ScienceAsia 30 (2004): 9-15

Micropropagation of Krawan (*Amomum krervanh* Pierre ex Gagnep)

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Received 27 Oct 2003
Accepted 24 Dec 2003

ABSTRACT: Krawan, one of the common spices and medicinal plants of Thailand, is conventionally propagated by vegetative means. The technique of micropropagation could be used as a possible means to overcome the problem of shortage of planting materials. Excised and surface disinfested axillary buds were cultured on plant growth regulator-free Murashige and Skoog[1] (MS) basal medium prior to culturing on MS, half-macro nutrient MS, half-nitrogen MS and Schenk and Hildebrandt (SH) media. The highest rate of multiplication was obtained from MS medium. When 0, 5, 10, 15 and 20% v/v coconut water (CW) were added, it was found that 5% CW was the best for shoot growth and development, i.e. shoot number, as well as fresh and dry weights. The effects of 0, 2 and 4 mg/l imazalil (IMA) were simultaneously studied in combination with 0, 0.1, and 0.5 mg/l 6-benzyladenine (BA) or thidiazuron (TDZ). The best media for shoot growth and development was the one with 2 mg/l IMA and 0.5 mg/l TDZ. Rooting was accomplished with a relative ease using a Plant Growth Regulator (PGR)-free MS medium. The *in vitro* rooted plantlets could be established in the soil with more than 90 percent success after transfer to *ex vitro*.

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

INTRODUCTION

Krawan (*Amomum krervanh*), also known as Siam cardamom or best cardamom, is a common spice and medicinal plant species native to Thailand and Cambodia. Its seed has a very close resemblance in flavor to that of the Indian cardamom (*Elettaria cardamomum*) which makes it the best substitute for the latter.[2] Previously, Thailand together with some other Southeast Asian countries were annually exporting about 400-1000 tones of krawan seed to the world market. However, there has been a significant reduction of export in recent years.[3] Currently, the crop is facing a serious threat of extinction, due to devastation of its natural habitat by deforestation.[4] Besides, there are no visible activities to expand new plantations, which could mainly be due to the absence of encouraging situations with respect to sustainable market outlet, supply of elite cultivars, provision of sufficient planting materials, etc. Compared to cardamom, krawan has better adaptation and a nearly three- to six-fold productivity.[5] It can grow well between altitudes of 60-1300 masl, though the best results are obtained from the higher altitudes.[4]

Krawan is commonly propagated by vegetative means.[4] The technique poses considerable loss of potential productive plant stands. To alleviate this and other related problems, in many instances, the technique of micropropagation is believed to be the best alternative. It offers the potential to produce thousands, or even millions, of plants of the desired clone per annum based on the available capacity. Besides, it also serves as a corner stone for future crop improvement by molecular biotechnology. In line with this, successful efforts have been made and encouraging results have been recorded elsewhere on different species of the family Zingiberaceae, including cardamom,[6] large cardamom,[7] ginger[8] and turmeric.[9] But, to our knowledge, no comprehensive work has so far been undertaken to optimize micropropagation protocols for krawan.

Nutrient media, including their mineral constituents together with qualitative and quantitative aspects of plant growth regulators play a decisive role in micropropagation.[10] Therefore, optimization of these factors is the basis for any *in vitro* related work. This study was thus undertaken with the objective of developing an effective micropropagation protocol for krawan.

Materials and Methods

Plant Material and Disinfections

Rhizomes of krawan brought from the Chanthaburi Horticultural Research Institute were grown at a nursery in the Kamphangsaen campus, Kasetsart University, Thailand to initiate sprouting. Rhizome pieces bearing the sprouting axillary buds (3 -5 mm) were collected and some scale leaves were removed prior to cleaning. The rhizomes were washed using tap water and laboratory detergent and then kept under running water for 90 minutes. Axillary buds were excised and washed. Some outer bud scales were removed, followed by rinsing with 70% ethanol under aseptic conditions for one minute. Further disinfections were carried out aseptically using 20 and 10% Hyter (ai: 6% v/v sodium hypochlorite) mixed with 2 ml/l Tween-80 for 10 and 5 minutes, respectively. Then, explants were rinsed four times using sterile distilled water and were further trimmed before transfer to the culture tubes.

Culture Conditions

Single disinfested explants were cultured on Murashige and Skoog [1] (MS) basal medium for culture initiation and served as explant source for subsequent experiments. In all the three experiments, 3% sucrose was used as a source of carbohydrate and the respective plant growth regulators used in each experiment were added to the medium prior to autoclaving. The basal media were gelled with 0.7 % agar-agar after adjusting the pH to 5.7. Twenty ml of the respective medium was dispensed to each 100 ml baby food jar, covered with a plastic cap, and autoclaved for 20 minutes at 121°C (1.06 kg/cm^2). In all cases, cultures were kept in the culture room at a constant temperature of 25 ± 2°C, under cool-white fluorescent light (28 m mol m^{-2}s^{-1}) at 16-hour photoperiod.

Experiment I: Comparison of Basal Media

Four different media including MS, half-macro nutrient MS, MS with half-nitrogen (i.e. MS medium with half of its original nitrate (NO_3^-) and ammonium (NH_4^+) ions), as well as Schenk and Hildebrandt (SH) [11] were evaluated for their effects on *in vitro* growth and development of krawan. All the basal media types used in this study were supplemented with 3 mg/l 6-benzyladenine (BA) and 1 mg/l Kinetin, following the recommendations of Bajaj *et al*[6] for cardamom.

Experiment II: Effects of Coconut Water

To determine the effect of coconut water (CW) supplement on growth and development of krawan, CW at five concentrations (0, 5, 10, 15 and 20% v/v) were also evaluated. For this study, the best basal medium selected from experiment I, MS, with 3 mg/l BA and 1 mg/l Kinetin was employed.

Experiment III: Effects of Plant Growth Regulators

In this part, the experiment was conducted using the best basal medium (MS) and coconut water concentration (5%) selected from experiments I and II. Three levels of imazalil, IMA, (0, 2 and 4 mg/l) in combination with three levels of thidiazuron, TDZ, (0, 0.1 and 0.5 mg/l) were studied. IMA at these same three levels in combination with three levels of BA (0, 0.1 and 0.5 mg/l) were simultaneously evaluated. In addition, a PGR-free medium and a medium containing 3 mg/l BA and 1 mg/l Kinetin were employed as blank and standard control, respectively.

Statistical Analyses

All the experiments were conducted using complete randomized design (CRD) with ten replications and repeated at least three times. Two explants were used per treatment in each replication. Only data from the last two repetitions were used for the analysis. Data for the numbers of shoots, leaves, and roots, as well as mean shoot and root lengths, and fresh and dry weights were collected after eight weeks of culture. To fulfill the basic assumptions of ANOVA, data for root number and lengths from all experiments were transformed using the square root transformation prior to analysis, though the actual means were used for reporting. Data from experiments I and III were analyzed using the ANOVA, followed by the Duncan's multiple range test (DMRT) and the Student-Newman-Keuls' (SNK) mean separation test, respectively. However, data from the CW experiment were analyzed using the General Linear Model (GLM) and contrast analysis, in order to establish a dose response relationship between the additive and the growth and development parameters evaluated. In all cases, data analyses were carried out using a PC-SAS program (Version 6.12 SAS Institute Inc., Cary, NC, USA).

Results and Discussion

Comparison of Basal Media

In this experiment, the composition of basal media significantly affected the numbers of shoots, leaves and roots, as well as root length of krawan. Non-significant effects were observed on the rest of the parameters, i.e. shoot length, as well as fresh and dry weights of *in vitro* plantlets. The highest number of shoots was obtained from MS medium followed by SH medium. This could be ascribed to the relative concentrations of nitrate (NO_3^-) in the respective media. The number of leaves, however, was observed to relate to the total nitrogen content of the respective medium.

Table 1. Effects of basal media types on *in vitro* growth and development of krawan after eight weeks of culture.

Media*	Shoot no.	Leaf no.	Root no**	Root lg.** (cm)	Shoot lg. (cm)	FW (gm)	DW (gm)
MS	5.7a	13.3a	4.3b	5.57b	6.02	0.911	0.116
HMS	3.2c	8.9b	7.3a	10.26a	6.10	1.850	0.177
HNMS	3.0c	8.9b	6.5a	8.17a	5.62	0.587	0.077
SH	4.4b	9.3b	3.9b	3.59b	4.08	0.426	0.047
Prob.	0.0001	0.0206	0.0003	0.0001	0.0726	0.2771	0.1038
% CV	30.63	34.93	16.82	19.48	34.28	184.54	115.14

*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.

The root number and length showed an inverse relationship with the nitrate concentration in the basal medium (Table 1). Thus, both half MS macro and half MS nitrogen media gave relatively numerous and longer roots.

As nitrogen is well known to serve as a constituent of many plant cell components, its deficiency inhibits plant growth. In addition to this total nitrogen content, the ratio of nitrate to ammonium (NH_4^+) is a very important aspect in nitrogen nutrition.[12] This is because the ratio strongly influences pH of the medium, which in turn determines the absorption of different other nutrients. Thus, as in most plant species, the relatively higher supply of nitrate-nitrogen within the MS medium could have exerted the profound effect on shoot growth of this plant species. In contrast to the report of Bajaj and his associates[7] that stated SH as the best basal medium for cardamom, MS medium produced the highest number of shoots in this experiment of krawan.

On top of the relative ratio of these two forms of inorganic nitrogen, the considerably reduced total nitrogen content in the three media types (½-MS, ½-nitrogen MS and SH) could have resulted in the production of fewer leaves in these three than in MS. On the other hand, the findings regarding root number and length were in accordance with that of Drew *et al*,[13] who observed fewer but longer roots in nitrate-deficient solutions during their work in barley.

Effects of Coconut Water

Addition of coconut water significantly affected *in vitro* growth and development of krawan explants, except shoot and root lengths. Basal medium supplemented with 5% coconut water was the best for krawan shoot growth and development, i.e. shoot number, as well as fresh and dry weights (Table 2). Contrast analysis showed the relationships between shoot and leaf numbers, as well as fresh and dry weights of krawan to be quadratic. However, root number was observed to have a linear relationship, while root and shoot lengths were not significantly affected by the use of CW (Table 2).

Several reports have also revealed the positive effects of CW supplement (5 - 20%) for the *in vitro* growth and development of different plant species in the family Zingiberaceae, including *E. cardamomum*,[6,14] large cardamom,[7] ginger[8] and turmeric[9] as well. Our result was in agreement with the reports of Bajaj *et al*[6] and Nadgauda *et al*[14] for cardamom, as well as Shirgurkar *et al*[9] for turmeric, who used 5% CW for shoot multiplication in their respective studies.

Table 2. Effects of coconut water (CW) on *in vitro* growth and development of krawan after eight weeks of culture.

% CW	Shoot no.	Leaf no.	Root no*	Root lg.* (cm)	Shoot lg. (cm)	FW (gm)	DW (gm)
0%	2.9	5.2	5.6	1.76	4.14	0.393	0.048
5%	5.3	10.3	4.6	2.91	6.12	0.825	0.098
10%	4.2	11.8	3.2	3.28	4.72	0.642	0.084
15%	3.7	8.3	3.9	2.05	4.04	0.689	0.083
20%	3.4	7.3	2.4	2.60	5.64	0.661	0.082
Model							
Prob.	0.0013	0.0058	0.0153	0.2718	0.1424	0.0071	0.0022
% CV	31.47	45.89	27.89	29.89	43.54	38.42	33.41
Linear	0.6871	0.5776	0.0014	0.7432	0.6733	0.1191	0.0541
Quadratic	0.0027	0.0008	0.8166	0.1231	0.9904	0.0244	0.0071
Cubic	0.0042	0.1299	0.1299	0.1904	0.0117	0.0342	0.0209

*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.

Effects of Plant Growth Regulators

Effects of imazalil (IMA) and thidiazuron (TDZ) on growth and development of krawan

The use of IMA and TDZ exerted a significant effect on shoot, leaf, and root numbers, as well as root and shoot lengths of krawan. Among these, however, only the numbers of shoots and leaves, and lengths of shoots were affected by the combined use of IMA and TDZ. The highest numbers of shoots and leaves were gained from the medium supplemented with 2 mg/l IMA and 0.5 mg/l TDZ, while the PGR-free medium gave the highest number of roots, as well as the longest shoots and roots. IMA had no independent effect on root number, while TDZ had a highly significant effect on all these five parameters. Inclusion of TDZ in the culture medium reduced root number and gave rise to comparatively shorter roots and shoots, as well. No statistical differences were observed between the effects of 0.1 and 0.5 mg/l TDZ on number and average length of krawan roots. However, the use of 0.5 mg/l TDZ resulted in the shortest shoots (Table 3).

Effects of IMA and BA on growth and development of krawan

In this part of the experiment involving IMA and BA, shoot number, as well as root and shoot lengths were statistically different. However, no interaction between the two chemicals was observed on the latter two, except for shoot number. The highest number of shoots was yielded from IMA and BA at 2.0 mg/l and 0.5 mg/l, respectively. Addition of IMA to the culture medium reduced shoot and root lengths of krawan. On the other hand, inclusion of BA to the medium increased shoot number but reduced their length (Table 4). This was in agreement with the reports of Werbrouck and Debergh.[15]

Comparison of different combinations of plant growth regulators on growth and development of krawan

In vitro growth and development of Krawan was highly affected by the type of growth regulator used for propagation. All the seven parameters studied showed highly significant differences with the type of the plant growth regulator used. The highest numbers of shoots (9.72) and leaves (19.72) were obtained from the culture medium supplemented with 2 mg/l IMA and 0.5 mg/l TDZ (IMATDZ). On the other hand, both root and shoot length revealed significant variation depending on the type of growth regulator used. The shortest roots and shoots were recorded from TDZ supplemented medium (IMATDZ). Higher fresh weight values were recorded from medium supplemented with 3 mg/l BA and 1 mg/l Kinetin (BAK), as well as with 2 mg/l IMA and 0.5 mg/l BA (IMABA). However, comparatively higher dry weights were obtained from those media supplemented with 2 mg/l IMA and 0.5 mg/l BA (IMABA), 2 mg/l IMA and 0.5 mg/l TDZ (IMATDZ) and the PGR-free control (PGRF) (Table 5).

Among the different phenyl urea derivatives so far studied for plant tissue culture use, TDZ is believed to be the most potent.[16] It induces organogenesis at much lower concentrations via a reduced dominance of the

Table 3. Effects of Imazalil (IMA) and Thidiazuron (TDZ) on *in vitro* growth and development of krawan after eight weeks of culture.

Treatment	Concentration (mg l^{-1})	Shoot no.	Leaf no.	Root no*	Root lg.* (cm)	Shoot lg. (cm)
IMA	0	4.38	10.94	3.54	5.78a	6.72
	2	6.91	15.16	3.21	3.97b	5.23
	4	6.67	14.02	3.22	4.03b	5.57
TDZ	0	3.39	9.28	5.26a	6.81a	7.82
	0.1	6.64	14.17	2.95b	4.03b	5.40
	0.5	7.91	16.65	1.80b	2.97b	4.32
IMA X TDZ	0 X 0	2.83	9.06	6.44	9.42	9.42
	0 X 0.1	5.32	10.42	2.47	4.24	5.54
	0 X 0.5	4.94	13.39	1.78	3.78	5.26
	2 X 0	4.05	10.26	4.53	4.74	7.02
	2 X 0.1	7.10	15.74	3.21	4.19	5.49
	2 X 0.5	9.72	19.72	1.83	2.92	3.07
	4 X 0	3.24	8.41	4.82	6.38	7.02
	4 X 0.1	7.45	16.25	3.15	3.67	5.18
	4 X 0.5	9.06	16.83	1.78	2.20	4.63
Prob.	IMA	0.0001	0.0001	0.7811	0.0145	0.0001
	TDZ	0.0001	0.0001	0.0001	0.0001	0.0001
	IMA X TDZ	0.0035	0.0202	0.3930	0.1306	0.0128
MODEL	Prob.	0.0001	0.0001	0.0001	0.0001	0.0001
	% CV	33.44	34.71	42.88	36.65	32.69

*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. Effects of Imazalil (IMA) and 6-benzyladenine (BA) on *in vitro* growth and development of krawan after eight weeks of culture.

Treatment	Concentration (mg/l)	Shoot no.	Root lg.* (cm)	Shoot lg. (cm)
IMA	0	3.42	6.11a	7.26a
	2	4.89	3.44b	6.04b
	4	4.46	3.20b	5.97b
BA	0	3.75	4.28	7.08a
	0.1	4.25	4.13	6.35b
	0.5	4.73	4.34	5.87b
IMA X BA	0 X 0	2.94	6.10	8.26
	0 X 0.1	3.53	5.84	7.04
	0 X 0.5	3.74	6.39	6.58
	2 X 0	4.11	3.62	7.00
	2 X 0.1	4.56	3.48	6.06
	2 X 0.5	6.00	3.22	5.07
	4 X 0	4.17	3.22	6.04
	4 X 0.1	4.68	3.05	5.94
	4 X 0.5	4.53	3.35	5.92
Prob.	**IMA**	0.0001	0.0001	0.0003
	BA	0.0001	0.9248	0.0029
	IMA X BA	0.0086	0.9885	0.2644
MODEL	**Prob.**	0.0001	0.0001	0.0001
	% CV	25.17	27.58	29.25

*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.

apical meristem. As reported by different workers,[16-18] the biological activity of TDZ was higher than or comparable to that of the most active adenine-type cytokinins at unusually low concentrations. Ponchia and Zanin[19] also reported BA to show an intermediate efficacy between TDZ and other synthetic cytokinins, even at increased concentrations. This could be ascribed to the requirements of higher levels of cytokinins to compensate the degradation effect of cytokinin oxidase, the enzyme that is known to reduce the effects of natural cytokinins to a large extent. Though much less active, adenine-type cytokinins, such as Kinetin and BA, are known to be less susceptible to this enzyme than the naturally occurring cytokinins such as zeatin. However, TDZ and some other phenyl urea derivatives are resistant to oxidases, stable, and biologically active at lower concentrations than the adenine-type cytokinins.[17]

The influence of IMA in enhancing the multiple shoot producing effect of cytokinins, including BA and TDZ has been reported by Werbrouck and Debergh.[15,20] Inducing changes upon the metabolism of exogenously applied cytokinin was suggested as a possible mechanism to explain the potentials of IMA in enhancing cytokinin actions.

In line with the effects of TDZ on the length of roots and shoots, different authors[16,21] have reported the suppression of shoot elongation due to the use of TDZ.

Table 5. Combined analysis of the effects of different plant growth regulators on the *in vitro* growth and development of krawan after eight weeks of culture.

Treatment*	Shoot no.	Leaf no.	Root no**	Root lg.** (cm)	Shoot lg. (cm)	FW (gm)	DW (gm)
PGRF	2.74c	9.53b	5.00a	8.36a	8.75a	0.797b	0.104a
IMATDZ	9.72a	19.72a	1.83b	2.92b	3.07c	0.828b	0.094a
IMABA	6.00b	11.89b	4.44a	3.22b	5.07b	1.134ab	0.119a
BAK	5.53b	10.21b	3.00ab	2.90b	5.25b	1.346a	0.061b
Prob.	0.0001	0.0001	0.0005	0.0001	0.0001	0.0077	0.0059
% CV	27.56	35.52	33.42	25.27	24.61	53.25	52.98

*PGRF = Plant growth regulator free medium; IMATDZ = Imazalil (2 mg/l) and Thidiazuron (0.5 mg/l); IMABA = Imazalil (2 mg/l) and BA (0.5 mg/l), BAK = BA (3 mg/l) and Kinetin (1 mg/l),
** 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, DW =Dry weight.

From their work on lentil, Fratini and Ruiz[22] have reported the average length of regenerated shoots and rooting to be inversely proportional to the number of shoots formed with TDZ and BA, though to a different degree. However, the observed shoot stunting effect of TDZ was not a permanent phenomenon and could be overcome by transferring the explants to BA containing or PGR-free media.[18,21] This was also the case in the present experiment. This phenomenon could be attributed to the effect of TDZ in inducing accumulation of endogenous cytokinin.[16,23] It could also be due to the comparatively higher cytokinin activity of TDZ even at a lower concentration.[23] Sahoo and Chand[24] associated this shoot stunting effect of TDZ to the presence a phenyl group in TDZ.

In another instance, Inoue et al[25] tried to relate the reduction of root length in plants cultured on TDZ supplemented medium to increased sensitivity of plant species to elevated cytokinin activities. Similar to our findings, Werbrouck and Debergh[15] also reported reduced root length and number from IMA supplemented media.

Rooting, Acclimatization and Plant Recovery

Roots were sporadically formed on the BAK medium. For profuse formation of roots, plantlets were transferred to PGR-free MS medium, where they produced a large number of healthy and fine roots. In the present study, rooted plantlets with well developed root systems were washed with tap water to remove the media and transplanted to pots containing peat moss and river sand at a 1:1 proportion. The pots were covered with plastic bags to retain high humidity. After seven days under such condition the plantlets produced new leaves and some new shoots, at which time the bags were left open for three days, so that they can adapt to a less humid condition. Then, the pots were transferred to a screen house nursery. Using this technique plants were observed to acclimatize well with more than 90 % survival (data not presented).

Acknowledgements

The authors would like to thank Ass. Professor Yingyong Paisooksantivatana, the first author's major subject committee, for his consistent encouragement, supply of different literature and provision of the krawan planting materials. We would like to acknowledge the Agricultural Research and Training Project (ARTP) of the Ethiopian Agricultural Research Organization (EARO) for the financial support it provided for undertaking this research.

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