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In vitro protocol optimization for micro-propagation of turmeric (*Curcuma Longa* L.) Variety of dame via sprout bud culture

Yeshambel Emewodih[1*], Berihu Mengs[2]
[1] Plant Biotechnology Lab, Jimma Agricultural Research Centre, Jimma, Ethiopia
[2] Ethiopia Institute of Agricultural Research, Addis Ababa, Ethiopia

Abstract
Turmeric (*Curcuma longa* L.) is an Asian native species used for ornamental, medicinal and food purposes. However, its conventional propagation method is costly and inefficient. Therefore, this study aimed to verify appropriate asepsis methods for shoots from rhizomes fortified with Murashige and Skoog (MS) medium, 3% sucrose, 0.7% plant propagated agar, plant hormones and the pH was adjusted at 5.75 for turmeric growth. Rhizomatous explants from two months old buds were cultured on Murashige and Skoog's (MS) medium supplemented with different concentrations of cytokinins and auxins for micro-propagations of turmeric cultivar. The highest plant height of 7.9 cm was recorded at growth regulator 1mg/L BA (Benzyl Adenine) combined with 0.25 mg/L NAA (α-Naphthalene Acetic Acid). Whereas the highest yield of shoots 23.3 were obtained from 2mg/L BA combined with 1mg/L NAA. In addition to that, the highest root length of 5.1 cm and yield of root number 13.6 were obtained from 0.5mg/L NAA as compared to the control. Hence the plant growth regulator have been grate tendency to hardening off in the green house on the soil media of top soil, coffee husk and sand at the ratio 2:1:1respectively were 100% survival as compared to Top soil: vermin compost: sand at 2:1:1 ratio respectively 90% survival has been showed.

Keywords: turmeric, *Curcuma longa*, *In vitro*, MS media and micro-propagation

1. Introduction
The genus *Curcuma* L. of the family Zingiberaceae is well known as the turmeric genus, because of *Curcuma longa* L. *C. longa* is the most investigated species of this genus, although there are over 100 others in this genus [2]. It is broadly used as a medicine because it is rich in compounds like curcumin, sodium curcuminate and methyl-curcumin [5] that have been studied due to their anti- inflammatory, antioxidant and antitumor potential. Moreover, in the food industry rhizomes were used to manufacture colorings that are usually used in pasta, in the preparation of sauces and breads [6]. Turmeric is conventionally propagated vegetatively through rhizome bits carrying one or two buds. This technique presents some limiting factors for seedling production, such as cost, production time, labor, and utilization of the commercialized part the rhizomes [4]. In addition to that, the contamination of the propagation material with soil fungi, bacteria and nematodes present in soil [1] is another obstacle that results in loss of the propagation material. However, *In vitro* regeneration or micro-propagation is the best alternative to overcome these hurdles and it holds tremendous potential for rapid multiplications and production of high quality medicines from them [3].

1.1 Justification
Conventional methods of propagating turmeric via rhizome seed were not granted due to the incidence of diseases, inadequate agricultural management practice and dynamic nature of the pathogen. As a result, there is poor productivity and limitation for expanding turmeric production making Ethiopia unable to benefit from its rich agro-ecological niche adopted to nationally release turmeric cultivars. However, mass propagation of disease free turmeric through tissue culture techniques is recommended and planted in clean and agro-ecologically adopted. In addition to that, true-to-type and disease free plantlets have been maintained *In vitro* using growth retardants. This study was assured that, *In vitro* mass propagation of disease free turmeric (*Curcuma longa* L.) cultivar via tissue culture techniques.

2. Materials and Methods
Rhizomes of variety Dame were collected from the Jimma Agricultural Research Center (JARC) fields were first washed and then disinfected by 0.3% (w/v) Mancozeb solution for 30 minutes. Although, rhizomes were completely covered with polyethylene black plastic sheeting and kept in glass house for one week having a temperature of 40ºC. The rhizomes were waited until bud sprouting.
After one month, the sprouting buds were subjected to explants sterilization and antioxidants using a combination of different chemicals such as copper sulfate, Poly Vinyl Pyridoxine (PVP), ascorbic acid, citric acid, antibiotics, alcohol, and 5% active chlorine bleach (Berekina). Explants sterilization by 70% (v/v) ethanol for 1 min, synergistic disinfection by 4% (w/v) copper sulfate and 0.1% (w/v) antioxidants for 1 hr, 0.2% (w/v) Streptomycin for 2 hrs, 70% (v/v) ethanol for 1 min and 25% (v/v) bleach for 20 min respectively were showed excellent result with more than 60% of the explants become asepsis. Therefore, Cultures were incubated in the dark for the first two weeks of establishment at 25 ± 2°C followed by 16/8 h photoperiod conditions and the light intensity of 40 µmol $m^{-2}s^{-1}$ provided by cool white fluorescent lamps.

2.1 Multiplication and Rooting
Multiplication experiments have conducted using full strength MS medium supplemented with five levels of

Benzyl adenine (BA) and four levels of α-Naphthalene Acetic Acid (NAA) hormone combinations. Whereas for rooting experiments have conducted using full strength MS medium supplemented with five level of Indole 3 butyric acid (IBA) and α-Naphthalene Acetic Acid (NAA) were applied. Therefore, Cultures were incubated in the dark for the first two weeks of establishment at 25 ± 2°C followed by 16/8 h photoperiod conditions and the light intensity of 40 µmol $m^{-2}s^{-1}$ provided by cool white fluorescent lamps. A total of 21 treatments were used in which one treatment is considered as a control. All treatments were replicated three times in Completely Randomized Design (CRD) and each replication contains five plantlets. There was a highly significant difference between treatments with regard to shoot length and shoot number.

2.2 Data analysis

Treatment effects in all experiments were analyzed using the analysis of variance procedure (ANOVA) via SAS statistical software (version 9.2) and the Least Significance Difference (LSD) is used for comparison among treatment means. For all the data analysis, probability level of less than 5% ($P < 0.05$) was considered for statistical significance.

3. Results and Discussion

Rhizomes are underground stems in direct contact with soil microorganisms, including pathogens like bacteria and fungi. This probably contributed to a greater infection of the buds and consequently affected the contamination rate at the time of *In vitro* establishment. For other studies that evaluated asepsis in Zingiberaceae buds, stronger substances were required, such as ethanol immersion (70%) for 1 min and 30% (V/V) bleach for 15 min in the laminar flow hood in turmeric asepsis [4]. Multiplication experiments have conducted using MS medium supplemented with five levels of Benzyl adenine (BA) and four levels of α-Naphthalene Acetic Acid (NAA) hormone combinations. A total of 21 treatments were used in which one treatment is considered as a control. The result showed that 2 mg/l BA in combination with 1mg/l NAA gave maximum number of shoots which are 23.3 per a single explant with shoot length of 7.68cm (Table 1) as compared to the control. There was a highly significant difference between treatments with regard to shoot length and shoot number. The stimulating effectivity of BA and NAA on multiple shoot formation has been reported earlier for several medicinal plants [7].

Table 1: Effect of BA and NAA on shootlets multiplication of Turmeric (data taken: after four weeks)

Treatments (mg/l)	Shoot length (cm)	Shoot number/explant
Hormone free (Control)	4.8 ^{ij}	6.5 ^{g}
1BA+0.25NAA	7.9 ^{a}	18 ^{abc}
1BA+0.5NAA	7.4 ^{ab}	18.1 ^{abcd}
1BA+0.75NAA	7.1 ^{abcde}	12 ^{degf}
1BA+1.00NAA	7.1 ^{abcde}	12 ^{cdefg}
2BA+0.25NAA	6.5 ^{cdefg}	11.7 ^{efg}
2BA+0.5NAA	5.8 ^{fghi}	13.9 ^{bcdef}
2BA+0.75NAA	6.6 ^{bcdefg}	12.4 ^{cdef}
2BA+1NAA	7.7 ^{ab}	23.3 ^{a}
3BA+0.25NAA	6.1 ^{efgh}	11.4 ^{efg}
3BA+0.5NAA	6.9 ^{abcdef}	12.7 ^{cdef}
3BA+0.75NAA	6.7 ^{abcdef}	13.2 ^{cdef}
3BA+1NAA	7.3 ^{abcd}	16.9 ^{abcde}
4BA+0.25NAA	6.6 ^{bcdefgh}	9.4 ^{fg}
4BA+0.5NAA	6.1 ^{efgh}	12.4 ^{cdef}
4BA+0.75NAA	5.6 ^{ghi}	15.6 ^{bcde}
4BA+1NAA	6.3 ^{defgh}	14.7 ^{bcdef}
5BA+0.25NAA	6.1^{efgh}	13.8 ^{bcdef}
5BA+0.5NAA	5.6 ^{hi}	15.5 ^{bcde}
5BA+0.75NAA	6.6 ^{bcdefg}	14.1 ^{bcdef}
5BA+1NAA	5.0 ^{hij}	20.2 ^{ab}
Mean	6.47	14.18
LSD(0.05)	1.61	5.95
CV (%)	5.1	13.2

Mean with same letter within the column are not significantly different at p<0.05 ANOVA, least significance difference (LSD). CV = coefficient variation (%)

Rooting experiments was conducted using half strength MS medium supplemented with α-Naphthalene Acetic Acid (NAA) and Indole-3 Butyric Acid (IBA) hormones alone. A total of 11 treatments were used in which one treatment was considered as a control. The result showed that the maximum number of root number observed in 0.5 mg/l NAA which was 13.6 per a single explant with root length of 5.1cm (Table 2) as compared to the control. There was a highly significant difference between treatments with regard to root length and root number.

The acclimatization experiment has performed using different substrates such as top soil, coffee husk, vermin compost and sand in different combinations of unsterilized soil media. The treatments were top soil: coffee husk: sand in 1:1:1 ratio; top soil: coffee husk: sand in 2:1:1 ratio; top soil sole (as a control); top soil: vermin compost: sand in 1:1:1 ratio and top soil: vermin compost: sand in 2:1:1 ratio.

The top of each pot was also completely covered with polyethylene white plastic sheeting in the first one week with periodic watering three times a week. In the second week, the polyethylene white plastic sheeting was removed from each pot for two hours per a day and covered again by the sheeting for the rest of the hours to provide sufficient amount of sunlight. In the third week, the sheeting was completely

removed from the pot but in the same green house. Starting from the first day of the fourth week, the pots were transferred to another polyethylene greenhouse having a temperature of 30-40ºC depending on the weather condition. Starting from the second week, plantlets were wetted when they need water.

The result showed that substrates containing top soil: coffee husk: sand in 2:1:1 ratio gave the highest survival percentage which was 100% after one month of transfer (Table 3) as compared to top soil: vermin compost: sand 2:1:1 90% survival rate respectively.

Table 2: Effect of NAA and IBA on rooting of Turmeric (data taken: after four weeks)

Treatments (mg/l)	Root length (cm)	Root number/shoot
Hormone free (Control)	1.98 ^{ef}	4.5 ^{e}
0.25 mg/l NAA	2.6 ^{de}	12.36 ^{abc}
0.5 mg/l NAA	5.1^{a}	13.6 ^{a}
1 mg/l NAA	3 ^{cd}	12.5 ^{ab}
1.5 mg/l NAA	3.6 ^{bc}	11.2 ^{bc}
2 mg/l NAA	2.6 ^{de}	9 ^{bcd}
0.25mg/l IBA	1.7^{f}	4.5 ^{e}
0.5mg/l IBA	2.4 ^{de}	6.8^{de}
1mg/l IBA	3.9 ^{bc}	6.7 ^{de}
1.5mg/l IBA	2.4 ^{def}	7.3 ^{d}
2mg/l IBA	4^{b}	8.1 ^{cd}
Mean	3.03	8.89
LSD (0.05)	0.95	2.7
CV (%)	8.46	10.65

Mean with same letter within the column are not significantly different at p<0.05 ANOVA, least significance difference (LSD). CV = coefficient variation (%)

Table 3: Turmeric acclimatization (data taken: after four month)

Substrates	No. of plantlets planted	Survived	Percentage (%)	Remark
Top soil sole (control)	30	30	100	Dried leaves on the tip
Top soil: coffee husk: sand in 2:1:1 ratio respectively	30	30	100	Completely normal leaves
Top soil: coffee husk: sand in 1:1:1 ratio respectively	30	29	96.7	
Top soil: vermin compost: sand in 1:1:1 ratio respectively	30	28	93.3	
Top soil: vermin compost: sand in 2:1:1 ratio respectively	30	27	90	

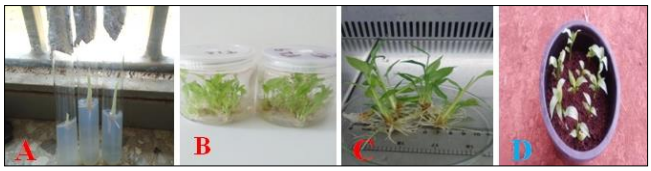

Fig 1: *In vitro* and *Ex vitro* protocol for mass multiplication of turmeric var. Dame (a) initiation (b) multiplication, (c) rooting and (d) acclimatization

4. Conclusion

In this experimental study, we have been established an efficient and reliable micro-propagation protocol for *In vitro* regeneration of *Curcuma longa* L. from rhizomal explants, protocol optimized, which can ensure large scale propagation, as well as diseases free and true to type plants, which is very important for the sustainable supply of plant materials to the pharmaceutical industries and for conservation multiplications of elite turmeric germplasm.

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