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Contact activities of *Piper guineense* (Schum and Thonn) and *Eugenia aromaticum* (L). (Merril and Perryl) extracts against larvae of hide beetles, *Dermestes maculatus* (Degger) (Coleoptera: Dermestidae)

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Contact activities of *Piper guineense* (Schum and Thonn) and *Eugenia aromaticum* (L). (Merril and Perryl) extracts in the control of *Dermestes maculatus* larvae infesting stored fish (*Clarias gariepinus*) were investigated under laboratory condition (28 ±3°C and 65±5% RH). The extracts were tested by application of 2.0 µL each to ten third instars larvae using micro pipette at a concentration of 6.00, 10.00 and 20.00% of each of plants extracts (methanol, n-hexane and ethyl acetate). Mortality was recorded at 1, 2, 3 and 7 days of post treatments. The observed mortality was dose and exposure-dependents. All the extracts significantly enhance larval mortality (P>0.05) when compared with control. The n-hexane and ethyl-acetate extracts of *P. guineense* at 20% concentration induced the highest mortality of 86.66%, lowest mortality of 56.66% was observed on methanol fraction treated larvae after 7days of post treatments. The n-haxane of *E. aromaticum* extracts recorded the highest mortality (80.0%), followed by ethyl-acetate (76.66%) and methanolic (7.00%) fractions treated larvae at 20% concentration after 7 days of post-treatments. The results showed strong insecticidal activity in control of larvae of hide beetles infesting dried fish.

Key words: Plant extracts, *Piper guineense*, *Eugenia aromaticum*, *Dermestes maculatus.*

INTRODUCTION

Hide Beetle *Dermestes maculatus* (Degeer) (Coleoptera; Dermestidae) is one of the most destructive insect pests of stored smoked-dried fish in Nigeria (Tejumade, 2019). These pests generally infest dried fish during storage, transportation and marketing, thus responsible for extensive damage to marketed fish leading to enormous weight loss (Don-Pedro, 1989; Amadi and Dimkpa, 2018). Tejumade (2019) reported *D. maculatus* account for about 71.5% of the observed infestation with substantial loss in dry weight of about 43 to 62.7% from both larvae and

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adult.

The control of these pest in Nigeria is primarily dependent upon repeated application of synthetic chemicals such as chloripyripos-methyl, permetrin, cypermetrin, BHC, and "Otapiapia" (locally formulated) onto fish carton for protection against insect pest (Igene et al., 1998; Abolagba et al., 2011). Although many synthetic chemicals are effective, the general use of such chemicals to protect stored fish has been hampered by the report of health hazard, high cost of purchase, lack of availability, illiteracy of fish handler for right application and less susceptibility of Dermestid larvae (Booke et al., 2001; Amusan and Okorie, 2002). With these demerits of synthetic chemicals currently world-wide interest is centered on search for alternative pesticide to stored product by the use of botanical pesticide.

Botanical pesticide tends to have broad spectrum activity which are relatively specific in their mode of action and easy to process and use (Viglianco et al., 2008). To minimize use of synthetic pesticide, several plants extract have been reported as effective against *D. maculatus* on dried fish by several researchers (Fasakin, 2003; Akinwumi et al., 2006, 2007; Akpotu and Adebote, 2013; Olayinka, 2014). These extracts provide a solution to the problem emanating from the use of synthetic chemicals. The present studies have been chosen to investigate the effects of methanolic extracts fractions of *Piper guineense* and *Eugenia aromaticum* against *D. maculatus* larvae as an alternative strategy to synthetic chemicals method of pest control.

MATERIALS AND METHODS

Collection, identification of plant material and preparation of plant powders

The sample of dried fruit of *P. guineense* and *E. aromaticum* were obtained from Sokoto Central Market, Nigeria. The plants were identified and authenticated in the Herbarium of Biological Sciences Department of Usman Danfodiyo University, Sokoto. Voucher specimens (UDUH/ANS/0258 and 0221) were deposited. Samples were milled into fine powders using mortar and pestle, sieved with 0.2 mm mesh following the methods of Adedire and Lajide (2000), Akinwumi et al. (2006) and Jose and Adesina (2014). Each of the plant powders was labeled and kept in a separate plastic container and placed in a cool dry place prior to use.

Preparations of feed

The samples of dried fish, *Clarias gariepinus* was purchased from fish mongers at Sokoto Central Market, identified and authenticated in Hydrobiology Laboratory, Biological Sciences Department of Usmanu Danfodiyo University, Sokoto. The fish samples were disinfected by heat treatment in the laboratory-drying cabinet at 60°C for 1 h and allow cooling at room temperature as adopted by Onu and Baba (2003).

Collection of hide beetle and maintenance of insect culture

Different stages of hide beetle were obtained from naturally infested fish collected from Sokoto Central Market fish stalls. Several adult pairs of *D. maculatus* were obtained and kept in transparent plastic containers (19.0 cm height and 21.2 cm in diameter) fed with dried fish. The containers were covered with Muslin cloth and tight with rubber band. Wet cotton wool was supplied in each jar to provide water requirements for oviposition as suggested by Hill (1990). The adult's laid eggs which hatched into larvae and changed to pupae, pupae were picked up and transferred into separate container to obtained newly emerged adult, which were used for regular supply of larvae for the experiment in line with Akinwumi et al. (2006).

Preparation of methanol extracts and solvent fractionation

Four hundred grams (400 g) of *E. aromatica* and *P. gueneense* were homogenized with 95% methanol (1 L) in plastic container and kept at room temperature for 24 h and filtered. The methanol crude extract was collected and concentrated almost to dryness in drying cabinet at 40°C for 48 h. The dried extracts were stored in freezing medium until used for fractionation (Akinwumi et al., 2006).

The dried methanol crude extract of *E. aromatica* (19.47 g) and *P. guineense* (26.17 g) were suspended in distilled water and then partitioned with 500 ml of n-Hexane, ethyl acetate and water in increasing order of polarity, following method of Bakele et al. (2016).

Effect of extract fractions on *D. maculatus*

Effects of each plant extract fraction were conducted according to Talukder and Howse (1994). 20% stock solution was prepared for each solvent (methanol, n-haxane and ethyl acetate). Lower concentrations (6 and 10%) were obtained from dilution of the stock solution with distilled water. Ten third instars larvae were chilled for 5 min to immobilize them and then picked up individually by the use of camel hair brush and 2 µl of each of the solution was applied to the dorsal surface of the larvae. Experiments were in three replicates (each replicate contains ten treated larvae). In addition, the same number of larvae (10) was treated with distilled water only as control. After treatment insects were transferred into transparent plastic containers (19.0 cm height and 21.2 cm in diameter) containing dried fish. Observations were made daily and those that did not move or respond to gentle touch was consider as dead. Mortality was recorded at 1st, 2nd, 3rd and 7th days of post treatment.

Data analysis

Data obtained were subjected to one-way analysis of variance (ANOVA) using General Linear Model (SPSS, 2007) and means found to be significant were separated using Duncan multiple range test at 5% level of significant ($p<0.05$).

RESULTS

Effects of methanol fractions on mortality of *D. maculatus* larvae

The effect of *P. guineense* methanolic extracts applied to *D. maculatus* larvae by topical application are presented in Table 1. All the three extract of *P. guineense* exhibited insecticidal activity against *D. maculatus* larvae as dose and time-dependent variables. At day 1 all the three extract of *P. guineense* showed less than 50% mortality

Table 1. Mortality among D. *maculatus* larvae by topical application with *P. guineense* methanolic extracts fractions.

Solvent	No. of Larvea introduced	Mean larval mortality ± SE					
		Period of exposure (in days)					
		Concentration (%)	1st	2nd	3rd	7th	Mortality (%)
Methanol	10	20.00	2.33 ± 0.88^{bcde}	3.33 ± 0.88^{bc}	4.33 ± 0.33^{cd}	5.66 ± 0.88^{cd}	56.60
	10	10.00	2.33 ± 0.33^{bcde}	3.33 ± 0.88^{bc}	4.33 ± 1.20^{cde}	5.66 ± 0.33^{cde}	56.60
	10	6.00	1.33 ± 0.66^{cde}	1.66 ± 0.88^{cd}	2.00 ± 1.73^{ef}	3.33 ± 1.20^{ef}	33.30
N-hexane	10	20.00	4.66 ± 0.33^{b}	6.33 ± 0.33^{ab}	$\mathbf{8.00}\pm0.57^{ab}$	8.66 ± 0.66^{bc}	86.60
	10	10.00	4.33 ± 1.45^{b}	6.00 ± 2.08^{abc}	7.00 ± 2.08^{abc}	8.00 ± 2.00^{abc}	80.00
	10	6.00	1.00 ± 0.57^{cd}	2.00 ± 0.57^{cd}	3.66 ± 0.33^{cde}	5.66 ± 0.33^{de}	56.60
Ethylacetate	10	20.00	6.67 ± 0.88^{a}	$\mathbf{8.00}\pm0.57^{a}$	8.33 ± 0.66^{ab}	8.66 ± 0.33^{bc}	
	10	10.00	4.33 ± 0.33^{b}	5.66 ± 0.33^{ab}	5.66 ± 0.33^{bcd}	7.33 ± 0.33^{abc}	86.60 73.30
	10	6.00	3.66 ± 0.88^{bc}	4.00 ± 1.15^{bc}	5.66 ± 0.33^{bcd}	6.3388^{bc}	63.30
Cypermetrin	10	0.05	3.00 ± 0.57^{bcd}	6.33 ± 0.33^{ab}	9.33 ± 0.33^{a}	9.66 ± 0.33^{a}	96.60
Control	10	-	0.00 ± 0.00^{e}	0.33 ± 0.46^{d}	1.33 ± 0.33^{e}	1.33 ± 0.33^{e}	13.30
p-level	-	-	-	-	-	-	-

Means that have the same super script within a column are not significantly different at 5%level using Duncan's multiple range test.
Source: Author 2020.

of larvae except ethyl-acetate extract at the highest concentration (20%) which caused 66.70% mortality of larvae. At 2nd and 3rd day, mortalities in all the treatment at all concentration increased compared to day 1 of exposure. The ethyl-acetate extract at 20% concentration remain the highest 80% motality of larvae. However, at 7th day of exposure all the three extract at all concentration except 6% concentration of methanol showed a significant ($p<0.05$) mortality of larvae compared to control. The ethyl-acetate extract recorded the highest mortality range of 63.30 to 86.66%, followed by n-Hexane (56.60-
86.6%) and methanol extract (33.30-56.60%).

The contact activity of *E. aromaticum* methanolic extracts fractions applied *D. maculatus* larvae presented in Table 2. All treatments except 6% concentration of methanol and 10 and 6% of n-Hexane were significantly more toxic than control at 1st day of exposure. Efficacy was dosage-dependent with significant higher mortality occurring with increase dosage. No mortality occurred in control (0.00%). Highest mortality was recorded in ethyl-acetate extract at 20% concentration with a percentage mortality of 63.30%. At 2nd day of exposure only 6.0% concentration of methanol extract was statistically similar ($p>0.05$) with control, all other treatment showed significant mortality of larvae compared with control, the highest mortality was recorded in ethyl-acetate (63.33%) followed by n-Hexane (56.60%) and methanol (53.30%). However, at 3rd day of exposure mortality in all the treatment followed a similar trend with 2nd day of exposure with ethyl-acetate extract was the highest with a mortality range of 50 to 70%. At 7th day of exposure, the highest mortality of 80.00% was recorded from n-Hexane fraction at 20% concentration, other concentration of n-Hexane also showed higher mortality of larvae of 53.30 and 46.66%. In addition, the ethyl acetate and methanol extract fraction recorded mortality of larvae ranging from 53.30 to 76.60% and 43.30 to 70.00%, respectively.

DISCUSSION

In the current study, the three (3) extract of *P. guineense* demonstrated contact efficacy to *D. maculatus* larvae. The results indicated that the reported ethanol extract of *D. tripetala* and *P. guineense* resulted in 100% mortality of *D. maculatus* larvae after 3 days of post treatment. Ajayi (2015) stated that acetone extract is more effective in reducing oviposition and adult emergence of *Callosobruchus maculatus* than methanolic and ethanolic extract of the same plant, while methanolic and ethanolic extract were significantly more effective than aqueous extract.

In the current study, the higher activity of ethyl acetate fraction observed might be due to the presence of polar and no polar bioactive component against larval stage of *D. maculatus,* as ethyl acetate is a semi polar solvent that had ability to extract polar and non-polar compound in the extract of *P. guineense.* Variation in the bioactivities of different solvent fraction observed in the study confirmed the finding of Sun et al. (2001). That crude extract that

Table 2. Mortality among *D. maculatus* larvae by topical application with *E. aromatica* methanolic extracts fractions.

Solvent	No. of Larvea introduced	Mean larval mortality ± SE					
		Period of exposure (days)					
		Concentration (%)	1st	2nd	3rd	7th	Mortality (%)
Methanol	10	20	3.33±0.33^{ed}	5.33±0.33^{abc}	6.00±1.15^{b}	7.00±1.15^{bcd}	70.00
	10	10	4.44±0.66^{bc}	4.00±0.57^{bcd}	5.66±0.33^{b}	6.33±0.66^{bcd}	63.30
	10	6.0	1.33±0.33^{efg}	1.33±0.33^{ef}	2.66±0.66^{cd}	4.33±0.66^{e}	43.30
N-Hexane	10	20	5.33±0.33^{ab}	5.66±0.33^{ab}	6.33±0.33$^{\mathbf{b}}$	8.00±1.00^{ab}	80.00
	10	10	1.00±0.66^{fg}	3.33±0.66^{cd}	4.66±0.66^{bc}	5.33±0.88^{cde}	53.30
	10	6.0	1.66±0.88^{defg}	2.33±0.88 ^{de}	2.33±0.88^{d}	4.66±0.33^{de}	46.60
Ethylacetate	10	20	6.33±0.66^{a}	6.33±0.57^{ab}	7.0±1.15^{b}	7.00±0.57^{bcd}	70.00
	10	10	4.33±1.20^{bc}	6.00±0.57^{ab}	6.33±0.33^{b}	7.66±0.33^{abc}	76.60
	10	6.0	2.00±0.57^{def}	4.33±1.20^{abcd}	5.00±1.15^{b}	5.33±0.88^{cde}	53.30
Cypermetrin	10	0.05	3.00±0.57^{cde}	6.33±0.33^{a}	9.66±0.33^{a}	9.66±0.33^{a}	96.60
Control	10	0.00	0.00±0.00^{g}	0.33±0.33^{f}	0.66±0.33^{d}	1.33±0.33^{f}	13.30

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Source: Author 2020.

was screened with ethyl acetate, n-butyl alcohol and water fractions of alcoholic extract of leaves and stem of *Vanilla fragrans* against *Culex pipiens* larvae found that n-butyl alcohol and ethylacetate fractions were active in the bio assay, while the water fraction appeared to contain no substance that inhibited the larval growth. Overgaard et al. (2014) showed that mortality rates of mosquitoes declined with increasing polarity of the solvent, the water extract of *Zanthoxylum heitzii* (Rutaceae) produce the lowest adult mortalities whereas its ethyl-acetate and hexane extracts produce high mortalities against *Anopheles gambiae.*

Furthermore, the classes of phytochemical compound contained in the fractions might be responsible for insecticidal actions. Lale and Alaga (2000) reported *P. guineense* extract is known to contain at least three different alkaloids responsible for its insecticidal activity (piperine, chavicine and piperidine).

The result of the study also revealed the efficacy of *E. aromaticum* extracts in which all three extract fractions (was solid, not oily) gave high mortalities of larvae which could be due to its important secondary metabolite such as tarpenes, linoleic acid and oleic identified as the main active compound in *E. aromatica* (Golob et al., 1999). This supports the finding of Akinwumi (2010) who reported 100% mortality of *D. maculatus* adults when 1 ml of oil is mixed with 10.00 g of powder of *E. aromaticum* after seven days of post treatment; the finding also supports the work of Ajayi (2015) who reported that clove and west African black pepper was significantly more toxic to adult of *Tribolium castaneum* than ginger at dosage of 100 mg/50 of seed. Clove and West African black pepper and ginger oil caused 96.3, 100 and 13.2% adult mortalities, respectively and 65.7 and 9.6 larval mortalities, respectively. Akinwumi et al. (2007) also reported 0.5 g of *E. aromaticum* recorded 50.00% larval mortality and 51.67% adult mortality and concentration of 1.0, 2.0 and 2.5 g recorded 100% larval and adult mortality.

Conclusion

The study demonstrated the contact toxicity of *P. guineense* and *E. aromaticum* against *D. maculatu*s larvae. The maximum mortality was recorded on the highest dose of ethyl acetate fraction for both plants, followed by n-Haxane, while methanolic extract recorded the least activity. Hence, ethyl-acetate extract of both plants have potential insecticidal activity against *D. maculatus* larvae.

Recommendations

The findings revealed that both methanolic fractions of *P. guineense* and *E. aromatica* could be used as fish protectant against *D. maculatus*. Therefore, the use of this plant extracts for control of *D. maculatus* infestation during processing, storage, transportation and market of smoked dried fish is recommended.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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

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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 ≤ 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 ≤ 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

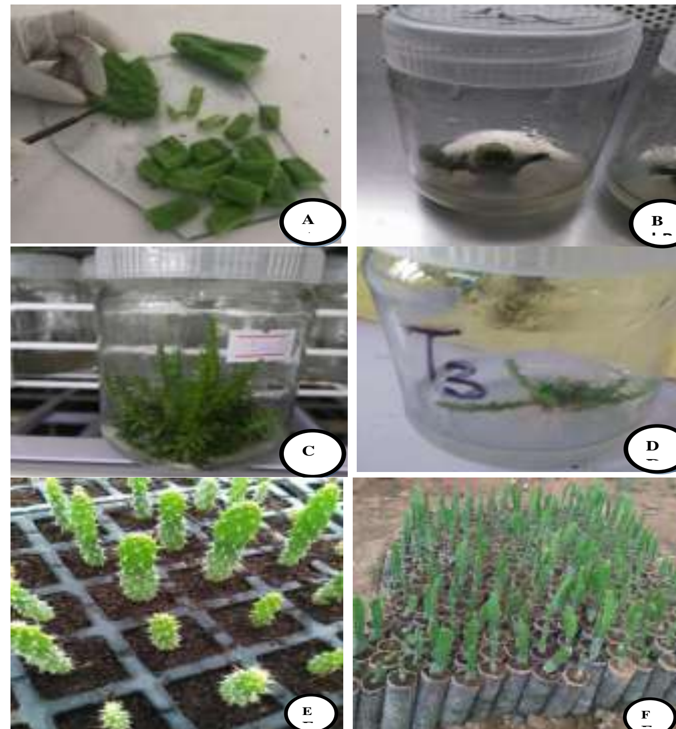

Figure 1. Micro propagation of elephant ear cactus. (A) Cladode explant with one areole, (B) shoot initiation on 0.5 mg/l BAP, (C) Shoot regeneration on MS media supplemented with 1.0 mg BAP, and (D) Root induction on ½ MS media supplemented with 0.5 mg/l NAA (E, F) Primary and secondary acclimatization 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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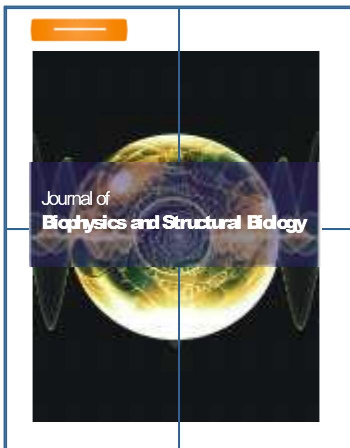

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