EVALUATE THE BEST STERILIZATION METHOD AND THE HORMONAL COMBINATION FOR *IN-VITRO* PROPAGATION OF *Curcuma longa* (TURMERIC)

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INTRODUCTION

Curcuma longa (Turmeric) is a herbaceous perennial plant belonging to the family Zingiberaceae. Turmeric is a perennial herb to 1m tall with underground rhizomes. The leaves of the turmeric plant are long and oblong in shape. The rhizome is tuberous with rough and segmented skin. Turmeric produces beautiful white flower spikes. Turmeric is native to South East Asia. Turmeric is grown in India, Pakistan, Malaysia, Thailand, Philippines, Japan, Sri Lanka, China, Africa, and Central America. In Sri Lanka *Curcuma longa* (Turmeric) is grown in wet and intermediate zones as a mono-crop and intercrop under coconut. Major growing areas in Sri Lanka are Kurunegala, Gampaha, Kalutara, Kandy, Kegalle and Matale districts.

The rhizome of turmeric is used for growing new plants and for consumption. After banning importing turmeric annual production is not enough for consumption as well as to use as seeds for the next season. The only possible option is micropropagation of Turmeric to increase the seed production for the next crop. The success of *in vitro* propagation depends on explant surface sterilization and medium supplemented with hormonal combination for best growth and development. It is important to produce a contamination-free large number of healthy plants to compensate for the annual demand in the country. The present study is important to find the best sterilization method and the best hormonal combinations for shoot induction under *in-vitro* propagation.

METHODOLOGY

The healthy, disease-free rhizome (1-2 cm length) of *Curcuma longa* (Turmeric) were collected from mature one year old healthy mother plants. The explant (Rhizome) was cleaned using a sharp knife and thoroughly washed under running water (10-15 min). Then explant was dipped in liquid detergent (Teepol) and fungicides (Captan) for 30 min and will be shaken using a conical flask. The basic tissue (rhizome) was thoroughly washed five times with sterilized distilled water to remove the traces of detergent and fungicides. Surface sterilizing of basic tissues was done inside the laminar flow cabinet under aseptic conditions. The sterilized explants were inoculated into the basal Morishige and Skoog (MS) medium (1962) under aseptic conditions. All cultured explants were incubated at 25 ^{0}C with 16 hr photoperiod in a growth room.

Determining the best sterilization method

Surface sterilizing of basic tissues was done inside the laminar flow cabinet under aseptic conditions by using eighteen sterilization treatments structuring factorial combination with Ethanol (70%, 75%, 80%) for 30 seconds and Clorox bleach solution (20%, 30%, 40%) or Hydrogen peroxide (2%, 4%, 6%) for 30 minutes to evaluate the best sterilization method.

The data were recorded on the contaminations and activated shoot buds. All treatments were replicated five (05) times. The treatment (T) combinations were,

T1 - 70% ethanol + 20% Clorox
T2 - 70% ethanol + 30% Clorox
T3 - 70% ethanol + 40% Clorox
T4 - 75% ethanol + 20% Clorox
T5 - 75% ethanol + 30% Clorox
T6 - 75% ethanol + 40% Clorox
T7 - 80% ethanol + 20% Clorox
T8 - 80% ethanol + 30% Clorox
T9 - 80% ethanol + 40% Clorox
T10 - 70% ethanol + 2% hydrogen peroxide
T11 - 70% ethanol + 4% hydrogen peroxide
T12 - 70% ethanol + 6% hydrogen peroxide
T13 – 75% ethanol + 2% hydrogen peroxide
T14 - 75% ethanol + 4% hydrogen peroxide
T15 - 75% ethanol + 6% hydrogen peroxide
T16 - 80% ethanol + 2% hydrogen peroxide
T17 - 80% ethanol + 4% hydrogen peroxide
T18 - 80% ethanol + 6% hydrogen peroxide
T19 – Sterilized distilled water (Negative control)

Determining the best hormonal combination

Explants were established in the basal MS medium supplemented with fifteen treatments structuring a combination of hormones with BAP (0, 2, 3, 4, 5 mgl^{-1}) and NAA (0, 0.25, 0.5 mgl^{-1}) to evaluate the best hormonal combination for Turmeric. All treatments were replicated five (05) times. The data were recorded on the number of shoot buds, shoot length, and length of longest shoot per one explant. The treatment (T) combinations were,

T1 - 0 mg/L BAP + 0 mg/L NAA
T2 - 2 mg/L BAP + 0 mg/L NAA
T3 - 3 mg/L BAP + 0 mg/L NAA
T4 - 4 mg/L BAP + 0 mg/L NAA
T5 - 5 mg/L BAP + 0 mg/L NAA
T6 - 0 mg/L BAP + 0.25 mg/L NAA
T7 - 2 mg/L BAP + 0.25 mg/L NAA
T8 - 3 mg/L BAP + 0.25 mg/L NAA
T9 - 4 mg/L BAP + 0.25 mg/L NAA
T10 - 5 mg/L BAP + 0.25 mg/L NAA
T11 - 0 mg/L BAP + 0.5 mg/L NAA
T12 - 2 mg/L BAP + 0.5 mg/L NAA
T13 - 3 mg/L BAP + 0.5 mg/L NAA
T14 - 4 mg/L BAP + 0.5 mg/L NAA
T15 - 5 mg/L BAP + 0.5 mg/L NAA

Completely Randomized Design (CRD) experimental design was applied for experiments one and two. The data were analyzed by ANOVA using Minitab 17 software. Means were compared by the Least Significant Difference (LSD 0.05).

RESULTS AND DISCUSSION

Evaluate the best sterilization method

Explants obtained from turmeric rhizomes were subjected to surface sterilization by two different methods, wherein in the first group different concentrations of ethanol followed by different concentrations of commercial bleach were used and the second group was sterilized with different concentrations of ethanol followed by different concentrations of Hydrogen Peroxide. The results from an evaluation of post-inoculation contamination following applying different sterilization methods are shown in Table 01. Table 02 presents the % of the explants with activated shoot buds without contaminations; it can be clearly seen that the results varied between the treatment methods. However, as can be seen clearly from table 02, all three treatments were successful in leading to efficient surface sterilization of the explants compared to the untreated control.

Table 01. Number of activated shoot buds and success explants as a percentage without contaminations with different sterilization methods

Sterilization method	No. activated shoot buds without contaminations*	Success explant percentage %
70% Ethanol + 20% Clorox bleach solution	0 ± 0^{c}	0
70% Ethanol + 30% Clorox bleach solution	0 ± 0^{c}	0
70% Ethanol + 40% Clorox bleach solution	0 ± 0^{c}	0
75% Ethanol + 20% Clorox bleach solution	0 ± 0^{c}	0
75% Ethanol + 30% Clorox bleach solution	0.2 ± 0.45^{bc}	20
75% Ethanol + 40% Clorox bleach solution	0.4 ± 0.59^{bc}	40
80% Ethanol + 20% Clorox bleach solution	0 ± 0^{c}	0
80% Ethanol + 30% Clorox bleach solution	0 ± 0^{c}	0
80% Ethanol + 40% Clorox bleach solution	0.4 ± 0.59^{bc}	40
70% Ethanol + 2% Hydrogen peroxide	0.8 ± 1.79^{bc}	20
70% Ethanol + 4% Hydrogen peroxide	0.6 ± 1.34^{bc}	20
70% Ethanol + 6% Hydrogen peroxide	1.4 ± 2.19^{ab}	40
75% Ethanol + 2% Hydrogen peroxide	0 ± 0^{c}	0
75% Ethanol + 4% Hydrogen peroxide	1.0 ± 1.73^{abc}	40
75% Ethanol + 6% Hydrogen peroxide	0.4 ± 0.89^{bc}	20
80% Ethanol + 2% Hydrogen peroxide	0.2 ± 0.45^{bc}	20
80% Ethanol + 4% Hydrogen peroxide	2.2 ± 2.39^{a}	80
80% Ethanol + 6% Hydrogen peroxide	0 ± 0^{c}	0
Control (only distilled water)	0 ± 0^{c}	0

*Means followed by the same letter(s) are not significantly different at $p > 0.05$.

Effect of hormonal combination

Table 02 explain the number of shoot buds, length of shoot buds and length of longest shoot bud with different hormonal combinations after 4 weeks after culturing. The bud induction was observed without growth hormone however, the best bud induction rates were higher in culture media with growth hormone. Number of shoot buds were significantly different (3.6 ±1.67) in treatment added with 0.5 NAA mg/L and 2 BAP mg/L. However, the length of shoot buds or length of longest shoot bud did not show statistically significant differences. The numerical maximum mean number of shoot buds (3.6) with a mean shoot length of 0.46 cm were observed in the MS medium supplemented with 2 mgl^{-1} BAP and 0.5 mgl^{-1} NAA.

Table 02. Number of shoot buds, length of shoot buds, and length of longest shoot bud with different hormonal combinations after 4 weeks of culture

Treatment		Number of shoot buds*	Length of shoot buds (cm)*	Length of longest shoot bud (cm)*
NAA (mg/L)	BAP (mg/L)			
0	0	0.8 ±0.84 ^{b}	0.12±0.11 ^{bc}	0.12 ± 0.11^{bc}
0	2	1.6 ±2.07 ^{b}	0.16±0.15 ^{bc}	0.22 ±0.23 ^{abc}
0	3	1.8±1.79 ^{b}	0.16±0.18 ^{bc}	0.24±0.34 ^{abc}
0	4	1.2 ±0.84 ^{b}	0.19±0.11 ^{abc}	0.26±0.18 ^{abc}
0	5	0.8 ±0.84 ^{b}	0.13±0.13 ^{bc}	0.14±0.13^{bc}
0.25	0	1.4 ±1.34 ^{b}	0.20±0.23 ^{abc}	0.30±0.35 ^{abc}
0.25	2	0.6 ±1.34 ^{b}	0.12±0.27 ^{bc}	0.2±0.45^{bc}
0.25	3	0.2 ±0.45 ^{b}	0.06±0.13 ^{c}	0.06±0.13 ^{c}
0.25	4	0.6 ±0.89 ^{b}	0.14±0.19 ^{bc}	0.22±0.30 ^{abc}
0.25	5	0.8 ±1.3 ^{b}	0.38±0.52 ^{ab}	0.7±1.09 ^{a}
0.5	0	0.4 ±0.89 ^{b}	0.04±0.09 ^{c}	0.04±0.09 ^{c}
0.5	2	3.6 ±1.67 ^{a}	0.46±0.29 ^{a}	0.6±0.43 ^{ab}
0.5	3	1.4 ±2.19 ^{b}	0.12±0.17 ^{bc}	0.2±0.27^{bc}
0.5	4	1.6±1.82 ^{b}	0.14±0.13 ^{bc}	0.2±0.21^{bc}
0.5	5	1.4 ±0.89 ^{b}	0.28±0.21 ^{abc}	0.38±0.23 ^{abc}

*Means followed by the same letter(s) are not significantly different at $p > 0.05$.

Similar results were reported by Kambuska et al (2010) reported the highest rate of shoot multiplication from Turmeric explants cultured on MS + 2.00 mgl^{-1} BAP and 0.5 mgl^{-1} NAA and Rahman et al., (2004) reported the best shoot proliferation from rhizome bud explants in MS + 2.0 mg/l BAP and gave 6.2 average lengths of shoots per culture and Swarnathilaka and Nilantha (2012) was shown the optimum medium to produce multiple shoots was MS medium supplemented with 4.00 mgl^{-1} BAP and 0.25 mgl^{-1} NAA while for the shoot elongation and the best medium was medium supplemented with 2.00 mgl^{-1} BAP

and 0.5 mgl^{-1} NAA. Like that Rahman et al., (2004) reported best shoot proliferation from rhizome bud explants in MS supplemented with 2.0 mg/l BAP.

CONCLUSION

In the present study, the highest results were found in explant surfaces sterilized with 80% ethanol followed by 4% hydrogen peroxide combination. When studying the whole experiment, we realized that Hydrogen peroxide is more effective than Clorox bleach solution. The highest effective hormonal combination for shoot generation was found in the MS medium supplemented with 2.0 mgL^{-1} BAP and 0.5 mgL^{-1} NAA. Further studies are needed for evaluating the best hormonal combination with faster growth accumulating with different other hormone combinations for *In-Vitro* Propagation of Curcuma *longa* (Turmeric).

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