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In vitro Rapid Regeneration of Betel Vine (*Piper betle* L.)

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Authors' contributions

This work was carried out in collaboration between all authors. Author MME designed the study and performed the statistical analysis. Authors HH and MEH managed the analyses of the study. Author FK wrote the protocol, wrote the first draft of the manuscript and managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

The experiment was conducted on betel vine (*Piper betle* L.) for *in vitro* regeneration in Biotechnology Lab. of the Department of Biotechnology, Sher-e-Bangla Agricultural University, Bangladesh from the period of July 2014 to April 2015 to investigate the effect of BA, KIN, IAA and IBA in MS medium, where nodal segment was used as explants. The highest percent of shoot proliferation (70%) was achieved with 1.5 mg/L BA + 3.0 mg/L KIN whereas the highest number of shoots and leaves were obtained in 1.0 mg/L BA + 3.0 mg/L KIN. The maximum 14.40 roots/explants were observed with 3.0 mg/L IBA compare with IAA. For hardening the regenerated plantlets were transferred to growth chamber where maximum survival rate (90%) was observed whereas 75% plantlets were survived in shade house in soil which was higher than in open atmosphere (50%).This experiment can be used as a reliable protocol for *in vitro* regeneration of betel vine.

Keywords: In vitro; rapid regeneration; BA; KIN; IAA; IBA.

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1. INTRODUCTION

The Betel (*Piper betle* L.) is the leaf of a vine belonging to the Piperacae family, which includes pepper and Kava. It is a creeper with shiny, green heart-shaped leaves. The vine is a dioecious (male and female plants are different), shade loving perennial root climber. The most probable place of origin of betel vine is Malaysia [1]. There are about 100 varieties of betel vine in the world, of which about 40 are found in India and 30 in West Bengal [2-4].

The leaves of betel vine are commonly known as *Pan* in Bangladesh. It is an important cash crop in Bangladesh. Most of the people in Bangladesh like chewing betel vine as habit, sometimes it is used as an item of rituals, etiquette and manners. In Bangladesh most cultivated varieties of betel vine are Desi Bangla, Bangla, Kali Bangla, Jhali, Sanchi, Bhabna, Mitha, Geso, Bonhoogly etc. Bangladesh is the second largest grower of betel vine on about 14,175 hectare. Total annual production of the crop in Bangladesh is about 72,500 tons. The average yield is 2.27 tons per acre [5].

A well-prepared betel quid is regarded as an excellent mouth freshener and mild vitalizer, routinely served on the social, cultural and religious occasions like marriage, religious festivals. For such traditional use of betel leaf in our society, the leaf really stands alone without any parallel even today [2,6].

Betel leaf is traditionally known to be useful for the treatment of various diseases like bad breath, boils and abscesses, conjunctivitis, constipation, headache, hysteria, itches, mastitis, mastoiditis, leucorrhoea, otorrhoea, ringworm, swelling of gum, rheumatism, abrasion, cuts and injuries etc as folk medicine while the root is known for its female contraceptive effects [7,8]. The leaves are very nutritive and contain substantial amount of vitamins and minerals and therefore, six leaves with a little bit of slaked lime is said to be comparable to about 300 ml of cow milk particularly for the vitamin and mineral nutrition. Betel leaf is a second most popular daily consummation item in Asia, which contribute the best oral hygiene to oral cavity [9]. The fresh betel leaves possess antimicrobial, act against ringworm, antifungal, so it act as antiseptic and antihelminthic effects [10]. The leaves have wound healing property [11]. The leaf has the great potency to act as natural anti-oxidant. Chewing betel leaves has also shown to prevent oral cancer by maintaining the level of ascorbic acid in the saliva [12]. Extracts of betel leaves are known to have gastro protective activity and help in preventing gastric ulcers [13]. Moreover, extracts of betel leaves are known to control blood sugar levels and have an effective anti-diabetic property [14]. Betel leaves are a major component in various Ayurvedic (herbal) medicines used in treating warts [15].

In spite of its enormous potentiality in both domestic and international market, the acreage of betel vine is decreasing fast because of some physical and socioeconomic barriers like unavailability of credit facilities, uncontrolled marketing system and infestation of diseases and pest and low quality planting materials [16].

The common propagation of *Piper betle* L. is conventional method by means of stem cutting which is inefficient to meet the demand of *Piper betle* L. leaves. The occurrence of diseases like foot and leaf rot, anthracnose, stem or collar rot and bacterial leaf spot and infestation of pests including betelvine bugs (*Disphinctus politus*), mealy bugs (*Ferrisia virgata*), scales (*Lepidosaphes cornutus*) and whiteflies (*Dialeurodes pallida*) are the important constraints in betel pepper cultivation. As a result, the yield of betel vine decreases day by day.

In vitro regeneration holds tremendous potential for the production of high-quality planting materials as compare to conventional propagation. The problem of low productivity associated with conventional method can be minimized by using micropropagation techniques [17]. Auxin and cytokinin are the most common plant growth regulators used *in vitro* regeneration [18]. The cytokinin type and concentrations are important factors for successful *in vitro* multiplication. The best shoot proliferation response of nodal explant was observed with a cytokinin combination of N_6-benzyladenine and kinetin in *Piperacae* [19]. Auxin play important role in root induction in tissue culture plantlets. Though several attempts was taken for last few decades to develop tissue culture systems of *Piper* sp but still the efficient regeneration protocols are requisite to develop a rapid, less expensive, efficient and easy method of micropropagation of *Piper betle* L. Hence, the present study has been carried out with the following objectives:

1. Establishment of an efficient *in vitro* regeneration protocol of betel vine.
2. Assessment of hormonal effect for *in vitro* response.
3. To regenerate the plants that is genetically identical to the source material.

2. MATERIALS AND METHODS

The present study was carried out during July, 2014 to April, 2015 at the Biotechnology Laboratory, Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e Bangla Nagar, Dhaka-1207, Bangladesh, using healthy, disease free *Piper betle* L. as experimental materials that was were collected from farmers' field in Kalkini, Madaripur and the nodal part of 1-1.5 cm was used as explants.

MS [20] medium supplemented with different phytohormones as per treatments such as IBA (1.0, 1.5, 2.0 and 3.0 mg/L), IAA (1.0, 1.5, 2.0 and 3.0 mg/L), BA (0.5, 1.0, 1.5 and 2.0 mg/L) and KIN (1.0, 2.0, 3.0 and 4.0 mg/L) were used for shoot induction, shoot multiplication and maintenance and regeneration of roots from multiplied shoots. Hormones were added separately to different media according to the requirements. The pH was adjusted to 5.8 before placing in microwave oven which was used for melting agar (semisolidifying agent). After autoclaving the media were stored in at 25±2℃ for several hours to make it ready for inoculation with explants.

The vines were collected from healthy and disease free betel vine plants grown under field conditions were washed thoroughly under running tap water. The roots and outer tissues of the vine were removed with the help of a sharp knife. Then the nodal segments of 2 to 3 cm size (Plate 1A) were taken for surface sterilization with 70% ethanol for 1 minutes followed by washing with sterilize distilled water for 3 to 4 times. After that the explants were cut into suitable size (1 to 1.5 cm) then immersed in 0.1% $HgCl_2$ solution with 3 to 4 drops of Tween-20 for 5 minutes with constant shaking in clockwise and anticlockwise direction. Finally the nodal segments were rinsed with sterilize distilled water for at least 3 times.

The isolated and surface sterilized nodal segments were inoculated (Plate 1B) carefully to each of the culture tube containing 50 mL of MS medium supplemented with different concentrations of hormones as per treatment through maintaining aseptic condition inside the laminar air flow cabinet. The culture vials transferred to culture racks and allowed to grow within 25±1℃ temperature by an air conditioner and 16 hour photoperiod was maintained along with light intensity of 3000 lux for proper growth and development of culture. The observations on development pattern of shoots were made throughout the entire culture period. Data recording was started after 3 weeks from inoculation.

For the maintenance of proliferating shoots, the entire samples of *in vitro* shoot were cut into small pieces so that each piece would contain about one shoot and subcultured into a similar fresh medium. The subculturing was done at the interval of 20-25 days.

In vitro proliferated micro shoots were separated and each of the micro shoot was placed on culture medium, which was supplemented with particular concentration of hormone for shoot differentiation. Newly formed shoots with adequate length were excised individually from the culture vial and transferred to rooting media.

Regenerated plantlets were transplanted to pots (10×15 cm) containing sandy soil and cow dung in 1:1 ratio. Occasional spray of water was done to prevent sudden desiccations and maintain high humidity (98%) around the plantlets. Initially the plantlets were hardened in growth chamber. Then after 2 weeks, exposed to lower humidity and higher light intensity. Finally, after 20 days plantlets were transferred to natural environment.

Data were recorded at 3, 5 and 8 weeks after inoculation (WAI) on the effect of different treatments on shoot and root proliferation. The experiment was conducted in Completely Randomized Design (CRD) with three replications in culture room. Data were statistically analyzed by analysis of variance (ANOVA) technique and differences among treatment means were compared by using Duncan's multiple range test (DMRT) at 5% probability level using MSTAT-C program.

3. RESULTS AND DISCUSSION

The effects of BA, KIN, IAA and IBA were investigated with different concentrations for *in vitro* regeneration of betel vine using nodal segments as explants. Micropropagation exploits the morphogenetic potential of existing growing points or meristems within the plant [21]. The

results are discussed based on the nature of morphogenetic response of variety, hormones with different concentrations and their combination.

3.1 Effect of BA on Shoot Induction Potentiality in *Piper betle* L.

The effect of BA on shoot proliferation and elongation from nodal segments of betel vine was investigated by adding different concentrations of BA to a basal MS medium (semi solid). Significant variations at 5% level were observed among different treatments of BA on percent of explants (%) showing shoot induction, number of shoots and leaves per explant in the laboratory condition the results are presented in the Figs. 1- 3.

Maximum percentage of shoot (55%) was obtained in 1.0 mg/L BA and minimum percentage (25%) was observed in hormone free MS medium (Fig. 1). The results of present findings are partially supported by Parida and Dhal [22] where they reported the highest percentage of growth response was induced from nodal segments of *Piper* sp. in MS medium with 3.0 mg/L BA. The variation may be due to the age, nature, origin and the physiological state of the explant and seasonal variation play a crucial role in the establishment of cultures and subsequent plant regeneration [23].

The treatments of 2.0 mg/L BA showed the highest number of shoots 1.46 and 2.00 at 5 WAI and 8 WAI (Plate 1C), respectively whereas the lowest number of shoots 1.13 and 1.26 at 5 WAI and 8 WAI, respectively, was found in control (Fig. 2). In case of leaf, the maximum 3.33 leaves were recorded with 2.0 mg/L BA and the minimum 2.10 in control (Fig.3). This observation is consistent with Qusay et al. [24] where they found the BAP at 1.0 mg/L was the best concentration to induce shoot multiplication from *Piper betle* explants. Likewise, Hussain et al. [25] observed that shoot regeneration was excellent on MS media supplemented with 0.5 mg/L BA. This may be due to an excess of growth regulators in the culture media, might lead to genetic, physiological and morphological change resulting in a reduction of the proliferation rate *in vitro* [26].

3.2 Combined Effect of BA and KIN on Shoot Induction Potentiality in *Piper betle* L.

In present study, the combined effects of different concentrations of BA and KIN have been investigated. The cytokinin combination of BA and KIN are most effective than sole dose of cytokinin in Piperacae [27]. There was significant variation of BA and KIN concentrations on percent of explants showing shoot induction, number of shoots and number of leaves per explants. Maximum percentage of shoot induction (70%) was noticed in 1.5 mg/L BA + 3.0 mg/L KIN and minimum percentage (25%) was induced in control (Table 1). The present findings are not similar with Padhan [27]. He found 98% of explants showed shoot proliferation on MS supplemented with 1.0 mg/l Kinetin and 1.5 mg/l BA. The variation may be due to the age, nature, origin and the physiological state of the explant and seasonal variation play a crucial role in the establishment of cultures and subsequent plant regeneration [23].

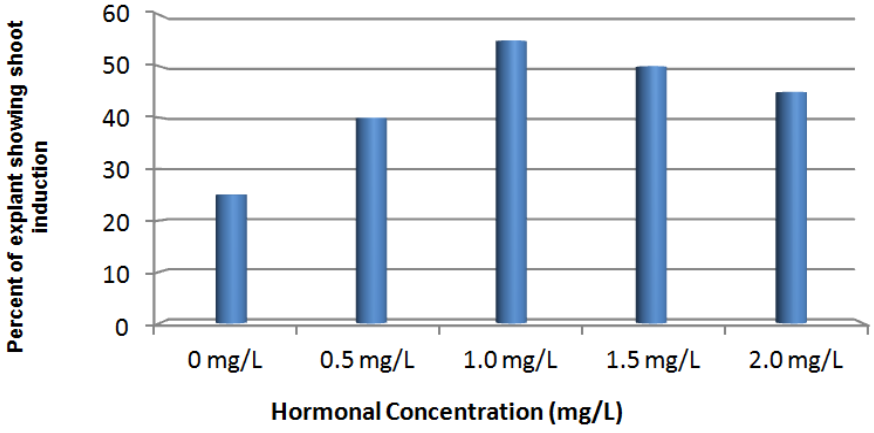


Fig. 1. Effect of BA on percent of explants showing shoot induction using nodal segment as explants in *Piper betle* L.

Table 1. Combined effect of BA and KIN on shoot induction potentiality

Treatments	Name of the phytohormones	Phytohormones concentration (mg/L)	Number of explants Inoculated	Shoot induction potentiality				Average no. of leaves per explant
				Percent (%) of explants showing shoot induction	No. of shoot per explant (3 WAI)	No. of shoot per explants (5 WAI)	No. of shoot per explants (8 WAI)	
T_1	Control	0.0	20	25	1.00i	1.13i	1.26i	2.1h
T_2	BA+KIN	0.50+1.00	20	35	1.03hi	2.13h	2.76h	3.3g
T_3	BA+KIN	1.00+1.00	20	40	1.13gh	2.23gh	2.86h	3.2g
T_4	BA+KIN	1.50+1.00	20	50	1.23fg	2.36fg	3.03g	3.6f
T_5	BA+KIN	2.00+1.00	20	45	1.23fg	2.40efg	3.10fg	3.5f
T_6	BA+KIN	0.50+2.00	20	55	1.33ef	2.56de	3.16fg	3.6f
T_7	BA+KIN	1.00+2.00	20	45	1.43de	2.56de	3.23f	3.7ef
T_8	BA+KIN	1.50+2.00	20	50	1.40de	2.50def	3.23f	4.0cd
T_9	BA+KIN	2.00+2.00	20	50	1.47d	2.63cd	3.46e	4.1bc
T_{10}	BA+KIN	0.50+3.00	20	55	1.50d	2.76bc	3.90c	4.3b
T_{11}	BA+KIN	1.00+3.00	20	60	2.03a	3.46a	4.63a	4.8a
T_{12}	BA+KIN	1.50+3.00	20	70	1.86b	2.93b	4.13b	4.2b
T_{13}	BA+KIN	2.00+3.00	20	55	1.83b	2.86b	3.96c	4.2b
T_{14}	BA+KIN	0.50+4.00	20	60	1.83b	2.76bc	3.83c	4.2b
T_{15}	BA+KIN	1.00+4.00	20	55	1.63c	2.60cd	3.86c	3.9de
T_{16}	BA+KIN	1.50+4.00	20	45	1.80b	2.76bc	3.66d	3.7ef
T_{17}	BA+KIN	2.00+4.00	20	50	1.66c	2.66cd	3.63d	3.7ef
CV%		-			4.68	3.88	2.26	1.66
LSD value		-			0.1173	0.1659	0.1285	0.1892

**WAI=Weeks After Inoculation. In a column, mean values with the same letters are not statistically different from each other at 5% probability by DMRT*

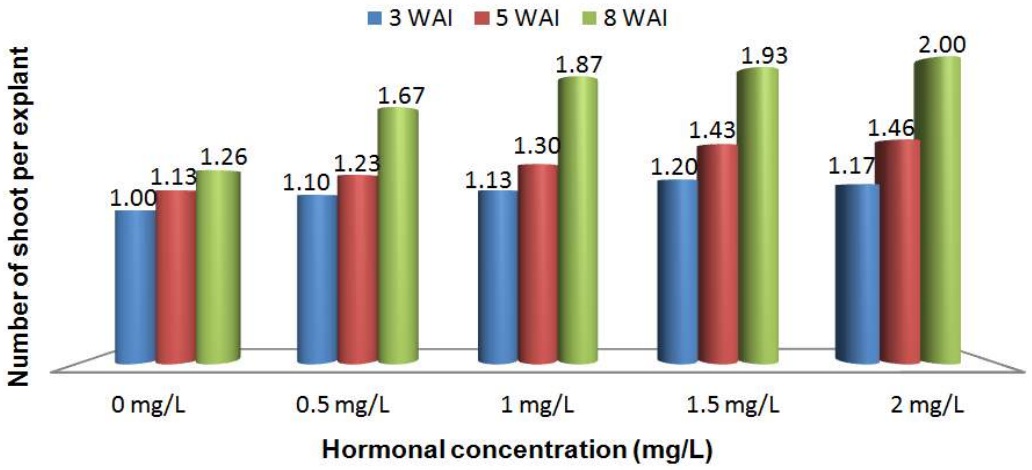


Fig. 2. Effect of BA on the number of shoots per explant in *Piper betle* L.

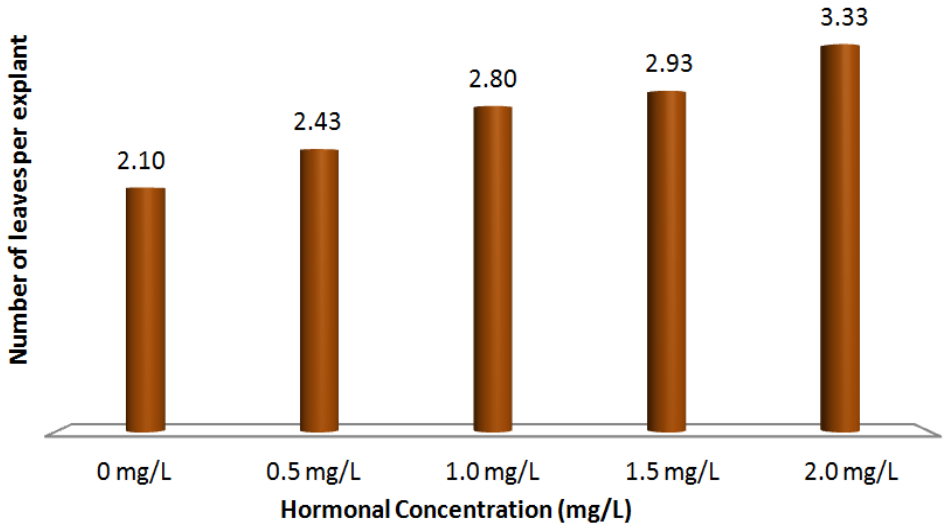
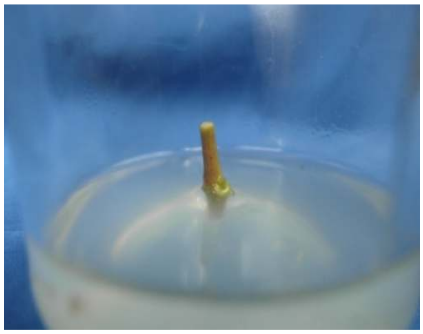


Fig. 3. Effect of BA on the number of leaves in *Piper betle* L.

The treatment 1.0 mg/L BA+3.0 mg/L KIN gave the highest number of shoots 2.03, 3.46 and 4.63 at 3 WAI, 5 WAI and 8 WAI (Plate 2), respectively, whereas the lowest number of shoots 1.0, 1.13 and 1.26 at 3 WAI, 5 WAI and 8 WAI, respectively, were found with control (Table 1). The results of present study are not consistent with Padhan [27] where he reported 5-6 shoots per explants were obtained in MS with combination of 1.0 mg/l Kinetin and 1.5 mg/l BA. Similarly, Soniya and Das [28] showed that *Piper* sp produced maximum number of shoots in MS medium with 2 mg/l BA and 1 mg/l KIN. MS basal medium supplemented with 1mg/l of IAA and 0.5mg/l BAP is suitable for induction of multiple shoots in shoot tip and leaf base explants in *Piper betle* instead of nodal explants [29]. This may be due to the factors involved in the control of organogenesis in culture are more complex and plant hormones, organic and inorganic nutrient and osmotic concentration exert a performed influence on organogenesis [30]. Besides, *in vitro* growth and regeneration is a complex phenomenon and is influenced by a number of genetic and environmental factors [31]. The maximum number of leaves per explant (4.8) were noticed in 1.0 mg/L BA+3.0 mg/L KIN, statistically different from rest of treatments, whereas the minimum was 2.1 in control (Table 1).

A. Desirable size of explants (1 to 1.5) for placement



B. Explant inoculation

C. Multiple shoots of *Piper betle* L

Plate 1. *In vitro* regeneration of *Piper betle* L. with 2.0 mg/L BA in MS medium at 8 WAI.

Plate 2. Combined effect of 1.0 mg/L BA+3.0 mg/L KIN on the number of shoots per explant in *Piper betle* L. at 8 WAI

3.3 Effect of IAA and IBA on Root Formation Potentiality of Micro Propagated Shoots in *Piper betle* L.

To develop root in the regenerated shoots, they were excised and transferred to rooting media supplemented with IAA and IBA separately. The significant variations of different concentrations of IAA and IBA on the number of roots per explants were studied at 5% level of significance.

3.3.1 Effect of IAA on root formation

The treatment 2.0 mg/L IAA showed the highest number of roots 4.70, 7.83 and 13.07 at 3 WAI, 5 WAI and 8 WAI, respectively, whereas the lowest number of roots 1.13 and 2.73, 4.86 at 3 WAI, 5 WAI and 8 WAI, respectively, were found with control (Table 2). Our observation is not congruent with Padhan [27] results. He obtained profuse rooting with 0.5 mg/L IAA in MS medium. Besides, Bhat et al. [32] reported the rooting response *Piper betle* in B5 medium containing 0.175 mg/l IAA. Rooting of micropropagated plants of *Piper longum* in half strength MS medium with 0.1 mg/l IAA was also reported by Bhat et al. [33]. The variation may be due to influence of a number of genetic and environmental factors [31].

3.3.2 Effect of IBA on root formation

The maximum number of roots (6.53, 14.87 and 22.40) were obtained with 3.0 mg/L IBA at 3

WAI, 5 WAI and 8 WAI (Plate 3), respectively, whereas the lowest number of roots (1.13 and 2.73, 5.86) were found at 3 WAI, 5 WAI and 8 WAI, respectively with hormone free media (Table 3). The findings of our study are completely opposed by Qusay et al. [24] where they showed, 1000 - 2000 mg/L of IBA treatments were effective in increasing the rooting percentage of the *P. betle* cuttings. This may be due to the factors involved in the control of organogenesis in culture are more complex and plant hormones, organic and inorganic nutrient and osmotic concentration exert a performed influence on organogenesis [30]. Besides, *in vitro* growth and regeneration is a complex phenomenon and is influenced by a number of genetic and environmental factors [31].

3.4 *Ex vitro* Acclimatization and Establishment of Plantlets on Soil

After 35 days of culture on rooting media, the plantlets were taken in growth chamber for acclimatization where 90% of plantlets were survived (Table 4 & Plate 4A). Then the small plantlets were brought out from culture vessel carefully without damaging any roots. Excess media around the root was washed off by running tap water to prevent microbial infection. The plantlets were shifted to shade house in plastic pots filled with sand: sterilized soil: cowdung (1:1:1) with less humidity (70% RH) and indirect sunlight. In the shade house, the top of the pots were covered with transparent plastic sheet and grew at room temperature with periodic irrigation (2 days interval) and the survival rate was 83% (Table 4). Finally, in open atmosphere, 64% plantlets were survived (Table 4 & Plate 4B). The technique is labour intensive, involving several *in vitro* steps in which plants must be gradually acclimatized from culture to the greenhouse and finally to the field [34]. Padhan [27] observed 90% plantlets survived in soil in shade house that is very close to our present findings. The result of current investigation is partially supported by Anand and Rao [19] where they found 75% plantlets were survived in natural condition.

Plate 3. Effect of IBA on number of roots per explant in *Piper betle* L. at 8 WAI

A **B**

Plate 4. Acclimatization of regenerated plantlet (A) in growth chamber and (B) in natural condition

Table 2. Effect of IAA on number of root *in Piper betle* L.

Treatments	Name of the phytohormone	Phytohormone concentration (mg/L)	Number of root per explants		
			No. of root per explant (3 WAI)	No. of root per explant (5 WAI)	No. of root per explant (8 WAI)
T_1	Control	0.0	1.13e	2.73e	4.86e
T_2	IAA	1.0	1.73d	4.93d	9.47c
T_3	IAA	1.5	3.06b	6.43b	11.67b
T_4	IAA	2.0	4.70a	7.83a	13.07a
T_5	IAA	3.0	3.20c	4.80c	8.13d
CV %			6.70	7.74	2.27
$LSD_{0.05}$			0.3355	0.9223	0.5366

**WAI=Weeks After Inoculation. Values in the column are the means of five replicates. In a column, mean values with the same letters are not statistically different from each other at 5% probability by DMRT*

Table 3. Effect of IBA on number of roots *in Piper betle* L.

Treatments	Name of the phytohormone	Phytohormone concentration (mg/L)	Number of roots per explants		
			No. of roots per explants (3 WAI)	No. of roots per explants (5 WAI)	No. of roots per explants (8 WAI)
T_1	Control	0.0	1.13 e	2.73e	4.86e
T_2	IBA	1.0	3.73d	5.86d	9.80d
T_3	IBA	1.5	4.86c	7.66c	11.67c
T_4	IBA	2.0	5.07b	9.87b	13.37b
T_5	IBA	3.0	6.53a	10.87a	14.40a
CV %			3.87	2.16	3.56
$LSD_{0.05}$			0.2989	0.3593	0.9864

**WAI=Weeks After Inoculation. In a column, mean values with the same letters are not statistically different from each other at 5% probability by DMRT*

Table 4. Survival rate of *in vitro* regenerated plantlet of *Piper betle* L.

Acclimatization	No. of plantlet transplanted	No. of plantlet survived	Survival rate (%)
In growth chamber	20	18	90
In shade house	18	15	83
In natural condition	15	10	64

4. CONCLUSION

The present study showed that the overall single dose 2.0 mg/L BA for shooting and 3.0 mg/L IBA for rooting showed better response in *in vitro* regeneration of *Piper betle* L. Combined effect of BA+ KIN seems to be better than individual response of BA based on average performance of growth parameters. The treatment 1.0 mg/LBA+3.0 mg/L KIN was the best for shooting among all the treatments. Therefore, suitable protocol was established which could be used for *in vitro* rapid propagation of betel vine plantlets. Future experiment should be carried on different type of genotype of *Piper betle* L.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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