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Micropropagation of anchote [*Coccinia abyssinica* (Lam.) Cogn.]: High calcium content tuber crop of Ethiopia

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Anchote [*Coccinia abyssinica* (Lam.) Cogn.] is an endemic plant with high calcium content grown for its edible tuberous roots in Ethiopia. It is difficult to produce true-to-type anchote plants to sustain tuber quality through propagation by seeds as the plant pollinates both self and cross. Therefore, the objective of this study was to develop micropropagation protocol for anchote and to investigate the effect of table sugar on the rate of shoot multiplication as compared to laboratory grade sucrose. Culture was initiated from seedlings on MS-basal medium and the highest number of shoots per explant (13.13±3.90) was obtained on MS medium containing 0.25 mg/L BAP and 0.1 mg/L IBA. The maximum mean root number (7.00±2.75) and root length (5.97±1.13 cm) per explant were obtained on MS medium containing 0.025 mg/L and 0.05 mg/L IBA, respectively. No significant difference was observed in the rate of shoot multiplication between shoots cultured on the medium containing table sugar or laboratory grade sucrose. Among acclimatized plantlets, 68.75% survived. This work showed that BAP and IBA combination is superior to BAP alone in anchote *in vitro* shoot multiplication and this protocol can be used to produce clean and true-to-type anchote planting materials.

Key words: Anchote dishes, *in vitro* propagation, table sugar, seed uncoating.

INTRODUCTION

Anchote [*Coccinia abyssinica* (Lam.) Cogn.] belongs to the family *Cucurbitaceae*. The genus *Coccinia* contains 30 species, of which eight occur in Ethiopia (Jeffrey, 1995). Among these species, *C. abyssinica* is the only species grown for its edible tuberous roots, and the young shoots are used as vegetables (Fekadu, 2011). It is an annual trailing herbaceous plant whose tubers vary in shape depending on environmental conditions, but generally spherical or elongated at maturity (Figure 3D). Based on tuber colors, two anchote cultivars are known by local names as '*diimaa*' and '*adii*' meaning red and white, respectively. Anchote is distributed in the western and southwestern parts of Ethiopia. Particularly, it is widely cultivated and used in Jimma, Illu-Abba-Bora and Wallaga areas of the Oromia Regional State (Fufa and Urga, 1997; Getahun, 1969). It was also indicated that it occurs in Gonder, Gojam and Bale areas although its extent of cultivation and utilization was not pointed out (Asfaw, 1997). The plant seems to have its center of origin and diversity in the western and southwestern parts of Ethiopia (Edwards, 1991). Anchote has nutritional, medicinal, economic and social importance. Cooked and

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spiced anchote paste is recommended for people with fractured bones and displaced joints may be because of its high protein and calcium contents (Hora, 1995). From a total of 100 g fresh weight, anchote tuber consists of 74.93 g moisture, 3.25 g protein and 327 mg calcium (Fekadu, 2011). It was also reported that anchote juice can be used to treat gonorrhea, tuberculosis and cancer. Anchote's saponin content was pointed out as active ingredient for its medicinal use in this case (Getahun, 1969). Three parts of anchote are harvestable: young shoots to be used as vegetable, seeds for propagation and tubers for the preparation of different anchote dishes. Anchote can be propagated both vegetatively and by seeds. In the vegetative propagation, tubers are planted and used as seed sources during the next growing season. Some tubers may also be left in soil for regrowth (as *'guboo'*) for the coming season. When this method is used from generation to generation, the plants are attacked by diseases due to accumulation of fungi, bacteria, viruses and nematodes. The commonest way of anchote propagation is via seeds, and as both cross- and self-pollination may occur in anchote (Getahun, 1969; Hora, 1995; Jeffrey, 1995), it may be difficult to obtain true-to-type plants.

Despite the high value of anchote, few studies were conducted on the plant. Micropropagation is important for the production of large number of plants of the same clone within relatively short period of time. *In vitro* regeneration and genetic transformation as well as disease-free plant production also require micropropagation. The high level of some anti-nutritional factors in anchote (Fekadu, 2011), may be removed through genetic transformation. Therefore, developing micropropagation protocol for this very useful crop is important. There was not any previous work on micropropagation of this crop.

The objectives of this study were to develop an efficient micropropagation protocol using seedling explants, and to investigate the effect of laboratory grade sucrose and table sugar on *in vitro* shoot multiplication.

MATERIALS AND METHODS

Evaluation of germination percentage of anchote seeds

Anchote seeds were bought in August, 2011 from three sellers (A, B and C) in Dembi Dollo town, 652 km west of Addis Ababa. Different seed sources were used to be on the safer side as seeds obtained from market usually fail to germinate. Seeds from each of the three sellers were sown on filter paper in Petri dishes (ANUMBRA, 100 × 15 mm). In another experiment, the seeds were soaked in water in a beaker. Those seeds that floated after thorough stirring were considered dead and discarded. Those that sank were considered to be viable, and used in further germination experiments. The viable seeds were sown on filter paper in Petri dishes and watered as necessary. Twelve seeds per Petri dish in five replications were used. Data were collected on the tenth day after sowing. In another experiment, the viable seeds were sown in pots containing a mixture of red soil, compost and sand in a 2:1:1 ratio, respectively and maintained in greenhouse. Ten seeds were sown per pot and watered every other day.

Anchote fruits and seeds assessment

Anchote fruits were obtained from Bako Agricultural Research Center and classified into four categories: ripe, semi ripe, unripe and insect infested. Each group was assessed for its color, texture and intactness. Finally, they were all washed and crushed by hand to extract the seeds, and to investigate their internal features. The seeds from each group of fruits were thoroughly washed under running tap water to remove the internal flesh. The size, hundred-seed weight, texture, color and other features of each group were assessed. The seeds from each group were sun dried for 10 days. Then, they were wrapped separately with paper and stored in lab. After six months, seeds from each group were sown on filter paper in Petri dishes, and watered as necessary to evaluate their percentage of germination.

Culture initiation

Viable seeds from sellers A and B were thoroughly washed under tap water for 30 min with powder detergent (Arial®) and the seed coat was removed and washed in the same way for 5 min followed by rinsing with double distilled water twice. The seeds were surface disinfected with 0.15% (w/v) $HgCl_2$ solution or 1% Clorox® for 5 or 10 min in each case followed by rinsing five times with sterile double distilled water. The seeds were then sown on filter paper in Petri dishes. In another experiment, coated seeds were disinfected with 1% Clorox® for 10 min after washing under tap water and rinsed with double distilled water. Seed coat was removed from some of the seeds and the rest remained with the coat. Each group was again disinfected with 1% Clorox® for 10 min followed by rinsing with sterile double distilled water five times and cultured in culture jars containing 40 ml growth regulators free full strength MS (Murashige and Skoog, 1962) basal medium. On the tenth day, roots were removed from the sterile seedlings that were obtained from uncoated seeds cultured on MS medium and transferred to fresh MS basal medium. The culture was maintained at temperature of 24 ± 2°C, light intensity of 23 µmol $m^{-2}s^{-1}$ and 16 h photoperiod. All cultures were maintained under these culture conditions.

Shoot multiplication

Three to four-week-old shoots induced on growth regulators free MS basal medium were cultured on MS medium containing different concentrations of BAP (0.08, 0.1, 0.25, 0.5, 0.75 or 1.0 mg/L) in combination with IBA (0.00, 0.05, 0.075 or 0.1 mg/L). Subculturing was done every four weeks and number of shoots per explant was recorded.

Effect of table sugar and laboratory grade sucrose on rate of shoot multiplication

Shoots were cultured on MS medium containing 0.25 mg/L BAP in combination with 0.1 mg/L IBA supplemented with 2% laboratory grade sucrose or table sugar. A total of 30 shoots per treatment was used. Shoot number, length of the main shoot, and fresh and dry weight per explant were recorded after four weeks.

Rooting and acclimatization

Three-week-old shoots raised on multiplication medium were

Figure 1. Anchote fruits and seeds at different maturity stages: A, Ripe fruit; B, semi-ripe fruit; C, unripe fruit; D, insect infested fruit; E, seeds from ripe fruit; F, seeds from semi-ripe fruit; G, seeds from unripe fruit; H, seeds from insect infested fruit; I, rotten fruit. Bars = 1 cm.

transferred to MS medium containing IBA (0.025 or 0.05 mg/L) for root induction. Growth regulators free MS basal medium was used as a control. After two weeks on rooting medium, the plantlets were removed from the culture jars and the agar was thoroughly washed away from the roots. The number of roots and main root length per plantlet was recorded. They were then, planted in polyethylene pots filled with red soil, compost and sand in a 2:1:1 or 2:2:1 ratio, respectively. Each pot was covered with perforated transparent polyethylene bag for a week. The plantlets were watered every day for the first week, and every other day afterwards. The polyethylene bag was removed after one week and the number of survived plants was recorded after a month.

Experimental design and data analyses

A total of 30 explants were used per treatment and all experiments were repeated once. Completely Randomized Design (CRD) was used. Statistical analysis of quantitative data was carried out by SPSS computer software, version 15.0. Tukey's PostHoc multiple mean comparison test was used, and a difference at probability level of $p \leq 0.05$ was considered significant for all mean comparison analyses.

RESULTS

Evaluation of germination percentage of anchote seeds obtained from local market

Non-screened seeds from sellers A and B germinated within 5 to 10 days after sowing on Petri dishes in laboratory, with only 9% germination and none of the seeds from seller C germinated. The seeds that were screened by water soaking method and sown in Petri dishes in laboratory also germinated within 5 to 10 days and among 54, 97, and 90 seeds of sellers A, B and C, 66.7, 64.9 and 15.5% germinated, respectively. However, all the germinants from seller 'C' exhibited aborted radicle and/or inability to get rid of their testa for emergence, and finally died. The screened seeds that were sown in greenhouse germinated within 10 to 15 days and among 28, 30, and 30 seeds of sellers A, B and C, 46.4, 43.3 and 10.0% germinated, respectively (Figure 3B).

Anchote fruits and seeds assessment

The ripe and semi-ripe fruits started rotting due to microbial contamination resulting from mechanical damages. The unripe ones were intact, while the insect infested ones were broken at many places on the surface and insect bored entrances were observable (Figure 1A to D). When the fruits were crushed and seeds extracted, it was easier to detach the seeds from the succulent flesh in ripe and semi-ripe fruits, while it was difficult to extract the seeds from unripe fruits, and the insect infested fruits had internally destructed features including their seeds. It was simple to clean seeds from the ripe and semi-ripe ones by washing. The seeds from unripe fruits could

Figure 2. *In vitro* culturing of anchote: A, Seeds with seed coat; B, uncoated seeds; C, uncoated seeds cultured on growth regulators free MS basal medium; D, seedlings from uncoated seeds; E, shoot multiplication on MS medium containing 0.25 mg/L BAP in combination with 0.1 mg/L IBA; F, rooting of shoots on growth regulators free MS basal medium; G, rooting of shoots with root branches on MS medium containing 0.025 mg/L IBA; H, rooting of shoots without root branches on MS medium containing 0.05 mg/L IBA; I, acclimatized plantlets after one month. Bars: A to H = 1 cm, I = 10 cm.

hardly be cleaned, and maintained the greenish internal flesh, even after washing. Seeds from the ripe fruits were vigorous, full, smooth, fibrous and good looking (Figure 1E and 3A). Those from semi-ripe fruits were shrunk and constricted (Figure 1F), while the ones from unripe fruits were more constricted than semi-ripe seeds and smaller in size (Figure 1G). The insect infested ones have been damaged (Figure 1H). The seeds were 7 to 10 mm long. Mean seed number per fruit in ripe, semi-ripe and unripe was 149, 128 and 118, respectively. The seeds from ripe and semi-ripe fruits failed to germinate in laboratory on filter paper.

Seeds from the unripe fruits germinated with percent germination of 36.7. The hundred-seed weight of seeds obtained from ripe, semi-ripe and unripe fruits was found to be 3.45, 2.83 and 2.78 g, respectively. Some ripe fruits were rotten (Figure 1I).

Culture initiation

Percent germination was increased almost by fold in the uncoated seeds as compared to the coated seeds that were treated twice with Clorox® for 10 min and sown on MS-basal medium (Figure 2A to C). The uncoated seeds exhibited 42.1% germination (Figure 2D) while only 22.2% of the coated seeds germinated and grew well. Similarly, 71.1% of uncoated seeds were free from microbial contaminants while all of the coated seeds were found to be contaminated in culture after sterilization (Table 1). This percentage germination of uncoated seeds sown on MS-basal medium was slightly lower than that of uncoated seeds that were treated only once with Clorox® and sown on filter paper on Petri dishes.

Shoot multiplication

Growth regulators free MS-basal medium did not induce multiple shoots, but supported the growth and development of good-looking shoots. The medium containing different concentrations of BAP alone exhibited different rate of shoot proliferation. Vigorous and good-looking shoots were produced on the medium containing 0.08 mg/L or 0.1 mg/L BAP. Although, shoots were multiplied on the medium containing 0.25, 0.5, 0.75 or 1.0 mg/L BAP, the shoots were vitrified, especially in the 0.25 mg/L BAP. Callus induction was also increased

Table 1. Germination response of anchote seeds after uncoating and treatment with $HgCl_2$ or Clorox®.

Treatments	Exposure time (min)	Medium of sowing	Total seeds sown	Germination (%)	Decontamination (%)
0.15% $HgCl_2$	5	Filter paper	52	61.5	9.6
	10	Filter paper	57	71.9	14
1.0% Clorox®	5	Filter paper	58	51.7	17.2
	10	Filter paper	56	48.2	21.4
	10*	MS medium	36	22.2	0
	10**	MS medium	38	42.1	71.1

*Coated seeds that were disinfected with 1.0% Clorox® for 10 min, rinsed with sterile double distilled water five times and this was repeated.
**Coated seeds that were disinfected with 1.0% Clorox® for 10 min, rinsed with sterile double distilled water five times and then uncoated and the disinfection treatment was repeated.

with increasing BAP concentration from 0.08 to 1.0 mg/L. However, when eighteen additional different treatments were used by combining each of the above six BAP concentrations with three different IBA concentrations (0.05, 0.075 and 0.1 mg/L), well multiplied normal shoots were obtained. The highest shoot number per explant (13.13±3.90) was obtained on medium containing 0.25 mg/L BAP in combination with 0.1 mg/L IBA (Table 2 and Figure 2E). BAP concentrations at 0.25 or 0.5 mg/L in combination with these three concentrations of IBA, exhibited higher shoot number per explant.

Effect of table sugar and laboratory grade sucrose on rate of shoot multiplication

No significant difference was observed between the shoots raised on ordinary table sugar and laboratory grade sucrose with regard to shoot number, shoot length and fresh weight per explant. Dry weight of shoots multiplied on medium containing laboratory grade sucrose was found to be significantly lower than those multiplied on medium containing table sugar (Table 3).

Rooting and acclimatization

Generally, the number and length of main roots per explant increased with the duration of time the shoots kept on the rooting media. However, shoots that were kept on the rooting media for only 15 days showed better survival rate during acclimatization than those kept longer on the rooting media. Consequently, data of root number and main root length per explant was recorded after 15 days of culturing on the rooting media. Although, there was no significant difference among the control (Figure 2F), 0.025 or 0.05 mg/L IBA in root number and main root length per plantlet, shoots rooted on medium supplemented with 0.025 mg/L IBA produced the highest root number per plantlet (7.00±2.75), and branched roots (Figure 2G) while others were without branches (Figure 2H). The mean longest main root per plantlet (5.97±1.13 cm) was produced in the rooting medium containing 0.05 mg/L IBA (Table 4). Plantlets taken from the rooting medium containing 0.025 mg/L IBA showed better survival rate and growth than the others during acclimatization. Plantlets planted in the soil mixture containing red soil, compost and sand in the 2:1:1 ratio, respectively, exhibited 60.01% survival. Plantlet survival in the glasshouse was increased to 68.75% by using another soil mixture consisting of the aforementioned three components in a 2:2:1 ratio (Figure 2I and 3C).

DISCUSSION

Evaluation of germination percentage of anchote seeds obtained from local market

Prolonged seed storage, extracting seeds from unripe fruits as well as seed extraction from fruits dropped to ground may be some of the factors that reduce germination rate of anchote seeds as the present study resulted only in 9% of germination on Petri dishes. It is well known that duration of storage of seeds affects the growth and development of plants, even after germination (Negash, 1993). So, ripened anchote fruits should be collected on time before they start rotting and attacked by insects. Formal seed supply systems should also be designed for such marginalized indigenous root crops. Water-soaking screening method used might have helped in getting rid of most of the dead seeds with aborted embryos as the percentage of germination increased from 9% to more than 64%, though seeds with viable embryos, but reduced food storage may also float and be discarded as dead. Percent germination of screened seeds in greenhouse was reduced by about 30 to 35% as compared to the percent germination in laboratory. This implies that environmental factors can influence both rate and percent germination of seeds. The laboratory environment was relatively a controlled one than that of the greenhouse environment.

According to Negash (2002), *Erythrina brucei* seeds directly sown in the field showed reduced rate and

Table 2. Mean number of shoots produced per explant on MS medium containing different concentrations of *BAP and **IBA.

BAP (mg/L)	IBA (mg/L)	Shoot number per explant
0.00	0.00	1.00 ± 0.00^{i}
0.08	0.00	7.27 ± 2.98^{g}
0.08	0.05	7.07 ± 2.98^{g}
0.08	0.075	7.23 ± 2.37^{g}
0.08	0.1	8.43 ± 2.79^{defg}
0.1	0.00	7.53 ± 3.79^{fg}
0.1	0.05	7.53 ± 2.08^{fg}
0.1	0.075	8.27 ± 2.98^{efg}
0.1	0.1	7.63 ± 2.46^{fg}
0.25	0.00	8.33 ± 3.11^{efg}
0.25	0.05	11.63 ± 3.97^{abc}
0.25	0.075	12.33 ± 5.24^{a}
0.25	0.1	13.13 ± 3.90^{a}
0.5	0.00	4.70 ±2.26^{h}
0.5	0.05	11.50 ± 3.80^{abc}
0.5	0.075	12.13 ± 3.75^{ab}
0.5	0.1	12.43 ± 4.17^{a}
0.75	0.00	9.83 ± 3.30^{cde}
0.75	0.05	7.53 ± 3.15^{fg}
0.75	0.075	8.47 ± 3.78^{defg}
0.75	0.1	7.53 ± 2.53^{fg}
1.0	0.00	8.53 ± 4.31^{defg}
1.0	0.05	8.87 ±3.65^{defg}
1.0	0.075	9.37 ± 2.53^{def}
1.0	0.1	10.40 ± 3.02^{bcd}

(*BAP = 6-Benzylaminopurine; **IBA = Indole-3-butyric acid). Data are given as means ± SD; means followed by the same superscripts (a-i), are not significantly different at 5% probability level.

Table 3. Effect of laboratory grade sucrose and table sugar on rate of *in vitro* shoot multiplication using MS medium containing 0.25 mg/L *BAP and 0.1 mg/L **IBA.

Carbon source	Shoot number per explant	Shoot length per explant (cm)	Fresh weight per explant (mg)	Dry weight per explant (mg)
Table sugar	8.47 ± 1.28^{a}	5.50 ± 2.66^{a}	713.00 ± 285.47^{a}	48.37 ±16.69^{a}
Lab. grade sucrose	8.57 ± 1.79^{a}	4.85 ± 2.26^{a}	611.30 ±256.70^{a}	36.43 ±14.14^{b}

(*BAP = 6-Benzylaminopurine; **IBA = Indole-3-butyric acid).
Data are given as means ± SD; means with the same superscript in the same column, are not significantly different at 5% probability level.

percentage germination than those sown in pots in glasshouse.

Anchote fruits and seeds assessment

Although, seeds extracted from ripe fruits are expected to result in highest percentage of germination among the ripe, semi-ripe, unripe and insect-infested seeds, in this study, this was not the case. This might be due to the mismanagement in collection of fruits and extraction of seeds which results in rotting of fruits. The seeds from those rotten fruits are damaged and attacked by microbial pathogens that significantly affect percentage of germination. Therefore, a better way of fruit collection, seed extraction, processing, storage and distribution should be developed.

Culture initiation

When seeds were disinfected with Clorox® for 10 min,

Table 4. Mean root number and root length produced per plantlet in different rooting media.

IBA (mg/L)	Root number per plantlet	Root length per plantlet (cm)
0.00	6.60 ± 2.85[a]	5.87±1.29[a]
0.025	7.00 ± 2.75[a]	5.51±1.33[a]
0.05	5.80 ± 2.34[a]	5.97±1.13[a]

Data are given as means ± SD; means with the same superscript are not significantly different at 5% probability level.

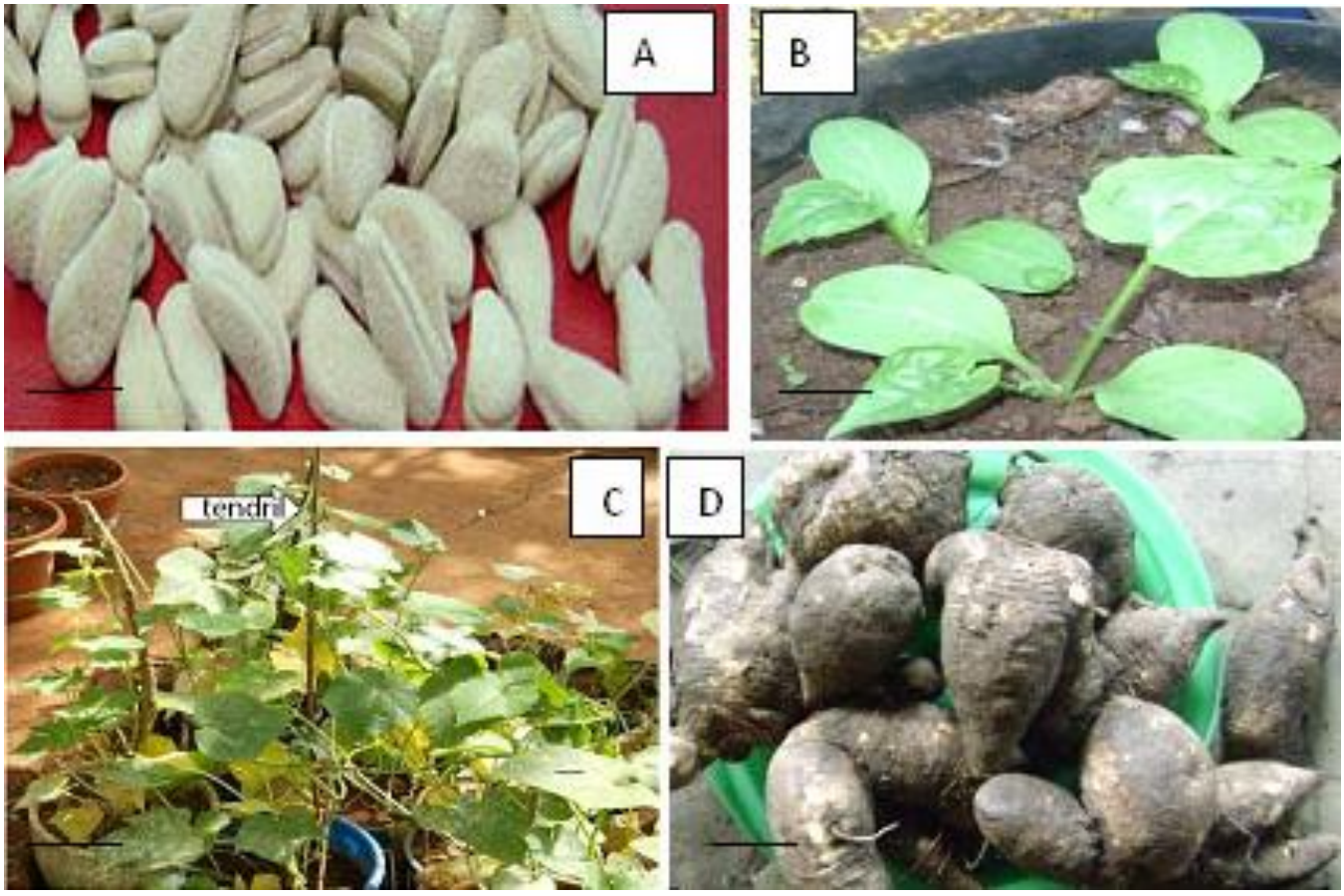

Figure 3. Morphological parts of anchote: A, Seeds; B, three-weeks old seedlings; C, anchote vines; D, differently shaped anchote tubers. Bars: A = 1 cm, B and D = 5 cm, C = 10 cm.

rinsed in sterile double distilled water, and then seed coats were removed from one group and the other group still with seed coat, and again disinfected with Clorox®, rinsed and cultured on growth regulators free MS medium, percent germination was increased almost by fold by the uncoated seeds as compared to the coated seeds. This better germination performance of uncoated seeds might be due to the fact that, on the MS-basal medium, these uncoated seeds get water and nutrients in a controlled steady way as opposed to those sown on filter paper in Petri dishes. Percent germination of coated seeds might have been affected by the sterilant (Clorox®) and microbes as the coat retains them despite repeated rinsing and disinfection, respectively.

Shoot multiplication

With increasing of BAP concentration alone from 0.08 to 1.0 mg/L, callus induction was promoted whereas when lower concentration of IBA (0.05, 0.075 or 0.1 mg/L) was combined with the BAP concentrations within this range, well multiplied normal shoots were obtained. The callus induction and inhibition of growth with increasing BAP concentration agrees with the micropropagation works of other tuber crops such as cassava (Beyene et al., 2010;

Onuoch and Onwubiku, 2007) and sweet potato (Dugassa and Feyissa, 2010). The present study showed that more than 0.75 mg/L BAP is supra-optimal concentration for anchote shoot multiplication and combination of lower concentration of BAP with IBA is essential for optimum shoot multiplication. For the improvement of the crop by modern biotechnological techniques such as genetic transformation, an efficient regeneration protocol should be developed.

Effect of table sugar and laboratory grade sucrose on rate of shoot multiplication

The similar performance of ordinary table sugar and laboratory grade sugar is a very interesting finding for micropropagation of anchote, since using table sugar as a substitute for the expensive laboratory grade sucrose reduces the cost of micropropagation dramatically. In addition, dry weight of shoots multiplied on medium containing laboratory grade sucrose was found to be significantly lower than those multiplied on medium containing table sugar. This may indicate that the former accumulated more water than those shoots grown on medium containing table sugar. The cost of laboratory grade sucrose was USD 25.85/kg, while that of the table sugar was USD 0.81/kg in Ethiopia during the progression of this study.

Rooting and acclimatization

Although, the number and length of roots per plantlet increased with the duration of time, shoots that were kept on the rooting medium for only 15 days showed better survival rate during acclimatization than those kept longer on the rooting medium. Although, there was no significant difference among the control, 0.025 and 0.05 mg/L IBA in root number and main root length per plantlet, shoots rooted on medium supplemented with 0.025 mg/L IBA produced branched roots that exhibited better survival rate during acclimatization. The mean longest main root per plantlet (5.97±1.13 cm) was produced in the rooting medium containing 0.05 mg/L IBA. According to Beyene et al. (2010), as IBA concentration in rooting medium increased, root number per plantlet in cassava also increased, but the roots became shorter and devoid of branching. Plantlets taken from the rooting medium containing 0.025 mg/L IBA showed better survival rate and growth than the others during acclimatization. This may be due to the better anchoring and absorptive capacities of the vigorous roots produced in that medium as well developed root systems are important for the successful development of acclimatized plantlets. Plantlets planted in the soil mixture containing red soil, compost and sand in the 2:1:1 ratio, respectively, had shown 60.01% survival rate and this percentage of survival was increased to 68.75% when the ratio of red soil, compost and sand was changed to 2:2:1, respectively. This might be an indication that anchote requires fertile soil as it is cultivated closer to residence areas where animal dung and other organic wastes are available.

The relatively low survival rate of plantlets could be due to the fact that the plantlets were kept under polyethylene bags only for one week.

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