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In Vitro Clonal Propagation of Korarima (Aframomum Corrorima (Braun) P.C.M. Jansen) on Improved Varieties of Bonga$_1$ and Benchmajji$_1$

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Abstract
Korarima (Aframomum corrorima (Braun) P.C.M. Jansen) is a diploid, 2n = 48, monocious, perennial and aromatic herb species under family Zingiberaceae. It is a native plant used as a spice in regular Ethiopian dishes and locally used in traditional medicine. However, commercial exploitation of this crop is hampered due to poor rate of seed germination and slow multiplication rate of the rhizomes along with other agronomic predicaments. Thus, the present study was aimed at *in vitro* micro propagations of two Aframomum corrorima cultivars of Bonga1 and Benchmajji1. Based on the results, better response was observed in rhizome bud explants with the highest mean number of shoots per explants, 4.33±0.33 and 4.67±0.33 for cv. Bonga1 and Benchmajji1 respectively, whereas 3.33±0.33 and 3.00±0.00 for cv. Bonga1 and Benchmajji1 respectively on the MS medium supplemented with BA 2.0mg/l and KN 0.5 mg/l. For multiplication, the highest mean number of shoots per explants (4.67±0.33) was obtained from the MS medium supplemented with combination of 3.0mg/l BA and 0.5 mg/l KN for cv. Bonga1, and the highest mean number of shoots per explants (4.00±0.00) was obtained from the MS medium supplemented with 2.0mg/l BA from shoot tip explants. For rooting induction, the highest mean number of roots per explants (15.00±1.15) was obtained in the MS supplemented with 1.0mg/l NAA for cv. Bonga1, and the highest mean number of roots per explants (17.33±0.88) was observed in the MS supplemented with 1.5mg/l IBA and 2.0mg/l for genotype cv. Benchmajji1, but the highest root length was obtained in *ex vitro* rooting with sand and vermi-compost at ratio of 2:1 with 1mg/l IBA three times per a week.
Keywords: In vitro clonal propagation, Shoot multiplication, Rooting induction and Ex-vitro
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Introduction

Korarima (*Aframomum corrorima* (Braun) P.C.M. Jansen) is a diploid, 2n = 48 (Wannakrairoj and Wondyifraw Tefera, 2013; Leweye Getie, 2017), monocious, perennial and aromatic herb species classified under monocotyledonous family Zingiberaceae (Jansen, 1981). The plant consists of an underground rhizome, a pseudostem and several broad leaves which morphologically resembles Indian cardamom (*Elettaria cardamomum*) (Jansen, 1981; Eyob Solomon, 2009). The diploid chromosome number of *A. corrorima* is also similar to that of its Indian close relative, *Elettaria cardamomum*, 2n = 48 (Leweye Getie, 2017).

Korarima is one of the native crop species that Ethiopia is known to be the center of its origin and diversity (Girma Hailemichael *et al*., 2016). It grows in various parts of the country; Kaffa, Jimma, East and West Wollega, Sidamo, Bale, South and North Omo, Illubabour, East and West Gojam, Gamugofa, etc. (Jansen, 1981; Simegn Kinfu *et al*., 2016), where its agro-ecological requirement is sustained (Eyob Solomon *et al*. 2007). It grows naturally at 1700–2000 m altitude in slightly shaded, more or less open sites in higher altitude rain forest having an annual rainfall ranging from 1300 mm to 2000 mm and the annual average temperature of about 20°C which is nearly the same agro-ecology as wild Arabica coffee species (*Coffea arabica*) (Jansen, 2002). Given the fact that korarima is a shade loving plant under natural forest condition, there is a claim that it can be also successfully intercropped with other horticultural crops like enset, banana, and coffee under small-scale production (Asfaw Zemede, 2001; Girma Hailemichael *et al*., 2008).

Used as a spice in a regular Ethiopian dishes and flavors different culinary tradition in Ethiopia (Girma Hailemichael *et al*., 2008; Eyob Solomon, 2009). Traditionally people use parts of korarima plants such as: pods, leaves, rhizomes and/or flowers in traditional medicine. Extracts and essential oils of korarima may be potentially used as good sources of antioxidants and it has antifungal properties (Eyob Solomon, 2008). Dried capsules and seeds of korarima have highly significant economic importance for local producers and as export commodity (Girma Hailemichael *et al*., 2008) given its managed production. Besides its importance as a spice, in traditional medicine and supporting local livelihood, korarima has invaluable contribution in environmental conservation (Israel Petros *et al*., 2019). Since korarima requires a shady environment and moisture soil for its growth the korarima plant can be used as a flagship species to preserve the endangered natural forests in the parts of the country where it inhabit (Girma Hailemichael *et al*., 2016). When it is cultivated on wetland, it forms a dense cover on the ground thereby avoiding excess evaporation and consequent drying of streams (Israel Petros

et al., 2019), which is another invaluable role in wetland preservation.

Conventionally, this multi-purpose crop can be propagated either by seeds or by cutting of its clumps or rhizomes. Propagation by seeds is advantageous to cover large areas of land while retaining the mother productive stand undamaged. However, it come with challenges of seed preparation, and the slow seed germination and growth of the subsequent seedlings. Given this challenges, a study by Eyob Solomon (2009) reported that exposure of seeds to 50% sulfuric acid (H_2SO_4) for 60 min, followed by soaking in 250 mgL-1 gibberellic acid (GA3) for 24 h was the most effective treatment for enhancing germination. Nonetheless, practicality of this method amid major korarima producers, farmers is still doubtful.

Another alternative conventional propagation method employed to propagate korarima is by vegetative means through cutting of clumps/ rhizomes, which is relatively more common. This method come with advantages of ensuring true-to-type ness and shortens the time period to reach production. But it also come with disadvantages of slow multiplication rate of the rhizomes, thus it does not meet the need of planting material for mass production, the destructive harvesting of the rhizomes from the mother stand is associated with the possibility of losing the mother plant, and the rhizomes are of perishable nature and prone to diseases when one has to transport to distant areas (Wondyifraw Tefera and Wannakrairoj, 2004; Eyob Solomon, 2009).

For a god-awful exploiter (human) such conventional propagation methods with constraint are not good alternatives. Thus, to come up with a better alternative which is a tissue culture method, a few studies have been conducted so far: Wendyifraw Tefera and Wannakrairoj (2004), (2006); Solomon Eyob (2009); Rahel Hagos and Hailay Gebremdhin (2015); Teferi Mekonnen (2016), and Leweye Getie, (2017), nonetheless further study is needed.

The first step in micro-propagation of any plant, the foundation for the next step is source of explants. For a micro-propagation of Korarima (*Aframomum corrorima*) two explants (rhizome axillary bud and *in vitro* regenerated seedlings' shoot tips) have been experimented so far and responded differently. The study by Eyob Solomon (2009) reported that shoot tips obtained from *in vitro* seedlings showed better performance with regard to percent of survival and shoot induction than rhizome buds obtained from crop grown under field condition, however the multiplication rate and other *in vitro* performances should be studied further.

The second critical stage in micro-propagation is multiplication stage, where the interplay of PGRs is evaluated for its rate of multiplication and growth of the plant. Different results were reported with different PGRs and its combinations. For instance, the basal medium supplemented with 0.5 mg/l TDZ in combination with 2 mg/l imazalil, 3 mg/l BA, and 3 mg/l paclobutrazol (PBZ) resulted 16.6, 14 and 26 respectively (Wondyifraw Tefera and Wannakrairoj, 2006). Nonetheless, those remarkable folds of shoot proliferation come with challenge associated with the use of TDZ, which is the very minuscule size of the shootlets. To counter this, one to three monthly-subcultures on a plant growth regulator (PGR)-free basal medium or BA supplemented medium to recover and attain normal growth was indicated as a measure in their study, but this requirement of repeated subculture and relatively longer time to elongate and produce functional roots for acclimatization is accompanied with huge cost that is not efficient as far as the protocol is needed to be cost effective even in terms of time.

Then another work comes by other investigators. The study by Rahel Hagos and Hailay Gebremdhin (2015) reported the use BA alone at 6.0 mg/l increased the number of regenerated shoots from hypocotyls. However, increased concentration of BA is associated with decreased shoot length, and has impacts on destroying plantlets via verification and shoot tip necrosis. In another study by Teferi Mekonnen (2016) MS medium supplemented with 0.5 mg/l TDZ and 0.25 mg/l IBA was reported to produce 2.97 shoots per explant from shoot tip explants within four weeks' time, but it is still insufficient as far as mass production is concerned. The relatively recent study by Leweye Getie (2017) showed that highest mean shoot number per explants (5.13) was obtained using 1.5 mg/l BAP in combination with 2.0 mg/l KN. Though the rate of multiplication is low in the latter three studies in comparison with the two former pioneer studies by (Wondyifraw Tefera and Wannakrairoj, 2004 and 2006), there was no problem reported with respect to miniaturization of the shootlets that can cost much as was explained earlier.

Given all the discussed scenarios with *in vitro* propagation of korarima, the present study was aimed to conduct an *in vitro* propagation of the two most recommended cultivars of korarima (Bonga1 and Benchmajji1), and to compare the *in vitro* responses of rhizome bud and *in vitro* regenerated seedlings shoot tip explants in terms of shoot proliferation. The present study is with an ample significance of providing information for further studies with different productive genotypes of korarima.

Statement of the problem

Korarima as an indigenous spice crop having tremendous values from economic to environmental, is not yet utilized to its possible potential. The production and productivity of korarima in Ethiopia where it is native, is facing challenges. The main challenge is destruction of plant's natural habitats (Jansen 1981, Eyob Solomon *et al*. 2007). Threats on its natural habitat are directly affecting the genetic diversity of korarima (Dagmawit

Chombe and Endashaw Bekele, 2018). The other major production constraint in korarima is lack of high yielding improved varieties accompanied with improved agronomic practices for sufficient planting materials (Jansen, 2002; Girma Hailemichael *et al*., 2008; Fissiha Gebreyesus and Zemed Mizan, 2015; Girma Hailemichael *et al*, 2016).

The agronomic practice conventionally employed for propagation of korarima is accompanied with shortage of planting materials due to slow seed germination and growth of the subsequent seedlings, and slow multiplication rate of the rhizomes had been a crucial hinder for wider dissemination of these crop species to major growers (Eyob Solomon *et al*., 2009; Girma Hailemichael *et al*, 2016). Thus, to counter these problems, *in vitro* mass propagation of planting materials should be enhanced further to meet the ever growing demand for quality planting materials (Girma Hailemichael *et al*, 2016) yet developing *in vitro* propagation protocol is with its predicaments as was detailed in introduction.

In addition to certain limitations with the previous study in an *in vitro* propagation of korarima as discussed in introduction, yet there is no verified and released variety of korarima to establish and optimize tissue culture protocol where reproducibility of previous study is not guaranteed so long as different cultivars of korarima exist. Currently the Tepi National Spice Research Center evaluated the top productive korarima genotypes for verification and release, and six genotype candidates are on pipeline. For the purpose of present study, two improved cultivars (promising pipeline genotypes (Bonga1 and Benchmajji1) were experimented *in vitro* propagation where no previous work was conducted on those cultivars.

General objective

In vitro protocol optimization of two improved cultivars of korarima (*Aframomum corrorim*) via rhizome buds and seeds explant types

Specific objectives

- To evaluate the *in vitro* responses of rhizome bud explant and *in vitro* regenerated seedling shoot tip explant in terms of shoot multiplication rate
- To investigate the effects of different concentrations of N-benzyladenine (BA) alone and in combinations with kinetin (KN) on shoot multiplication of *A. corrorima*
- To investigate the effects of different concentrations of IBA (indole-3-butyric acid), and NAA on rooting of *A. corrorima*
- To calculate percent of successfully hardened plantlets under greenhouse conditions for each cultivar.

Materials and Methods

MS Stock solution preparation

Murashige and Skoog (1962) basal medium was used throughout this study. Initially, full strength stock solutions of macronutrients, micronutrients, iron and vitamins were prepared separately. The recommended amount of each component nutrient was measured in volume for a liter of stock solutions (macronutrients, micronutrients, iron and vitamins), and it was dissolved in distilled water using magnetic stirrer and adjusted to final volume with distilled water. Exceptional, potassium nitrate (KNO_3) was dissolved with hot water before mixing with other components of the macronutrient stock. After all the component nutrients of each stock were completely dissolved, the solution of each stock was poured into plastic conical flasks and stored at 4°C until use.

Plant Growth Regulators Stock Solution Preparation

The plant growth regulators (PGRs) employed for this study was N-benzyladenine (BA), kinetin (KN), α-naphthalene acetic acid (NAA) and indol-3-butyric acid (IBA). Each of these growth regulators stock was prepared at a concentration of 1.0 mg/ml by weighting the required amount using precision balance followed by dissolving in few drops of 1.0 M NaOH and it was adjusted to final required volume with distilled water. After complete dissolution, the solution was transferred to bottle and stored at 4°C until use.

Preparation of growth culture conditions

Culture medium was prepared taking the recommended amounts of MS (Murashige and Skoog, 1962) stock solutions supplemented with 3% (w/v) sugar as a carbon source, and 0.7% (w/v) agar as solidifying agents. For each experiment the desired concentrations and combinations of auxins and cytokinins was added to the culture medium accordingly. Prior to addition of agar, the medium were adjusted to the final volume and the pH of the culture medium was adjusted to 5.8 with either 1% N HCl or 1% N NaOH, and 7g of agar was added. Then to melt the agar and dissolve the whole solution, it was boiled using boiler before dispensing to culture jar. Then, about 40-50 ml of the media was dispensed into 350 ml jar and covered with a plastic cap. Finally, the medium was autoclaved at 121°C for 20 min. After cooling, the entire autoclaved medium was maintained in the media storage room for a minimum of four days prior to use in order to check contamination over the media.

Plant Material Preparation and Sterilization

Auxiliary bud preparation and sterilization

Sprouting rhizomes of korarima (of two cultivars) having intact buds were collected from the garden of the horticulture division of Jimma Agricultural Research Center (JARC, Ethiopia). The buds were kept under running tap water for half an hour to remove the soil and other dirt. Then the buds were thoroughly washed with tap water using laboratory detergent. Then the outer bud scales were removed until the bud with single shoot tip remains, which was followed by washing using detergent. The cleansed buds were transferred to laminar air flow hood and it was rinsed with 70% ethanol for 1 min, followed by a surface sterilization using 5% active chlorine commercial bleach of 30% (v/v) added with 3 drops/l Tween-20 for 15 min in laminar air flow hood. Finally, the explants were thoroughly rinsed three times using sterile distilled water, and the dead and damaged tissues by chemicals were trimmed off and discarded. Surface sterilized explants consisting of a bud with a small portion of the rhizome were cultured on a basal MS medium for initiation.

Seed preparation and sterilization for *in vitro* regeneration

Well ripened capsules of *A. corrorima* were collected from horticulture garden of JARC. The capsules were washed under tap water using detergent. After it was sun dried of the moisture, the capsules were cut open to release seeds. The seeds were in aggregate form of three or four clusters. Then the clusters were separated and aggregates of seeds were disintegrated to bear individual seeds out. Seeds were washed under tap water with detergent three times. Then the seeds were soaked overnight in GA_3 (250mg/l). After 24 hours of treatment with GA_3, it was rinsed three times with distilled water, and then it was treated with a fungicide, Sabozeb (40g/l) for 20 minutes. Consecutively, it was treated with copper sulphate ($CuSO_45H_2O$) again for 20 minutes for further disinfection after three times rinse with distilled water. Again, it was rinsed three times with distilled water. Then under the laminar air flow hood, further sterilization with 70% alcohol for 60 seconds was undertaken. After three rinses with sterile distilled water, it was finally treated with 30% of commercial bleach containing 5% of active chlorine added with two drops of tween twenty for 30 minutes followed by five rinses with sterile distilled water. The surface sterilized seeds were put over dry sterilized filter paper in order to remove traces of moistures from the surface of seeds. Finally the seeds were cultured in culture jars containing 40-50 ml plant growth regulators free MS (Murashige and Skoog, 1962) medium. Fifteen seeds per culture jar in twenty replications were used.

Figure 1: Ripened, red capsules of *A. corrorima* (A). Seeds of *A. corrorima* in aggregates (B) *A. corrorima* seeds soaked in GA3 (250mg/l) in (C)

Establishment of aseptic culture and Initiation

For establishment of cultures with *in vitro* regenerated shoot tip explants, seeds were sown on the basal MS solid media. Seeds and seedlings of *A. corrorima* were monitored for the fungal and bacterial contamination as well as for germination of the seeds. The culture jars with cultured seeds were sealed properly with plastic cap, labeled for cultivars and placed under dark growth room until the seeds are germinated. After the seeds are germinated, the cultures were transferred to the growth room with 16/8 hours photoperiod and a temperature of 25 ± 2°C. The seedlings were maintained to grow until the shoot tip is well off for the subsequent use as explants.

In case of axillary bud explants, the bud explants were cultured on the basal MS media for four weeks for initiation. Similar to above, the fungal and bacterial contamination was monitored to obtain aseptic culture. Successfully initiated aseptic buds were cultured on the MS media supplemented with 2mg/l BA and 0.5mg/l KN

for six weeks. Then its response in terms of shoot multiplication was compared to cultures containing the same PGRs (2mg/l BA and 0.5mg/l KN) and *in vitro* regenerated seedlings' shoot tip explants.

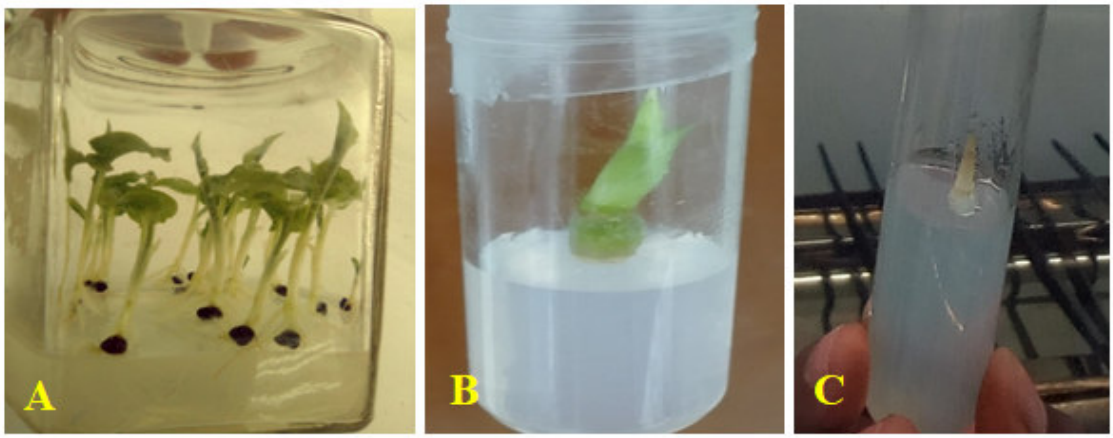

Figure 2: Aseptic in vitro germinated seedlings of *A. corrorima* (A). Rhizome bud explant cultured on MS basal media for initiation (B). Initiated rhizome bud explant after 5 weeks of culture (C)

Shoot multiplication

Shoot tip explants obtained from the *in vitro* germinated seedlings was used as explants for shoot multiplication experiments. The shoot tips was aseptically cut off and cultured on shoot multiplication medium. The shoot multiplication mediums were full strength MS medium containing different concentrations of BA (0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l) alone and in combination with KN (0, 0.5 and 1.0mg/l). Five shoot tip explants per culture jar and three replications for each treatment and cultivar was used. The culture jars were properly sealed, labeled and randomly placed in the growth room with 16/8 hours photoperiod and a temperature of 25 ± 2°C. The number of shoots per explant, mean shoot length and mean number of leaves per shoot were recorded after six weeks of culture.

Root Induction

Rooting of the shootlets of *A. corrorima* was conducted on ½ MS medium supplemented with different concentrations of IBA and NAA alone (0, 0.5, 1.0, 1.5, and 2.0 mg/l). All the medium used for the above experiments were supplemented with 3% (w/v) sucrose, the pH was adjusted to 5.8 with either 1% N HCl or 1% N NaOH and it was solidified with 0.7% (w/v) agar followed by autoclaving at 121°C and 15 kpa for 15 min. The number of roots per explant, mean root length and mean number of roots per shoot were recorded after six weeks of culture.

Acclimatization

For acclimatization process, well-developed *in vitro* rooted shoots were removed from the rooting medium and carefully washed off with hot water to remove adhering gel, and it was transferred to the pot in which sand and vermi-compost are mixed at a ratio of 2:1 by weight. Shoots were thoroughly acclimatized by covering the pots with polyethylene bags for a week and then uncovered for four weeks in greenhouse, and it was watered as necessary. After five weeks, percent of plantlets successfully hardening off were recorded.

Experimental design and Statistical data analysis

The design used for all the experiments was a complete randomized design (CRD) and the collected data were subjected to the statistical data analysis software SPSS, version 27.0. Data were analyzed using one way ANOVA, followed by Post-Hoc Multiple Comparisons tests "Tukey" at $P < 0.05$ level of significance.

Results and Discussion

In vitro responses of different explants for shoot multiplication of A. corrorima

In vitro responses of rhizome bud explants and *in vitro* regenerated seedling shoot tip explants of *A. corrorima* (cv. Bonga1 and Benchmajji1) were evaluated for shoot multiplication on MS media supplemented with the same combination and concentration of PGRs (BA 2.0mg/l; KN 0.5 mg/l). The highest mean number of shoots per explants, 4.33±0.33 and 4.67±0.33 was obtained from the rhizome bud explants for both cultivars, Bonga1 and Benchmajji1 respectively (Table1 and figure 4) when compared to mean number of shoots per explants,

obtained from *in vitro* regenerated seedling shoot tip explants.

Table 1: *In vitro* response of different explants of *A. corrorima* with regard to shoot multiplication

Treatment	Explant type	Cultivars	Mean no. of shoots/ explants
2.0 mg/L BA & 0.5 mg/L KN	Rhizome bud explants	Bonga1	4.33±0.33
		Benchmajji1	4.67±0.33
	In vitro regenerated seedling shoot tip explant	Bonga1	3.33±0.33
		Benchmajji1	3.00±0.00

Shoot multiplication

Effect of BA alone and in combination with KN on shoot multiplication via shoot tip on cv. Bonga1

The explants on MS medium supplemented with different concentrations of BA alone and in combination with KN resulted in different responses in shoot number and shoot length, but there was no significant difference on leaf number across treatments (Table 2 & Figure 3). The highest mean number of shoots per explants (4.67±0.33) was obtained from the MS medium supplemented with combination of 3.0mg/l BA and 0.5 mg/l KN and followed (4.33±0.33) was obtained on the MS medium supplemented with 3.0 mg/l BA alone. The multiplication rate was less on the MS medium containing 0.5 mg/l and 1.5 mg/l BA in combination with 1.0 mg/l KN which produced 1.00±0.00 mean number of shoots per explants that is not significantly different from control. The maximum and minimum shoot length per explants (4.67±0.09 and 1.63±0.03) were observed on the basal MS medium, control and MS medium containing 3.0 mg/l BA and 0.5 mg/l KN respectively. Increasing BA from 0.5 to 3.0 mg/l did not resulted in significant increase in mean shoot number per explants, but increasing BA from 0.5 to 2.0 mg/l with 0.5mg/l KN resulted in significant increase in mean shoot number per explants from 2.00±0.00 to 3.67±0.33. Also increasing BA from 2.5 to 3.0 mg/l with 0.5mg/l KN resulted in significant increase in mean shoot number per explants from 3.00±0.00 to 4.67±0.33. Further increase in BA concentration from 0.5 to 2.5 mg/l with 1.0 mg/l KN did not resulted in significant difference in mean shoot number per explants, but increasing BA from 1.5 to 3.0mg/l with 1.0 mg/l KN did resulted in significant difference in mean shoot number per explants. Increasing BA from 0.5 to 3.0 mg/l with 0.5 and 1.0 mg/l KN did resulted in significant decrease in mean shoot length per explants. There was no significant difference observed in mean leaf number per explants across all treatments and control.

Table 2: Effect of BA alone and in combination with KN on shoot multiplication via shoot tip on cv. Bonga1

PGRs				
BA	KN	Mean no. of shoots/explant	Shoot length (cm)	Mean no. leaves/explants
0.0	0.0	1.00±0.00^{d}	4.67±0.09^{a}	4.00±0.00^{a}
0.5	0.0	3.00±0.00^{bc}	3.10±0.06^{bc}	3.67±0.33^{a}
1.0	0.0	3.33±0.33^{abc}	3.10±0.11^{bc}	3.33±0.33^{a}
1.5	0.0	3.33±0.33^{abc}	2.70±0.06^{bc}	3.67±0.33^{a}
2.0	0.0	3.00±0.00^{bc}	2.47±0.12^{cd}	3.67±0.33^{a}
2.5	0.0	3.67±0.33^{ab}	2.63±0.09^{cd}	3.67±0.33^{a}
3.0	0.0	4.33±0.33^{ab}	2.43±0.09^{cd}	3.33±0.33^{a}
0.5	0.5	2.00±0.00^{cd}	2.37±0.09^{cd}	4.00±0.00^{a}
1.0	0.5	2.33±0.33^{bcd}	2.47±0.03^{cd}	4.33±0.33^{a}
1.5	0.5	2.67±0.33^{bc}	2.63±0.07^{cd}	4.00±0.58^{a}
2.0	0.5	3.67±0.33^{ab}	2.07±0.03^{de}	3.33±0.33^{a}
2.5	0.5	3.00±0.00^{bc}	2.50±0.06^{cd}	4.00±0.00^{a}
3.0	0.5	4.67±0.33^{a}	1.63±0.03^{e}	3.33±0.33^{a}
0.5	1.0	1.00±0.00^{d}	2.33±0.09^{cd}	3.67±0.33^{a}
1.0	1.0	2.00±0.00^{cd}	2.23±0.07^{de}	4.00±0.00^{a}
1.5	1.0	1.00±0.00^{d}	1.97±0.14^{de}	4.00±0.58^{a}
2.0	1.0	2.33±0.33^{bcd}	2.33±0.09^{cd}	4.00±0.00^{a}
2.5	1.0	2.33±0.33^{bcd}	1.93±0.09^{de}	3.67±0.33^{a}
3.0	1.0	3.33±0.33^{abc}	1.77±0.03^{e}	3.00±0.00^{a}
CV%		**7.89**	**9.56**	**6.91**

Mean difference within columns having different letters in superscript are significantly different at $P < 0.05$. The values represent mean ± standard error

Figure 3: Control (A), shoot multiplication on MS medium supplemented with BA 2.0 mg/l and KN 1.0 mg/l from *in vitro* regenerated seeding shoot tip explants (B), BA 3.0mg/l and KN 0.5mg/l (C)

Effect of BA alone and in combination with KN on shoot multiplication via shoot tip on cv. Benchmajji1

Similar to Bonga1 (Table 2), the explants on MS medium supplemented with different concentrations of BA alone and in combination with KN resulted in different responses in shoot number and shoot length, but there was no significant difference on leaf number across treatments (Table 3 & Figure 4). The highest mean number of shoots per explants (4.00±0.00) was obtained from the MS medium supplemented with 2.0mg/l BA which is not significantly different across concentration of BA from 0.5 to 3.0 mg/l. Increasing BA from 0.5 to 1.5 mg/l with 0.5 mg/l KN did not significantly increased the mean shoot number per explants, but increasing BA from 1.5 to 2.0 mg/l with 0.5 mg/l KN did significantly increased the mean shoot number per explants. Further increasing of BA from 2.0 to 3.0 mg/l with 0.5 mg/l did not significantly increase the mean shoot number per explants. Increasing BA from 0.5 to 3.0 mg/l with 1.0 mg/l KN significantly increased the mean shoot number per explants. The maximum and minimum shoot length per explants (4.80±0.06 and 1.60±0.06) were observed on the basal MS medium, control, and MS medium containing 1.5 and 3.0 mg/l BA with 0.5 mg/l KN respectively. An increase in BA concentration from 0.5 to 3.0 mg/l did not resulted significant difference in mean shoot length per explants, but increasing BA from 0.5 to 3.0 with 0.5 mg/l KN did resulted significant decrease in mean shoot length per explants. There was significant increase in mean shoot length per explants when BA increased from 1.5 to 2.5mg/l with 1.0mg/l KN. There was no significant difference observed in mean leaf number per explants across all treatments and control which was similar to Bonga1 (Table 2).

Table 3: Effect of BA alone and in combination with KN on shoot multiplication via shoot tip on cv. Benchmajji1

PGRs				
BA	KN	Mean no. of shoots/explant	Shoot length (cm)	Mean no. leaves/explants
0.0	0.0	1.00±0.00^{d}	4.80±0.06^{a}	4.00±0.00^{a}
0.5	0.0	3.33±0.33^{ab}	3.03±0.12^{b}	4.33±0.33^{a}
1.0	0.0	3.00±0.00^{ab}	2.67±0.19^{bc}	4.00±0.58^{a}
1.5	0.0	2.67±0.33^{ab}	2.70±0.06^{bc}	3.33±0.33^{a}
2.0	0.0	4.00±0.00^{a}	2.70±0.06^{bc}	4.33±0.66^{a}
2.5	0.0	3.67±0.33^{ab}	2.83±0.03^{bc}	4.33±0.33^{a}
3.0	0.0	3.33±0.33^{ab}	2.60±0.06^{bc}	3.67±0.33^{a}
0.5	0.5	1.33±0.33^{cd}	2.37±0.07^{cd}	2.67±0.33^{a}
1.0	0.5	1.33±0.33^{cd}	1.80±0.06^{de}	4.00±0.00^{a}
1.5	0.5	1.33±0.33^{cd}	1.60±0.06^{e}	3.33±0.33^{a}
2.0	0.5	3.67±0.33^{ab}	2.10±0.06^{cd}	3.33±0.33^{a}
2.5	0.5	3.00±0.00^{ab}	2.07±0.09^{d}	3.67±0.33^{a}
3.0	0.5	3.00±0.00^{ab}	1.60±0.06e	3.33±0.33^{a}
0.5	1.0	1.00±0.00^{d}	2.07±0.19^{d}	3.00±0.00^{a}
1.0	1.0	1.33±0.33^{cd}	2.53±0.03^{cde}	3.33±0.33^{a}
1.5	1.0	2.33±0.33^{bcd}	1.73±0.03^{de}	3.67±0.33^{a}
2.0	1.0	2.00±0.00^{bcd}	2.30±0.06^{cd}	3.67±0.33^{a}
2.5	1.0	2.33±0.33^{bcd}	2.43±0.03^{cd}	3.67±0.33^{a}
3.0	1.0	2.67±0.33^{ab}	1.90±0.06^{de}	3.33±0.33^{a}
CV%		**8.95**	**7.56**	**5.91**

Mean difference within columns having different letters in superscript are significantly different at $P < 0.05$. The values represent mean ± standard error

Figure 4: Shoot multiplication on MS medium supplemented with BA 1.0mg/l (A), BA 2.5mg/l and KN 1.0mg/l (B), BA 2.0mg/l (C)

Root Induction

The effect of IBA and NAA on rooting

Shoots having height greater than about 1.5cm in height were taken and transferred onto rooting medium that contains ½ MS supplemented with different concentrations of IBA and NAA (Table 4 & Figure 5). The highest mean no. of roots per explants (15.00±1.15) was observed in the MS supplemented with 1.0mg/l NAA for cv. Bonga1, and the highest mean no. of roots per explants (17.33±0.88) was observed in the MS supplemented with 1.5mg/l IBA and 2.0mg/l for cv. Benchmajji1. Increasing IBA concentration from 0.5 to 2.0mg/l did not significantly increased mean number of roots per explants for Bonga1, but significantly increased mean number of roots per explants for cultivars of Benchmajji1. Increasing NAA concentration from 0.5 to 2.0mg/l did significantly increased mean number of roots per explants for Benchmajji1, but not for Bonga1. The highest mean root length per explants (4.17±0.24cm) was observed in *ex vitro* rooting for Bonga1, and the highest mean root length per explants (3.47±0.09cm) was observed in 0.5mg/l NAA for Benchmajji1. The least mean root length (0.43±0.09cm and 0.80±0.06cm) was observed in 2.0mg/l NAA for cv. Bonga1 and Benchmajji1 respectively.

Table 4: The effect of IBA and NAA alone on rooting

PGR (mg/l)	Bonga1		Benchmajji1	
	Mean no. of roots per explants	Mean root length (cm)	Mean no. of roots/explants	Mean root length (cm)
IBA				
0.0	6.33 ± 0.33^{c}	2.77 ± 0.09^{b}	5.33 ± 0.33^{c}	3.23 ± 0.14^{a}
0.5	14.00 ± 0.58^{ab}	2.43 ± 0.09^{bc}	12.33 ± 0.88^{b}	1.60 ± 0.11^{b}
1.0	9.33 ± 0.66^{bc}	1.77 ± 0.09^{c}	10.67 ± 0.88^{b}	1.37 ± 0.09^{b}
1.5	10.67 ± 0.88^{b}	0.93 ± 0.12^{d}	17.33 ± 0.88^{a}	1.10 ± 0.10^{c}
2.0	14.00 ± 1.15^{ab}	0.57 ± 0.09^{d}	15.00 ± 1.15^{ab}	1.30 ± 0.06^{bc}
NAA				
0.0	6.33 ± 0.33^{c}	2.77 ± 0.09^{b}	5.33 ± 0.33^{c}	3.23 ± 0.14^{a}
0.5	13.33 ± 0.33^{ab}	3.77 ± 0.03^{a}	5.33 ± 0.33^{c}	3.47 ± 0.09^{a}
1.0	15.00 ± 1.15^{a}	1.93 ± 0.09^{c}	11.33 ± 0.33^{b}	1.20 ± 0.06^{bc}
1.5	14.00 ± 0.58^{ab}	1.50 ± 0.06^{c}	9.67 ± 1.20^{b}	0.83 ± 0.09^{c}
2.0	8.00 ± 0.58^{bc}	0.43 ± 0.09^{d}	17.33 ± 0.33^{a}	0.80 ± 0.06^{c}
CV%	**5.66**	**8.32**	**9.11**	**10.42**

Mean difference within columns having different letters in superscript are significantly different at $P < 0.05$. The values represent mean ± standard error

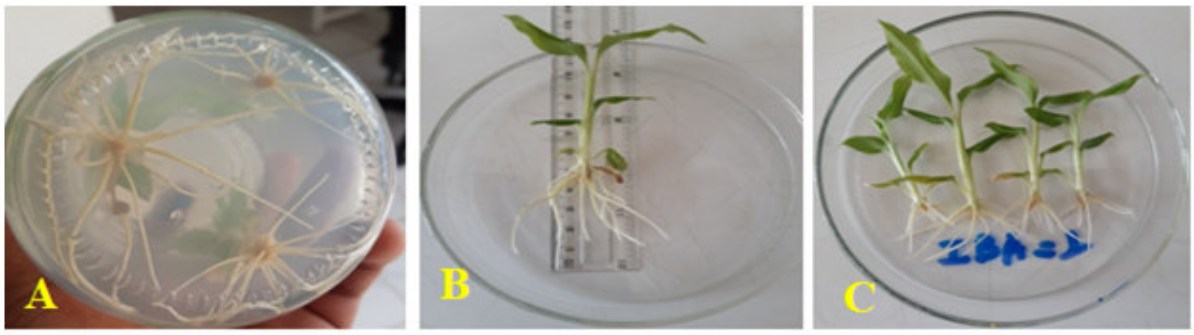

Figure 5: Rooting: Rooting on ½ MS medium supplemented with: NAA 0.5mg/l (A) and (B); IBA 1mg/l (C)

Acclimatization

A total of 100 (60 plantlets for cv. Bonga1 and 40 for cv. Benchmajji1) well developed plantlets with roots were transferred to greenhouse to hardening (10 plantlets per pot). 89 % of plantlets survived after five weeks of hardening off.

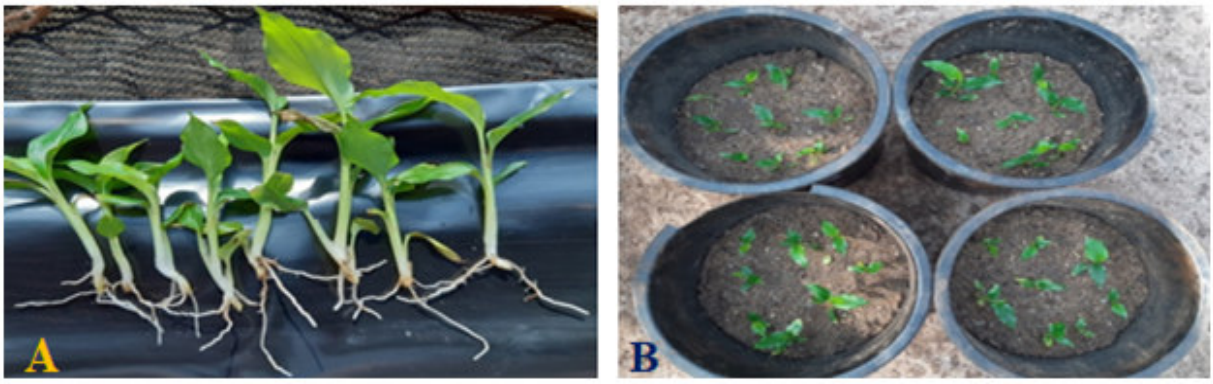

Figure 6: *Ex vitro* hardening off (A) Removing of gel from the root and (B) planting in sand soil

Discussion

In vitro responses of different explants of A. corrorima in terms of shoot multiplication

The success of *in vitro* technique in any plant propagation at first stage is highly dependent on the choice of the right explants (Bhojwani and Dantu, 2013). Hence, *in vitro* responses of rhizome bud explants and *in vitro* regenerated seedling shoot tip explants of *A. corrorima* (cv. Bonga1 and Benchmajji1) were evaluated for shoot multiplication on MS media supplemented with the same combination and concentration of PGRs (BA 2.0; KN 0.5 mg/l). In the table 1, the highest mean number of shoots per explants, 4.33±0.33 and 4.67±0.33 was obtained from the rhizome bud explants cv. Bonga1 and Benchmajji1 respectively, which is remarkably high when compared to mean number of shoots per explants (3.33±0.33 and 3.00±0.00) obtained from *in vitro* regenerated seedling shoot tip explants for cv. Bonga1 and Benchmajji1 respectively.

Eyob Solomon (2009) reported that shoot tip explants of *A. corrorima* obtained from *in vitro* regenerated seedlings have better performance with respect to percent of survival and shoot induction than rhizome buds obtained from crop grown under field condition, but from this study it was implied that with respect to rate of multiplication, rhizome bud explants was better off.

Shoot multiplication and In vitro responses of different A. corrorima genotypes

The type and concentration of cytokinins used in the shoot multiplication depends on the genotypes of plant species and explants type. For the purpose of the present study the cytokinin BA was used alone and in combination with KN to evaluate its effect on shoot multiplication of *in vitro* regenerated seedlings' shoots tip explants for two cultivars of *A. corrorima.* It was observed that there is a remarkable effect on shoot multiplication, but there was no remarkable difference in effects of BA alone and along with KN across two cultivars. The highest mean number of shoots per explants (4.67±0.33) was obtained from the MS medium supplemented with combination of 3.0mg/l BA and 0.5 mg/l KN for cv. Bonga1, and The highest mean number

of shoots per explants (4.00±0.00) (Table 3) was obtained from the MS medium supplemented with 2.0mg/l BA which is not significantly different across concentration of BA from 0.5 to 3.0 mg/l. Here it is noticeable that highest mean shoot number was obtained at different concentrations and combinations for two genotypes that can be simply referred to genotype-PGRs interaction effect. Shoot length was the parameter that is significantly affected with different concentration of BA and/with KN. On the treatments where the effects of PGRs on mean shoot number was high, shoot length was highly impacted (Table 2 & 3). But the mean leaf number was not affected across different concentrations of BA and/with KN. The results from this study are somehow correlated with the previous study by Rahel Hagos and Hailay Gebremdhin (2015), and Leweye Getie (2017).

Rooting and Acclimatization

Remarkably both IBA and NAA had induced rooting for both genotypes of *A. corrorima*, nonetheless the number of roots and the length of roots varied with their concentrations, but nearly the same between the genotypes (Table 4). Though the responses in terms of mean root number is low for both genotypes (Table 4), the root length is relatively the highest in this *ex vitro* rooting with many noticeable root hairs that is not attributed to *in vitro* rooting (Figure 5). It was observed that IBA and NAA at higher concentration had induced plenty roots for both genotypes but it was associated with short root length that was significantly different with the root length induced in half strength basal MS medium, control and *ex vitro* rooting with IBA 1mg/l. Similar to this study, Eyob Solomon (2009) reported that 1.0 mg/l IBA was the best to induce roots from in vitro culture of *A. corrorima*. In the study by Leweye Getie (2017), it was reported that the maximum rooting percentage per treatment (92.36±3.32 and 90.56±3.63) were recorded in ½ MS media supplemented with 1.5 mg/l IBA and 2.0mg/l respectively, which is in line with this study.

After sufficient development of roots for *in vitro* shoots, plantlets were successfully transplanted into plastic pots containing sand and vermin-compost at ratio of 2:1(Figure 6). After five weeks of hardening of plantlets in the greenhouse, 89% were survived. In previous studies by Wondyifraw Tefera and Wannakrairoj (2004) and Leweye Getie (2017) well rooted *in vitro* plantlets have survived with better survival record. There was no associated problem reported in these studies in terms of hardening off of *A. corrorima* plantlets that was well recognized in this study.

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