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RESEARCH ARTICLE

DEVELOPMENT OF *IN-VITRO* PROTOCOL TO ENHANCE MASS PRODUCTION OF TURMERIC (*Curcuma longa* L.)

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ABSTRACT

Conventionally, turmeric (*Curcuma longa* L.) is propagated through rhizomes. However, its multiplication rate is very low where a single turmeric rhizome approximately produces 6-8 lateral buds and nearly 20-25% of the harvest should be retained as planting materials for the next season. Therefore, the study focuses on the development of an *in vitro* regeneration protocol of turmeric for the year-round provision of disease-free planting material. Commercially grown sprouted rhizome buds were surface sterilized with fungicide followed by 70% ethanol, and with different concentrations of Clorox (10, 20, 30 and 40%). Different exposure times (5, 10, 15 and 20 minutes) were tested to develop the best sterilization procedure. MS medium supplemented with different concentrations of hormones BAP (2.0, 3.0, 4.0 and 5.0 mg/l) and NAA (0.25 and 0.5 mg/l) for shoot regeneration, and IBA (1.0, 2.0, 3.0 and 4.0 mg/l) for root regeneration to find the best combination. The results showed that 30% Clorox and 20 minutes exposure time is suitable for surface sterilization of buds about 1.5-2.0 cm long. Shoot regeneration was the highest when the media were treated with 4.0 mg/l of BAP and 0.5 mg/l NAA. The best IBA concentration that gives the highest number of roots in the least number of weeks is 2.0mg/l. Additionally, 58.33% of the plantlets survived after field acclimatization. The study concluded that the protocol can be used for *in vitro* propagation of turmeric using rhizome buds for large-scale production of plant materials.

Keywords: *Curcuma longa* L., *In vitro* propagation, Root regeneration, Shoot regeneration, Sterilization

INTRODUCTION

Turmeric is an important commercial crop that is highly used in Asian cuisine as a key spice. It belongs to the family *Zingiberazeae*, which is a rhizomatous herbaceous perennial plant (Prasanth *et al.* 2019). In addition to its extensive use as a spice, turmeric is also used as a food preservative, colouring agent and in Ayurvedic medicine due to its biological importance (Chanda and Ramachandra 2019).

Turmeric is conventionally propagated through underground rhizome buds. Well-developed turmeric rhizomes are selected at the end of the growing season when the leaves turn yellow. Dirt is removed and fleshy rhizomes are stored in dark, cool, and dry places until the appearance of sprouts from the rhizome. Turmeric is very rarely propagated through true seeds because of its poor flowering ability (Naz *et al*. 2009; Shylaja *et al*. 2016).

There is a high commercial demand for turmeric in the Sri Lankan market. However, their multiplication rate is very low from conventional propagation. Typically, 6-8 lateral buds are produced from a single

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rhizome during propagation and almost 20-25% of the harvest should be retained as planting materials for the next season (Sinchana *et al*. 2020). Also, since underground rhizomes are commercially valuable parts, they are highly susceptible to soil pathogenic microorganisms and result in loss of harvest. Furthermore, the availability of sprouting rhizome bud for conventional propagation is highly seasonal as turmeric is a seasonal plant. Therefore, for all these problems, *in vitro* propagation would ensure the year-round provision of disease-free planting materials for farmers while conserving the harvest and eliminating the soil pathogenic effect on planting materials.

In vitro regeneration or micro-propagation of plants is a technique used in plant tissue culture to produce disease-free bulk plantlets by using a small explant under aseptic conditions in a defined culture medium under controlled environmental conditions (Altman 2003).

Turmeric is conventionally propagated through rhizomes. Therefore, it is usually recommended to use sprouting rhizome buds for *in vitro* culture of turmeric (Sunitibala *et al*. 2001; El-Hawaz *et al.* 2015; Naz *et al*. 2009). Apart from rhizome buds, some researchers have used turmeric leaves, inflorescence, shoots, roots, and basal region of the shoots for callus induction (Salvi *et al*. 2000; Zapata *et al*. 2003; Raju *et al*. 2015). Generally, explants of 1 to 1.5 cm in size consisting of the shoot tip and a small portion of the rhizome are used for culture (Ashkanani *et al*. 2013). Smaller explants have a lower ability to transmit contaminants but they have a higher tendency toward damage during washing, sterilization, and handling, whereas big-sized explants are difficult to purify effectively (George *et al*. 1993). Therefore, selecting explants that are of the proper size to withstand washing and handling steps as well as which have a lesser tendency to transmit diseases is a must. Studies have shown that BAP concentrations of 2.0 - 4.0 mg/l can be used in the effective regeneration of the family *Zingiberaceae* in combination with auxins. However, a minimum percentage of shoot initiation would be observed when the BAP and IAA levels are decreased (Gomathy *et al*. 2014). The root induction was observed from IAA, IBA, and NAA, when they were incorporated into the basal medium. The literature survey revealed that IBA is the most promising growth regulator among the three frequently used auxins (Sinchana *et al*. 2020). The environmental conditions are maintained under the *in vitro* conditions to ensure the growth of plantlets. Hence, the transfer of *in vitro* plantlets to *ex vivo* conditions and fields should be carefully maintained due to changes in environmental conditions and the morphology of plantlets (Seran 2013). Therefore, this study aimed to develop an *in vitro* regeneration protocol of turmeric for the year-round provision of disease-free planting material for future requirement.

MATERIALS AND METHODS

The study was carried out in the research laboratory of the Department of Agricultural Biology, Faculty of Agriculture, the University of Ruhuna from November 2020 to April 2022.

Planting material and selection of the best sterilization process

The sprouting rhizome buds were taken from commercially grown turmeric (*Curcuma longa*) seed rhizomes and washed with running tap water for 1 hour in a beaker closed with a muslin cloth. The rhizome buds were then washed with one or two drops of commercial Teepol solution for 10 minutes while swirling and then thoroughly washed using distilled water until all Teepol was washed off. They were then washed in a 0.8w/ w Carbendazim fungicide solution for 20 minutes followed by washing the fungicide off with distilled water. The buds were taken inside the laminar flow and washed by immersion in a 70% alcohol solution for 2-3 minutes followed by washing three times with autoclaved distilled water.

The inoculation of cultures with explants from the rhizome bud was carried out inside the Laminar flow hood. The rhizome buds were washed with different concentrations of commercial Clorox (10, 20, 30 and 40%) and different time periods of exposure to the

sterilant (5, 10, 15 and 20 minutes) to select the best sterilization protocol. They were then washed three times with autoclaved distilled water before being transferred to half MS medium (Murashige and Skoog medium). Finally, the buds were inoculated under 25±2°C temperature and for a photoperiod of 16 hours light and 8 hours dark at a light intensity of 2000-3000LUX and 70% relative humidity. The uncontaminated percentage was calculated after four weeks of culture. The experiment was arranged in a two-factor (Clorox percentage and Exposure Time) factorial Complete Randomized Design with ten replicates for each treatment.

Selection of the best hormone combination for the shoot regeneration

Properly sterilized buds with the correct concentration of Clorox, were transferred to the culture medium treated with different concentrations of plant growth regulators, 2.0, 3.0, 4.0 and 5.0 mg/l BAP and 0.25 and 0.5 mg/l NAA to select the best concentration for shoot regeneration. They were also allowed to inoculate in a culture room under 25±2°C temperature and for a photoperiod of 16 hours light and 8 hours dark at a light intensity of 2000-3000LUX and 70% relative humidity. The number of regenerated shoots was counted after six weeks of culture. Each treatment consisted of 10 replicates and a two -factor factorial (cytokinin – BAP and auxin - NAA concentrations) Complete Randomized Design was used as the experimental design.

Selection of the best hormone combination for the root regeneration

The regenerated shoots from turmeric buds were then subcultured in a media containing different concentrations of IBA (1.0, 2.0, 3.0 and 4.0 mg/l) for root regeneration, which was inoculated under the same culture room conditions used for shoot regeneration. The number of weeks taken to generate roots and the number of roots regenerated were counted.

Acclimatization

Acclimatization of fully grown plantlets with roots was started by opening the lids of the culture bottles for 25%, 50%, 75% and 100% for a period of one week at each stage. The survived plantlets were then transferred to a sterilized potting mixture with coir dust under shade house conditions. Finally, they were transferred to pots filled with compost and topsoil manure mixture to grow into mature plants. The number of plants that survived after 4 weeks was counted and the percentage of survived plants was calculated.

Statistical analysis was done using SAS statistical software for Analysis of Variance (ANOVA) and Tukey's Studentized Range Test for the post hoc test. For the non-parametric data analysis, Wilcoxon Rank Sum Test and Kruskal-Wallis Test were used.

RESULTS AND DISCUSSION

Determination of the best sterilization process

Sterilization is the most crucial step in *in vitro* propagation. The underground turmeric rhizome buds that were taken as planting materials for *in vitro* propagation in the study are highly susceptible to soil pathogens. Therefore, a proper sterilization protocol must be followed to maintain a contamination-free culture.

The ANOVA test results of the sterilization showed that the p-value is equal to 0.0026 for the interactive effect between the exposure time and the concentration where it is greater than 0.0001 at 95% confidence level. This indicates both exposure time and concentration are statistically significant predictors of the uncontaminated rhizome buds. Therefore, Tukey's post hoc test was performed to determine which levels of Clorox concentration and exposure time are most suitable for the sterilization of the rhizome buds. The Tukey's grouping for means of Clorox concentration showed that 30% and 40% Clorox do not show any significant differences and 10% - 20% Clorox (Figure 1a) also does not show a significant difference in uncontaminated rhizome buds, while exposure time of 15 and 20 minutes and 5- and 10-minutes exposure time (Figure 1b) also does not have a significant difference with each other. Therefore, the results showed that either 30% or 40% Clorox and 15 or 20

minutes of exposure time can be used for the sterilization of the rhizome buds before inoculation. However, it is recommended to use a lower concentration of disinfectants as a higher concentration may result in a loss of viability of the explants. Therefore, 30% Clorox concentration and 20 minutes exposure time (73.33% uncontaminated rhizome buds) can be used to surface sterilize the rhizome bud explants (1.5-2.0 cm long) of turmeric for *in vitro* propagation.

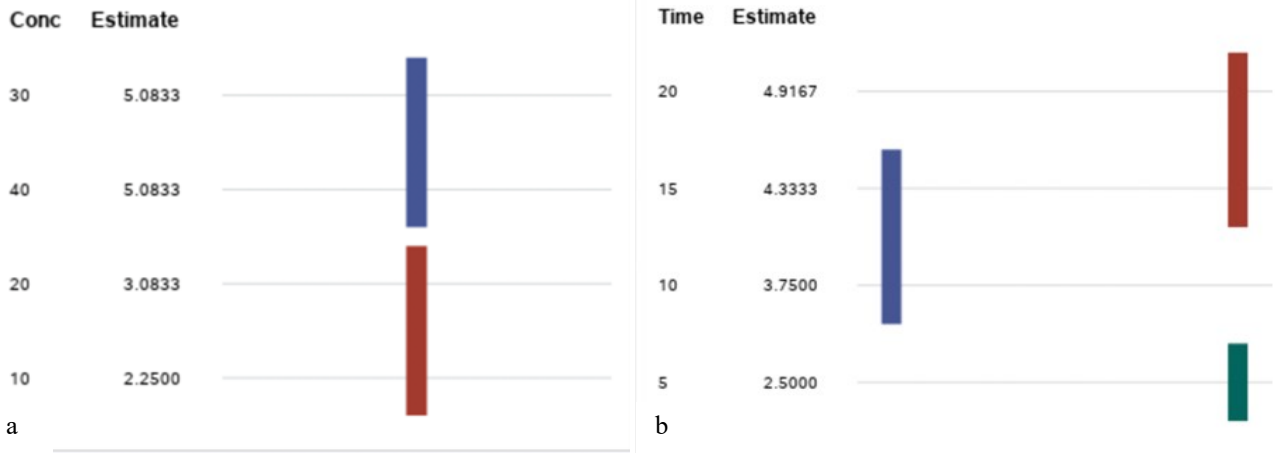

Figure 1: a) Tukey's grouping for means of Clorox concentration on uncontaminated rhizome buds, b) Tukey's grouping for means of Time on uncontaminated rhizome buds. The means covered by the same bar are not significantly different.

Determination of the best explant and BAP concentration for shoot regeneration

During the development of a shoot regeneration protocol for turmeric *in vitro* regeneration, different combinations of BAP and NAA were used in eight treatments (Plate 01). Auxins, cytokinin, and gibberellins are the most common plant growth regulators available naturally, and the interactive effect of auxins and cytokinin is considered to have a greater effect on the growth and development of plant tissues and organs (Gasper *et al*. 1996).

The p-value according to the results of the ANOVA test for BAP is <0.0001 and for NAA is also <0.0001 where they are statistically significant predictors of shoot regeneration. Therefore, the Tukey post hoc test was performed to determine which concentrations of BAP and NAA are the most suitable for shoot regeneration. Tukey's grouping of means for combination effect of 4 mg/l BAP and 0.5 mg/l NAA gives the highest shoot regeneration rate (Figure 2). Therefore, 4 mg/l BAP and 0.5 mg/l NAA can be used for shoot regeneration of turmeric rhizome buds (Plate 1).

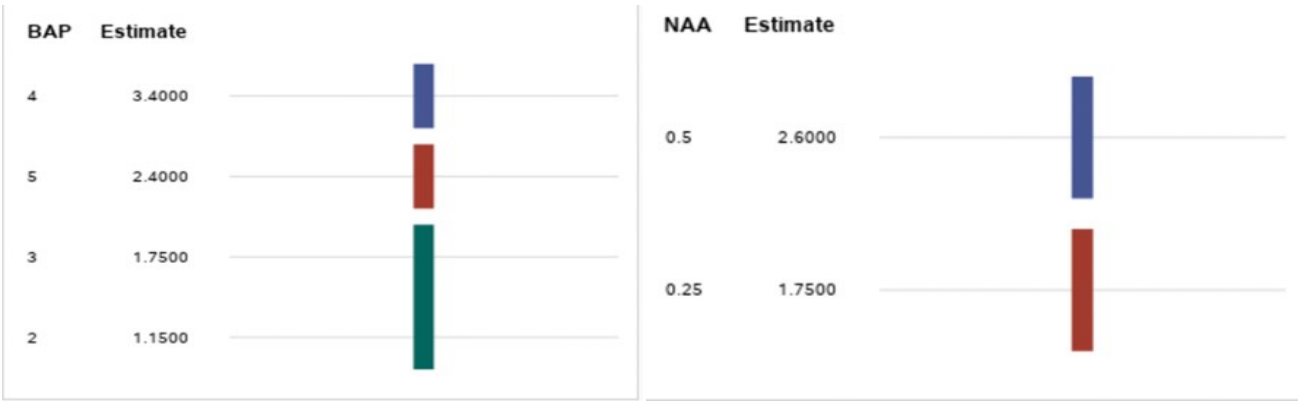

Figure 2: Tukey's grouping for the means of BAP and NAA concentration on the number of regenerated shoots. The means covered by the same bar are not significantly different

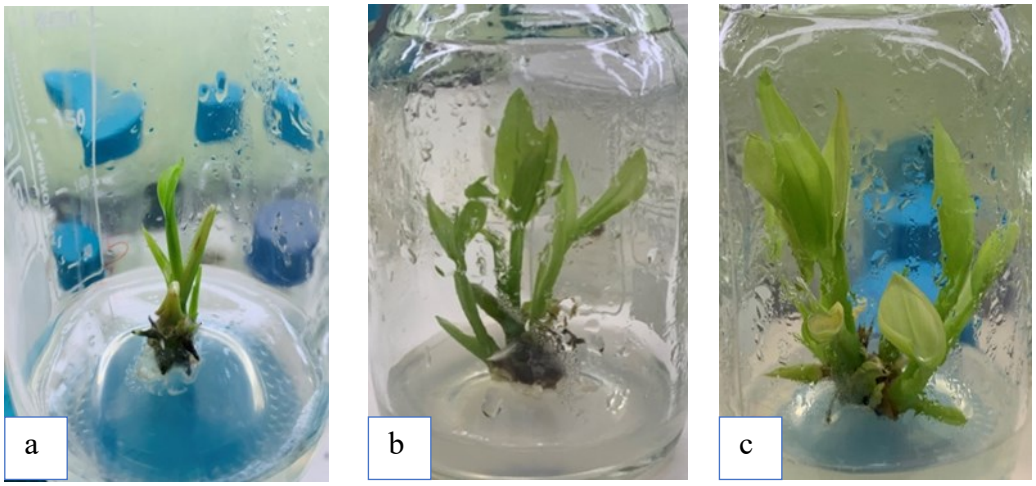

Plate 1: Rhizome bud in 4 mg/l BAP + 0.5 mg/l NAA culture after a) 6 weeks; b) 10 weeks and c) 12 weeks of culture

Determination of the best explant and BAP concentration for root regeneration

Indole-3-butryic acid (IBA) is responsible for root regeneration, regulation of root apical meristem size, root hair elongation, lateral root development, and formation of adventitious roots in plants (Frick and Strader 2018). The p-value of the Kruskal-Wallis test for the number of weeks taken to regenerate roots is 0.0156. As this value is less than 0.05, we rejected the null hypothesis which tells that the median number of weeks is the same for all four concentrations of IBA. Hence, this test has enough evidence to conclude that IBA concentration significantly affects the number of weeks taken for the emergence of roots from the turmeric plantlets.

The pairwise comparison conducted to find which means are statistically significant shows that the p-value of 2 mg/l IBA vs. 4 mg/l IBA is less than 0.05 (0.0229), indicating that there is a significant difference between 2 mg/l IBA vs. 4 mg/l IBA concentration but not any other. However, the descriptive analysis shows that 2 mg/l IBA concentration is helpful to regenerate roots in the lowest time, approximately two weeks compared to 4 mg/l IBA, which took three and half weeks.

Furthermore, the analysis of the number of roots regenerated from the different concentrations gives the results where the Kruskal-Wallis test p-value was recorded as 0.0005 which is less than 0.05 indicating that there is enough evidence to show that the IBA significantly affects the number of roots regenerated from the turmeric plantlets. The pairwise comparison shows that the p-values of 1 mg/l IBA vs. 2 mg/l IBA (0.0164), 1 mg/l IBA vs. 3 mg/l IBA (0.0155), 1 mg/l IBA vs. 4 mg/l IBA (0.0432) and 3 mg/l IBA vs. 4 mg/l IBA (0.0327) are less than 0.05 concluding that these are significantly different. The descriptive data analysis shows that the highest number of roots regenerated from 2 mg/l IBA concentration. Therefore, all the analysis concludes that 2mg/l IBA concentration is the most suitable one for the root regeneration in turmeric (Plate 2).

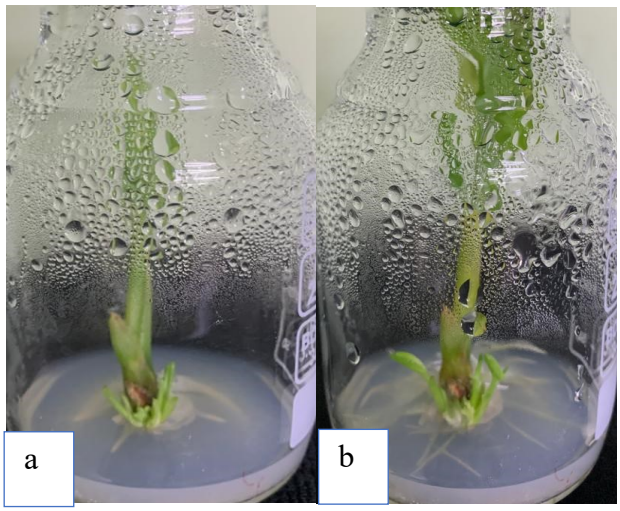

Plate 2: a) Root initiation and b) Root growth from the culture media containing IBA

Determination of the acclimatization process

A total of 50 plantlets were selected for the acclimatization process (Plate 3). Among them, 72% of the plantlets survived for the field adaptation. These plants were adapted to field conditions and the percentage of plantlets that survived after four weeks of field adaptation was 58.33% (Plate 4). However, the acclimatization process should be further optimized with large-scale planting of *in vitro* cultured turmeric plants.

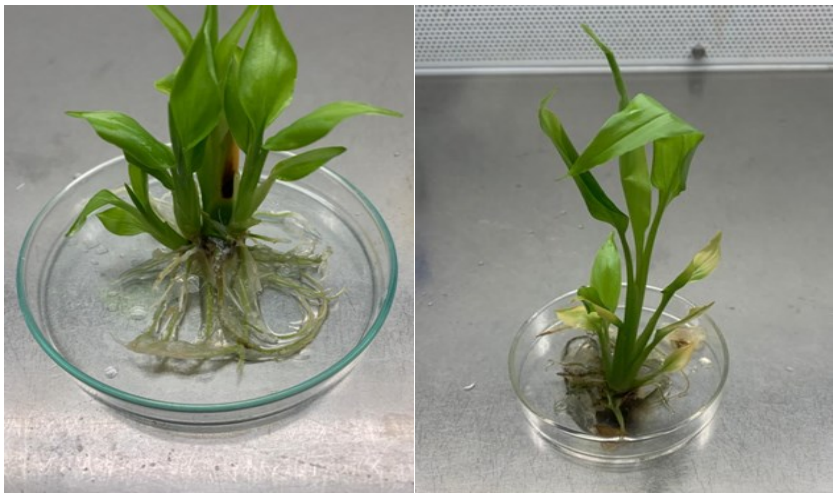

Plate 3: Cleaning of the roots of well-developed plantlets prior to field acclimatization

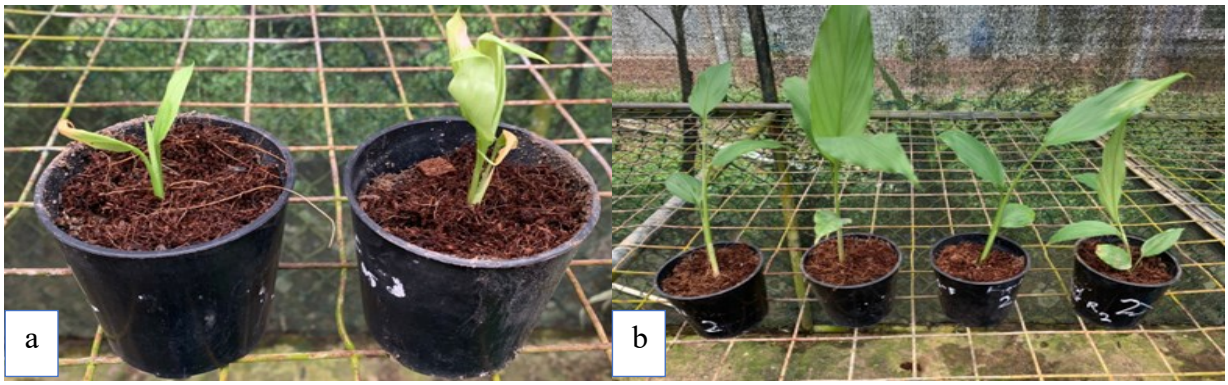

Plate 4: Field acclimated plants a, after one week and b, after 4 weeks

CONCLUSION

The study was able to develop a sterilization protocol, shoot and root regeneration protocols for the *in vitro* regeneration of turmeric while the acclimatization procedure should be further optimized. It was found that Clorox plays a successful role in sterilization and the concentration of 30% Clorox for a 20-minute exposure time sterilizes turmeric rhizome buds of 1.5-2.0 cm long, successfully. The highest shoot regeneration rate was obtained from the media supplemented with 4 mg/l BAP and 0.5 mg/l NAA concentration. The root regeneration was effective when treated with a concentration of 2 mg/l IBA where the highest number of roots were regenerated at the lowest time. The percentage of acclimatized plants was obtained as 58.33% after four weeks of field adaptation. Therefore, the study concluded that the protocol obtained from the study can be used for *in vitro* propagation of Turmeric using rhizome buds for large-scale production of plant materials.

AUTHOR CONTRIBUTION

ND, PCDP and SS designed the study. MMNTB performed the experiments. MMNTB and PCDP analysed the data. MMNTB wrote the paper with input from all

the authors. All authors discussed the results and commented on the manuscript.

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