Bangladesh J. Bot. **48**(3): 559-566, 2019 (September)

IN VITRO MASS PROPAGATION OF *PIPER BETLE* L.

SALIM KHAN, BARNA GOSWAMI, SHAHINA AKTER, MOUSONA ISLAM, AFSANA HUQ NOON, AHASHAN HABIB AND TANJINA AKHTAR BANU*

Plant Tissue Culture Section, Biological Research Division, Bangladesh Council of Scientific and Industrial Research, Dhanmondi, Dhaka-1205, Bangladesh

Keywords: Piper, In vitro mass propagation, Callus induction, Multiple shoot

Abstract

An efficient *in vitro* regeneration system was developed for *Piper betle* L. through direct and indirect organogenesis from nodal segment, leaf segment and petiole explants. Highest direct regeneration was recorded when nodal explants were cultured on MS with 1.0 mg/l BAP and 1.0 mg/l Kn where 80% explants produced multiple shoots and the average number of shoots per explants were 3.20. Remarkable results on callus induction and shoot initiation were observed when the explants cultured on MS + 2.0 mg/l BAP + 0.5 mg/l Kn + 1.0 mg/l IAA. It was observed that nodal explants were showed best response on shoot/explants 13.2 ± 4.5 after 8 weeks of callus culture on MS medium with 0.5 mg/l BAP. The best response towards root induction was observed on half strength of MS with 0.25 mg/l IBA. The well rooted plants were successfully acclimatized and transferred to soil.

Introduction

Piper betle L. is deep green heart shaped perennial creeper. It is popularly known as Paan in India and Bangladesh (Chowdhury *et al.* 2011). Significance of *P. betle* leaves has been explained in relationship to every sphere of human life including social, culture and religious and is very much relevant even in modern days (Guha 2006). In Bangladesh, various types of consumers eat different varieties of betel leaves (Paan). Among those, Dhakai, Khilipan, a ready pack of betel leaf processed in Dhaka is famous in the sub-continent. Moreover, with known ethnomedicinal properties (Nagori *et al.* 2011), this plant is widely used in Bangladesh, India, Indonesia and other countries of the Indo-China region (Pradhan *et al.* 2013). *P. betle* is one of the important plants in the Asiatic region, which ranks second to tea and coffee in terms of daily consumption (Chowdhury *et al.* 2011). At present, betel leaves have a worldwide market. Two factors are responsible for the expansion. First, the global dispersion of the betel leaves chewing South Asians and second, scientific recognition of the medicinal value of betel leaves. But in competition with India and other betel leaf producing countries, Bangladesh has a very small share of the world betel leaf market.

P. betle is vegetatively propagated crop Bangladesh. But it is susceptible to various bacterial diseases such as foot and leaf rot (*Phytophthora palmivora*), bacterial leaf spot (*Xanthomonas campestris pv. betlicola)* which are transmitted from generation to generation due to vegetative propagation through stem cuttings (Sengupta *et al.* 2011). Therefore, an alternative process is necessary to produce large scale healthy and quality planting material. Tissue culture can help in getting infection free true-to-mother type plants in large numbers and in a very short time. Considering this, the present study has been undertaken to establish a suitable protocol for large scale production of *in vitro* plantlets.

*Author for correspondence: <tanzinabcsir@yahoo.com>.

Materials and Methods

The experiment was conducted at Plant Tissue Culture Section, Biological Research Division, BCSIR Laboratories Dhaka, Bangladesh. Different types of explants (nodal segment, leaf segment and petiole) were collected from the plants grown in BCSIR research field. Explants were primarily kept under running tap water for 5 min. Then surface sterilized initially with mild detergent (Tween-20) and washed with tap water for 30 min. After that, explants were surface sterilized with 70% (v/v) ethanol for 1 min, followed by with 0.1% $HgCl_2$ by gentle shaking for 7 min and rinsed four to five times with autoclaved distilled water. Isolated explants were inoculated and cultured on MS and WPM (Woody Plant medium, Lloyd and McCown 1980) containing BAP, NAA, IAA and Kn singly or in combinations for direct and indirect (through callus) *in vitro* regeneration of shoots. *In vitro* regenerated shoots were subcultured regularly to fresh medium at an interval of 21 - 28 days for further multiplication. About 3 - 4 cm long shoots were separated and cultured on rooting medium containing full and half strengths of MS without hormonal supplement or with different concentrations of IBA. All *in vitro* grown cultures were maintained under illumination on a 16 hrs photoperiod at 25 ± 2°C. The well rooted plantlets were then transferred to plastic pot containing garden soil and compost (2 : 1) and moist them adequately for proper hardening.

Results and Discussion

The development of an efficient and reproducible *in vitro* regeneration protocol depends on several factors. Selection of suitable explants, sterilization of culture conditions and regeneration media with proper hormonal supplements directly related for proper regeneration system. *In vitro* regeneration of *P. betle* is severely hampered by high incidence of bacterial contamination. Even very healthy looking cultures also showed contamination after about one-two months, similarly reported by Bhat *et al.* (1995). Over exposure of sterilizing agents like $HgCl_2$ treatments (more than 7 mins) along with large amount of phenols also limited the regeneration capacities of explants. So, at first sterilization procedure is optimized for *in vitro* establishment of *P. betle* (data not shown). In this experiment nodal, internodal and leaf with petiole segments (medium aged) were used as explants for *in vitro* regeneration. Among the different explants nodal explants showed the best response towards shoot regeneration and subsequent plant development of *P. betle* (upper 2 - 6 nodes). Some literature showed that nodal segments of upper 4 - 5 nodes were suitable as the explants (Misra *et al.* 2004). In the present study it was observed that inter-nodal segments did not show any sign of shoot regeneration, very few explants produced slight callus.

Some studies demonstrated that MS was a suitable medium for shoots and root induction in *P. betle* (Misra *et al.* 2004). During this study, shoot regeneration was obtained through direct organogenesis from nodal explants using MS containing different hormonal supplements. At first MS with only BAP (0.5 to 5.0 mg/l) were applied to examine their effect on shoot regeneration different explants of *P. betle.* Responses of explants using BAP (0.5 to 5.0 mg/l) are presented in Fig. 1. Maximum response was observed on MS medium supplemented with 3.0 mg/l BAP. Shoot initiation was found to occur within 5 to 19 days of inoculation (Fig. 2a). These initiated shoots did not elongate properly. In this case 0.2 mg/l GA_3 was added with 3.0 mg/l BAP for shoot elongation during subculture (Fig. 2b), but the elongation rate was not satisfactory. Philip *et al.* 1992 showed that the best establishment and proliferation of shoot tip explants of *P. nigrum* was obtained on MS medium containing 1.5 mg/l BAP alone.

It was observed that BAP alone was not more effective for multiple shoot formation of *P. betle*. To obtain better response MS with different concentrations and combinations of BAP, NAA, IAA, Kn were tried. Results of this experiment are presented in Table 1. Among the various

hormonal combinations used for regeneration, most of the cases direct organogenesis was observed. Very often compact callus was formed at the base of the explants. But the callus did not show any sign of regeneration when they were subcultured on the same media combinations. Highest direct regeneration response was recorded when explants were cultured on MS with 1.0 mg/l BAP and 1.0 mg/l Kn (Table 1). In this combination initiation was found to occur after 5-11 days (Fig. 2c). The mean number of shoots per explants (3.20) (Fig. 2d). In *P. longum*, Soniya and Das **(**2002) also showed that maximum number of shoots were induced with 8.9 M BA and 4.64 M Kn. Next to this better response was observed on MS supplemented with 2.0 mg/l BAP, 0.5 ml

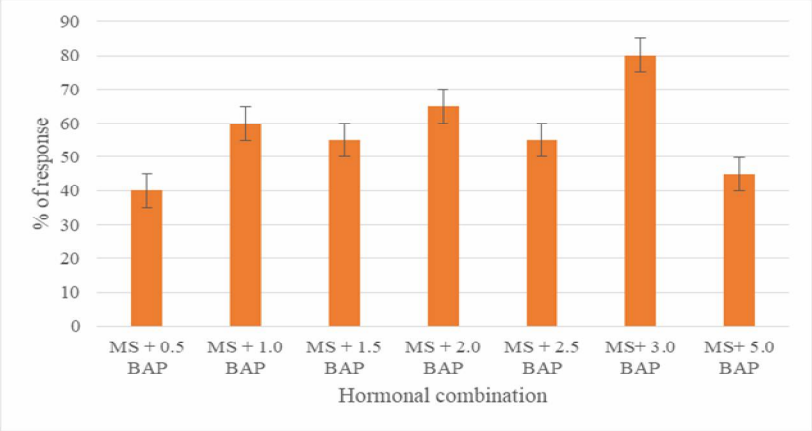

Fig. 1. Responses of explants of *P. betle* in MS with BAP (0.5 to 5.0 mg/l).

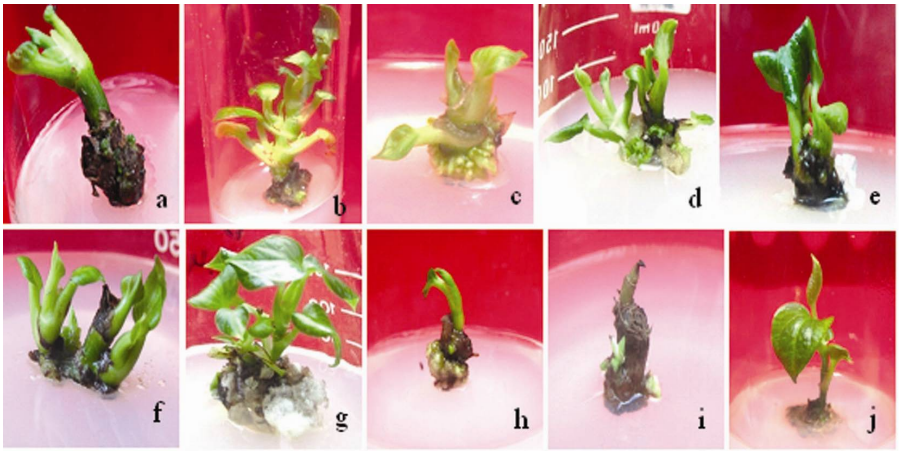

Fig. 2(a-j). Different stages of direct *in vitro* shoot regeneration of *P. betle* from nodal explants. (a) Formation of shoot from nodal explants on MS with 3.0 mg/l BAP, (b) Elongated shoot from node on same media composition as mentioned in Fig. a with addition of 0.2 mg/l GA_3, (c) Shoot initiation on MS with 1.0 mg/l BAP + 0.5 mg/l Kn, (d) Multiple shoot formation from node explants on same media as mentioned in Fig. c, (e) Shoot initiation on MS medium supplemented with 2 mg/l BAP + 1 mg/l IAA + 0.5 mg/l Kn, (f) Multiple shoots formation on same media composition as mentioned in Fig. e, (g) Multiple shoots with callus formation at the base of the explants on formation on same media composition as mentioned in Fig. e, (h) Shoot initiation on MS supplemented with 2.0 mg/l BAP and 0.2 mg/l NAA, (i) Shoot initiation on WPM media supplemented with 3.0 mg/l BAP and 1.0 mg/l Kn and (j) Single elongated shoot on WPM with 3.0 mg/l BAP and 1.0 mg/l Kn.

Kn and 1.0 mg/l IAA (Table 1). Direct organogenesis (Fig. 2e) as well as callus formation were also observed in this medium combination. Mean number of shoots/explants was found to be 1.75 (Fig. 2f) with average shoot length 4.01 in these hormonal combinations of BAP, Kn and IAA. Initiation of callus was found to occur at the base of the explants (Fig. 2g) in this combination. But the callus did not turned towards shoot initiation when they were subcultured on the same media combinations. Some reports showed that *P. betle* was successfully micropropagated with various concentrations of BA and IAA (Bhat *et al*. 1995). Parida and Dhal (2011) demonstrated that in *P. longum* the optimal medium for shoot multiplication was MS supplemented with 1.0 mg/l BAP and 1.0 mg/l IAA. But in the present study, when the explants were cultured on MS medium that

Table 1. Effects of different concentrations of BAP, NAA, IAA and Kn on MS medium on *in vitro* shoot development of *P. betle*.

Plant growth regulators (mg/l)				Frequency of shoot initiation (%)	Days to shoot initiation	Shoot/ex plants	Average shoot length (cm)
BAP	NAA	IAA	Kn				
0.5	0.2	-	-	45	5-16	1.00	1.50
1.0	-	-	0.5	60	5-13	1.25	2.75
1.00	-	0.2	-	55	6-13	1.00	2.15
1.00	-	0.5	-	50	6-15	1.00	2.25
1.00	**-**	**-**	**1.0**	**80**	**5-11**	**3.20**	**2.80**
1.50	**0.2**	**-**	**-**	**60**	**7-15**	**1.00**	**3.00**
2.00	-	-	0.5	50	5-11	1.53	2.25
2.00	**-**	**0.2**	**-**	**75**	**4-17**	**1.35**	**3.00**
2.00	**-**	**0.5**	**-**	**70**	**4-14**	**1.65**	**2.75**
2.00	**-**	**1**	**0.5**	**80**	**6-15**	**1.75**	**4.10**
2.00	-	-	1.0	55	5-11	1.53	2.25
2.00	**0.2**	**-**	**-**	**70**	**5-17**	**1.00**	**3.20**
3.00	**-**	**-**	**1**	**75**	**5-9**	**1.70**	**2.50**

Table 2. Responses of explants towards multiple shoot regeneration through callus formation on MS medium with 2 mg/l BAP, 0.5 m/l Kn and 1.0 mg/l IAA.

Hormonal composition of callus formation	Explants	% of responsive explants towards callus formation	Days required for initiation of callus formation	Hormonal composition for shoot formation	% of explants showed shoot initiation	Shoots/ explant after 40 days in SEM
MS+2 mg/l BAP+0.5 mg/l Kn+1.0 mg/l IAA	**Node**	63.33	14 - 22	MS	-	-
				MS+0.5 mg/l BAP	**71.42**	**13.2 ± 4.5**
				MS+1.0 mg/l BAP	42.85	9.66 ± 2.08
	Leaf with petiole	30	18-32	MS	-	-
				MS + 0.5 mg/l BAP	-	-
				MS + 1.0 mg/l BAP	40.0	9.25±2.5

supplemented with 1.0 mg/l BAP and 0.5 mg/l IAA very poor regeneration response was observed (Table 1). MS medium supplemented with different concentrations and combinations of BAP with NAA were applied in this experiment, but they did not show good response for shoot initiation as well as multiplication. In this hormonal combination almost, single shoot (Fig. 2h) was produced and sometimes the explants produced only white or green callus which did not show any sign towards shoot initiation. But Misra *et al.* (2004) showed that 2.0 mg/l BAP and 0.2 mg/l NAA was the most suitable combination for obtaining maximum number of axillary shoots which is contrast with present investigation.

Some reports revealed that callus formation and shoot initiation were easily formed on woody plant medium (WPM, Lloyd and McCown 1980) with combinations of BAP and Kn of some *Piper* spp. Therefore, WPM supplemented with different concentrations and combinations of BAP (1.0 to 3.0 mg/l) and 1.0 mg/l Kn were applied to examine their effect on shoot regeneration from nodal explants of *Piper betle*. Among the different concentrations and combinations of BAP and Kn used for shoot regeneration, maximum multiple shoot response was obtained on WPM media supplemented with 3.0 mg/l BAP and 1.0 mg/l Kn (Fig. 2i). Using this medium combination, it was observed that single shoot was elongated rapidly rather than the multiple shoot formation (Fig. 2j). The percentage of responsive explants towards shoot initiation was 80 (Fig. 3). Highest 1.31(data not shown) mean number of shoots was observed in this hormonal combinations. Initiation of shoots was found to occur within 4 to 13 days of inoculation in this medium combination (WPM + 3.0 mg/l BAP + 1.0 mg/1 Kn). Results of these experiments are shown in Fig. 3. In case of *P. barberi* Babu *et al.* (1992) showed that WPM supplemented with 3.0 mg/l BA and 1.0 mg/1 Kn was the best for both multiple shoot production and regeneration of plantlets.

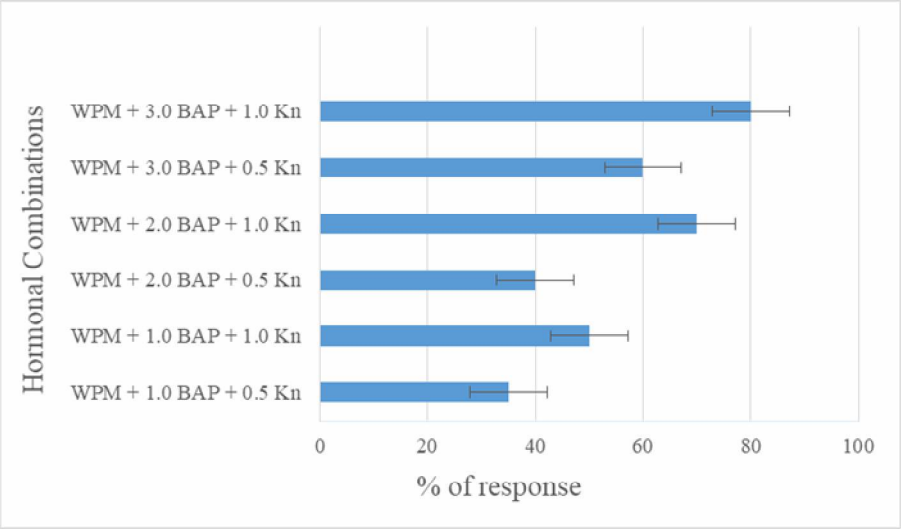

Fig. 3. Effects of WPM medium supplemented with BAP and Kn (mg/l) on shoot regeneration response of *P. betle*.

Although various responses were observed on different combinations and concentrations of hormone with different basal media (MS and WPM), but number of shoots per explant was not adequate (1.0 - 3.20). To obtain better response towards multiple shoot formation special focus was given to produce multiple shoots through callus formation from node and leaf with petiole explants. For this purpose, some combinations were chosen in which callus formation was

observed in previous experiments. Remarkable result was observed when the explants were cultured on MS+ 2 mg/l BAP + 0.5 mg/l Kn + 1 mg/l IAA. In this combination callus was formed at the base of node and petiole part of leaf explants. When the explants were subcultured in various lower concentrations of BAP containing MS medium the callus started to initiate shoot regeneration (Table 2). Almost a period of 14 - 22 days was required for callus formation from node explants (Fig. 4a) and 18 - 32 days were required for callus formation from leaf with petiole explants. It was observed that nodal explants showed best response with mean number of shoot/explant 13.2 ± 4.5 (Table 2) after 8 weeks of culture on MS medium supplemented with 0.5 mg/l BAP (Fig. 4b, c). On the other hand, leaf with petiole explants showed best shoot initiation when the calluses were transferred on MS medium supplemented with 1 mg/l BAP. Banu *et al.* (2017) also observed better response in *Gynura procumbens* on MS with BAP, Kn and IAA supplemented medium. Khan *et al*. (2018) also reported that combination of 2.0 mg/l BAP, 0.5 mg/l IAA and 0.02 mg/l NAA was best for the multiple shoot formation from nodal segments in *Rauvolfia serpentina*. Bhat *et al.* (1995) showed that BA alone was more effective than Kn for different *Piper* spp. and its optimum concentration for *P. longum*, *P. betle* and *P. nigrum* were 1 - 2, 10 and 10 µM, respectively. Philip *et al.* (1992) showed that the best establishment and proliferation of shoot tip explants of *P. nigrum* were obtained on MS medium containing 1.5 mg/l BAP alone.

Table 3. Effect of half and full strength of MS medium with IBA on induction of roots from the *in vitro* regenerated shoots.

Media composition	Days required for root initiation	% of response for root induction	Roots per plant
MS + 0.25 IBA	8 - 16	90	10.5
MS + 0.5 IBA	7 - 14	70	11.0
MS + 0.75 IBA	7 - 15	30	09
MS + 1.0 IBA	7 - 13	50	10
1/2MS + 0.25 IBA	**5 - 12**	**100**	**12.5**
1/2MS + 0.5 IBA	5 - 14	80	05

After successful development of shoots, a number of experiments were conducted to induce effective roots at the base of the isolated elongated shoots. Full and half strengths of MS media with various concentrations and combinations of IBA were used for induction of roots from *in vitro* grown shoots. In the present study, the best response towards root induction was observed on half strength of MS with 0.25 mg/l IBA. In this combination 100% (Table 3) regenerated shoots formed well developed roots in 5 - 12 days where mean number of roots per shoot was 12.5 (Fig. 4d). In this hormonal combination roots were healthy and formed at the base of the cut end (Fig. 4e). Half strength of MS with 0.50 mg/l IBA also showed 80% root formation response (Fig. 4f). Misra *et al.* (2004) showed that MS with 0.25 mg/l IBA was the best medium for root induction and development of *P. betle*. In case of *P. longum*, Soniya and Das (2002) showed that the maximum percentage of root formation was obtained in MS supplemented with 2.46 M IBA. In the present study full strength of MS medium supplemented with 0.25 mg/l IBA also showed good rooting response. About 90% shoots were formed roots and it took 8 - 16 days for root induction where mean number of roots was 10.5 (Fig. 4g). In this combination sometimes, roots were formed at the side of the shoots and not from the cut end. The well-developed rooted plantlets of

P. betle were successfully transplanted into small plastic pots containing autoclaved soil (Fig. 4h, i and j). Using this method, the survival rate of the transplanted plantlets was found to be almost 90% (data not shown). For their further growth and establishment, the survived plantlets were transferred to larger clay pots.

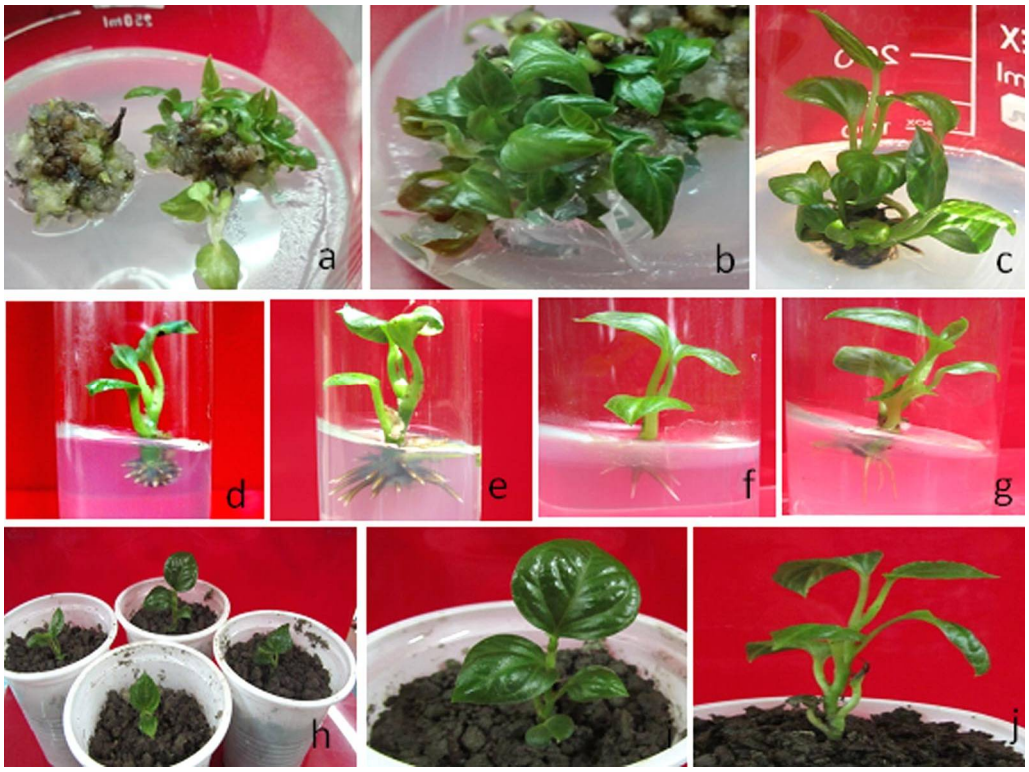

Fig. 4a-j. Indirect shoot regeneration and formation of roots from *in vitro* regenerated shoots of *P. betle*. (a) Callus formation as well as initiation of multiple shoots from nodal explants using on MS + 2 mg/l BAP + 1 mg/l IAA + 0.5 mg/l Kn, (b) Formation of multiple shoots from nodal explants on MS medium supplemented with 0.5 mg/l BAP, (c) Elongated shoots on the same media and explants as mention in Fig. b, (d) Initiation of roots from *in vitro* regenerated shoots on half strength of MS with 0.25 mg/l IBA, (e) Formation of mature roots on same media as mention in Fig. d, (f) Induction roots on same media as mention in Fig. d, but in 0.50 mg/l IBA, (g) Formation of roots from *in vitro* regenerated shoots on full strength of MS with 0.25 mg/l IBA, (h, i & j) Transplantation and acclimatization of *in vitro* regenerated plantlets of *P. betle*.

Based on the above discussion it may be concluded that the *in vitro* mass propagation protocol developed in the present investigation could be used for the large-scale cultivation and production of *P. betle*. It may be mentioned that as a young nation, Bangladesh is striving hard for the future and facilitation for promotion of investment and export. Using only conventional propagation techniques it is really not possible to fulfill the requirements of elite product demands. In this respect, mass commercial production of *P. betle* using *in vitro* techniques could be accelerated the production and helped to conserve this medicinal plant.

References

Babu KN, Rema J, Pillai SG, Divakaran M, Ravindran PN and Peter KV 1992. Micropropagation of betel vine (*Piper betle* L.). J. Spices Arom. Crops. **1**(2): 160-162.

Banu TA, Goswami B, Akter S, Islam M, Tanjin T, Habib A and Khan S 2017. High frequency *in vitro* regeneration of *Gynura procumbens* (Lour.) Merr. Plant Tissue Cult. & Biotech. **27**(2): 207 - 216.

Bhat SR, Chandel KPS and Malik SK 1995. Plant regeneration from various explants of cultivated *Piper* species. Plant Cell Rep. **14**: 398-402.

Chowdhury I, Amin R and Binzaid S 2011. Optimal control on environments for improving the *Piper betle* (Paan) growth, Life Sci. Leaflet. **60**(17): 605- 615.

Guha P 2006. Betel leaf: The neglected green gold of India. J. Hum. Ecol. **19**(2): 87-93

Khan S, Banu TA, Akter S, Goswami B, Islam M, Hani U and Habib A 2018. *In vitro* regeneration protocol of *Rauvolfia serpentina* L. Bangladesh J. Sci. Ind. Res. **53**(2): 133-138.

Lloyd G and McCown B 1980. Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. Proc. Int. Plant Prop. Soc**. 30**: 420-427.

Misra P, Datta SK, Johri JK, Singh HB and Srivastava A 2004. An improved method for *in vitro* large scale propagation of *Piper betle* L. J. Plant Biochem. & Biotechnol. **13**: 161-164.

Murashige T and Skoog F 1962. A revised medium for rapid growth and bioassays. Physiol.Planta **15**: 473-499.

Nagori K, Singh MK, Alexander A, Kumar T, Dewangan D and Badwaik H 2011. *Piper betle* L.: A review on its ethnobotany, phytochemistry, pharmacological profile and profiling by new hyphenated technique DART-MS (Direct analysis in real time mass spectrometry). J. of Pharmacy Res. **4**(9): 2991-2997.

Parida R and Dhal Y 2011. A study on the micro-propagation and antioxidant activity of *Piper longum* (an important medicinal plant). J. Medicinal Plants Res. **5** (32): 6991 - 6994.

Philip VJ, Joseph D, Treggs GS and Dickonson NM 1992. Micropropagation of black pepper (*Piper nigrum* L.) through shoot tip culture. Plant Cell Rep. **12**: 41-44.

Pradhan D, Suri KA, Pradhan DK and Biswasroy P 2013. Golden heart of the nature: *Piper betle* L. J. Pharmacog. Phytochem. **1**(6): 147.

Sengupta DK, Dasgupta B and Datta P 2011. Management of foot rot of betel vine (*Piper betle* L.) caused by *Phytophthora parasitica*. Journal of Crop and Weed **7**(2): 179-183.

Soniya EV and Das MR 2002. *In vitro* micropropagation of *Piper longum* - An important medicinal plant. Plant Cell, Tiss. Org. Cult.**70**: 325-327.

(Manuscript received on 2 September, 2018; revised on 20 January, 2019)