a sterile reproductive plant that must only be propagated by rhizomes, which produce a lower proliferation rate, considering that the rhizomes are the economically used part of the plant. Anthropogenic pressure reduces the availability of suitable planting material for cultivation. Moreover, Soil-borne pathogen infection of rhizomes constitutes the most important constraint for reducing yield (5). Rhizomes may degenerate under long-term vegetative propagation, and breeding could not be effective because of poor flowering and sterility problems. To overcome the problems associated with ginger production, the use of modern vegetative propagation methods, mainly *in vitro* tissue culture, is a promising alternative. The success *of in vitro* ginger regeneration depends on explant disinfection (6, 7) and the type and concentration of growth regulators in the medium (8,9, 10). Recent work on in vitro ginger propagation provides important information about the types of explants and culturing factors, such as disinfection chemicals and plant growth regulators (11, 12). Sodium hypochlorite was used alone (13, 14) or along with mercuric chloride (15) for ginger explant disinfection. A high infection rate of explants (about 35%) has resulted from this treatment (15). Improvement of the disinfection procedure of ginger explants is required to optimize the aseptic culture rate to 70%. Concerning bud regeneration and proliferation, Naphthalene α-Acetic Acid (NAA) in combination with different doses of 6-Benzylaminopurine (BAP) (16, 17) or kinetin was tested without examining the effects of zeatin, a natural cytokinin. Although several studies have reported the use of auxins in ginger plantlet rooting (1, 15), it is important to optimize root development for particular genotypes because these genotypes respond differently in tissue culture. Finally, successful adaptation of plantlets to natural environments is the key to achieving the objectives of mass propagation of ginger plants. The present study

was conducted to determine the dose of mercuric chloride for optimal aseptic culture establishment and highlight effective and reliable doses of cytokinins/auxins for ginger *in vitro* proliferation and rooting for successful acclimatization.

MATERIALS AND METHODS

Plant Material

The plant material included ginger rhizomes obtained from an agro-pastoral farm in Athiémè Township, Benin. The experiment was carried out in the Central Laboratory of Plant Biotechnologies and Plant Breeding of the Department of Genetics and Biotechnologies at the University of Abomey-Calavi (Republic of Benin) from May 2021 to July 2022.

Explant Disinfection and Establishment:

Ginger rhizomes were carefully washed with soap and tap water and placed in a white bag lined with black for a week to facilitate the emergence of buds (14). After 1 week, the induced bud sprouts were washed with tap water followed by distilled water. The axillary buds, approximately 15 mm in length, were excised and washed with soap and distilled water. The buds were briefly immersed in 70% ethanol for 2 min for surface sterilization, followed by immersion in mercuric chloride at different concentrations (0.05%; 0.1%; 0.15%) (w/v) dropped with 20 (as a wetting agent) for 5 and 10 min for each mercuric chloride concentration. Finally, the explants were rinsed (3 to 4 times) with sterile distilled water (1). These explants were reduced to 2 mm and cultured on *MS* medium containing 30 g/l of sucrose, 7 g/l of agar, and 0.75 g/l ($MgCl_2$) (18 Samuel et al., 2017) for explant germination.

Shoot Proliferation:

At this stage, the sprouted buds were incubated with MS-based medium (19) containing sucrose (30g/l) and agar (7g/l) supplemented with the same concentration (4mg/l) of 6-benzyladenine (BAP), Kinetin (Kin), and Zeatin and their combination with 0.5 mg/l of Naphthalene Acid Acetic (NAA). After six weeks of culture, growth parameters, such as the number and length of the shoots, and leaf number/shoot, were recorded.

Plantlets Rooting

Shootlets obtained from proliferations >3cm were individually cultivated in MS-based media with sucrose (30mg/l) and agar (6mg/l) supplemented with different concentrations of NAA (0.1, 0.3, 0.5, 0.8, 1 mg/l). Parameters such as root number and root length were recorded six weeks after culturing.

The Media Preparation Process

MS nutrient elements with sucrose were mixed using distilled water for the media preparation. The pH of the mix was adjusted to 5.7 before the agar addition to solidify the media. The prepared media were sterilized using an autoclave at 121 ^{o}C and 1.2 kg/cm^2 for 20 min. The cultures were incubated in a growth chamber under a photoperiod of 16 h light and 8 h dark at 80% relative humidity and 3000 lux approximatively for light intensity.

Acclimatization Stage:

The plantlets were delicately extracted from the jars and thoroughly washed with tap water to remove the nutrients and agar from the roots (20). The plantlets were then transplanted in

small pots containing a mixture of a sterilized substrate composed of compost, mold, and cocomat in proportions of 1:2:2 (1C2M2C) respectively (21). The culture was performed under two culturing conditions: room condition (Temperature (TM) 26.54 °C / Relative Humidity (RH) 96.16%) and tunnel condition (Temperature (TM) 29.94 °C /Relative Humidity (RH) 67.38%). Pots were covered with polythene bags and sprayed with water every two days to maintain humidity. The pots were moved to the greenhouse after four weeks of acclimatization. Shives and Robbins solution (1942) was prepared and used to water plantlets at 2-day intervals (22). The plastic coverings were gradually removed to expose the plantlets to natural conditions.

Statistical Analysis

In all stages of regeneration, five explants were considered for each treatment, and the experiment was replicated three times. The collected data concerning each evaluated parameter was for variance analysis (ANOVA) using Statistica software (Ver 6.1). The mean values were compared, and significant differences among treatments were determined using the Fisher's exact test (*F-test*) at the 5% threshold. The graphics were designed using EXCEL 2010. Regarding the plantlet acclimatization experiment, the χ^2 *independence test* was used following the generalized linear model to determine the relationship between survival rate, temperature, and relative humidity during the process.

RESULTS

Mercuric Chloride (*$HgCl_2$*) Effectiveness in Ginger Aseptic Culture *in vitro*

There was a significant difference (p = 0.003) among mercuric chloride treatments on the survival of buds. In the overall experiment, 57% of the explants were cultured aseptically and 43% were necrosed. Among the survival explants, 25% were infected. The immersion duration of the explants and their interaction with the disinfectant concentration showed no significant influence on the survival rate of the explants.

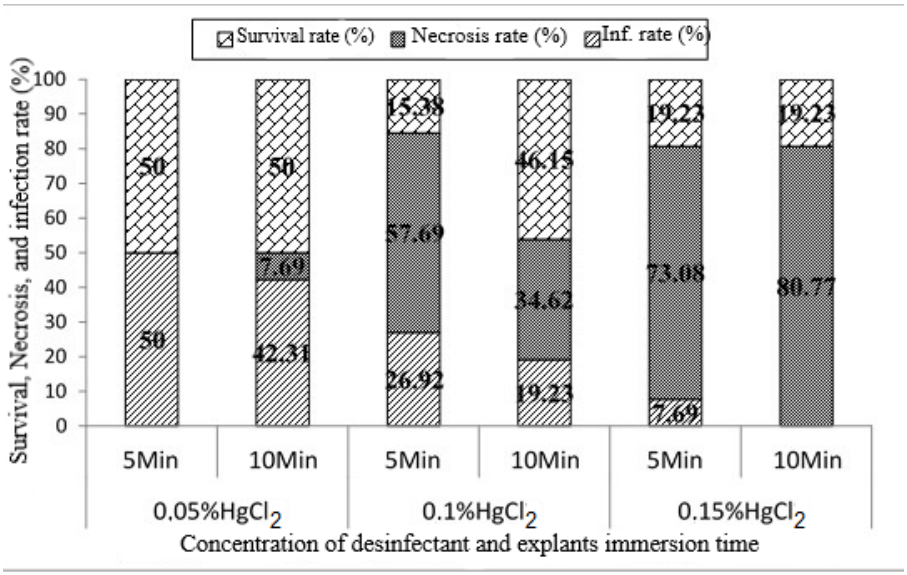

Figure 1: Effect of mercuric chloride on ginger buds' survival after six weeks of culture on MS Medium.

Indeed, the highest survival rate (50%) of infection was obtained with 0.05% of $HgCl_2$ regardless of immersion durations of 5 and 10 min, whereas the lowest survival rate (15.38%) of infection was observed with 0.1% of $HgCl_2$ for 5 min of immersion. Furthermore, regardless of the immersion duration (5 min, 10 min), 0.15% of $HgCl_2$ resulted in 19.23% survival compared with 46.15% survival among those with 0.1% of $HgCl_2$ for 10 min of immersion (Fig. 1).

Exogenous 6-Benzylaminopurine, Kinetin, and Zeatin Stimulate Ginger Proliferation in vitro:

There was a significant difference ($P< 0.0001$) among 6-Benzylaminopurine, Kinetin, and Zeatin treatments on proliferation parameters, such as the number of shoots/explants, shoot height, and leaf number. Considering the actions of growth regulators alone, MS supplemented with Zeatin (M4) recorded maximum shoot numbers/explants and leaves respectively 4.8 and 6.87 (Table 1). The highest shoot height was recorded on M3 (3.54±0.6 cm), whereas the lowest height was obtained on M2 (3.12±0.6 cm). The lowest shoot numbers/explants and leaves were obtained on MS supplemented with Kinetin (M3) at levels of 3.0 and 4.6, respectively. The combination of the previous growth regulators with 0.5 mg/l of NAA has significantly changed the number of shoots, explants, and leaves. The highest numbers of shoots/explants and leaves were recorded with the combination of NAA and BAP (M5) at 6.27 and 9.53, respectively, while the height of shoots was high on M6 (Table 1). High coefficient of variation values of 51.47%, 50.37%, and 65.57% were observed for the number of shoots, number of leaves, and shoot height, respectively (Table 1). Figure 2 shows the developmental steps during the proliferation stage of vigorous shoots and green leaves.

Table 1: Mean number of shoots/explants, number of leaves, and shoot height on MS media supplemented with various concentrations of NAA, BAP, kinetin, and zeatin.

Media	PGR content	Number of shoots	Numbers of leaves	Shoot height (cm)
M1	0 PGR	1.33±0.4a	2.47±1.3a	2.20±0.6a
M2	4mg/l (BAP)	3.73±0.4b	4.67±1.3b	3.12±0.6b
M3	4mg/l (Kinetin)	3±0.4b	4.6±1.3b	3.80±0.6b
M4	4mgl (Zeatin)	4.8±0.4b	6.87±1.3c	3.54±0.6b
M5	0.5mg/l (NAA)+4mg/l (BAP)	6.27±0.4c	9.53±1.3d	6.05±0.6c
M6	0.5 mg/l (NAA)+4mg/l (Kinetin)	4±0.4b	8,33±1.3bd	6.80±0.6c
M7	0.5 mg/l (NAA)+4mg/l (Zeatin)	3.87±0.4b	6.6±1.3ac	5.73±0.6d
	R^2 (%)	21	65.6	53
	Mean	4.14±2.13	6.15±6.40	4.46±2.27
	CV (%)	51.47	50.37	65.57
	Pr > F, where	0.0002	< 0.0001	< 0.0001

Legend: Means followed by the same letter are not statistically different at the 5% threshold; R^2: Coefficient of determination; Avg.: Average; CV: Coefficient of variation; Pr: probability; F = test of fisher; PGR: Plant growth regulators

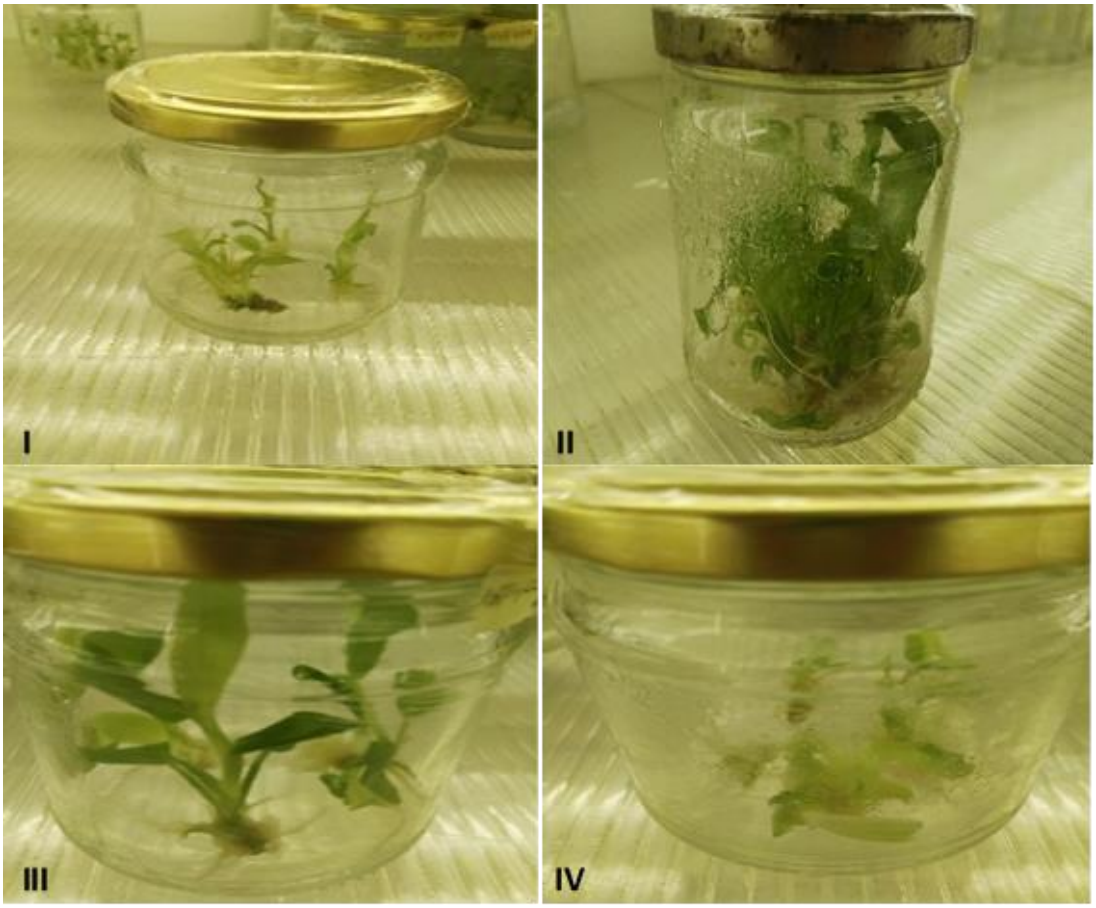

Figure 2: Organogenesis and proliferation of ginger plantlets.
Legend: I) *Plantlets in proliferation on MS without growth regulators*; II) *Plantlets in proliferation on MS supplemented with 0.5 mg/l NAA + 4 mg/l BAP;* III) *Plantlets in proliferation on MS supplemented with 4 mg/l (Zeatin)*; IV) *Plantlets in proliferation on MS supplemented with 4 mg/l (Kinetin).*

1-Naphthalene Acetic Acid (NAA) Controls the Rooting of Ginger Plantlets

There was a significant difference ($P < 0.0001$) between the effects of different doses of NAA on the root number and length of plantlets. The coefficients of variation were 52.71% and 60.73% for root length and root number, respectively (Table 2). *Figure* 3 shows that the medium containing 0.5 mg/l NAA had the highest root number (20.2/ explant) and root length (6.4 cm). The lowest numbers of roots (10.47) and root lengths (2.55 cm) were observed in the medium containing 0.8 mg/l of NAA (*Figure* 3). Although the difference in parameters was significant, the value of the coefficient of determination (R^2) was low (17.36%) for the number of roots and root length average (29.17%) (Table 2). Figure 4 shows root formation and elongation during the rooting stage of the plantlets.

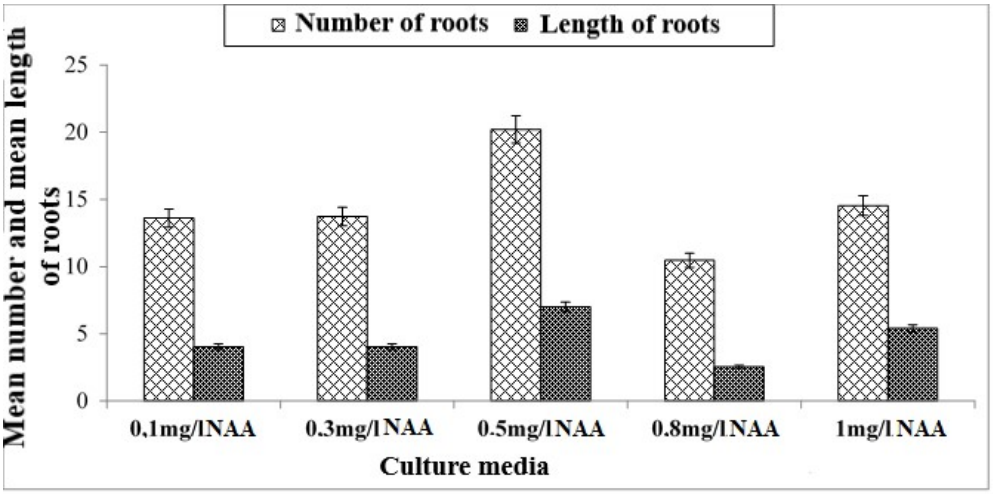

Figure 3: Roots number and length average of ginger according to NAA concentration in the media

Table 2: Number of fish roots and average length of ginger

Statistique	Numbers of roots	Root length
R^2	17.36**	29.17***
Mean.	14.51±7.65	4.61±2,79
CV (%)	52.71	60.73
F	3.675	7.209
Pr > F	0.008**	< 0.0001***

R^2: Coefficient of determination; Avg.: Average; CV (%): Coefficient of variation; Pr: Probability;
***: very highly significant difference at the 0.1% threshold **: highly significant difference at the 1% threshold.

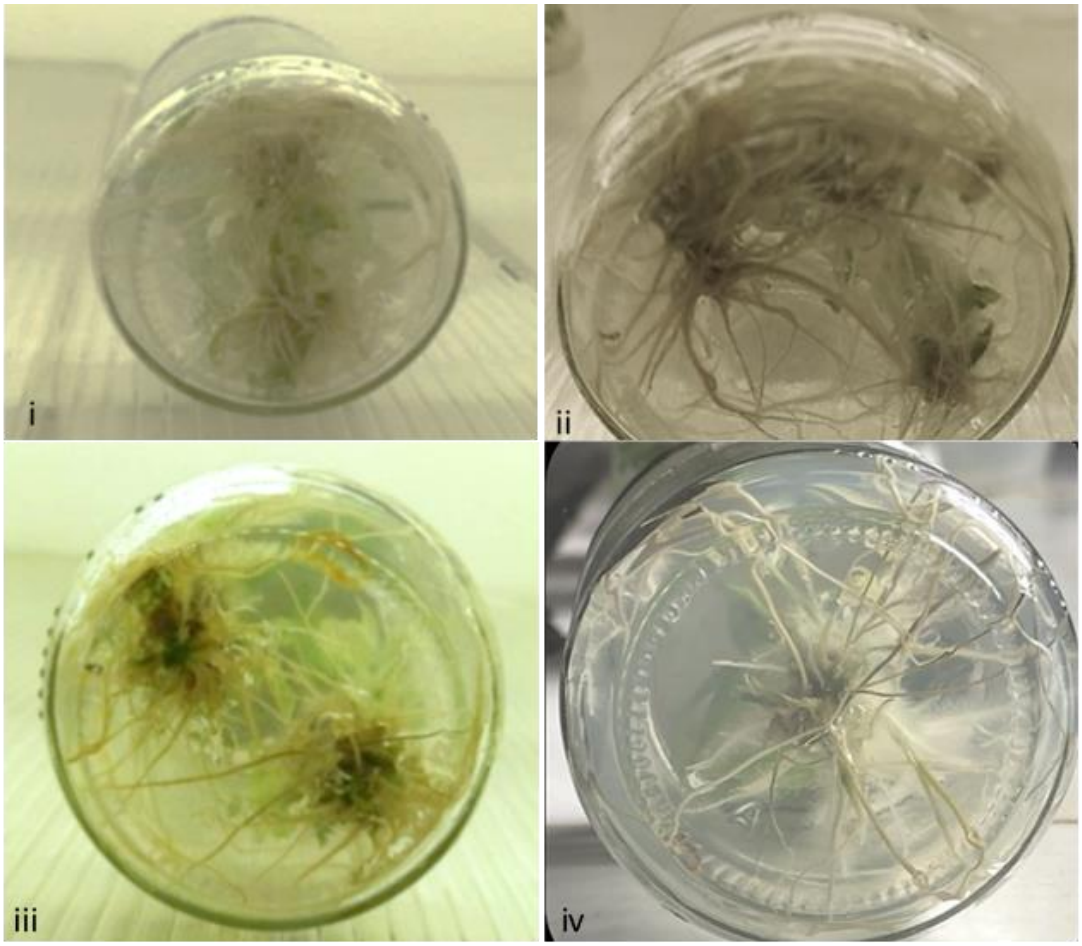

Figure 4: Root development of ginger plantlets on MS supplemented with NAA.
Legends: i) *Plantlets rooting on MS supplemented with 0.8 mg/l* of NAA; ii) *Plantlets rooting on MS supplemented with 0.5 mg/l of NAA;* iii) *Plantlets rooting on MS supplemented with 0.3 mg/l of NAA*; iv) *Plantlets rooting on MS supplemented with 1 mg/l of NAA.*

Physical Factors Determine the Success Rate of Ginger Plantlet Acclimatization

There was a significant difference ($p = 0.003$) in the survival of the plantlets according to the time of culture (Figure 5A). Likewise, physical factors such as temperature and relative humidity significantly influenced ($p = 0.025$) the survival rate of plantlets. The interaction of physical factors and culture duration had no significant ($p=0.999$) influence on the survival of plantlets four weeks after weaning. 100% of survival plantlets were obtained during the first week of culture under both culture room conditions (TM 26.54 °C / RH 96.16%) and tunnel (TM29.94° C /RH 67.38%). After the second week, the rate of survival of the plantlets was 67.38% under tunnel conditions, whereas under room conditions, the rate of survival of the plantlets was increased to 96.16% (weaning) (Figure 5A). The highest survival rate (82.5%) was obtained under room conditions, whereas the lowest survival rate (62.5%) was obtained under tunnel conditions (Figure 5 B). Figure 6 shows the acclimatization process of the plantlets.

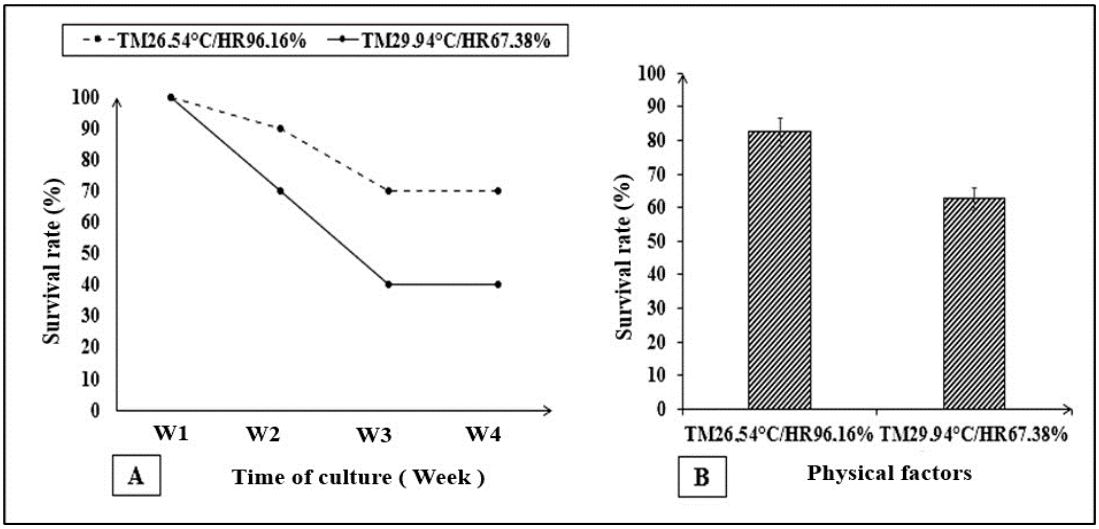

Figure 5: Percentage of survival plantlets according to the time, temperature, and relative humidity.

Legends: *W = week; TM = temperature, relative humidity,*

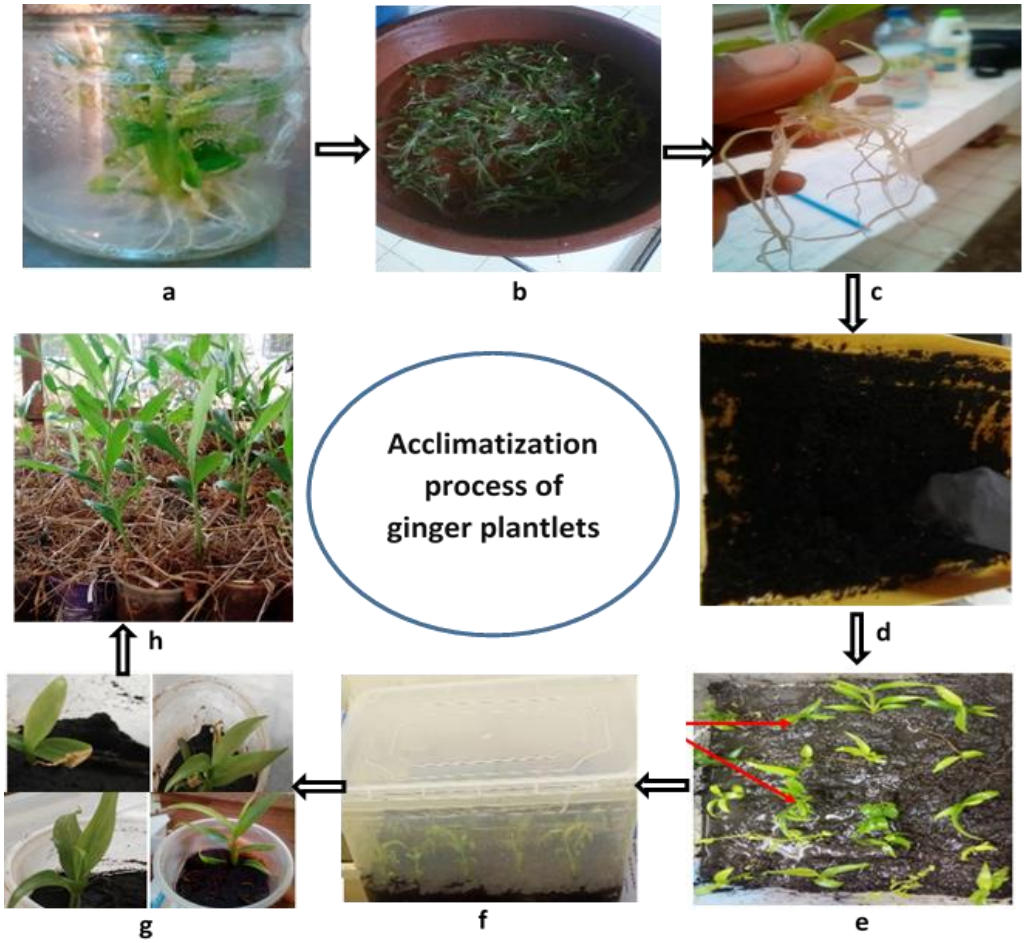

Figure 6: Ginger plantlets acclimatization steps

Legends: a) Plantlets ready to be acclimatized; b) Plantlets removed from medium and rinsed; c) Plantlets with developed roots; d) Substrate preparation; e) Plantlets sowing on the substrate; f) progressive culture in

transparent plastic; g) Plantlets transplanted into new pots for growth; h) Acclimatized ginger plantlets in development.

DISCUSSION

The *in vitro* disinfection, proliferation, and acclimatization of ginger survival plantlets as part of mass production are important findings in connection with several neighboring species. Adequate disinfection of explants and appropriate culture media are the main factors determining *in vitro* culture success (7). Mercuric chloride has been shown an efficient disinfectant of explant in *Tectona grandis*, *Discoscora sp* (23). In ginger, the significant positive effect of mercuric chloride obtained according to the duration of immersion (5 min or 10 min) in the explants showed the efficacy of mercuric chloride in ginger explant disinfection. Disinfection of rhizome buds was performed using 0.1% mercuric chloride ($HgCl_2$) solution for 10-20 minutes to establish aseptic cultures in *Curcuma longa* (24). The low survival rate (19%) obtained with the 0.15% ($HgCl_2$) treatment after 5 min of immersion is attributable to the strong necrotizing power of the disinfectant. The use of high-dose mercuric chloride could have an effect inducing the death of the explants (23). In taro, apical buds disinfection with 0.15% $HgCl_2$ for 5 min controls the infection with a better survival rate (25). Regarding organogenesis and proliferation, significant differences were observed in leaf, root, and shoot numbers according to culture media. The media containing NAA (0.5 mg/l) and BAP (4 mg/l) was found to be more suitable for shoot proliferation, with 6.27 shoots per bud and 9.53 leaves per plant. A similar combination of NAA (0.5 mg/l) and BAP (3 mg/l) in the yellow ginger variety showed low performance (2 shoots/ bud et five leaf/shoot) (17). MS-medium containing 4.5 mg/l of BAP induced the highest percentage of shoot multiplication (26). We found better proliferation and growth rates in media supplemented with NAA (0.5mg/l) and BAP (4mg/l). This observation is related to the synergistic action of auxins and cytokines during ginger plant cell division. Furthermore, kinetin (4mg/l) responds better to rhizogenesis than the other cytokinins. This finding highlights the synergistic effects of NAA and cytokinin in organogenesis, (23, 27).

Likewise, Akplogan et al. (22) study on *Colocasia esculenta* proliferation and observed better proliferation with the combination of 4 mg/l (BAP) + 0.5 mg/l (NAA) in media and better leaf and root formation with the combination of Kinetin (4mg/l) and NAA (0.5mg/l). The best proliferation (6.27) and shoot growth (7.5 cm) on the combined NAA (0.5mg/l) + BAP (4mg/l) medium were approximately equal to those obtained with Ethiopian ginger on the medium containing the combined two cytokinins BAP (2 mg/l) and kinetin (1 mg/l) (28). The combined effect of the two cytokines is efficient for ginger root regeneration. The effects of zeatin are comparable to those of Thidiazuron (TDZ), which is already observed in ginger seedlings. Despite the effectiveness of kinetin, it was preferred to evaluate the effects of NAA with BAP to guarantee better development of aerial organs. For underground organs, culture media with different concentrations of NAA have a significant influence on the number and length of roots formed. In addition, NAA alone was more favorable for root induction (20.2) than each cytokinin. The work of Hossain et al. (27) on Indian ginger genotypes revealed a significantly lower root initiation response (43.46%), even with a higher NAA dose (2 mg/l) but in combination with IBA (2 mg/l). A concentration of 0.5 mg/l of NAA is optimal for ginger rhizogenesis. Media supplementation with cytokines (BAP, Kinetin, or Zeatin) even without

NAA led to the development system with a lower average root. This observation is attributable to the presence of endogenous auxins in the buds that stimulate the formation of roots (29).

CONCLUSION

The study carried out within the framework of ginger micropropagation identified an efficient method for ginger seed production that accelerates its cultivation. The disinfection of ginger rhizome buds, the combinations of growth regulators for plantlet proliferation, and the survival conditions during acclimatization were understood. Buds immersed in 0.1% mercuric chloride for 10 min or 0.05% mercuric chloride for 5 min or 10 min was more efficient for the *in vitro* disinfection of initiated buds. The MS medium supplemented with NAA (0.5 mg/l) and BAP (4 mg/l) generated better proliferation, whereas the combination of NAA (0.5 mg/l) and kinetin (4 mg/l) generated shoots of better quality and height. Finally, the culture room conditions were optimal for the survival of the plantlet. The identification and quantification of essential oils must be continued to assess the impact of technology on this spice, which has global nutritional, therapeutic, and economic importance.

Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the work and the publication of this paper.

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Author Contributions

Original draft preparation by CGHT, JAH, and FDK; work conducted by JRUK and FDK, validation; analysis, and visualization by SSH; review and editing by JAH and CGHT; Revision by CA. Supervision by CA and CGHT. All authors have read and agreed to the submission of the final version of the manuscript.

Data Availability

Data presented in this study will be available upon reasonable request to the corresponding author.

Ethics Approval

Not applicable to this manuscript

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