EVALUATING BEST SURFACE STERILIZATION METHOD AND HORMONE COMBINATION FOR SHOOTS INITIATION OF *Zingiber officinale*

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INTRODUCTION

Ginger (*Zingiber officinale)* is an herbaceous perpetual, monocot plant from the family Zingiberaceae. It has been one of the important minor commercial crops in Sri Lanka for decades. It is found to be everywhere throughout the world as a spice and medicine. Ginger is planted in the tropics for its palatable rhizomes filling culinary and therapeutic needs. The ginger rhizomes are small while the fibrous flesh is ash white in color. The ginger's taste and aroma are comparatively higher than other species and it is largely used for beverages. Several local and imported ginger cultivars namely Local, Rangun and Chinese are grown in Sri Lanka in large scale. Rhizomes of local ginger are small and the fibrous flesh is somewhat ash white in color. Its' taste and aroma are comparatively higher than Rangun and Chinese. It is largely used as a spice, beverage, and basic material in ayurvedic medicine and the pharmaceutical industry (Swarnathilaka *et a*l., 2017). The *in vitro* technique's success largely depends on the aseptic culture establishment, shoot regeneration capacity, rooting, and acclimatization. Rhizome buds, which are often used as the source of explants in Zingiberaceae, have been proven to be more responsive. However, the initial establishment of contamination-free culture is difficult owing to the exposure of rhizomes to various soil pathogens. The present study is an attempt to develop a successful protocol for the sterilization and regeneration of plantlets from ginger buds with the idea of developing a commercial protocol for ginger *in vitro* propagation. Accordingly, there were two objectives; evaluating the best surface sterilization method for Ginger seeds and the best hormonal combination for in-shoot multiplication during *in vitro* propagation.

METHODOLOGY

Healthy and mature rhizomes of ginger were collected from the farms located in the Low Country Wet Zone. Uprooted rhizomes were collected in sealed, labeled polythene bags before transporting to the laboratory. The experiment was carried out in the Tissue culture laboratory, Department of Agricultural and Plantation Engineering at the Open University of Sri Lanka.

Evaluating the best surface sterilization method

Fresh healthy rhizomes collected from mother plants were cleaned to remove soil debris by washing them in running tap water for 20 minutes followed by immersing in Captan fungicide with a few drops of Teepol for 30 minutes. Partially cleaned rhizomes were washed 4 -5 times dipping in distilled water, while shaking mildly for 5 minutes at a time to remove the traces of the disinfectant.

Table 1: Treatment combinations of sterilizing agents and their concentrations

Treatment No	Sterilizing agents with concentration
T1	Dipped in Sterilized distilled water 30 minutes(control)
T2	Dipped in 70% ethanol followed by 10% bleaching.
T3	Dipped in 70% ethanol followed by 20% bleaching
T4	Dipped in 70% ethanol followed by 30% bleaching

T5	Dipped in 75% ethanol followed by 10% bleaching
	Dipped in 75% ethanol followed by 20% bleaching
T7	Dipped in 75% ethanol followed by 30% bleaching
T8	Dipped in 80% ethanol followed by 10% bleaching
T9	Dipped in 80% ethanol followed by 20% bleaching
T10	Dipped in 80% ethanol followed by 30% bleaching

Then the cleaned buds were separately immersed in different concentrations of sterilization agents added with 2 - 4 drops of Tween 20. Table 01 explains the treatment combinations of sterilizing agents and their concentrations applied to each combination. Explants were washed 4 -5 times using sterile distilled water separately to remove the traces of bleaching powder and ethanol followed by transferring to the laminar floor after carefully being placed in a sterile Petri dish for media culture.

Evaluating the best hormonal combination for shoot multiplication

Morishige and Skoog (MS) medium was prepared for each treatment using stock solutions with different hormone concentrations. The stock solutions with macro, micro, ferrous, and vitamins stored in the refrigerator were used. Hormonal combinations were added to all solutions separately followed by 0.1 g/L of myoinositol and 30 g/L of sucrose with the measured volumes of stock solutions to prepare the medium later adjusted pH to 5.8. The solution was heated and stirred before and after adding 8 g of tissue culture agar until dissolved the agar completely. Culture vessels were added with 30 ml of media and autoclaved at 121º C for 20 minutes. Sterilized ginger buds were transferred to the culture vessels inside the laminar floor under sterilized conditions. The cultures were incubated at 26 °C with 16 h photoperiod. After four weeks, data on the number of healthy surviving cultures percentage was recorded. Cultures were checked regularly for contaminations and those that revealed apparent infection symptoms were immediately discarded.

Table 02: Hormone combination of the treatments added for shoot multiplication

Treatment number	Hormone Combination
T1	Without hormones (Control)
T2	2 mg/L BAP
T3	3 mg/L BAP
T4	4 mg/L BAP
T5	5 mg/L BAP
T6	0.25 mg/L NAA only without BAP
T7	2 mg/L BAP and 0.25 mg/L NAA
T8	3 mg/L BAP and 0.25 mg/L NAA
T9	4 mg/L BAP and 0.25 mg/L NAA
T10	5 mg/L BAP and 0.25 mg/L NAA
T11	0.5 mg/L NAA without BAP
T12	2 mg/L BAP and 0.5 mg/L NAA
T13	3 mg/L BAP and 0.5 mg/L NAA
T14	4 mg/L BAP and 0.5 mg/L NAA
T15	5 mg/L BAP and 0.5 mg/L NAA

$$\text{Contamination \%} = \frac{\text{Num. of explants contaminated}}{\text{Total num of explants inoculated}} \times 100$$

Shoot multiplication was evaluated by examining the cultured explants periodically, and by recording the morphological changes such as the number of buds/shoots, shoot length (cm),

number of leaves, and leaf length (cm) on the basis of visual observations by using a pasted ruler in a side of the culture vessel. The experiment was conducted in Completely Randomized Design with five replicates from each treatment in the experiment done on surface sterilization as well as hormonal combinations. Data Analysis was done by ANOVA one-way analysis using Minitab 17 software and MS excel spreadsheet. Means will be compared by the Least Significant Difference (LSD 0.05).

RESULTS AND DISCUSSION

Evaluation of the best surface sterilization method

Table 3 represents the mean survival rate of ginger (*Zingiber officinale)* buds after surface sterilization with different concentrations of ethanol and bleaching solution. A statistically significant difference was observed in T6 in the survival rate and percentage of Ginger buds when applied sterilization with 75% ethanol followed by 20%(w/v) bleaching solution. Sterilization with sterilized distilled water (control) and 70% ethanol with 10%(w/v) bleaching powder both showed the least effect on reducing contamination in micro-propagation of ginger. Additionally, in this study, treatments 3, 4, 5, 7, 8, 9 and 10 showed no significant difference in survival percentage when compared with the control treatment.

Table 03: Mean survival rate of ginger (*Zingiber officinale)* buds after surface sterilization with different concentrations of ethanol and bleaching solution.

Sterilizing agents with concentration	Mean Survival Rate*
Distilled water (control)	0.00^{b}
70% ethanol + 10 % bleaching solution	0.00^{b}
70% ethanol + 20 % bleaching solution	0.20^{ab}
70% ethanol + 30 % bleaching solution	0.20^{ab}
75% ethanol + 10 % bleaching solution	0.40^{ab}
75% ethanol + 20 % bleaching solution	0.60^{a}
75% ethanol + 30 % bleaching solution	0.40^{ab}
80% ethanol + 10 % bleaching solution	0.20^{ab}
80% ethanol + 20 % bleaching solution	0.40^{ab}

*Means followed by different letters in each column are significantly different at $p < 0.05$

However, increasing the bleaching solution concentration did not show satisfactory results with all ethanol concentrations. Furthermore, 75% ethanol with 20% bleaching solutions had shown a numerical increase in survival rate so as the 80% ethanol followed by 20% bleaching solution.

After surface sterilization and its results 75% ethanol with 20% bleaching powder was used for evaluating the best hormone concentration for shoot multiplication. Common surface disinfectants include ethanol, sodium hypochlorite, hydrogen peroxide, and mercury chloride. Among those 70% (v/v) ethanol concentration is a common sterilization agent routinely used in the laboratory. As per Mehaboob et al in 2019, 70–75% ethanol inactivates some bacteria by infiltrating through their cell membranes to denature various proteins. Further, as per the investigation of Zuraida et al in 2016, the bactericidal effect of 75% ethanol is higher when used with other disinfectants, rather than using alone. However, the present research revealed that 80 % ethanol had numerically better results than the 70% ethanol use treatments.

Evaluating the best hormonal combination for shoot formation

Figure 01 explains the influence of different concentrations of BAP and NAA on bud/shoot formation and shoot length and figure 03 explains the influence of BAP and NAA on the formation of leaves per shoot and leaf length 3 weeks after culture.

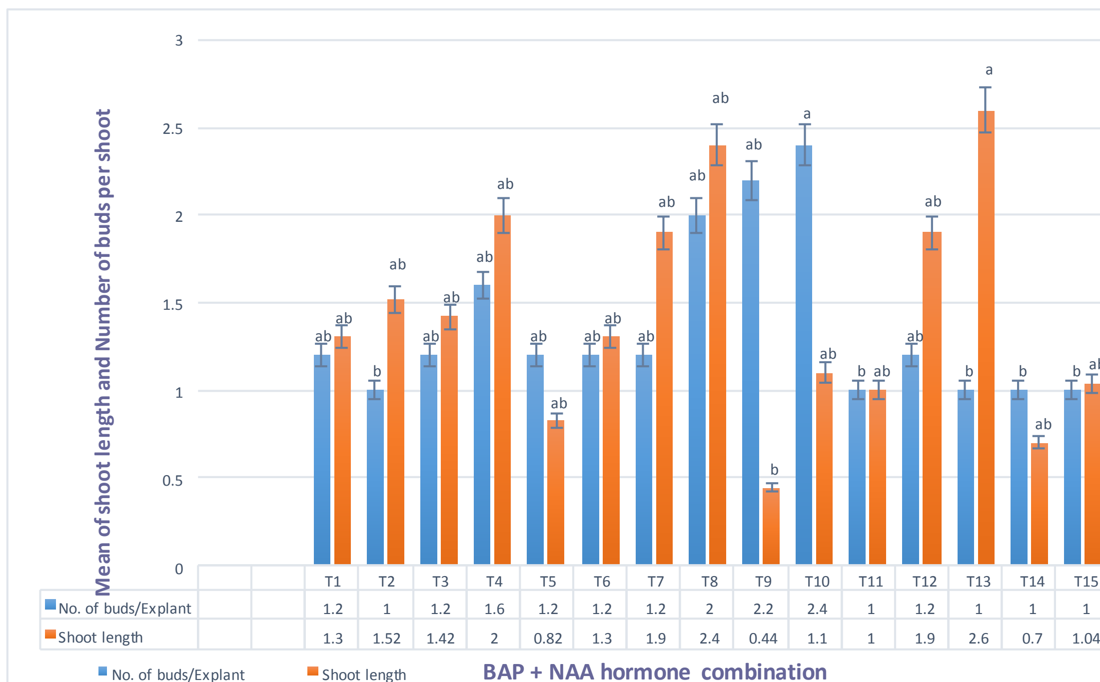

Figure 01: Influence of BAP and NAA on no. of bud/shoot and shoot length after 3 weeks of culture

Treatment number 10 where MS media was added with 5 + 0.25 (BAP + NAA mg/L) showed a statistically significant difference among the treatments having the best buds/explant formation 3 weeks after culturing. Least bud/shoots formation was observed from treatments T2, T11, T13, T14 and T15 with no significant deterrent among the treatments and the control. However, higher shoot elongation was observed in treatment number 13, added with 3mg/L BAP + 0.5mg/L NAA combination to the MS media.

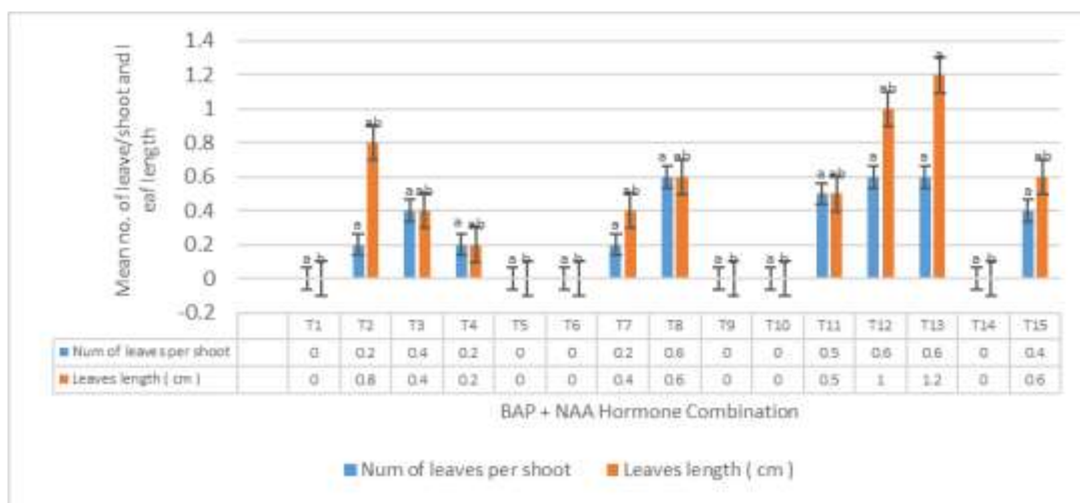

Figure 02: Influence of BAP and NAA on leaves per shoot and leaf length after 3 weeks of culture

Statistically, a significant leaf length was observed in treatment number 13 MS media supplemented with 3mg/L BAP and 0.5 mg/L NAA having the highest mean leaf length of 1.2 cm. Most of the treatments 1, 5, 6, 9, 10, and 14 had not shown a measurable number of leaves

per shoot. Our results are in line with the observations made by Sharma and Singh in 1997, as well as Kambasaka and Santilata in 2009; resulting in the best response of ginger for shoot multiplication, obtained on MS medium added with BAP and NAA and is further revealed that the *in-vitro* plantlets having normal shoots have been mostly obtained on MS medium supplemented with BAP (1-5 mg/L) in combination with low concentration (0.5 mg/L) of NAA. Moreover, Hiremath in 2006; Behera et al in 2010 as well as Swarnathilaka et al in 2017 resulted in better observations from the medium added with BAP 2.0 mg/L together with NAA 0.5 mg/L than in treatments added BAP alone. However, in our research 2.0 g/L BAP and 0.5 mg/L NAA did not show a significant difference when compared with other treatments as well as the control.

CONCLUSION

As per the results of the present study, the best sterilant combination for the sterilization of Ginger (*Zingiber officinale*) is 75% ethanol with 20% (w/v) bleaching solution. These results were used for the hormone combination procedure as a surface sterilization protocol. 5 + 0.25 (BAP + NAA mg/L) hormone combination is the best for *in vitro* bud/shoot formation. This study showed the locally available Bleaching powder ($Ca(ClO)_2$) in the form of a solution can be used as low-cost sterilizing agent after using ethanol and commercial bleaching solution as the surface sterilant agents.

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