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Propagation of cochineal scale insect free cactus (*Opuntia ficus-indica*) by *in vitro* regeneration culture technique in Tigray, Ethiopia

Tsehaye Kidus Ebuy[1]* and Kiros–Meles Ayimut[2]

[1]Department of Quality Assurance, Research and Development, Tigray Biotechnology Center Pvt. Ltd. Co, Mekelle, Ethiopia.
[2]College of Dry land Agriculture and Natural Recourse, Mekelle University, Mekelle, Ethiopia.

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Cactus in northern Ethiopia is an endangered plant due to the attack by cochineal scale insect (Dactylopius coccus). The aim of this study was to micro propagate disease-free and cochineal resistance cactus pear by in vitro regeneration culturing technique. The study started with young cladodes carefully removed from mother plants. The surface-sterilized 1 cm2 cladode with one areole was cultured on shoot initiation MS media supplemented with 0, 0.5, 1.0, and 1.5 mg/l BAP alone. The already established explants were cultured on shoot multiplication media fortified with BAP at 0, 1.0, 2.0, and 3.0 mg/l. The proliferated cultures were inoculated for rooting on half-strength MS media supplemented with NAA alone at 0, 0.5, 1.0 and 1.5 mg/l. The MS medium appended with 0.5 mg/l BAP produced significantly the highest shoot number per explant (3 ± 1) and highest micro shoot length (3.27 ± 0.40). The highest multiplication factor (9.93 ± 2.25) was observed on a medium containing 1 mg/l BAP while the highest shoot lengths or elongation (3.03 ± 0.26) were observed on the medium containing 2 mg/l BAP. The best highest root number (6.06 ± 0.92) was recorded on the half MS Basal medium containing 0.5 mg/l NAA and highest root length (3.03 ± 0.27) was verified on the half MS Basal medium containing 1.0 mg/l NAA. The well-rooted plantlets were transferred for acclimatization purposes using coco peat substrate and 100% of the plants survived and established as vigorous plants under modern greenhouse conditions. The creation of a successful micro propagation method that allows for the production of more than 10,000 rooted plantlets from a single longitudinally divided shoot explant in just short period of time.

Key words: *Opuntia ficus-indica, in vitro*, tissue culture, areoles, cladodesba.

INTRODUCTION

The Cactaceae family includes approximately 130 genera and 1500 species (Rojas-Aréchiga and Vázquez-Yanes, 2000; Pérez-Molphe-Balch et al., 2015). Of these, the *Opuntia* and *Nopalea* genera are the most important due

*Corresponding author. E-mail: tsehayemkboy@gmail.com.

to their usefulness to man (Flores-Valde´z, 1995). *Opuntia* has a specialized photosynthetic mechanism known as Crassulacean Acid Metabolism (CAM), whereby these plants open their stomata and take up CO_2 at night, when temperatures are lower and humidity higher than during the daytime. This invariably results in reduced water loss (Nobel, 1995) and offers exceptional possibilities for large quantities of biomass in water-limited areas that are useful for livestock feed (Felker et al., 2006). Within the genus *Opuntia*, *Opuntia ficus-indica* (L.) Mill is the most important species for the production of edible fruits and cladodes, which can be used as a vegetable and valuable forage resource in arid and semiarid lands during periods of drought and shortage of herbaceous plants (Scheinvar, 1995; Le Houérou, 2000; Juárez and Passera, 2002).

Cactus pear, *O. ficus-indica* (L.), is an introduction to Ethiopia. There are diverse views as to the ways of cactus pear introduction to northern Ethiopia. According to Kibra (1992), missionaries introduced cactus to Northern Ethiopia around 1847 and recently Habtu (2005) reported that Muslim pilgrimage from the Middle East introduced cactus to Southern Tigray of Northern Ethiopia in 1920. It might be possible that multiple introductions to have happened.

Cactus pear is adapted to many parts of Northern Ethiopia. In Tigray alone, wild cactus covered about 32,000 ha of land (Tesfay et al., 2011). Farmers also maintain cactus backyards, but most of the fruit harvest comes from the wildly growing cactus plantation. Cactus pear has now become an integral part of the culture and economy of the Tigray people. Cactus fruits are eaten fresh from July to September. Cladodes are used as livestock feed and are planted for soil and water conservation purposes. Recently, other uses like nopalitos, jam, and carmine have been introduced. Cactus fruits have also become a source of income as it is currently sold at prices (15 birrs kilogram) well above bananas and oranges in the supermarkets of Addis Ababa. Meaza (2009) also found a strong association of cactus holding with increased income of farmers in Kihen Tabia of Eastern Tigray. The same author reported cactus pear production in backyards as the second most important option of coping with drought for farmers in Kihen Tabia, safety-net being the first. Efforts that improve the management and utilization of the cactus crop in the Tigray region could help attain food security and improve the livelihood of the cactus farmers.

In general, prickly pear cactus species can be sexually and asexually propagated. Seed propagation is only used for scientific research to study genetic variability and factors that affect the germination process (Rojas-Are and Vasquez Yanes, 2000). Normally, cactus are slow emerging plants that occasionally have limited reproductive capacities and often have specific and limited conditions for seed germination, flowering, and seed production (Guadalupe et al., 1999). Although the conventional propagation has been attempted for *Opuntia*, genetic segregation and slow growth and development represent serious practical problems (Malda et al., 1999). Cactus seeds are often challenging to be obtained (Mauseth, 1977) and plantlets are reported to be susceptible to damping-off (Mauseth, 1979; Ault and Blackmon, 1987). There have been several reports on the micro propagation of many cactus species (Hubstenberger et al., 1992; Lema-Ruminska et al., 2014). Nevertheless, the procedures for *in vitro* culture of cacti are still not well advanced and *in vitro* morphogenetic behavior is not well understood (Fay and Gratton, 1992; Palomino et al., 1999; Llamoca-Za´ rate et al., 1999) although each different species will require separate hormone, media formulation, treatments and better optimization of micro propagation methods. Besides, the existing cultivars found in northern Ethiopia, Tigray are attacked by scale cochineal insect (*Dactylopius coccus*) they are sap-sucking insects that only eat cactus species (plants in the family Cactaceae). When the adult or juvenile cochineal insects feed on the plant sap, the plant dies because it becomes discolored and swollen around the feeding site (Moran and Cobby, 1979). And now almost all the wild cactus coverage in the northern region of Ethiopia is heavily infested by the insect pest. The production of plantlets using *in-vitro* plant tissue culture techniques could be effective for providing disease-free, resistance, and sufficient planting materials of cactus. The fore most aim of this study was to micro propagate disease-free and cochineal resistant cactus pear (*O. ficus-indica*) variety Elephant ear by *in vitro* regeneration culturing technique of areoles, and to distribute them to growers in northern Ethiopia, Tigray as a tool to against the damage by the cochineal insect.

MATERIALS AND METHODS

Description of study area

The experiment was conducted at the Tigray Biotechnology Center Pvt. Ltd. Co. (Commercial Plant tissue culture laboratory with a capacity of producing more than 40 million plantlets per annum). The center is located in Mekelle, Tigray, Ethiopia, at an altitude of 2034 masl; latitude: 13°30″0′N; longitude: 39°28″11′E about 200 km southeast of the historic city of Aksum.

Preparation of explants

The mother plants were acquired from the fields of Mekelle University's main campus. The explants were kept under a modern greenhouse for maintenance purposes. The newly established young cactus cladodes about three months old were used as explants in this experiment. The selected young cladodes were carefully removed from mother plants using sterile blade. The young cactus cladodes size was abridged and prepared up to 1 cm^2 with one areole (Figure 1-A).

They were rinsed with sterilized reverse osmosis water 3x time and surface sterilized again with 5% sodium hypochlorite solution for 5min with a few drops of Tween 20. The explants were again

Table 1. Effect of BAP on shoot initiation of *in vitro* elephant ear cactus.

PGRs (mg/l)	Days required for shoot initiation (Mean)	Bud forming explant (%)	Shoot No./explant	Micro shoot length (cm)
			Mean ± standard deviation	Mean ± standard deviation
BAP				
0	0.0^{d}	0.0	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}
0.5	6^{c}	100	3 ± 1^{a}	3.27 ± 0.40^{a}
1	10^{a}	100	1.67 ± 0.6^{b}	2.93 ± 0.3^{b}
1.5	8^{b}	100	1.33 ± 0.6^{b}	2.63 ± 0.2^{b}
Coefficient of variation (CV %)			36.6	10.2

Means represented by different letters are significantly different at $p \leq 0.05$, LSD: Least significant difference.
Source: Authors

rinsed with sterilized reverse osmosis water 3x times to remove the chemical residue outside of the laboratory. After this, the explants were immersed in 0.1% mercury chloride solution for 5 min under laminar airflow and then the explants were rinsed 5x using sterilized reverse osmosis water and the surface-sterilized explants were trimmed and inoculated on the surface of the culture bottle with MS media prepared for initiation stage.

Experimental treatment and design

MS medium (Murashige and Skoog, 1962) was used for the establishment stage as well as the shoot multiplication and rooting stage. For the establishment stage MS Basal media supplemented with merely 6-benzyl amino purine (BAP: 0, 0.5, 1.0 and 1.5 mg/l) was used. For the multiplication stage MS Basal media supplemented with BAP (0, 1.0, 2.0 and 3.0 mg/l) were used. Whereas, for rooting stage ½ MS. Basal media supplemented with naphthalene acetic acid (NAA: 0, 0.5, 1.0, and 1.5 mg/l) were used. All treatments used were additionally accompanied by 30 g/l sugar and 5% agar in constant mode. All cultures were maintained at 22°C with a light/dark cycle of 16/8 h, and at 55 to 80% relative humidity. White fluorescent light with an intensity of 120 µmol m^{-2} s^{-1} was used for illumination. After six weeks of inoculation, the rooted plantlets were acclimatized under a modern greenhouse with 100% coco peat substrate. Each experiment was laid out as a complete randomized design and the experiment for each treatment was replicated three times. The experiment was conducted from November 2019 to June 2020.

Data collection and analysis

The data such as a number of days required for shoot initiation, shoot numbers per explant, shoot length, number of leaf per explant, number of roots per plantlet, root height, and survival rate were recorded after an explicit interval of time. Statistical analysis of the data was carried out using analysis of variance (ANOVA) and differences among treatment means were compared using univariate least significant difference. Test at 5% probability level using SAS/SPSS.

RESULTS AND DISCUSSION

Effect of diverse concentrations of BAP on shoot initiation/establishment stage

The retorts of *cactus* explants (cladodes) cultured on free hormone (control) and MS medium supplemented with different concentrations of BAP alone, showed a significant difference ($P<0.05$) in terms of days required for the shoot initiation, percentage of bud forming explants, number of shoots per explant and micro shoot length (Table 1). The MS basal media supplemented with 1 and 1.5 mg/l BAP was not statically different among each other on shoot number per explant and micro shoot length. Fewer days were required for the shoot initiation on MS medium containing 0.5 mg/l BAP and extended days were recorded on the MS medium containing 1 mg/l BAP. The average revealed that the MS medium fortified with 0.5 mg/l BAP produced considerably the highest shoot number per explant (3 ± 1) (Figure 1-B) while the MS medium fortified with 1.5 mg/l BAP produced significantly the lowest shoot number per explant (1.33 ± 0.6). The lowest shoot length (2.63 ± 0.2) was observed on MS medium containing 1.5 mg/l BAP, while the highest micro shoot length (3.27 ± 0.40) was considerably shown on the MS medium supplemented with 0.5 mg/l BAP (Figure 1-B). This is in agreement with the earlier report by Clayton et al. (1990) in which the addition of concentration more than 1.0 mg/l IAA to the BAP supplemented medium inhibited the rate of multiple shoot formation. In contrast, Bhau and Wakhlu (2015) and Khalafalla et al. (2007) found high shoot number per explant on 1.5 and 5 mg/l BAP, respectively.

Due to the occurrence of high BAP concentration in medium, which decreases the role of endogenous auxin in stimulating cell elongation in salmon and AL Dabagh, the intention for the small numbers of shoots at high BAP concentration in medium (2000).

Effect of different concentrations of BAP on shoot multiplication stage

The already established culture was inoculated on MS basal medium with hormone-free (control) and MS medium supplemented with different concentrations of BAP. The result showed significant difference in multiplication factor, but there was no statistical

Table 2. Effect of BAP on shoot multiplication of *in vitro* elephant ear cactus.

PGRs (mg/l)	Multiplication rate	Shoot Length/explant (cm)
	Mean ± Standard deviation	Mean ± Standard deviation
BAP		
0	2.22 ± 0.59^{c}	2.86 ± 0.49^{a}
1.0	9.93 ± 2.25^{a}	2.70 ± 0.07^{a}
2.0	6.24 ± 0.68^{b}	3.03 ± 0.26^{a}
3.0	1.53 ± 0.71^{c}	2.11 ± 0.22^{b}
Coefficient of variation (CV %)	21.2	9.7

Means represented by different letters are significantly different at $p \leq 0.05$, LSD: Least significant difference.
Source: Authors

Table 3. Description of root number per shoot.

Source	Sum of Squares	df	Mean Square	F	Sig.
Contrast	20.704	3	6.901	1.085	0.409
Error	50.876	8	6.36	-	-

Df: Degrees of freedom: F value, LSD: Least significant difference.
Source: Authors

difference on shoot length except the treatment with MS basal medium containing 3 mg/l BAP (Table 2). The highest multiplication factor or shoot proliferation rate (9.93 ± 2.25) was observed on the medium containing 1 mg/l BAP (Figure 1-C) and the lowest multiplication rate was observed on the medium containing 3 mg/l BAP. Also, the highest shoot lengths (3.03 ± 0.26) were observed on the medium containing 2 mg/l BAP while the lowest shoot length (2.11 ± 0.22) were recorded on MS basal medium supplemented with 3 mg/l. Shoots with a high concentration of BAP responded to a deterioration in the shoot multiplication rate and shoot length similarly observed that following to reduction in the occurrence of BAP, nearby shoots were developed as normal shoots. Our finding on the effect of BAP on the shoot proliferation was contrary to other researchers such as Bhau and Wakhlu (2015), Martinez-Vazquez and Rubluo (1989), and Akram et al. (2013) who obtained the best multiplication rate and shoot length on the high concentration of BAP that was between 1.5 and 5 mg/l BAP. All different types of explants had a drop in the number of shoots as a result of the medium's higher cytokinin concentrations (BAP > 1 mg/l). When grown on MS medium supplemented with 1 mg/l BAP, shoots exhibited more robust growth than when grown on BAP at high concentrations. This suggests that the drop in shoot proliferation rate may have been caused by a larger concentration of exogenous BAP reaching a super optimum cytokinin level in the tissue in addition to the endogenous cytokinin, as observed in *O. amyclaea* (Escobar et al., 1986).

Effect of NAA on root induction and acclimatization

The proliferated cultures were inoculated on half MS basal medium with hormone-free (control) and half MS medium supplemented with different concentrations of NAA and there was no significant difference in root number per shoot and root length (Table 3). The highest root number (6.72 ± 3.66) was recorded on the half MS Basal medium containing 0.5 mg/l NAA (Table 4) (Figure 1-D) and the lowest root number (3.06 ± 0.92) was observed on media containing free hormone whereas the highest root length (3.03 ± 0.27) was verified on the half MS Basal medium containing 1.0 mg/l NAA and the lowest root length (2.11 ± 0.36) was observed on media containing the free hormone (Table 5). It was observed that all the concentrations of NAA a responded to rooting which reached a 100% including the shoots that were inoculated on half MS basal medium without plant growth hormone which is control. This suggests that the cactus species produces high endogenous Auxin (Hubstenberger et al., 1992) and this was exhibited in the rooting potential of the specific cactus cultivar in the current study. These findings are in agreement with the research reported by Khalafalla et al. (2007) and García-Saucedo et al. (2005). After four weeks, the rooted cactus plants were removed from the culture bottle and were washed using moderately hot running reverse osmosis water to remove

Table 4. Description of root length.

Source	Sum of Squares	df	Mean square	F	Sig.
Contrast	0.045	3	0.015	0.087	0.965
Error	1.378	8	0.172	-	-

Df: Degrees of freedom: F value, LSD: Least significant difference.
Source: Authors

Table 5. Effect of NAA on root induction of *in vitro* elephant ear cactus.

PGRs (mg/l)	Root number per shoot	Root Length (cm)
	Mean ± Standard deviation	Mean ± Standard deviation
NAA		
0	3.06 ± 0.92[a]	2.86 ± 0.66 [a]
0.5	6.72 ± 3.66[a]	2.70 ± 0.29[a]
1	5.37 ± 2.89[a]	3.03 ± 0.27 [a]
1.5	5.23 ± 1.69[a]	2.11 ± 0.36[a]
Coefficient of variation (CV %)	44.9	14.8

Means represented by different letters are significantly different at $p \leq 0.05$, LSD: Least significant difference.
Source: Authors

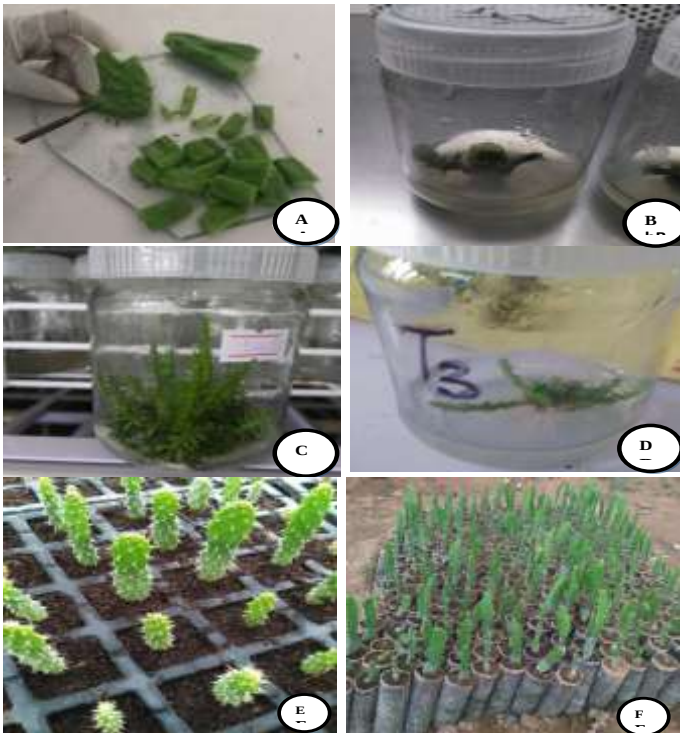

Figure 1. Micro propagation of elephant ear cactus. (A) Cladode explant with one areole, (B) shoot initiation on 0.5 mg/l BAP, (C) Shoot regeneration on MS media supplemented with 1.0 mg BAP, and (D) Root induction on ½ MS media supplemented with 0.5 mg/l NAA (E, F) Primary and secondary acclimatization under the polycarbonate greenhouse.
Source: Tigray Biotechnology center Tissue culture laboratory

the agar. Then the rooted plantlets were shifted to a 48 cell pro trays containing coco peat and placed on a modern polycarbonate greenhouse with 70 to 80% humidity. Acclimatization of *in vitro* raised rooted cactus plantlets was successful. There was 100% survival of the plantlets when shifted to coco peat and established vigorous and healthy cactus plants (Figure 1-E, F). The provision of a suitable substrate (Coco peat) and good acclimatization management, such as effective controlling of environmental conditions in greenhouses, can be achieved to fully acclimate cactus pear, even though a number of factors, including temperature, substrate, humidity, and others, can hinder the success of *in vitro*-raised cactus plantlets in their acclimatization process. The current finding was similar and in agreement with the report of pear cactus cultivars of Khalafalla et al. (2007), Estrada-Luna et al. (2008), and Akram et al. (2013) and other micro-propagated cacti (Bhau, 1999).

Conclusion

The *in vitro* micro propagation technique for the elephant ear genotype of cactus cochineal resistance was designed and developed in this work, and the findings of this study indicate that, regardless of the method, micro propagation is characterized by the efficiency of the multiplication rate. By using the methodology outlined earlier, this criterion is met. The typical method produces roughly 10 tubercles per plant annually from cacti. On the other hand, the micro propagation method described here can result in the quick growth of many plants. The micro propagation of the elephant ear cactus (*Opuntia ficus-indica*) was carried out using an *in vitro* regeneration culture approach.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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