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A Micropropagation Method for Korarima (*Aframomum corrorima* (Braun) Jansen)

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ABSTRACT: An efficient method for micropropagation of korarima (*Aframomum corrorima* (Braun) Jansen, an important culinary and medicinal plant species native to Ethiopia, was developed using axillary bud explants obtained from the rhizome. Murashige and Skoog[1] (MS) medium proved to be the best for the establishment stage. Addition of 5% coconut water to the culture medium was effective in enhancing shoot proliferation. Basal medium supplemented with 2 mg/l imazalil in combination with 0.5 mg/l thidiazuron gave 7.5-fold higher shoot multiplication compared to Plant Growth Regulator (PGR)-free medium within eight weeks of culture period. The shoots developed roots readily when transferred to PGR-free MS medium. Rooted plantlets were easily acclimatized by transplanting to a potting mix substrate of river sand and peat moss (1:1), and then covered with polythene bags for a week. Acclimatized plants successfully grew (93%) when transferred to screen-shaded nursery.

KEYWORDS: 6-benzyladenine, coconut water, imazalil, korarima, micropropagation, thidiazuron, Zingiberaceae.

INTRODUCTION

Korarima (*Aframomum corrorima*) or the Ethiopian cardamom is a renowned spice and medicinal crop of the family Zingiberaceae native to Ethiopia. The dried fruits are part and parcel of the daily dishes of the Ethiopians. They are also used as a carminative, purgative and tonic in the traditional medicine.[2] According to Sebsebe,[3] korarima oil has similar chemical composition with that of its famous relative, the Indian cardamom (*Elettaria cardamomum*), except for its reduced content of terpinyl acetate, which is the major component in the latter.

Previously Ethiopia was well known for its considerable exports of korarima capsules to the world market, mainly as a substitute for the Indian cardamom.[2] However, the supply has greatly fluctuated during the past few decades that the total annual korarima export has decreased to less than 60 tones in the years 1994 - 1998, fetching only some 2.1 million USD.[4] This situation could mainly be ascribed to the reduction of production as a result of the ever-increasing destruction of the natural habitat, which is even threatening the mere existence of the crop in the country. Compared to cardamom, korarima has a relatively wider adaptation and higher productivity (ca 5.5-fold), a factor that could have attracted producers' interest to expand its production. However, there are no visible activities regarding establishment of new plantations due to the varied problems associated with the sector. Among others, these include lack of a sustainable market outlet, absence of processing industries and high yielding cultivars of superior quality, and a shortage of planting materials.[5]

Vegetative propagation, involving rhizome splits with one old and another young sucker, is the conventional technique used in korarima. The method shortens the juvenile phase of the stand and also enables propagation of true-to-type plants of a desired clone. However, this particular technique is always accompanied with shortage of planting materials to cover large areas of land and involves sacrifice of potentially productive stands. As yield and chemical composition do show considerable variation with clone in korarima, it is imperative to devise ways and means to rapidly propagate promising lines. However, there has not been any technology to enable this. Development of micropropagation methodologies does not only enable production of sufficient amounts of planting material of a desired clone, but is also the basis for future improvement through genetic engineering, as well as modern germplasm conservation tasks. Among the prominent members of the family Zingiberaceae, micropropagation has so far been successfully carried out on cardamom,[6,7] large cardamom,[8] ginger[9] and turmeric.[10] However, unlike its close relatives, no work has so far been carried out on korarima to optimize the *in vitro* propagation protocols.

Therefore, this study was undertaken to identify the best basal media formula and plant growth regulator

combinations for effective *in vitro* propagation of korarima through axillary bud proliferation.

MATERIALS AND METHODS

Plant Material and Sterilization

Rhizomes of korarima (Jimma local) were obtained from the Jimma Agricultural Research Center (JARC, Ethiopia) and planted in a nursery at Kampangsaen campus, Kasetsart University, Thailand. Axillary buds (3 - 5 mm) were excised from actively growing rhizomes. The buds from the sprouting rhizome were thoroughly washed using laboratory detergent after removal of some outer bud scales. Subsequently, the buds were kept under running tap water for one and half hour. More scales were then removed, followed by washing with the detergent.

The buds were rinsed with 70% ethanol for 1 minute; followed by a two-step surface sterilization using 20 and 10% Hyter (6% v/v sodium hypochlorite) mixed with 2 ml/l Tween-80 for 10 and 5 minutes, respectively. Then, the explants were washed four times using sterilized distilled water and were further trimmed to remove dead and chlorine affected tissues. Single explants consisting of a shoot tip with a small portion of the rhizome were cultured on Plant Growth Regulator (PGR)-free Murashige and Skoog[1] (MS) medium for culture initiation. The *in vitro* initiated shoots were subcultured every month on this same medium until the desired quantities of explants were obtained for subsequent experiments.

Culture Conditions

In all cases, 30 gm/l sucrose was used as a source of carbohydrate and media were gelled with 0.7% agaragar, after adjusting the pH to 5.7. In each experiment, the desired concentrations and types of plant growth regulators were added before autoclaving and 20 ml of the respective medium was dispensed to each 100 ml baby food jar and covered with a plastic cap. The media were autoclaved for 20 minutes at 121°C (1.06 kg/cm^2). Cultures were incubated in the culture room at a constant temperature of 25 ± 2°C, under cool white fluorescent light of 28 mmol/m^{2}s photosynthetic photon flux density with 16 hour photoperiod for eight weeks.

Experiment I: Effects of Basal Media

In this experiment, comparisons were made among MS, modified MS using half-strength macronutrients (HMS), modified MS using half-strength nitrogen salt (HNMS: MS medium containing only half of its nitrate (NO_3^-) and ammonium (NH_4^+) ions), as well as Schenk and Hildebrandt (SH)[11] media. Following the recommendation of Bajaj *et al*[7] for cardamom, 3 mg/l 6-benzyladenine (BA) and 1 mg/l Kinetin were added to all the culture media studied.

Experiment II: Effects of Coconut Water

This experiment involved comparison of 0, 5, 10, 15 and 20% (v/v) coconut water (CW) using the best basal medium obtained from experiment I (MS). All media were supplemented with 3 mg/l BA and 1 mg/l Kinetin.

Experiment III: Effects of Imazalil in Combination with Thidiazuron and/or BA

To identify the best combinations of plant growth regulator concentrations for shoot multiplication of korarima, two simultaneous experiments were conducted using 0, 2 and 4 mg/l imazalil (IMA) in combination with 0, 0.1 and 0.5 mg/l of either *N*-phenyl N'-1,2,3-thidiazol-5-ylurea (thidiazuron, TDZ) or 6-benzyladenine (BA). A PGR-free medium and a medium supplemented with 3 mg/l BA and 1 mg/l Kinetin were also included as a blank and standard control, respectively. For these experiments, the best basal medium (MS) and coconut water level (5%) from experiments I and II were used.

Statistical Analyses

The design used for all the experiments was a complete randomized design (CRD) with ten replications. Every treatment in each replication contained two explants and all experiments were repeated at least three times. All parameters were recorded after eight weeks of culture and analyzed using the PC-SAS program (Version 6.12 SAS Institute Inc., Cary, NC, USA). Data from the last two repetitions were used for analysis. In all cases, to fulfill the basic assumptions of ANOVA, root number and mean root length of plantlets were transformed using the square root transformation prior to analysis. Data from experiment I were analyzed using ANOVA, followed by the Duncan's multiple range test (DMRT). On the other hand, data from the experiment II were analyzed using the General Linear Model (GLM) accompanied with contrast analysis, to come up with the best possible relationships between the different coconut water levels and the respective growth parameters. Data from the plant growth regulator study, however, were analyzed using ANOVA followed by the Student-Newman-Keuls' (SNK) mean separation test.

RESULTS AND DISCUSSION

Comparison of Basal Media

The type of basal media affected all shoot, leaf and root numbers, as well as shoot and root lengths of korarima. The highest number of shoots and leaves

Table 1. Growth of korarima after eight weeks of culture on four basal media.

Media*	Shoot no.	Leaf no.	Root no**	Root lg.** (cm)	Shoot lg. (cm)	FW (gm)	DW (gm)
MS	6.2a	13.5a	2.2b	1.92b	5.37a	0.843	0.094
HMS	2.8c	7.3b	7.3a	3.97a	4.45b	0.659	0.084
HNMS	3.4c	8.5b	6.6a	4.19a	4.34b	1.077	0.125
SH	4.8b	5.8b	4.3ab	2.66ab	3.45c	0.669	0.085
Prob.	0.0001	0.0001	0.0026	0.0077	0.0009	0.1294	0.1830
% CV	27.19	38.58	34.67	27.18	21.63	52.46	46.46

*MS= Murashige and Skoog (1962), HMS= Half macro MS medium, HNMS= Half Nitrogen MS medium, SH= Schenk and Hildebrandt (1972).
Root lg.= Root length, Shoot lg. = Shoot length, FW=Fresh weight, DW =Dry weight.
**Transformed using square root transformation.
Means within a column followed by the same letter are not significantly different using Duncan's multiple range test (DMRT) at the 5% level of probability.

were obtained from MS, while both HMS and HNMS media followed by SH gave the highest root number. Korarima explants cultured on MS medium produced significantly longer shoots, while those on SH were the shortest. However, the shortest roots were produced on the MS medium followed by SH medium (Table 1).

Nitrogen is involved in the synthesis of several principal components of plant cells, including amino acids, proteins, nucleic acids and chlorophyll, that are indispensable to plant growth and development,[12] thus its deficiency results in stunted growth. As stated by Pilbeam and Kirkby,[13] nitrate (NO_3^-) is the most preferred nitrogen source for plant growth. In addition to the total nitrogen content, the relative concentration of NO_3^- and ammonium (NH_4^+) has been reported to have a strong influence on plant growth and development by influencing the medium pH, which in turn determines availability of nutrients.[14]

The results of korarima shoot number obtained in the current study are in accordance with the NO_3^- concentration of the different basal medium used, while the number of roots and root lengths were inversely proportional to nitrate concentration. Therefore, the higher number of shoots recorded from MS medium in this experiment could be indicative of the preference of korarima plants for nitrate-nitrogen. The present finding regarding shoot proliferation is in accordance with that of Chaillou and Lamaze,[15] who reported better plant growth and development with higher proportions of nitrate to ammonium in plant nitrogen nutrition. On the other hand, both leaf number and shoot length of korarima seem to be related to the total nitrogen content of the respective medium used in the study. The results of root number and length obtained here are in agreement with the statements of Touraine *et al*,[16] who indicated a relationship between inhibition of root growth and high levels of tissue nitrate.

Effects of Coconut Water

In vitro growth and development of korarima plants were highly influenced by the concentration of CW supplemented to the culture medium. Addition of CW increased the number of shoots and leaves, mean lengths of shoots, and both fresh and dry weights of the plantlets. But inclusion of CW to the culture medium resulted in reduction of both root number and length. Contrast analysis showed the relationships between all these parameters to be quadratic, except that of shoot length and root number, which were linear (Table 2).

Several reports confirm the beneficial use of CW (5

Table 2. *In vitro* growth and development of korarima after eight weeks of culture on media with coconut water (CW).

% CW	Shoot no.	Leaf no.	Root no*	Root lg.* (cm)	Shoot lg. (cm)	FW (gm)	DW (gm)
0	2.9	6.7	6.4	2.72	3.08	0.534	0.074
5	6.5	14.5	2.3	1.88	4.45	1.256	0.136
10	6.0	18.0	1.2	0.68	4.32	1.449	0.153
15	5.2	13.8	2.2	2.29	4.02	1.096	0.119
20	3.0	9.2	1.8	1.75	4.39	0.755	0.092
Model							
Prob.	0.0001	0.0001	0.0013	0.0310	0.0028	0.0027	0.0038
% CV	39.06	39.41	44.17	37.05	20.34	52.81	41.77
Linear**	0.5538	0.3851	0.0031	0.2572	0.0109	0.6003	0.6579
Quadratic**	0.0001	0.0001	0.0052	0.0467	0.0311	0.0001	0.0002
Cubic**	0.1501	0.4305	0.1153	0.2212	0.0116	0.3206	0.2805

*Transformed using square root transformation.
**The linear, quadratic and cubic values are obtained from contrast analyses, which were carried out using the SAS PC program.
Root lg.= Root length, Shoot lg. = Shoot length, FW=Fresh weight, DW =Dry weight.

- 20% v/v) for micropropagation of plant species in the family Zingiberaceae, including the Indian cardamom (*Elettaria cardamomum*),[6,7] large cardamom (*Amomum subulatum*),[8] ginger[9] and turmeric,[10] as well. Among these, however, the reports of Bajaj *et al*[7] and Sajina *et al*[8] for Indian cardamom and large cardamom, respectively, that indicated 5% CW as the optimal level are in direct agreement with the results obtained here for korarima.

Effects of Plant Growth Regulators

Effects of imazalil and TDZ on growth and development of korarima

Unlike the fresh and dry weight of plantlets, all the remaining five *in vitro* growth and development parameters of korarima evaluated in the current study were highly affected by the use of IMA and TDZ. The interactions of these two plant growth regulators were also highly significant in four of these parameters, except number of leaves (Table 3). Nonetheless, IMA and TDZ independently had a strong influence upon the number of leaves. In general, the best result, with respect to shoot proliferation (16.6 shoots/ explant) was obtained from the combined use of 2 mg/l IMA and 0.5 mg/l TDZ.

Effects of imazalil and BA on growth and development of korarima

The use of IMA and BA affected the numbers of shoots, leaves, and roots. The lengths of roots and shoots, and fresh weight of korarima plantlets were also affected by these two plant growth regulators. However, interaction of the two chemicals was observed only with regard to shoot number and lengths of root and shoot. On the other hand, the use of each plant growth regulator in the medium gave rise to higher numbers of leaves. In contrast, the number of roots was observed to decrease with increasing concentrations of IMA. The addition of BA to the culture media, however, increased the fresh weight of plantlets. The highest multiplication of shoots (7.32 shoots/explant) was obtained from the use of 4 mg/l IMA in combination with 0.1 mg/l BA (Table 4).

The use of different types and concentrations of plant growth regulators highly affected the *in vitro* growth and development of korarima. The combination of 2 mg/l IMA and 0.5 mg/l TDZ (IMATDZ) gave the highest number of shoots and leaves. It produced 7.5- and 3.5-fold more shoots and leaves, respectively, than the PGR-free medium (Table 5). On the other hand, the latter gave the highest number of roots, as well as the longest shoots and roots. Maximum fresh weight of korarima plantlets was recorded from both BA supplemented media (IMABA: 4 mg/l IMA and 0.1 mg/ l BA, and BAK: 3 mg/l BA and 1 mg/l Kinetin).

Similar to these results, Chand *et al*[17] have observed more vigorous growth of taro explants on culture medium supplemented with TDZ at 2.6 µM (0.6 mg/l) than BA, even at concentrations above 3.0 mg/l. Rai[18] has also gained the highest rate of *Nothapodytes foetida* shoot multiplication on MS medium containing 2.2 µM (0.5 mg/l) TDZ, as compared to that of BA or Kinetin at

Table 3. *In vitro* growth and development of korarima after eight weeks of culture on media containing Imazalil (IMA) and Thidiazuron (TDZ).

Treatment	Concentration (mg l^{-1})	Shoot no.	Leaf no.	Root no*	Root lg.* (cm)	Shoot lg. (cm)
IMA	0	7.83	13.48b	4.17	2.61	4.42
	2	9.67	17.07a	3.74	1.73	3.16
	4	10.98	16.04a	2.77	1.70	3.36
TDZ	0	2.25	8.34b	10.45	5.96	6.67
	0.1	10.28	18.12a	0.24	0.12	2.60
	0.5	15.52	19.76a	0.25	0.12	1.82
IMA X TDZ	0 X 0	2.21	6.32	12.21	7.58	7.58
	0 X 0.1	7.25	16.15	0.35	0.30	3.80
	0 X 0.5	13.75	17.60	0.35	0.19	2.04
	2 X 0	2.38	10.28	11.5	5.44	5.56
	2 X 0.1	9.26	17.42	0.10	0.00	2.37
	2 X 0.5	16.6	22.85	0.20	0.04	1.76
	4 X 0	2.16	8.53	7.68	4.83	6.82
	4 X 0.1	14.72	21.06	0.28	0.05	1.52
	4 X 0.5	16.26	18.79	0.21	0.12	1.65
Prob.	IMA	0.0001	0.0103	0.0001	0.0001	0.0001
	TDZ	0.0001	0.0001	0.0001	0.0001	0.0001
	IMA X TDZ	0.0001	0.1415	0.0001	0.0001	0.0001
MODEL	Prob.	0.0001	0.0001	0.0001	0.0001	0.0001
	% CV	36.59	41.82	22.20	18.10	28.74

*Transformed using Square root transformation.
Treatment means within a column followed by the same letter are not significantly different using and the Student-Newman-Keuls' (SNK) mean separation test at the 5% level of probability.
Root lg.= Root length, Shoot lg. = Shoot length.

Table 4. *In vitro* growth and development of korarima after eight weeks of culture on media with Imazalil (IMA) and 6-benzyladenine (BA).

Treatment	Concentration (mg/l)	Shoot no.	Leaf no.	Root no*	Root lg.* (cm)	Shoot lg. (cm)	FW (gm)
IMA	0	2.60	10.14b	10.58a	5.98	6.54	0.879
	2	3.57	13.34a	6.82b	4.92	5.68	0.925
	4	4.81	13.16a	5.70b	5.10	6.05	1.001
BA	0	2.57	9.98b	8.52	6.63	6.79	0.800b
	0.1	4.65	13.74a	7.58	4.88	6.11	1.011a
	0.5	3.76	12.88a	7.05	4.53	5.42	0.992a
IMA X BA	0 × 0	2.16	7.42	11.47	7.80	7.47	0.763
	0 × 0.1	2.67	10.56	10.11	5.37	6.78	0.821
	0 × 0.5	2.95	12.35	10.15	4.78	5.44	1.041
	2 × 0	2.58	11.05	8.00	6.21	6.45	0.822
	2 × 0.1	3.83	15.11	7.22	4.72	5.83	1.072
	2 × 0.5	4.32	13.95	5.26	3.82	4.78	0.889
	4 × 0	3.00	11.56	5.94	5.83	6.44	0.818
	4 × 0.1	7.32	15.47	5.53	4.56	5.75	1.133
	4 × 0.5	4.05	12.40	5.65	4.96	6.00	1.040
Prob.	IMA	0.0001	0.0009	0.0001	0.0005	0.0008	0.2779
	BA	0.0001	0.0004	0.2102	0.0001	0.0001	0.0123
	IMA × BA	0.0001	0.2670	0.4879	0.0206	0.0155	0.2287
MODEL	Prob.	0.0001	0.0001	0.0001	0.0001	0.0001	0.0319
	% CV	42.20	41.50	25.78	13.09	19.73	43.33

*Transformed using Square root transformation.
Treatment means within a column followed by the same letter are not significantly different using SNK at the 5% level of probability.
Root lg.= Root length, Shoot lg. = Shoot length, FW=Fresh weight.

similar concentrations or more.

There is substantial evidence that confirms the efficacy of TDZ in the induction of axillary bud break and production of adventitious buds, at a relatively lower rate than the adenine type cytokinins.[19,20] These peculiar effects were mainly ascribed to the induction of synthesis or accumulation of endogenous cytokinins by TDZ.[21] This could further be associated with a variety of factors including an increase in synthesis, a decrease in catabolism,[22] or release of biologically active cytokinin molecules from non-active storage forms.[20]

From their work on Araceae, Werbrouck and Debergh[23] stated TDZ to be much more effective when combined with IMA than other cytokinins, including BA, zeatin and mT (6-3(-hydroxybenzyl) adenine). They also suggested that this effect of IMA may be attributed to its effect upon the general mechanism of cytokinin action, i.e. its effect on changing the metabolism of exogenously applied cytokinin. In *Spathiphyllum floribundum* culture, IMA increased the shoot inducing effect of BA, but resulted in shoots with diminished sizes,[24] a result that is in agreement with our findings in korarima. In another report, Werbrouck and Debergh[25] suggested the positive effect of IMA upon BA to be associated with its possible role in altering the metabolism of BA or its probable inhibitory effect on catabolic enzymes.

Production of high number of shoots by TDZ is

Table 5. Comparison of *in vitro* growth and development of korarima after eight weeks of culture on media with different plant growth regulators.

Treatment*	Shoot no.	Leaf no.	Root no**	Root lg.** (cm)	Shoot lg. (cm)	FW (gm)
PGRF	2.21d	6.56d	12.42a	7.82a	7.93a	0.716b
IMATDZ	16.6a	22.85a	0.20c	0.04c	1.76d	0.750b
IMABA	7.32b	15.47b	5.53b	4.56b	5.75b	1.133a
BAK	5.42c	11.05c	4.37b	4.51b	4.23c	1.066a
Prob.	0.0001	0.0001	0.0001	0.0001	0.0001	0.0043
% CV	34.46	41.85	29.49	11.38	20.77	40.35

*PGRF = Plant growth regulator free medium; IMATDZ = Imazalil (2 mg l^{-1}) and Thidiazuron (0.5 mg l^{-1}); IMABA = Imazalil (4 mg l^{-1}) and BA (0.1 mg l^{-1}), BAK = BA (3 mg l^{-1}) and Kinetin (1 mg l^{-1}).
** Transformed using Square root transformation.
Means within a column followed by the same letter are not significantly different using SNK at the 5% level of probability.
Root lg.= Root length, Shoot lg. = Shoot length, FW=fresh weight.

mostly accompanied with reduced numbers of roots, as well as shorter shoots and roots.[26] D' Arth *et al*[27] have stated that such reductions in root lengths are indications of the plant's sensitiveness to higher levels of cytokinin. In this experiment of ours, the small sized shoots produced from TDZ supplemented medium were easily elongated and produced roots when subcultured on a PGR-free MS medium. Similarly, Chand *et al*[17] have also obtained relatively smaller plantlets from TDZ treatment (as compared to BA supplemented medium), which successfully revived their vigorous growth when transferred to fresh PGR-free or BA supplemented media. The present finding related to the reduced root number and length of korarima plantlets grown on IMA supplemented media was in agreement with that of Werbrouck and Debergh.[23,25]

Rooting, Acclimatization and Plant Recovery

Rooting of explants was accomplished simply by culturing them on PGR-free MS medium, where they produced numerous very fine and healthy roots. Plantlets with well-developed roots were removed from culture bottles, rinsed under tap water to remove the adhering media, and transferred to 10.1 cm plastic pots containing a mixture of autoclaved peat moss and river sand (1:1) substrate. Potted plants were covered with polythene bags for maintaining high humidity for a week. After the initiation of new leaves in the plastic bags the plantlets were transferred to a screen-shaded nursery for further growth where around 93% were successfully grown (data not presented).

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