An efficient micropropagation protocol for an economically valuable medicinal plant *Kaempferia parviflora* Wall. Ex Baker

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Abstract

Kampferia parviflora is an important medicinal plant that is used in many popular systems of traditional medicine. Fresh plant material for medicinal uses and research purposes is critically unavailable around the year due to its long dormancy period. This study aimed to establish an aseptic culture of K. parviflora using silver nanoparticles. Explants from rhizome buds were cultured on Murashige and Skoog (MS) medium supplemented with different concentrations of individual plant growth hormone BAP, Kinetin, ADS and 2ip. Highest number of shoots was 6.33, highest multiplication rate was 3.33, highest number of leaves was 3.33 recorded at 6 mgl^{-1} BAP and highest length of shoots 2.7 were recorded on 8 mgl^{-1} BAP. When explants were inoculated on MS medium containing different concentrations and combinations of BAP with NAA, IAA, Kinetin and ADS, highest number of shoots was 5.67.

Thus, individual effects of BAP improved significantly the shoot growth and proliferation. MS medium supplemented with half strength MS media with 2 mgl^{-1} NAA and gave the highest number of roots (7.56). However, longest roots per explant were obtained with MS + 0.5 mgl^{-1} IAA alone. Plant tissue culture techniques have been applied to produce disease-free planting materials of black ginger to overcome these problems. Hence, the in vitro induced microrhizomes are considered as alternative disease-free planting materials for black ginger cultivation. Therefore, this study was conducted to optimize sucrose and plant growth regulators (PGRs) for its microrhizome induction. Microrhizomes were successfully induced in Murashige and Skoog (MS) medium supplemented with a high sucrose concentration 75 gml^{-1}sucrose). NAA at 1.4 mgl^{-1} was found more effective for microrhizome diameter, fresh and dry weight. The proliferated shoots were green and healthy in appearance. Finally, healthy and complete plants with well-developed roots were hardened, acclimatized and planted in the field successfully with a survival rate of 100%.

Keywords: *Kaempferia parviflora*, shoot multiplication, *in vitro*, acclimatization, rhizome.

Introduction

Kaempferia parviflora, also known as the Thai black ginger, Thai ginseng or krachaidum, is an herbaceous plant. *K. parviflora* is a perennial herb with short fleshy rhizomes, tuberous roots, erect leaves are 8-16 cm long, thin, rounded at the base, plain green with red margins. The leaf-stalk is short and channelled having white inconspicuous flowers with a purple labellum and few flowers are in a stalkless central cluster. The bracts are 2.5 cm, lance shaped and green. The calyx is longer than the bract. The flower-tube is 3 cm long, segments are greenish, 1 cm long, upper ascending and rather concave. The lip is 0.75 - 1.0 cm, somewhat notched with the anther-crest blunt and broad as the long. *K. parviflora* can reach up to 20 cm tall. The rhizomes are small and of deep purple colour, thus inspiring the name black ginger.

The rhizomes have been traditionally used in Thai folklore medicine for the treatment of leucorrhea[11], oral diseases[32], stomachache, flatulence, digestive disorders and gastric ulcers[38]. *K. parviflora* has anti-allergic properties[34-36], anti-peptic ulcer effects[28], anti-inflammatory[37], antimutagenic[5], anti-viral protease effects[30], antidepressive[39] and anticancer properties[6,20,35]. Black ginger is a herb that has some historical and medicinal usage for treating metabolic ailments improving vitality. *Kaempferia parviflora* has health promoting benefits and potential therapeutic functions. Despite the high demand for the rhizomes of *K. parviflora*, there is a scarcity of planting materials. This is due to sluggish natural regeneration of *K. parviflora* through rhizome and a long dormancy period. Under natural conditions, *K. parviflora* plants undergo a dormancy phase for five to six months from November to early May during a dry season.

The dormancy period affects cropping cycles, year round cultivation and is a major impediment in the commercial cultivation of this plant. Investigation into dormancy requirements is necessary to develop appropriate cultural practices for commercialization of *K. parviflora*.

Hence, microrhizomes have been considered as alternative disease-free planting materials. Microrhizomes produced under *in vitro* conditions are modified stems or storage organs of the rhizomatous plant species. They are the same as the mature rhizomes of these plant species and they can sprout and develop into a whole plant. Microrhizomes as planting materials minimize the cost and time of

acclimatization and they can be planted directly in the field without the process of acclimatization[13,33].

Microrhizomes can be easily stored and they are less vulnerable during transportation. Thus they are more suitable for international shipping and germplasm exchange[21]. Microrhizomes could also be used as a source of secondary metabolites[19].

Hence, microrhizome production is one of the most useful techniques for rhizomatous plant micropropagation. Tissue culture is one of the techniques in biotechnology, which has brought about significant impact in the field of plant breeding and conservation of many endangered plants.

Material and Methods

Plant materials and surface sterilization: Rhizomes of black ginger were procured from College of Horticulture and Forestry (CHF), Central Agricultural University, Pasighat, Arunachal Pradesh. Rhizomes were washed thoroughly under running tap water followed by tween-20 for 15 min. The rhizome buds were surface sterilised with silver nanoparticles for one hour and then finally rinsed three-times with sterile distilled water. The sterilised terminal buds were inoculated on Murashige and Skoog (MS) medium[23].

Culture condition and shoot initiation: Initially, rhizome explants were inoculated into different basal media with 3% sucrose and 0.5% CleriGar (Himedia, India) to investigate the effect of culture medium strength on shoot induction. In every experiment, pH of the medium was adjusted to 5.7 with the pH meter using different concentrations of the HCl or NaOH (0.1N, 1N, 10N). Culture media were sterilized by autoclaving at 121˚C at 15 lbs psi pressure for 15 minutes. All the cultures were maintained in an air-conditioned culture room at a temperature of 25 ± 1˚C with a relative humidity of 50 to 60%. The source of illumination consisted of 2.5 feet wide fluorescent tubes (40 watts), provided two in number for each rack. The intensity of the illumination was 2500 lux at the level of cultures.

The cultures were subjected to a 16-hour light regime followed by 8 hours of the dark period. Shoot regeneration rate and shoot morphology from different explants in different media were analyzed after 3 weeks of inoculation. Each treatment was replicated three times with 15 explants per treatment.

Shoot multiplication: The auxiliary buds produced from rhizome segments were cultured for 3 weeks on MS medium supplemented with individual plant growth hormone of 6-benzylaminopurine (BAP-2, 4, 6, 8, 10, 12 mgl^{-1}), kinetin (1, 3 mgl^{-1}), ADS and 2ip (1, 3, 5 mgl^{-1}) for shoot multiplication. In other experiment, *in vitro* response of the explants of *Kaempferia parviflora* to various combinations of BAP with kinetin, ADS and 2ip was assessed by recording the various morphological parameters *viz.* length of shoot, number of leaves, number of shoots and multiplication rate for three multiplication cycles achieved by repeated subculturing of shoot clusters on fresh culture medium with selected medium composition. All cultures were incubated under the laboratory conditions.

***In vitro* rooting and acclimatization:** Regenerated plants were transferred to full and half basal MS medium with 0.5, 1 and 2 mgl^{-1} of IAA, IBA and NAA for root induction. For acclimatization, *in vitro* raised plantlets were gently taken out from the culture bottles to avoid any damage to the root system. The roots of regenerated plantlets were then washed in distilled water for 5 minutes to remove media. Further, these plantlets were dipped in 0.2 % carbendazim solution for 10 minutes to avoid any fungal infection. Different types of potting substrates such as soil, cocopeat, sand, pindstrup® (readily available hardening substrate), vermiculate and perlite were used in acclimatization of regenerated plants.

Effect of Different Concentrations of Sucrose and NAA on Microrhizome Induction: In this experiment, aseptic *in vitro* grown shoots approximately 4-5 cm long, which were derived from the established culture of *Kaempferia parviflora*, were used as explants for induction of microrhizomes. Media with different level of sucrose and NAA were experimented (Table 10). Data on the number of rhizomes per explants, size of rhizome (width mm), rhizome fresh weight and dry weight were recorded after 90 days in culture.

Statistical analysis: Each treatment contained three replicates and the observations was recorded at the end of 3 weeks in all the experiments. All experiments were conducted in a completely randomized design[25]. The data were analyzed by Analysis of Variance (ANOVA) at the 5% significant level.

Results and Discussion

Initiation stage: During culture initiation, different basal media *viz*. MS[23], WPM (Woody Plant Media), Nitsch, B5 (Gamborg B5 medium), N6 basal medium were screened. However, MS basal medium was used throughout the study as it was found more responsive for various morphogenetic events based on this experiment (Table 1).

For initiation used different basal media and gelling agents, explants initiated on MS basal medium supplemented with gelrite provided good results after 3 weeks of initiation with higher number of sprouts of 3.67 along with 90% sprouting percent. Similar to our results, there are some reports that revealed that MS media were best for shoot induction.

Qahtan et al[26] reported that *Ruta chalepensis* MS media gave the largest number of 40.3 shoots per explant and the longest average shoot length of 4.8 cm. Jain et al[16] studied the effect of medium in *Harpagophytum procumbens*. Result shows that MS media produced highest number of shoots with WPM having significantly fewer shoots.

Table 1
Effect of different media combinations on sprouting response of Black ginger

Media composition*		Sprouting Percent	Days to sprout induction	No. of Sprouts	No. of leaves	Length of sprouts (cm)
Basal Media	BAP (mgl^{-1})					
WPM	2.0	40	9.67^{def}	1.00^{c}	1.00^{c}	0.47^{c}
Nitsch	2.0	30	8.67^{fg}	1.00^{c}	1.00^{c}	0.27^{d}
B5	2.0	40	10.33^{cd}	1.00^{c}	1.00^{c}	0.30^{d}
N6	2.0	50	9.00^{efg}	2.00^{b}	1.67^{c}	0.77^{b}
MS	2.0	90	8.33^{g}	3.67^{a}	3.00^{a}	1.23^{a}
WPM	-	60	11.67^{b}	1.00^{c}	1.00^{c}	0.50^{c}
Nitsch	-	40	13.67^{a}	1.00^{C}	1.00^{c}	0.43^{c}
B5	-	50	10.00^{cde}	1.00^{c}	1.00^{c}	0.23^{d}
N6	-	60	11.00^{dc}	1.00^{c}	1.00^{c}	0.23^{d}
MS	-	80	9.67^{df}	1.00^{c}	1.00^{c}	0.73^{b}
S.Em. ±			0.33	0.11	0.10	0.03
CD at 5%			0.98	0.31	0.31	0.08
CV (%)			5.66	12.45	14.414	9.52

Table represents pooled means from 5 replicates in each three repetitions; means within a column, followed by the same letters within a column, were not significantly different $p < 0.05$ by DMRT

Table 2
Effect of various concentrations of BAP, Kinetin, ADS, 2ip on multiple shoot induction after first multiplication cycle

Media composition*				Number of shoots	Number of leaves	Shoot Length (cm)	Multi plication Rate
BAP	Kinetin	ADS	2ip				
(mgl^{-1})							
-	-	-	-	1.17^{k}	1.50^{f}	2.23^{b}	0.83^{e}
2.0	-	-	-	3.50^{b}	1.67^{ef}	2.10^{b}	2.23^{bc}
4.0	-	-	-	2.5^{efg}	2.67^{b}	2.37^{ab}	1.33^{d}
6.0	-	-	-	6.33^{a}	3.33^{a}	2.40^{ab}	3.3a
8.0	-	-	-	3.17^{bcd}	2.50^{bc}	2.70^{a}	2.23^{bc}
10	-	-	-	3.33^{bc}	2.00^{de}	2.10^{b}	2.10^{bc}
12	-	-	-	2.83^{cde}	1.67^{ef}	2.30^{ab}	2.43^{b}
-	1.0	-	-	1.50^{jk}	1.50^{f}	2.27^{b}	1.00^{e}
-	3.0	-	-	2.33^{efgh}	2.17^{cd}	2.47^{ab}	2.33^{bc}
-	-	1.0	-	2.67^{de}	2.33^{bcd}	2.17^{b}	2.23^{bc}
-	-	3.0	-	1.67^{ij}	1.00^{g}	1.60^{c}	1.57^{d}
-	-	5.0	-	2.00^{hi}	2.00^{de}	1.57^{c}	1.4^{6d}
-	-	-	1.0	2.50^{efg}	2.33^{bcd}	2.23^{b}	2.13^{bc}
-	-	-	3.0	2.10^{fghi}	1.50^{f}	2.50^{ab}	2.03^{c}
-	-	-	5.0	2.8^{de}	2.50^{bc}	1.70^{c}	1.57^{d}
S.Em. ±				0.15	0.13	0.13	0.15
CD at 5%				0.45	0.37	0.37	0.44
CV (%)				9.71	11.24	9.96	9.72

Table represents pooled means from 5 replicates in each three repetitions; means within a column, followed by the same letters within a column, were not significantly different $p < 0.05$ by DMRT

Effect of individual plant growth hormone BAP, Kinetin, ADS and 2ip on shoot multiplication in MS media: *In vitro* response of the explants of *Kaempferia parviflora* to various concentration of BAP Kinetin, ADS and 2ip was assessed by recording the various morphological parameters *viz.* length of shoot, number of leaves, number of shoots and multiplication rate. Analysis of Variance (ANOVA) showed significant differences between the treatments for different characters. BAP stimulates the formation of adventitious shoots and promotes organogenesis especially in dormant rhizomes. Medium supplemented with 6 mgl^{-1} BAP reported highest number of shoots (6.33), number of leaves (3.33 cm) and multiplication rate (3.33). Medium supplemented with 8 mgl^{-1} BAP produced highest shoot length (2.70). The detailed results are presented in table 2 and fig. 1 B.

This type of production of shoots was reported earlier for *K. parviflora*[19] who used different concentrations of BAP (0, 8.88, 17.76, 26.64, 35.52 and 44.40 µM) for shoot multiplication in *Kaempferia parviflora*. Treatment with 35.52 µM N6-benzyladenine (BA) produced highest number of shoots (22.4 ± 1.84) and leaves (29.27 ± 1.30) per explant. The positive effect of MS medium supplemented with 4.5 mgl^{-1} BAP recorded the highest percentage of shootlets multiplication[1]. Zingiberaceae species[9] reported maximum numbers of plantlets/explant (4.33) observed on the media supplemented with 4 mgl^{-1} BAP. Panda et al[24] reported that the use of BAP alone with *Curcuma amada* gave the maximum number of shoots per explant.

According to the results, when BAP was used alone, the maximum number of shoots, no. of leaves and highest shoot length obtained were compared to other treatments. With an increase in the concentration of BAP, the shoot numbers as well as shoot length reduced (Table 2). Similarly, the role of BAP in shoots proliferation has been reported in other Zingiberaceae species[7] using different concentration of cytokinins including 2.2-22.2 µM benzyladenine (BA), 2.3-23.2 µM kinetin (Kin), or 2.4-24.6 µM 2-isopentenyladenine (2iP) for multiplication of *Eclipta alba*.

Buah et al[10] and Sparzak-Stefanowska et al[31] studied the effect of different concentrations of cytokinins, benzylaminopurine (BAP), kinetin and 2ip at two different concentrations (4.5 and 5.0 mgl^{-1}). Media supplemented with BAP had the highest shoot induction response, followed by kinetin and 2ip. In contrast, Mohamed et al[22] reported that MS medium fortified with 2.0 mgl^{-1} 6-benzylaminopurine (BAP), produced highest percentage of shoot response (80 ± 1.90), the maximum number (7.8 ± 1.40) of shoots per explant, maximum shoot length of (2.8 ± 1.01cm). Khairudin et al[18] observed that the treatment of 1.5 mgl^{-1} BAP produced the highest number of shoots and highest shoot length (4.3 cm).

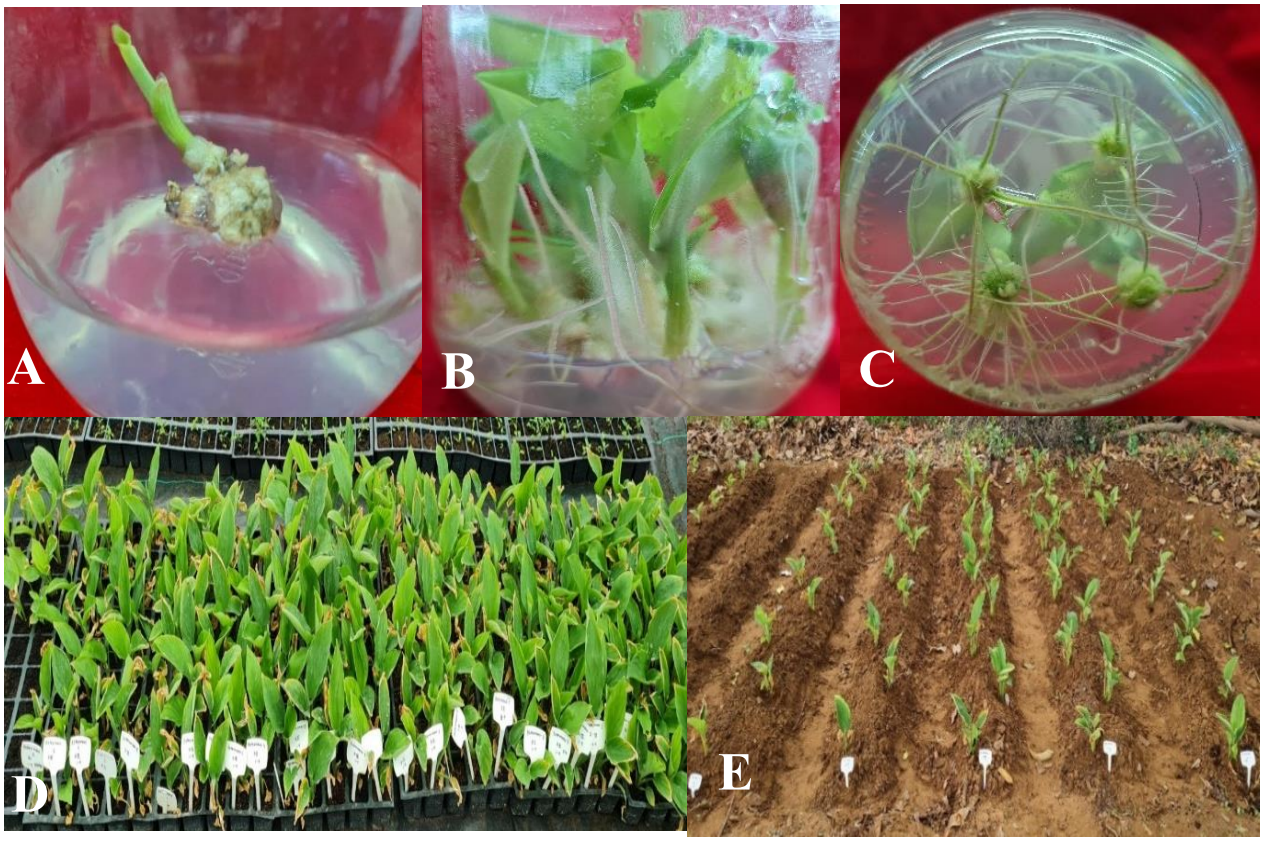

Figure 1: Different stages of micropropagation in black ginger. (A) Rhizome sprouted bud used as explants for initiation (B) Multiple shoots produced on MS medium containing 6.0 mgl^{-1} BAP (C) *In vitro* rooted plantlets derived from ½ MS medium containing 2.0 mgl^{-1} NAA (D) Acclimatization of rooted plantlets in green house (E) Successfully hardened plants in field

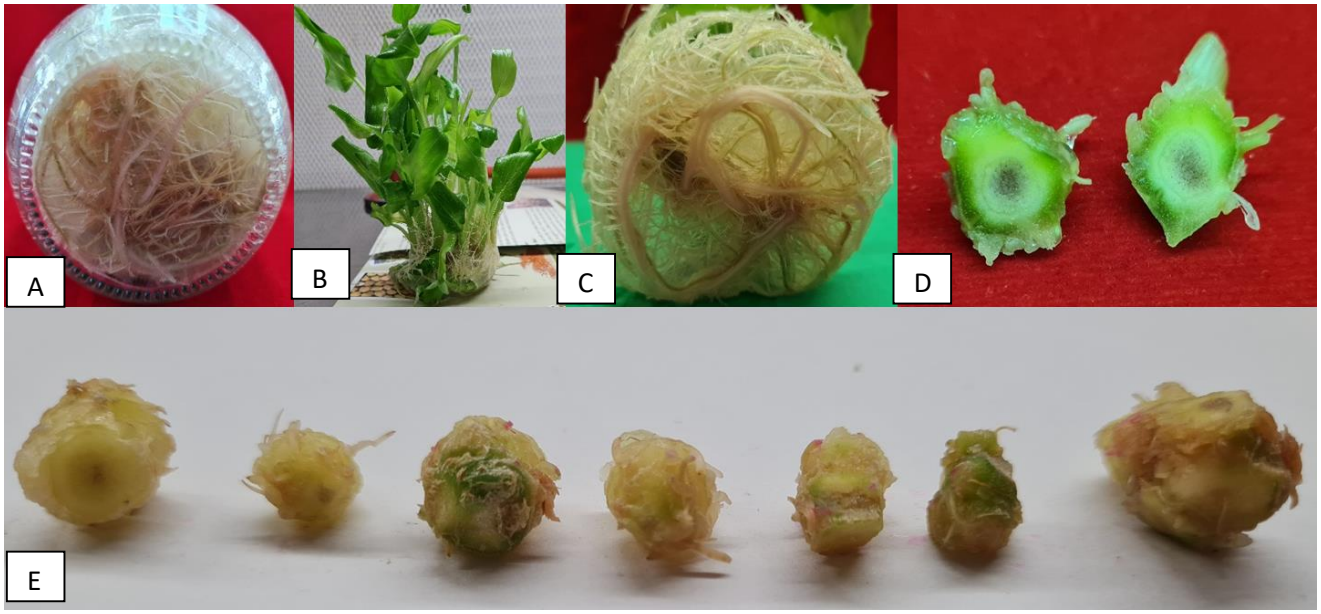

Figure 2: Morphological characters of *Kaempferia parviflora* inductive microrhizomes plantlets growing on MS medium fortified with 75g/l sucrose after 10 weeks of cultivation under light and darkness conditions at 25±1°C. (A) to (C) represent shoots showing swollen bases on microrhizome induction medium, (D) Cut section of the microrhizome (E) Microrhizomes of different sizes harvested after 10 weeks of incubation under light condition at 25±1°C

Table 3
Effect of various concentrations of BAP, Kinetin, ADS, 2ip on multiple shoot induction after second (42 days) multiplication cycle

Media composition*				Number of shoots	Number of leaves	Shoot Length (cm)	Multi plication Rate
BAP	Kinetin	ADS	2ip				
(mgl^{-1})							
-	-	-	-	1.33^{g}	1.67^{ef}	2.2^{abcd}	1^{f}
2.0	-	-	-	3.33^{d}	2^{de}	2.1^{bcd}	2.43^{b}
4.0	-	-	-	2.67^{e}	2.5^{bc}	2.27^{abc}	1.67^{e}
6.0	-	-	-	5.83^{a}	3.17^{a}	2.53^{ab}	3.67^{a}
8.0	-	-	-	3.33^{d}	2.33^{bcd}	2.6^{a}	2.17^{bcd}
10	-	-	-	3.83^{c}	2.17^{cd}	2.33^{abc}	2.17^{bcd}
12	-	-	-	5^{b}	1.17^{gh}	2.4^{abc}	2.43^{bc}
-	1.0	-	-	1.67^{g}	1.5^{fg}	2.47^{ab}	1.17^{f}
-	3.0	-	-	2.67^{e}	2^{de}	2.57^{a}	2.4^{bcd}
-	-	1.0	-	2.17^{f}	1^{h}	2.2^{abcd}	2.13^{bcd}
-	-	3.0	-	1.17^{g}	1.17^{gh}	2^{cde}	1.63^{e}
-	-	5.0	-	2.17^{f}	2.5^{bc}	1.8^{de}	1.5^{e}
-	-	-	1.0	2.17^{f}	2.33^{bcd}	2.47^{ab}	2.17^{bcd}
-	-	-	3.0	1.67^{g}	1.67^{ef}	2.1^{bcd}	$2.1^{b\,d}$
-	-	-	5.0	2.5^{ef}	2.67^{b}	1.63^{e}	1.57^{e}
			S.Em. ±	0.15	0.13	0.13	0.10
			CD at 5%	0.45	0.37	0.37	0.29
			CV (%)	9.71	11.24	9.96	8.67

Table represents pooled means from 5 replicates in each three repetitions; means within a column, followed by the same letters within a column, were not significantly different $p < 0.05$ by DMRT

Effect of Different Combinations of BAP with other PGRs NAA, Kinetin, ADS, 2iP and IAA on Shoot Multiplication: Different concentrations of BAP with Kinetin, ADS and 2iP were supplemented on MS media for multiplication. *In vitro* response of the explants of *Kaempferia parviflora* to various combination of BAP with Kinetin, ADS and 2iP was assessed by recording the various morphological parameters *viz.* length of shoot, number of leaves, number of shoots and multiplication rate. The detailed results for shoot multiplication are presented in table 3. Data in table 3 showed significant differences concerning the number of leaves/shoot at the level of 1% among the

applied different treatments. Moreover, the data revealed that there is a positive relationship between BAP combined with NAA and kin on number of leaves/shoot. The maximum number of leaves (4.67) was recorded with 2.0 mgl^{-1} BAP + 0.2 mgl^{-1} NAA followed by 10 mgl^{-1} BAP + 2.5 mgl^{-1} Kin.

Similarly, Sathyagowri and Seran[29] observed the multiple shoot production from explants of ginger with a mean number of 5.25 shoots per explant on MS medium fortified with 5.0 mgl^{-1} BAP and 0.5 mgl^{-1} NAA. The highest growth of shoots per explants 4.5 with a higher shoot length of 5.2 cm was obtained in the medium MS + BAP (1.5 mgl^{-1}) + NAA (0.5 mgl^{-1}) by Kambaska et al[17]. Mohamed et al[22] cultured shoots of *Ammannia baccifera* on MS medium fortified with various concentration of BAP (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mgl^{-1}) in combination with NAA (0.2, 0.3, 0.4, 0.5, 0.6 and 0.7 mgl^{-1}) for multiple shoot development. The highest number of shoots multiplication response (85 ± 1.68), the maximum number shoot per explants (28.6 ± 1.63) and shoot length (3.0 ± 1.12 cm) were documented on MS medium supplemented with 2.0 mgl^{-1} BAP and 0.5 mgl^{-1} NAA.

Bharalee et al[8] obtained best response for shoot multiplication on MS basal medium supplemented with 4 mgl^{-1} BAP and 1.5 mgl^{-1} NAA for *C. caesia* (3.5 shoots per explant) and 1 mgl^{-1} BAP + 0.5 mgl^{-1} NAA for *C. zedoaria* (4.5 shoots per explant). Yusuf et al[40] reported the 2.0 mgl^{-1} BAP and 0.5 mgl^{-1} NAA gave the best result. Similar results were also reported by Ayenew et al[4]. The highest shoot multiplication of ginger was obtained on 2 mgl^{-1} BA and 1 mgl^{-1} kinetin. Bhattacharya and Sen[9] reported during their *in vitro* studies on *Kaempferia galanga* L. for shoot multiplication with combination of BAP and Kinetin. They found that the maximum numbers of plantlets were obtained in the media supplemented with 3 mgl^{-1} BAP + 4 mgl^{-1} kinetin. Ali et al[2] developed multiplication system for banana through meristem culture. MS medium containing 1.0 mgl^{-1} BAP + 0.25 mgl^{-1} kinetin provided the best multiplication response which was 8 shoot per culture vial within 21.6 days after inoculation into shoot multiplication medium.

Effect of plant growth hormone IBA, IAA and NAA on root induction in full strength and half strength of MS media: All the treatments showed 100 % root induction. Treatment comprising of MS medium supplemented with 1.0 mgl^{-1} IAA + 0.5 mgl^{-1} NAA produced highest number of roots (7.17) and root length (8.83cm) observed on 0.5 mgl^{-1} IAA and 1.0 mgl^{-1} NAA (Table 4 and fig. 1C). The second best response was seen in case of ½ MS media with 2.0 mgl^{-1} NAA which produced 7.56 roots.

Table 4
Effect of various concentrations of BAP, Kinetin, ADS, 2ip on multiple shoot induction after third (63 days) multiplication cycle

Media composition*				Number of shoots	Number of leaves	Shoot Length (cm)	Multi plication Rate
BAP	Kinetin	ADS	2ip				
(mgl^{-1})							
-	-	-	-	1.33^{h}	1.67^{d}	2.2^{cd}	1.17^{g}
2.0	-	-	-	$3.17^{b\ d}$	1.83^{cd}	2.53^{bc}	2.57^{b}
4.0	-	-	-	2.33^{ef}	2.17^{bc}	1.93^{d}	1.83^{ef}
6.0	-	-	-	6.17^{a}	3.07^{a}	2.9^{a}	3.83^{a}
8.0	-	-	-	3.67^{b}	2.17^{bc}	2.6^{ab}	2^{de}
10	-	-	-	3^{d}	1^{e}	2.33^{bc}	2.3^{bcd}
12	-	-	-	2.17^{efg}	1.17^{e}	2.4^{bc}	2.27^{bcd}
-	1.0	-	-	2^{efg}	1.67^{d}	2.5^{bc}	1.2^{g}
-	3.0	-	-	2.5^{e}	2.33^{b}	2.53^{bc}	2.33^{bc}
-	-	1.0	-	3.67^{bc}	2.83^{a}	2.2^{cd}	2.17^{cd}
-	-	3.0	-	1.67^{gh}	2^{bcd}	1.9^{d}	1.57^{f}
-	-	5.0	-	2.17^{efg}	2^{bcd}	2.2^{cd}	1.6^{f}
-	-	-	1.0	2.33^{ef}	2.17^{bc}	2.47^{bc}	2.13^{cde}
-	-	-	3.0	1.83^{fgh}	1.67^{d}	2.2^{cd}	2.03^{cde}
-	-	-	5.0	$3.17^{b\ d}$	1.83^{cd}	1.9^{d}	1.57^{f}
S.Em. ±				0.17	0.15	0.12	0.10
CD at 5%				0.48	0.43	0.36	0.29
CV (%)				13.01	13.06	9.63	8.61

Table represents pooled means from 5 replicates in each three repetitions; means within a column, followed by the same letters within a column, were not significantly different $p < 0.05$ by DMRT

Table 5
Effect of different combinations of BAP with NAA, Kinetin, ADS and IAA on multiple shoot induction after first multiplication cycle

Media composition*					Number of shoots	Number of leaves	Length of shoots (cm)	Multi plication Rate
BAP	NAA	IAA	Kn	ADS				
	(mgl^{-1})							
-	-	-	-	-	1.67^{f}	1.83^{f}	2.27^{ab}	0.67^{e}
2.0	0.2	-	-	-	4.33^{b}	4.67^{a}	1.67^{d}	2.17^{bc}
6.0	0.6	-	-	-	5.67^{a}	3.33^{cd}	1.80^{cd}	2.17^{bc}
10	1	-	-	-	4.00^{bc}	3.17^{d}	2.00^{bc}	2.50^{ab}
2.0	-	2.0	-	-	2.17^{e}	2.17^{ef}	1.63^{d}	2.30^{abc}
6.0	-	6.0	-	-	3.50^{cd}	3.50^{cd}	1.33^{e}	2.00^{c}
10	-	10	-	-	3.33^{d}	2.5^{e}	2.17^{ab}	2.00^{c}
2.0	-	-	0.5	-	4.10^{b}	4.33^{ab}	2.17^{ab}	2.30^{abc}
6.0	-	-	1.5	-	4.10^{b}	3.83^{bc}	2.03^{bc}	2.17^{bc}
10	-	-	2.5	-	5.67^{a}	4.17^{ab}	2.47^{a}	2.67^{a}
2.0	-	-	-	0.2	3.50^{cd}	3.50^{cd}	2.30^{ab}	1.33^{d}
4.0	-	-	-	0.4	3.83^{bcd}	3.83^{bc}	2.20^{ab}	2.07^{bc}
S.Em. ±					0.17	0.17	0.09	0.13
CD at 5%					0.49	0.49	0.26	0.39
CV (%)					7.53	8.48	7.85	11.51

Table represents pooled means from 5 replicates in each three repetitions; means within a column, followed by the same letters within a column, were not significantly different $p < 0.05$ by DMRT

Table 6
Effect of different combinations of BAP with NAA, Kinetin, ADS and IAA on multiple shoot induction after second multiplication cycle

Media composition*					Number of shoots	Number of leaves	Length of shoots (cm)	Multi plication Rate
BAP	NAA	IAA	Kn	ADS				
	(mgl^{-1})							
-	-	-	-	-	1.33^{f}	1.67^{f}	2de	0.5^{e}
2.0	0.2	-	-	-	4.17^{b}	2.67^{cde}	1.83^{ef}	2.2^{bc}
6.0	0.6	-	-	-	5.83^{a}	4.33^{a}	1.6^{fg}	2^{c}
10	1	-	-	-	3.83^{bc}	3^{bc}	2.03^{cde}	2.27^{bc}
2.0	-	2.0	-	-	2.33^{e}	2.17$^{d\,f}$	1.5^{g}	2.3^{bc}
6.0	-	6.0	-	-	3.33^{d}	3.17^{bc}	1.47^{g}	2.07^{c}
10	-	10	-	-	3.5^{cd}	2.67^{cd}	2.1^{cd}	2.17^{bc}
2.0	-	-	0.5	-	4.23^{b}	4^{a}	2.4^{b}	2.47^{b}
6.0	-	-	1.5	-	4.13^{b}	3.33^{b}	2de	2.17^{bc}
10	-	-	2.5	-	4^{b}	4.07^{a}	2.8^{a}	2.83^{a}
2.0	-	-	-	0.2	3.5^{cd}	3.4^{b}	2.27^{bc}	1.17^{d}
4.0	-	-	-	0.4	3.83^{bc}	4.17^{a}	2.37^{b}	2^{c}
S.Em. ±					0.14	0.16	0.09	0.12
CD at 5%					0.41	0.48	0.27	0.35
CV (%)					6.58	8.79	7.87	10.48

Table represents pooled means from 5 replicates in each three repetitions; means within a column, followed by the same letters within a column, were not significantly different $p < 0.05$ by DMRT

Similarly, Rahman et al[27] reported that *in vitro* proliferated shoots of *K. galanga* produced 100% roots in on modified medium (half strength of both major and minor salts) supplemented with 0.2 mgl^{-1} IBA. Das et al[12] studied rooting response of *Z. zerumbet* and *Z. moran* with half strength of both MS and MSR media along with three different auxins. Highest rooting percentage, number of roots and root length in *Z. moran* and *Z. zerumbet* were found at 0.5 mgl^{-1} NAA respectively. Kambaska et al[17] found best rooting percentage (95 %) when the shoots were cultured on half strength MS + 2.0 mgl^{-1} NAA with an average number of 8.5 ± 0.33 roots per plantlet and an average root length 3.5 ± 0.38 cm.

***In vitro* Microrhizome induction:** Microrhizome formation started after 7–8 weeks of culture by initial swelling at the base of the shoot. Both sucrose and NAA significantly increased ($p < 0.05$) the number, biomass and diameter of microrhizomes compared to the control treatment (Table 6). MS medium which was supplemented with 75 gml^{-1} sucrose and 1.4 mgl^{-1}NAA, resulted in the highest number of microrhizomes per explant (2.07) with the maximum biomass (0.61 g) and diameter (3.11 mm) of microrhizome. Further increase of sucrose more than 75 gml^{-1} caused a significant reduction in the number, biomass and diameter of microrhizomes.

Similar to present investigation, Zahid et al[41] developed microrhizome of ginger. MS medium supplemented with 60 gl^{-1} sucrose, 10 µM zeatin and 7.5 µM NAA was the optimum combination for the microrhizome induction of Bentong ginger. Anisuzzaman et al[3] produced *in vitro* microrhizome in *Curcuma zedoaria*. Maximum percentage (70%) of shoot induced microrhizome when it was cultured on 60 gl^{-1} sucrose containing medium.

Hardening and *ex vitro* acclimatization: Among the five different hardening mixtures tested for acclimatization of plantlets produced *in vitro*, cocopeat + pindstrup® (3:1) proved to be the best growing medium. The plants grown in cocopeat + pindstrup® (3:1) produced highest no. of leaves (3.8), length of shoots (11.7 cm), length of roots (11.1 cm) and no. of roots (18.6). It may be attributed to high level of nutrient content and good water holding capacity of cocopeat + pindstrub. Secondary hardened plants were shifted to field plantation after 45 days of hardening including primary and secondary hardening. The field survival of plants was found to 100 % with all the favorable growth characters (fig. 1 D, E).

Conclusion

The present study on *in vitro* shoot multiplication of *Kaempferia parviflora* demonstrates a successful step towards the *in vitro* propagation of the species. Rhizome explants inoculated with MS solid medium with 3% sucrose and 0.5 % clerigar fortified 2.0 mgl^{-1} BA were found be the best protocol for shoot bud induction. Multiple shoot production was found on 6 mgl^{-1}BAP. After different experiments, better rooting was observed on half strength MS medium supplemented with 2.0 mgl^{-1} NAA. Our developed protocol can produce a higher amount of large microrhizomes under *in vitro* conditions.

Table 7
Effect of different combinations of BAP with NAA, Kinetin, ADS and IAA on multiple shoot induction after third multiplication cycle

Media composition*					Number of shoots	Number of leaves	Length of shoots (cm)	Multi plication Rate
BAP	NAA	IAA	Kn	ADS				
	(mgl^{-1})							
-	-	-	-	-	1.57^{g}	1.5^{f}	2.57^{bc}	1^{c}
2.0	0.2	-	-	-	4.4^{c}	3.17^{c}	1.6^{e}	2.23^{b}
6.0	0.6	-	-	-	5.83^{a}	4.33^{a}	2^{d}	2^{b}
10	1	-	-	-	4.03^{cd}	3.23^{c}	1.63^{e}	2.33^{b}
2.0	-	2.0	-	-	2.43^{f}	2.23^{e}	2.03^{d}	2.37^{b}
6.0	-	6.0	-	-	3.47^{e}	2.67^{d}	1.3^{f}	2.17^{b}
10	-	10	-	-	3.27^{e}	2.17^{e}	2.33^{c}	2.03^{b}
2.0	-	-	0.5	-	5.83^{a}	4.33^{a}	2.83^{a}	2.13^{b}
6.0	-	-	1.5	-	4.33^{c}	3.67^{b}	2.03^{d}	2.1^{b}
10	-	-	2.5	-	4.43^{c}	4.27^{a}	2.63^{ab}	2.73^{a}
2.0	-	-	-	0.2	3.67^{de}	2.17^{e}	2.47^{bc}	1.17^{c}
4.0	-	-	-	0.4	3.33^{e}	2.67^{d}	2.4^{bc}	2^{b}
S.Em. ±					0.15	0.15	0.08	0.12
CD at 5%					0.43	0.44	0.24	0.35
CV (%)					6.58	8.62	6.62	10.11

Table represents pooled means from 5 replicates in each three repetitions; means within a column, followed by the same letters within a column, were not significantly different $p < 0.05$ by DMRT

Table 8
Effect of various concentrations of NAA, IAA and IBA on *in vitro* root induction

Media composition*			Days to root induction	Rooting (%)	Number of roots	Length of roots (cm)	No. of secondary roots
IBA	IAA	NAA					
(mgl^{-1})							
-	-	-	10.33^{ab}	100	6.00^{abc}	6.33^{d}	9.57^{cd}
0.5	-	-	10.33^{ab}	100	4.80^{c}	7.67^{bc}	11.17^{bc}
1.0	-	-	7.67^{de}	100	7.10^{a}	7.00^{cd}	13.33^{b}
2.0	-	-	9.33^{bc}	100	5.70^{bc}	4.90^{e}	8.43^{d}
-	0.5	-	11^{a}	100	6.03^{abc}	8.83^{a}	20.93^{a}
-	1.0	-	10.33^{ab}	100	5.53^{bc}	8.67^{ab}	18.83^{a}
-	2.0	-	8.33^{cd}	100	5.27^{bc}	3.30^{f}	8.80^{cd}
-	-	0.5	6.33^{ef}	100	5.43^{bc}	5.13^{e}	10.20^{cd}
-	-	1.0	6.5^{ef}	100	7.10^{a}	8.83^{a}	10.70^{cd}
-	-	2.0	6.0^{f}	100	6.10^{ab}	6.50^{d}	13.40^{b}
S.Em. ±			0.468	-	0.38	0.33	0.72
CD at 5%			1.382	-	1.13	0.96	2.12
CV (%)			9.42	-	11.21	8.40	9.95

Table represents pooled means from 5 replicates in each three repetitions; means within a column, followed by the same letters within a column, were not significantly different $p < 0.05$ by DMRT

Table 9
Effect of various concentrations of NAA, IAA and IBA on *in vitro* root induction

Media composition*			Days to root induction	Rooting (%)	Number of roots	Length of roots (cm)	No. of secondary roots
IBA	IAA	NAA					
(mgl^{-1})							
-	-	-	10.33^{ab}	100	6.00^{abc}	6.33^{d}	9.57^{cd}
0.5	-	-	10.33^{ab}	100	4.80^{c}	7.67^{bc}	11.17^{bc}
1.0	-	-	7.67^{de}	100	7.10^{a}	7.00^{cd}	13.33^{b}
2.0	-	-	9.33^{bc}	100	5.70^{bc}	4.90^{e}	8.43^{d}
-	0.5	-	11^{a}	100	6.03^{abc}	8.83^{a}	20.93^{a}
-	1.0	-	10.33^{ab}	100	5.53^{bc}	8.67^{ab}	18.83^{a}
-	2.0	-	8.33^{cd}	100	5.27^{bc}	3.30^{f}	8.80^{cd}
-	-	0.5	6.33^{ef}	100	5.43^{bc}	5.13^{e}	10.20^{cd}
-	-	1.0	6.5^{ef}	100	7.10^{a}	8.83^{a}	10.70^{cd}
-	-	2.0	6.0^{f}	100	6.10^{ab}	6.50^{d}	13.40^{b}
S.Em. ±			0.468	-	0.38	0.33	0.72
CD at 5%			1.382	-	1.13	0.96	2.12
CV (%)			9.42	-	11.21	8.40	9.95

Table represents pooled means from 5 replicates in each three repetitions; means within a column, followed by the same letters within a column, were not significantly different $p < 0.05$ by DMRT

Table 10
Effect of various concentrations of NAA, IAA and IBA on *in vitro* root induction using ½ MS media

Media composition*			Days to root induction	Rooting (%)	Number of roots	Length of roots (cm)	No. of secondary roots
IBA	IAA	NAA					
(mgl^{-1})							
-	-	-	8.33^{de}	100	4.43^{cde}	6.57^{b}	33.77^{a}
0.5	-	-	8.67^{cde}	100	6.53^{b}	4.43^{d}	25.73^{c}
1.0	-	-	10.0^{ab}	100	6.36^{b}	$7.2a^{b}$	30.23^{b}
2.0	-	-	9.0^{bcd}	100	4.43^{cde}	4.8^{cd}	6.77^{f}
-	0.5	-	10.33^{a}	100	4.03^{de}	7.3^{ab}	18.37^{d}
-	1.0	-	8.33^{de}	100	4.27^{de}	6.93^{ab}	10.07^{e}

-	2.0	-	9.67^{abc}	100	3.67^{e}	5.37^{c}	9.97^{e}
-	-	0.5	10.33^{a}	100	4.87^{cd}	7.37^{a}	8.7^{ef}
-	-	1.0	10.33^{a}	100	5.33^{c}	4.43^{d}	6.8^{f}
-	-	2.0	7.67^{e}	100	7.56^{a}	7.47^{a}	6.17^{f}
		S.Em. ±	0.350	-	0.29	0.23	0.92
		CD at 5%	1.031	-	0.87	0.69	2.70
		CV (%)	6.53	-	9.91	6.53	10.14

Table represents pooled means from 5 replicates in each three repetitions; means within a column, followed by the same letters within a column, were not significantly different $p < 0.05$ by DMRT

Table 11
Effect of different concentrations of sucrose and NAA on microrhizome induction

Media composition*		No. of microrhizome/ explants	Diameter of microrhizome	Fresh weight	Dry Weight
Sucrose (gm/l)	NAA (mgl^{-1})				
30	-	1.13^{d}	2.77^{bcde}	0.41^{e}	0.03^{e}
45	-	1.2^{cd}	3.00^{a}	0.58^{b}	0.05^{d}
60	-	1.93^{a}	3.00^{a}	0.42^{e}	0.05^{d}
75	-	2.07^{a}	2.87^{b}	0.37^{f}	0.05^{d}
90	-	0.93^{e}	2.57^{fg}	0.32^{g}	0.01^{g}
30	0.4	1.53^{b}	2.87^{b}	0.42^{e}	0.03^{e}
45	0.4	1.6^{b}	2.67^{ef}	0.27^{h}	0.01^{g}
60	0.4	1.6^{b}	2.67^{ef}	0.38^{f}	0.05^{d}
75	0.4	1.93^{a}	2.77^{bcde}	0.41^{e}	0.06^{c}
90	0.4	0.93^{e}	2.80^{bcde}	0.43^{e}	0.02^{f}
30	0.9	1.93^{a}	2.70^{de}	0.43^{e}	0.03^{e}
45	0.9	0.93^{e}	2.67^{ef}	0.33^{g}	0.03^{e}
60	0.9	1.13^{d}	2.83^{bc}	0.48^{d}	0.06^{c}
75	0.9	1.33^{c}	2.70^{de}	0.38^{f}	0.07^{b}
90	0.9	1.2^{cd}	2.73^{cde}	0.51^{c}	0.03^{e}
30	1.4	1.6^{b}	2.23^{h}	0.42^{e}	0.03^{e}
45	1.4	1.13^{d}	2.83^{bc}	0.32^{g}	0.03^{e}
60	1.4	1.13^{d}	2.53^{g}	0.51^{c}	0.06^{c}
75	1.4	1.2^{cd}	3.10^{a}	0.61^{a}	0.11^{a}
90	1.4	0.87^{e}	2.17^{h}	0.36^{f}	0.02^{e}
	S.Em. ±	0.35	0.03	0.01	0.23
	CD at 5%	1.03	0.10	0.02	0.69
	CV (%)	6.53	4.42	6.65	6.53

Table represents pooled means from 5 replicates in each three repetitions; means within a column, followed by the same letters within a column, were not significantly different $p < 0.05$ by DMRT

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