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EFFECTS OF CARBENDAZIM ON *IN VITRO* MICROPROPAGATION AND FLOWERING OF PEPPER ELDER HERBAL PLANT

E Mohan Raj[1], Teoh Lydia[2], Abdul Bakrudeen Ali Ahmed[1], Muneeb M. Musthafa[3], Vidya Devanathadesikan Seshadri[4], Vijay Lobo[1], R. Vidhyavathi[1], Faiz MMT Marikar[5]*

[1]Department of Biochemistry, Centre for Research and Development (CRD), PRIST Deemed University, Vallam, Thanjavur -613 403, Tamil Nadu, India

[2]Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia

[3]Department of Biosystems Technology, Faculty of Technology, South Eastern University of Sri Lanka, University Park, Oluvil, #32360, Sri Lanka

[4]Department of Pharmacology, College of Pharmacy, Prince Sattam Bin Abdul Aziz University, Kingdom of Saudi Arabia.

[5]General Sir John Kotelawala Defence University, Ratmalana, Sri Lanka

ABSTRACT

The present study reports on application of carbendazim for micropropagation and *in vitro* flowering of *Peperomia pellucida*. *In vitro* multiplication of shoot tips and axillary nodal explants were used in 25-100 mgL^{-1} carbendazim in 70% alcohol treatment for 5 min in MS (Murashige and Skoog) medium and MS hormone medium The explants were cultured on MS medium supplemented with auxins, cytokinins and additives (Casin hydrolyte and glutamine) used to proliferate the shoots. The maximum number of shoots (21.5/shoot tip) were developed on MS medium supplemented with 2.0 mgL^{-1} KIN (Kinetin) 0.5 mgL^{-1} NAA (α- Naphthalene acetic acid), 100 mgL^{-1} casein hydrolysate and 100 mgL^{-1} glutamine additives. The highest number of roots per shoot (17.6) and highest rooting frequency (94.2%) were obtained when the adventitious shoots were inoculated on MS medium with 1.5 mgL^{-1} IAA (Indole-3-acetic acid). The highest number of shoot development (75.4 %) was achieved on MS medium supplemented with 2.0 mgL^{-1} KIN 2.0 + 0.5 mgL^{-1} ABA (Abscisic acid) with 5% sucrose. It was found that MS medium devoid of 1.5 mgL^{-1} IAA promote the root formation and maturation of *in vitro* flower in *P. pellucida*. Acclimatized plantlets were transferred to botanical garden and the characters compared with mother plant of *P. pellucida*.

Keywords: Additives, flower bud, Plant growth regulators, Sucrose, Surface sterilization.

INTRODUCTION

Peperomia pellucida is a medicinal plant belongs to the family Piperaceae and commonly known as pepper elder, shining bush plant and man to man. It is a tropical annual fibrous shallow-rooted herb, which grows as succulent and erect plant (Wagner *et al*. 1999; Majumder 2012). The stem part is fleshy, round, delicate and glabrous (Majumder 2012), whereas bud like structures attached to several fruiting spikes. shiny heart-shaped fleshy leaves are arranged alternately. The flowers are numerous, scattered on the spike with only stamens and an ovary. It has fruits which are minute, and seeds produce mustard-like odor when crushed (Majumder 2012). It is widely distributed in many North American, South American and Asian countries (Bayma *et al*. 2000; Arrigoni-Blank *et al*. 2004) like Bangladesh, Philippines, Thailand, Vietnam, Indonesia, and Japan (Khan *et al*. 2010; Mutee *et al*. 2010).

Different parts of the *P. pellucida* plant have been used as a food and a medicine and hence played a significant role in human life (Craig 1999; Sheikh *et al*. 2013). The plant is used in traditional medicine to cure conditions like abdominal pain, boils, colic fatigue, abscesses,

*Corresponding author: faiz.marikar@fulbrightmail.org

acne, convulsions, fever, gout, headache, renal disorders, , conjunctivitis, skin diseases, breast cancer rheumatic joint pain and to lower blood cholesterol level (Khan *et al*. 2010).

The potential bioactive compounds were isolated and quantified from *P. pellucida* such as flavonoids, apiols, phytosterols, substituted styrenes, secolignans, tetrahydrofuranlignans, highly metaoxylateddihydronaphthalenone, arylpropanoiids, sesamin, isoswertisin, xanthone glycoside and peperomins (Khan *et al*. 2010). Peperomin showed anti-cancer activity, arylpropanoids shown antifungal activity (Wei *et al*. 2011) and flavonoids components showed the antioxidant, anti-neoplastic, anti-ulcer, anti-inflammatory and anti-microbial activities (Majumder 2011).

Plant tissue is widely used for conservation, commercial propagation and production bioactive compounds. The common challenges for plant tissue culture, especially stock plants derived from mature plants have been elimination of culture contaminants and failures of micropropagation protocols. Aseptic conditions has always been a key factor towards successful *in vitro* plant culturing and mass multiplication (Mug'omba *et al*. 2012). A wide range of elicitors have been employed to modify plant metabolism in order to enhance shoot regeneration and pharmaceutical compounds in plant tissue culture (Baskaran *et al*. 2013). *In vitro* flowering approach has proven to be a very useful strategy for the investigation of flowering physiology and the important factors influence the processes such as plant growth regulators (PGRs) , light, carbon source and pH of the medium (Heylen and Vendrig 1998) as well internal factors like explant position, genotype and gene expressed during flowering (Jumin and Nito 1996). Hence, no reports on *in vitro* propagation and *in vitro* flowering of *P. pellucida*.

The aim of this study was to investigate the potential of *in vitro* culture of *P. pellucida*, i) Surface sterilization with suitable surfactant for mass propagation of clonal material, *in vitro* milieu to engage in other applied biotechnologies; ii) PGRs induced the multiple shoot initiation and proliferation with suitable organic elicitors followed by root induction and acclimatization from shoot tip and axillary node explants; iii) this study deals with the development of *in vitro* flower system from shoot tip explants and the subsequent transplantation of the plantlets. Furthermore, we examined the safety of the *in vitro* and *ex vitro* plant material in pharmacological studies, in comparison with field grown plants of *P. pellucida*.

MATERIALS AND METHODS

Surface sterilization of explants

Plants of *P. pellucida* (Voucher Specimen No: KLU47794, University of Malaya Herbarium) were collected from the Botanical garden of Institute of Biological Science, Faculty of Science, Kuala Lumpur. Surface sterilization techniques were tested for the explants used in-shoot multiplication and *in vitro* flowering studies. Initially, axillary node and shoot tip explants of *P. pellucida* were immersed in a2% Teepol solution (4 min) and washed with running tap water (20 min) to remove adherent particles followed by a rinsed off with sterile distilled water (SDW). Further sterilization was done under aseptic conditions of laminar air flow cabinet. Both explants were treated with 70% ethanol (1 min), soaked in 5% sodium hypochlorite v/v (8 min) washed with DW (5 min). Then the explants were treated with a freshly prepared mixture of 0.1% HgCl2 (w/v) for 2 min. In addition, 0.4% (v/v) Carbendazim (Carbendazim powder; dissolved in 70% ethanol), a broad-spectrum systemic fungicide, for 5 min. After each treatment, explants were thoroughly washed with SDW for removal of disinfectants traces. Finally, washed with the distilled water with continuous shaking of explants upto 5 min.

Chemicals and Culture Conditions

Carbendazim (C9H9N3O2) (Kenso Corporation (M) Sdn. Bhd. Malaysia); the PGRs such N6-benzyladenine (BA), Kinetin (KIN), Indole -3-acetic acid (IAA), indole-3-butyric acid (IBA), α-Naphthalene acetic acid (NAA), 2,4-dichlorophenoxy acidic acid (2,4-D), casein hydrolysate (CH), glutamine (GM), Abscisic acid (ABA) and MS medium (Murashige and Skoog, 1962) power were purchased from Sigma-Aldrich Co, Kuala Lumpur, Malaysia and the soap solution Teepol (Anti-bacterial grade,

Teepol & Co, Malaysia). The surface sterilant such as sodium hypochlorite (Clorox, Malaysia), mercuric II chloride (HgCl2), sucrose, Agar-Agar, Ethanol and other basic chemicals were purchased from Hi-media, Kuala Lumpur, Malaysia.

The MS powder dissolved and fortified with 30 g L-1 sucrose (carbon source) and gelled with 0.8% (w/v) agar and the pH of the medium was adjusted to 5.7 ± 0.2 using 0.1N NaOH (Sodium hydroxide) or 0.1 N HCl (Hydrochloric acid) after addition of PGRs. The medium was autoclaved at 121ºC and pressure for 30 min and maintained. The explants were inoculated on MS (medium prepared in culture tube (25 X 150 mm) and plugged tightly with non-absorbent cotton. All the cultures were maintained at 25 ± 2ºC under 16/8 (light/dark) regime provided by cool white, fluorescent light (60 µmol-2 light intensity) with 55-60% relative humidity.

Shoot proliferation and rooting

Shoot tips (3-4 cm) and axillary nodes (4-5 cm length) collected from 4-5 months old *P. pellucida* plants were inoculated into MS medium with different concentrations of plant growth regulators and organic elicitors after surface sterilization and maintained under culture room conditions. The MS medium was supplemented with different concentrations of cytokinins 0.5-2.0 mgL^{-1} 6-benzyl adenine (BA) and kinetin (KN) with 0.1- 2.0 mgL^{-1} NAA, IBA or NAA for multiple shoot initiation and development. For the maximum proliferation of multiple shoots, the explants were treated with optimum concentration (KIN and NAA) combine with organic elicitors of 50- 200 mgL^{-1} casein hydrolyte and glutamine. The number of regenerated shoots per explant was observed and the shoots were visually evaluated after 12 weeks. For root induction, the full-strength MS medium supplemented with different Conc. of auxins, 0.5-2.5 mgL^{-1} IAA, IBA and NAA were used. Root induction (%), root number (mean ± SE), root length (cm), shoot length (cm) and morphological characters were observed at the end of eight weeks.

***In vitro* flowering and rooting**

A rapid and economic *in vitro* flowering protocol was developed from axillary node explants of *P. pelludica*. Explants (axillary nodes) were cultured on MS medium supplemented with 0.5-2.0 mgL^{-1} KIN and 0.1-1.0 mgL^{-1} ABA (Abscisic acid) with sucrose treatment (1-6% sucrose). Flowered shoots were transferred to a MS medium supplemented with 0.5-2.5 mgL^{-1} IAA with 3% sucrose for rooting. Flowered shoots with a well-developed root system were acclimatized and introduced to the field.

Acclimatization

For acclimatization, the rooted plantlets (From both experiments: micropropagation and *in vitro* flowering) were removed from the cultures vessels and rinsed with SDW to remove the adhering agar. Plantlets were established in pots containing sterile soil content mixture of manure, sterile soil and vermiculite [1:1:1 in ratio]. Survival rate (%), flower initiation and maturation were recorded after 6 weeks of hardening. Plantlets were initially covered with a polythene sheet to maintain relative humidity (90%). All survived plantlets were transferred to a greenhouse after 10 weeks and water sprayed in two days interval.

Statistical analysis

In vitro shoot induction, rooting, hardening, *in vitro* flowering and acclimatization were conducted in completely randomized design. The experiments were repeated thrice with a total of 30 replicates per treatment. Experimental data were recorded after two weeks from shoot regeneration of *P. pellucida*. All the data obtained were analyzed using analysis of variance. The means were compared using Duncan's Multiple Range Test at P= 0.05 using SPSS 14.0. The mean values with similar alphabets within columns are not significantly different. The values are the means ± standard error.

RESULTS AND DISCUSSION

Explant Surface sterilization

Explants (axillary nodes and shoot tip) were surface sterilized with different surfactants and carbendazim (0.1, 0.2, 0.4, 0.6 and 0.8% prepared by 70% ethanol). Surface steriliza-

tion with a soap solution (Teepol), 70% ethanol (1 min), 5% NaoCl2 (8 min), 0.1% HgCl2 (2 min) and followed by 0.4% Carbendazim (5 min) was suitable for explant survival (Fig. 1a). The surfactants can prevent initial contamination, but fungi often find their way into cultures at a later date (2 weeks) (Table 1). The contaminated explants were discarded after autoclaving. We have observed that fungal contaminants are faster than the growth of *in vitro* shoot multiplication and are easy to eliminate from explants using fungicides (0.4% carbendazim) and induced the multiple shoots without contamination.

Despite of using sterile techniques and aseptic conditions, contamination of plant cell and tissue cultures remains a persistent problem (Orlikowska *et al*. 2016). Carbendazim is the active ingredient derived from thiophanate methyl and benomyl breakdown process, which are commercially available as Topsin M and Benlate. Earlier days, benomyl has been added to the media and autoclaved; the media contain benomyl degraded to form carbendazim (Maxwell and Brody 1971). Many propagation studies recommended that the Carbendazim is an active surfactant. Successful results have been obtained for purple passion fruit (Prammanee *et al*. 2011) and Indian Siris (Saeed and Shahzad 2015) with0.1% carbendazim.

***In vitro* shoots induction**

Axillary buds and shoot tip explants were induced 2 or 3 shoots with roots and shoots are tall (3-4 cm) in free MS basal medium (Control) (Figure 1b). Explants of *P. pellucida* were cultured on MS medium with different cytokinins [benzyl adenine (BA) and kinetin (KIN); 0.5- 3.0 mgL^{-1}] with auxins (NAA, IAA and IBA; 0.5- 2.0 mgL^{-1}) to evaluate their potency on shoot multiplication (Table 2; Figure 1c- e). The shoot regenerated rate varied significantly between treatment after 12 weeks of culture initiation (Table 2). However, axillary node explants induced shoots which were taller (3.4 cm/ explant) (Figure 2a) than shoot tip explants (Table 2). On the other hand, BA alone, BA combine with NAA, KIN combine with NAA concentration induced the multiple shoots after 12 weeks of culture (Table 2; Figure c- e). The present study indicates that axillary nodes

Table 1: Optimization of disinfectant concentrations (surface sterilization) used in shoot tip and axillary explants of *P. pellucida* Kunth.

Disinfectant	*In vitro* shoot explant survival /Concentration, preparation and sterilization duration			
	Treatment 1 (*)	Treatment 2 (**)	Treatment 3 (***)	Treatment 4 (****)
EtOH	70% (v/v) (1 min) in SDW	70% (v/v) (1 min) in SDW	70% (v/v) (1 min) in SDW	70% (v/v) (1 min) in SDW
NaOCl	3% (v/v) (4 min) in SDW	3% (v/v) (6 min) in SDW	3% (v/v) (8 min) in SDW	3% (v/v) (10 min) in SDW
$HgCl_2$	0.1% (w/v) (30 s) in SDW	0.1% (w/v) (1 min) in SDW	0.1% (w/v) (2 min) in SDW	0.1% (w/v) (3 min) in SDW
Carbendizim	0.4% (w/v) (1 min) in 70% EtOH	0.4% (w/v) (2 min) in 70% EtOH	0.4% (w/v) (3 min) in 70% EtOH	0.4% (w/v) (4 min) in 70% EtOH

EtOH: Ethanol; NaOCl: Sodium hypochlorite; $HgCl_2$: Mercuric chloride; SDW: Sterile distilled water; %: percentage; w: Weight;
v: Volume; *: Multiple shoot initiation and contamination in 2nd week; **: Multiple shoot initiation and contamination in 3rdweek.
: Multiple shoot initiation and contamination absent in 4th week; *: Multiple shoot initiation failed and explant died in 6 week

Table 2: Effect of plant growth regulators on adventitious shoot from axillary node and shoot tip explants of *Peperomia pellucida* (L.)Kunth.

Plant growth regulator mgL^{-1}					Shoot tip explants			Axillary node explants		
					Shoot Development (%)	Multiple shoot / explant	Shoot length (cm) /explant	Shoot Responses (%)	Multiple shoot / explant	Shoot length (cm) /explant
MS free medium					**35.5h**	**2.1 ± 0.16 g**	**1.6 ± 0.16 d**	**29.5g**	**1.9 ± 0.14 g**	**2.1 ± 0.24f**
KIN	**BA**	**NAA**	**IBA**	**IAA**		**Mean ± SE**	**Mean ± SE**		**Mean ± SE**	**Mean ± SE**
1.5	-	-	-	-	64.5 bc	5.8 ± 0.52 d	1.7 ± 0.14 bc	52.6 d	5.2 ± 0.32 d	2.5 ± 0.28 bc
2.0	-	-	-	-	72.8 a	9.2 ± 0.48 a	2.6 ± 0.28 a	60.8 a	7.6 ± 0.28 a	3.4 ± 0.18 a
2.5	-	-	-	-	58.6 d	7.4 ± 0.26 b	1.9 ± 0.16 b	55.2 b	6.2 ± 0.14 b	2.7 ± 0.12 b
-	1.0	-	-	-	48.8 f	4.8± 0.14 f	1.4 ± 0.34 f	45.2 ef	4.6 ± 0.12 de	1.1 ± 0.14 g
-	1.5	-	-	-	65.5 b	6.4 ± 0.62 bc	1.5 ± 0.20 de	52.8 bc	5.4 ± 0.48 bc	2.3 ± 0.28 d
-	2.0	-	-	-	54.2 de	5.2 ± 0.48 de	0.8 ± 0.18 g	46.2 e	3.8 ± 0.72 f	2.2 ± 0.16 de
2.0	1.0	-	-	-	59.5 g	4.2 ± 0.12 ij	1.5 ± 0.16 h	51.0 i	4.8 ± 0.16 hi	2.4 ± 0.42 b
2.0	1.5	-	-	-	67.2 d	5.4 ± 0.20 ef	2.1 ± 0.84 c	55.4 f	6.1 ± 0.52 de	2.5 ± 0.64 a
2.0	2.0	-	-	-	53.4 ij	5.2 ± 0.16 g	1.6 ± 0.62 fg	43.8 l	5.2 ± 0.18 h	2.3 ± 0.18 bc
2.0	-	0.2	-	-	69.8 bc	10.2 ± 0.23 b	1.7 ± 0.38 f	63.4 b	7.9 ± 0.15 bc	1.7 ± 0.54 h
2.0	-	0.5	-	-	81.4 a	14.4 ± 0.28 a	2.4 ± 1.74 b	72.5 a	11.6 ± 0.16 a	2.1 ± 0.18 de
2.0	-	1.0	-	-	72.2 b	9.6 ± 0.56 bc	1.8 ± 1.16 de	59.8 c	8.4 ± 0.32 b	1.5 ± 0.22 jk
2.0	-	-	0.5	-	8.2 gh	6.2 ± 0.18 e	1.1 ± 0.16 l	51.6 gh	5.8 ± 0.48 f	1.7 ± 0.24 h
2.0	-	-	1.0	-	60.5 ef	7.6 ± 0.12 d	1.3 ± 0.14 j	58.8 cd	6.2 ± 0.74 d	2.2 ± 0.32 d
2.0	-	-	1.5	-	48.2 k	4.8 ± 0.24 gh	1.2 ± 0.22 jk	45.2 jk	3.4 ± 0.16 l	1.9 ± 0.14 f
2.0	-	-	-	0.5	62.6 e	3.5 ± 0.16 k	1.4 ± 0.48 hi	46.4 j	4.5 ± 0.32 jk	1.6 ± 0.42 j
2.0	-	-	-	1.0	54.0 i	4.6 ± 0.18 i	2.6 ± 0.24 a	52.0 g	5.4 ± 0.26 fg	1.8 ± 0.18 fg
2.0	-	-	-	1.5	47.2 kl	2.4 ± 0.08 l	1.9 ± 0.12 d	58.7 e	4.6 ± 0.28 j	1.4 ± 0.26 l
-	1.5	0.2	-	-	42.4 g	4.2 ± 0.16 d	1.7 ± 0.24 b	43.5 ef	3.8 ± 0.14 e	2.1 ± 0.16 c
-	1.5	0.5	-	-	51.8 a	5.6 ± 0.22 c	1.3 ± 0.82 f	65.8 a	4.4 ± 0.28 c	2.4 ± 0.32 a
-	1.5	1.0	-	-	45.4 c	8.2 ± 0.18 a	1.8 ± 0.64 a	53.2 b	5.6 ± 0.32 a	1.8 ± 0.44 d
-	1.5	1.5	-	-	43.6 e	6.6 ± 0.34 b	1.4 ± 0.38 de	44.2 e	3.6 ± 0.18 ef	1.3 ± 0.72 g
-	1.5	-	0.2	-	40.8 h	2.8 ± 0.14 h	1.1 ± 0.38 h	36.5 h	2.9 ± 0.42 h	1.6 ± 0.26 f
-	1.5	-	0.5	-	48.5 b	3.4 ± 0.26 de	1.2 ± 0.12 fg	52.6 bc	3.5 ± 0.72 g	1.7 ± 0.74 de
-	1.5	-	1.0	-	43.2 ef	3.0 ± 0.16 f	1.5 ± 0.14 d	48.4 d	5.2 ± 0.42 b	2.3 ± 0.64 b
-	1.5	-	1.5	-	44.6 cd	2.6 ± 0.12 fg	1.6 ± 0.26 bc	41.0 g	4.1 ± 0.18 cd	1.1 ± 0.24 h

Rs = Plant growth regulators, (%) = percentage, cm = centimetre; Values are mean ± standard error (SE). Mean followed by same letters in each column are not significantly different ($p = 0.05$) using Duncan's multiple range test.

produce taller shoots. Other physiological disorder commonly observed in *in vitro* cultures affecting a wide range of plants depends on the type and concentration of cytokinins in the culture medium.

Axillary node explants cultured on MS medium with 2.0 mgL^{-1} KIN and 0.5 mgL^{-1} NAA exhibit the multiple shoots (11.6 /shoot) and produced tall shoots with less number (Figure 2 b- c) than shoot tip explants (Table 2). After 12 weeks, cultured shoot tip explants 2.0 mg L-1KIN with 0.5 mgL^{-1} NAA produced the multiple shoots 14.4/ explant (Figure d and e) and the results were better than axillary node explants (Table 2). As well, we have tested without cytokinins of shoot multiplication in shoot tip and axillary node explants; explants were produced single shoot with branched roots (Figure 1f and g) in MS medium supplemented with IAA, IBA and NAA treatments (data not shown).

So far, no *in vitro* propagation report in the plants, but species protocols are different from the previous reports. A low auxin con-

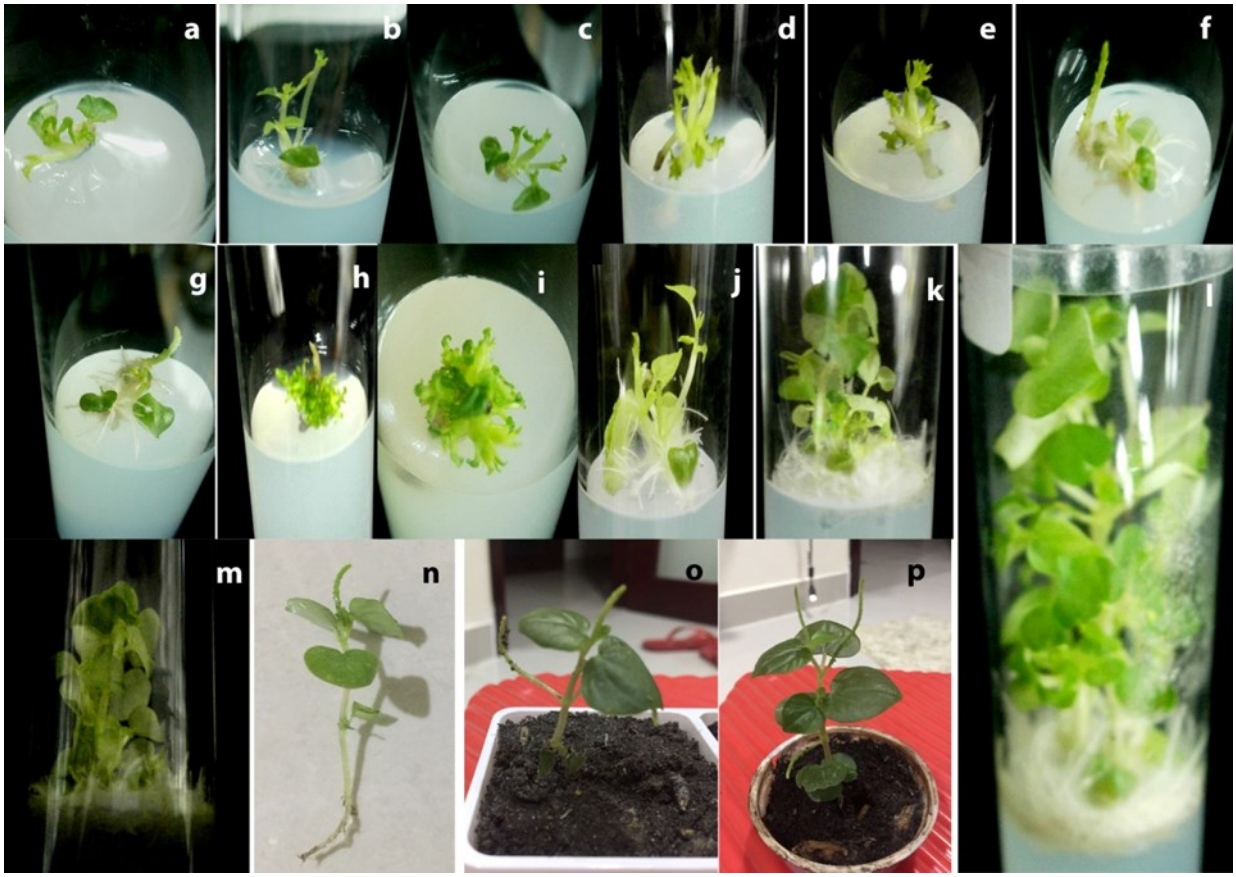

Figure 1: *In vitro* propagation from shoot-tip explants of *Peperomia pellucida*(L.) Kunth. (a) Multiple shoots developing from shoot-tip explants on MS medium with 2.0 mg L^{-1} KIN after 4 week of culture, (b) Shoot multiplication on MS medium free medium without plant growth regulators, (c) Shoot proliferation with shoot necrosis on MS medium with 1.5 mg L^{-1} BA, (d) Adventitious shoot regeneration on MS medium with 2.0 mg L^{-1} KIN and 0.5 mg L^{-1} NAA, (e) Shoot multiplication on MS medium with 1.5 mg L^{-1} BA and 1.0 mg L^{-1} NAA, (f) Multiple shoot proliferation absent in MS medium with 0.5 mg L^{-1}NAA Without KIN treatment, (g) Adventitious shoots were absent and root induction in MS medium with 1.0 mg L^{-1} IBA without BA treatment, (h) Multiple shoot proliferation and shoot necrosis on MS medium with 1.5 mg L^{-1} BA and 0.5 mg L^{-1} NAA with 100 mg L^{-1} casein hydrolysate and 100 mg L^{-1} glutamine, (i) Production of adventitious shoots development in MS medium with 2.0 mg L^{-1} KIN and 0.5 mg L^{-1} NAA with 100 mg L^{-1} casein hydrolysate and 100 mg L^{-1} glutamine, (j) Roots induction in MS medium with 1.5 mg L^{-1} IBA, (k) Roots proliferation and shoot development in MS medium with 1.5 mg L^{-1} IAA, (l-m) Maximum shoot length and root proliferation, (n) Rooting of shoot derived from the root culture, (o-p) Acclimatized plants with ex vitro flowering from shoot tip explants after 3 months.

centration combination with high cytokinins concentration promoted shoot proliferation in Crambetataria (Piovan *et al*. 2011)andP. Obtusifolia (Hany and Amira 2014)belongs to the family Piperaceae. *In vitro* propagation is an excellent technique to obtain maximum number of plants within a short period of time . Our studies showed that axillary node developed shoots were tall as well less shoot indicating that cytokinins-auxin have shown the synergistic effect in shoot proliferation (Table 2). Results were agreed with the previous reports of KIN and NAA was the most appropriate concentration for *in vitro* shoot multiplication of other species medicinal herbs of *Bauhinia racemosa* (Sharma et al. 2017).

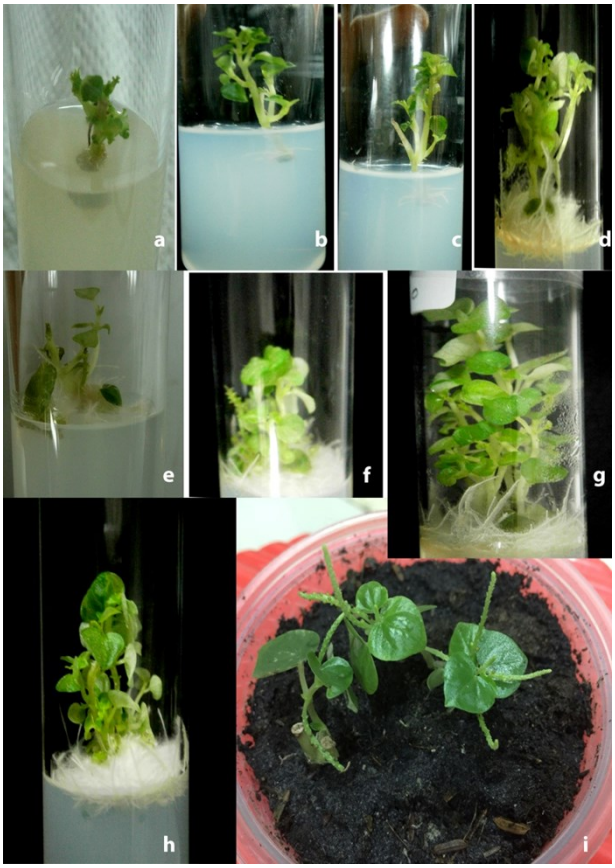

Figure 2: *In vitro* propagation from axillary node explants of *Peperomia pellucida* (L.) Kunth. (a) Multiple shoot initiation from axillary node explants on MS medium with 2.0 mg L^{-1} KIN after 6weeks of culture, (b) Adventitious shoot regeneration on MS medium with 1.5 mg L^{-1} BA, (c) Shoot multiplication on MS medium with 2.0 mg L^{-1} and 0.5 mg L^{-1} NAA, (d) Root induction and proliferation on MS medium with 1.5 mg L^{-1} IAA, (e) Root proliferation and shoot development on MS medium with 1.5 mg L^{-1} IBA, (f) Adventitious shoot and root development on MS medium with 1.5 mg L^{-1} NAA, (g-h) Maximum shoot and root development from axillary node explants; (i) Acclimatized plants with ex vitro flowering from axillary node explants after 3 months.

Multiple shoots proliferation with organic elicitors

Present study established the multiple shoot proliferation protocol with organic elicitors (casein hydrolysate and glutamine), cytokinins of KIN and BA combination with auxins of NAA was examined in shoot tip explants of *P. pellucida* (Table 3). The prolonged culturing in the same medium did not increase the shoot regeneration frequency (Table 2) and in order to achieve a higher shoot proliferation, effects of casein hydrolysate and glutamine were tested (Table 3). Optimum response was (21.5 shoots per explant after 12 weeks of culture; Figure 1i) obtained in MS medium supplemented with 2.0 mgL^{-1} KIN and 0.5 mgL^{-1} NAA with 100 mgL^{-1} glutamine and 100 mgL-1 casein hydrolysate. The results were significantly higher than the results of the treatment with 1.5 mgL-1 BA and 0.5 mgL^{-1} NAA (Fig. 1h). In many studies, the organic elicitors are regulating the biochemical entities which have prolific influence on plant growth and multiple shoot development as they control vital cellular processes including DNA replication, fruit development, senescence and secondary metabolite production under abiotic and biotic stress conditions (Baskaran and Van Staden, 2011; Cvrckova *et al*. 2014).

***In vitro* rooting**

In this study, the effect of different auxins (IAA, IBA, NAA and 2,4-D) were evaluated for *in vitro* rooting of *P. pellucida*. *In vitro* generated shoot tips were transferred to rooting MS medium supplemented with IAA, IBA, NAA and 2,4-D treatment. Our results showed that IAA induced the highest rooting percentages (94.2%), greater root numbers (17.6), longer root length (2.9 cm) and shoot length (10.5 cm) than NAA and IBA treatment after 8 weeks (Table 3; Fig. 1j-m). As well, root induction, proliferation and shoot length was observed in axillary node explants

(Figure 2 d-h); whereas NAA, IBA and 2,4-D treatment induced roots were lesser (Fig. 2e-f) than IAA treatment in axillary buds (data not shown). As well, multiple shoots of axillary node explants were tried for root induction and proliferation (Fig. 2g-h) and the results were significantly lower than shoot tip explant rooting treatment (data not shown). Without PGRs, free MS media induced the less roots at the end of 8 weeks (Table 3). The highest rooting percentage, up to 94.2%, occurred in 1.5 mg L-1 IAA (Figure 1l and m); while IAA concentrations either lower or higher 1.5 mg L-1 had significantly lower percentages (Table 4). Whereas the other rooting treatments such as IBA, NAA and 2,4-D (0.5-2.0 mg L-1) induced lesser roots and length of roots were poor than IAA treatment. The higher concentrations of IAA, IBA, NAA and 2,4-D induced basal callus and the shoot was shorten with less root number (data not shown). We have observed the root numbers (17.5 roots per shoot induced by 1.5 mg L-1 IAA and the mean root length 2.9 cm (Fig. 1m) and the shoot length (10.54 cm) / culture and the similar type of results were observed in *Salvadora persica* (Kumari et al. 2017).

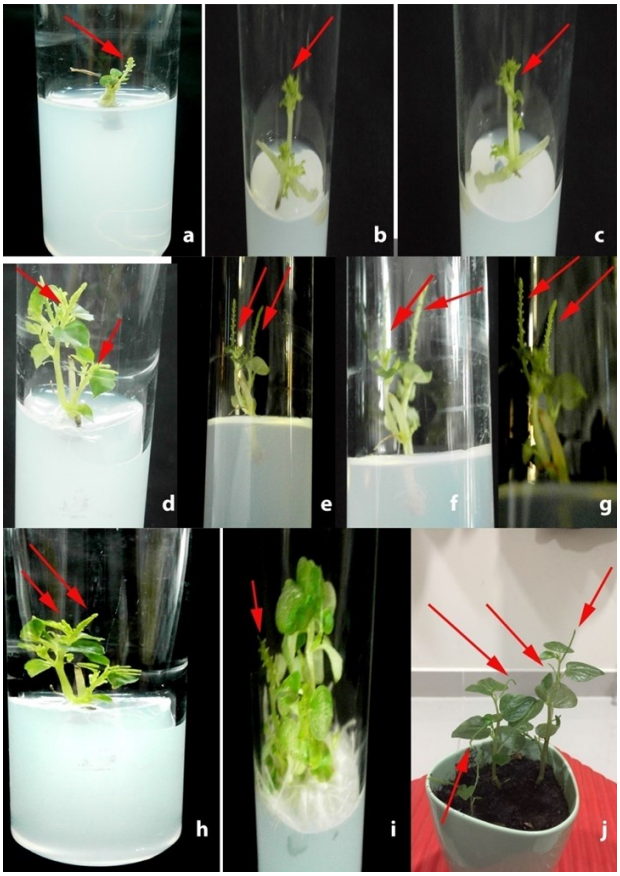

Figure 3: *In vitro* flowering from shoot tip explants of *Peperomia pellucida* (L.) Kunth. *In vitro* flower induction on MS medium with 2.0 mg L^{-1} KIN and 0.5 mg L^{-1} ABA with 4% sucrose, (b) *In vitro* flower bud initation on MS medium with 2.0 mg L^{-1} KIN and 0.5 mg L^{-1} ABA with 5% sucrose, (c) *In vitro* flower bud initiation on MS medium with 2.0 mg L^{-1} KIN and 0.5 mg L^{-1} ABA with 5% sucrose, (d) Multiple shoots with *in vitro* flower developing branches of flower on MS medium with 2.0 mg L^{-1} BA and 0.5 mg L^{-1} ABA, (e-g) Maximum length of *In vitro* flowers on MS medium with 2.0 mg L^{-1} KIN and 0.5 mg L^{-1} ABA with 5% sucrose after 12 weeks; (h) *In vitro* flower root induction on MS medium with 1.5 mg L^{-1} IAA, (i) *In vitro* flower with root proliferation on MS medium with 1.5 mg L^{-1} IAA after 16 weeks, (j) Acclimatized plants with *ex vitro* flower with new leaves, new flower initation and old flower maturation after 20 weeks.

In vitro flowering and rooting

A rapid and economic *in vitro* flowering protocol was developed from shoot tip explants of *P. pelludica* (Fig. 3a-j). Sterile explants (shoot tip) were cultured on MS medium supplemented with KIN, BA, and ABA with sucrose treatments and flowers were induced after 6 weeks of culture initiation (Fig. 3a). Maximum number of flowers were induced on MS medium containing 2.0 mgL^{-1} KIN and 0.5 mgL^{-1} ABA (Abscisic acid) with 5% sucrose after 12 weeks (Table 5; Fig. 3d-e). As well, we have noticed the flower bud induction on MS medium supplemented with 2.0 mg L^{-1} KIN and 0.5 mg L^{-1} ABA with 5% sucrose (Fig. 3b-c). This combination yielded the highest number of flowers (4.7) / shoot and response (75.4%) (Fig. 3d-g) after 12 weeks. Whereas KIN and ABA with different percentage of sucrose were (4% and 6%) induced flower buds which did not mature until 12 weeks. Flowers were emerged from the branch region of the developing shoots of *P. pellucida*. On the other hand, the flower production was absent in MS medium supplemented with KIN and ABA with 2% and 3% sucrose treatments (data not shown). Maximum number of roots were obtained from the medium supplemented with 1.5 mgL-1 IAA and 3% sucrose after 16 weeks (Fig. 3h-i). Well-developed plantlets were hardened in pots (100 x 125 mm) (Fig. 3j) after 20 weeks.

In the present study, the flower inducing agent ABA combination with KIN with 5% sucrose was found to be responsible for *in vitro* flowering. Cytokinins in medium are considered as the main component which induce flowering. The similar result was obtained in *in vitro* flower development in *Brachystelma glabrum* (Revathi *et al*. 2017). Flower induction and development in responses to exogenous cytokinins have been observed in few medicinal plants; as *Ipomoea sepiaria* (Cheruvathur *et al*. 2015). We have observed 5% sucrose was suitable for the flower initiation and maturation in *P. pellucida* than other sucrose treatments. Similar result was observed in Morusindica (Gogoi *et al.* 2017).

Acclimatization

Acclimatization was divided in to two experiments: i) Acclimatization of plantlets obtained from axillary node and shoot tip explants. (Fig. 1/p); ii) Acclimatization of plantlets obtained from the *in vitro* flowering experiment. The hardened plantlets were covered with transparent plastic polythene bags in order to retain moisture and avoid transient shock. To allow proper aeration, small pores were made on plastic bag and water sprayed at regular intervals.

Each set consisted of 25 pots (100 x 125 mm) (Experiment 1: without flower/pot; Experiment 2: with flower/pot. Shoot tip and axillary node derived plantlets were hardened and acclimatization in the green house (Fig. 1n-p and Fig. 2i) after 2 month , the acclimatized plants showed flowers (Fig. 1n-p and Fig. 2i).

In vitro flowered plantlets were produced new leaves and new flowers with shoot development (Fig. 3j). All plantlets were transferred to pots containing a mixture of manure, sterile soil and vermiculite [1:1:1 in ratio] and grown under culture room conditions with humidity level regulated at 50%, temperature at 25±2°C and photoperiod of 16 hours light and 8 hours dark. Experiment 1, the hardened plantlets survival rate was significantly higher than experiment 2 of *in vitro* flowering stud-

Table 3: Effect of organic elicitors (casein hydrolysate, glutamine) and optimum PGRs on adventitious shoot from shoot tip explants of *Peperomia pellucida* (L.) Kunth.

Multiple shoot proliferation with additives							Rooting							
PGRs (mg L^{-1})		Additives (mg L^{-1})			Shoot Response (%)	Multiple shoot / explant	Shoot length (c.m) / explant	PGRs (mg L^{-1})				Responses (%)	Root number	Root length (c.m)	Shoot length (c.m)
KIN + NAA	BA + NAA	CH	GL		Mean ± SE	Mean ± SE	Mean ± SE	IAA	IBA	NAA	2,4-D		Mean ± SE	Mean ± SE	Mean ± SE
								MS Free				36.4 j	4.9 ± 1.44 lm	1.3 ± 0.72	3.8 ± 0.86
2.0 + 0.5	-	50	-		81.4c	14.8 ± 1.28 b	2.6 ± 0.84 b	1.0	-	-	-	89.0 ab	12.4 ± 2.34 c	2.4 ± 0.32 bc	8.1 ± 1.16 bc
2.0 + 0.5	-	100	-		95.0a	21.5 ± 1.08 a	3.4 ± 0.38 a	1.5	-	-	-	94.2 a	17.6 ± 1.78 a	2.9 ± 0.26 a	10.5 ± 1.74 a
2.0 + 0.5	-	150	-		88.2b	12.4 ± 1.32 de	1.8 ± 0.16 fg	2.0	-	-	-	75.8 d	15.2 ± 1.82 b	2.5 ± 0.68 b	7.9 ± 1.14 d
2.0 + 1.0	-	-	50		64.2ij	10.6 ± 1.26 fg	1.9 ± 0.28 f	-	1.0	-	-	72.4 de	9.8 ± 1.42 ef	1.8 ± 0.86 e	7.1 ± 0.82 e
2.0 + 1.0	-	-	100		78.6e	14.2 ± 1.10 bc	2.1 ± 0.64 de	-	1.5	-	-	82.0 c	11.2 ± 1.72 d	2.2 ± 0.64 d	8.5 ± 1.32 b
2.0 + 1.0	-	-	150		71.4gh	9.0 ± 0.86 hi	1.7 ± 0.14 h	-	2.0	-	-	65.6 f	10.1 ± 1.26 e	1.7 ± 0.26 ef	6.3 ± 1.28 f
-	1.5 + 0.5	50	-		73.8g	9.4 ± 1.34 h	1.6 ± 0.26 hi		-	1.0	-	53.8 h	8.3 ± 1.54gh	0.8 ± 0.84 i	4.2 ± 0.54 j
-	1.5 + 0.5	100	-		80.6cd	12.8 ± 1.16 d	2.4 ± 0.14 bc		-	1.5	-	65.4 fg	9.1 ± 1.86 g	1.1 ± 0.62 gh	5.7 ± 0.82 fg
-	1.5 + 0.5	150	-		65.4i	8.8 ± 0.68 j	2.2 ± 0.68 d		-	2.0	-	51.2 hi	7.1 ± 1.38 i	0.6 ± 0.12 ij	4.5 ± 0.96 hi
-	1.5 + 1.0	-	50		63.8k	7.5 ± 0.54 k	1.5 ± 0.34 jk				1.0	24.0 lm	6.1 ± 1.65 j	0.4 ± 0.26 kl	2.6 ± 0.52 m
-	1.5 + 1.0	-	100		76.2ef	10.8 ± 0.72 f	1.6 ± 0.18 hi				1.5	32.6 jk	6.8 ± 1.05 ij	0.5 ± 0.44 k	4.6 ± 0.26 h
-	1.5 + 1.0	-	150		59.5l	7.2 ± 0.58 kl	1.2 ± 0.42 l				2.0	25.4 l	5.2 ± 1.28 l	0.3 ± 0.32 m	3.4 ± 0.34 l

ies after 2 months. Plant field growth (%) and survival rate were recorded until 6 months.

CONCLUSION

The present work studied *in vitro* shoot induction and multiplication from axillary and shoot tip explants of *P. pellucida*. A combination of 2.0 mgL^{-1} KIN, 0.5 mgL^{-1} NAA, 100 mgL^{-1} glutamine and 100 mgL^{-1}casein hydrolysate promoted the maximum shoot production. Successful rooting was achieved on full strength MS medium fortified with 1.5 mgL^{-1}

Table 4: Effects of sucrose and plant growth regulators with ABA for *in vitro* flowering in *Peperomia pellucida* (L.) Kunth.

PGRs (mg L^{-1})		ABA mgL^{-1}	Sucrose (Carbon source)					
			In vitro flowering (%)			Number of flower /shoot (Mean ±SE)		
KIN	BA		4%	5%	6%	4%	5%	6%
1.5	-	0.2	54.3 ij	61.8 k	59.4 i	1.2 ± 0.14 qr	2.8 ± 0.16 f	1.4 ± 0.26 k
1.5	-	0.5	63.6 bc	68.9 cd	65.0 bc	1.6 ± 0.26 jk	3.2 ± 0.14 d	1.8 ± 0.42 cd
1.5	-	1.0	47.8 j	55.6 p	61.8 h	1.5 ± 0.24 l	2.6 ± 0.82 ef	1.4 ± 0.32 g
2.0	-	0.2	63.8 b	67.2 e	65.6 b	1.4 ± 0.26 m	3.5 ± 0.84 c	1.2 ± 0.08 lm
2.0	-	0.5	67.4 a	75.4 a	69.8 a	2.8 ± 0.16 a	4.7 ± 0.64 a	2.4 ± 0.10 a
2.0	-	1.0	55.8 gh	71.4 b	64.2 d	2.6 ± 0.14 b	4.2 ± 0.52 b	1.6 ± 0.36 e
2.5	-	0.2	54.6 i	65.4 gh	62.5 f	1.6 ± 0.14 j	2.2 ± 0.42 i	1.2 ± 0.34 l
2.5	-	0.5	61.4 cd	68.8 cd	64.0 de	2.6 ± 0.18 bc	3.2 ± 0.16 de	2.2 ± 0.16 b
2.5	-	1.0	52.8 k	61.5 kl	58.4 k	1.2 ± 0.34 s	2.6 ± 0.18 g	1.4 ± 0.28 gh
-	1.5	0.2	48.5 op	53.4 r	54.6 mn	1.8 ± 0.16 hi	1.6 ± 0.54 jk	1.2 ± 0.36 n
-	1.5	0.5	56.8 g	65.6 g	62.4 fg	2.2 ± 0.18 f	1.8 ± 0.72 j	1.6 ± 0.12 ef
-	1.5	1.0	51.4 m	58.8 o	55.0 m	1.8 ± 0.32 h	1.2 ± 0.58 mn	1.2 ± 0.42 no
-	2.0	0.2	59.2 e	62.0 ij	54.2 o	1.2 ± 1.34 o	1.4 ± 0.16 m	1.4 ± 0.34 i
-	2.0	0.5	62.4 c	66.8 ef	59.0 ij	2.4 ± 1.16 d	2.4 ± 0.26 gh	1.8 ± 0.26 c
-	2.0	1.0	52.6 kl	59.6 m	48.6 p	1.2 ± 0.68 op	1.6 ± 0.38 l	1.2 ± 0.18 p
-	2.5	0.2	49.5 o	59.4 mn	43.2 r	1.4 ± 0.24 mn	1.2 ± 0.14 o	1.4 ± 0.14 ij
-	2.5	0.5	58.4 ef	62.8 i	56.8 l	1.8 ± 0.52 fg	1.4 ± 0.34 lk	1.2 ± 0.32 pq
-	2.5	1.0	51.2 mn	55.0 pq	45.6 q	1.2 ± 0.48 q	1.2 ± 0.42op	1.2 ± 0.24 r

PGRs = plant growth regulators; ABA = Abscisic acid; (%) = percentage; Values are mean ± standard error (SE). Mean followed by same letters in each column are not significantly different (p = 0.05) using Duncan's multiple range test.

Table 5: Influence of IAA and IBA on rooting and flower maturation of *In vitro* flowering shoots of *Peperomia pellucida* (L.) Kunth.

PGRs (mg L^{-1})		Root responses (%)	Rooting with shoot and *in vitro* flower development (Mean ±SE)			
IAA	IBA		Root number	Root length (cm)	Shoot length (cm)	No. of flower maturation
1.0	-	78.8 d	13.2 ± 1.46 bc	1.7 ± 0.42 ab	7.3 ± 1.14 b	2.8 ± 0.84 bc
1.5	-	89.2 a	15.2 ± 1.24 a	1.9 ± 0.26 a	8.4 ± 1.36 a	3.5 ± 0.78 a
2.0	-	80.8 bc	13.8 ± 1.18 b	1.6 ± 0.28 c	6.8 ± 1.08 d	2.4 ± 0.56 de
-	1.0	72.8 e	9.6 ± 1.32 e	1.3 ± 0.16 ef	5.4 ± 1.16 e	2.6 ± 0.56 d
-	1.5	83.4 b	11.4 ± 1.44 d	1.5 ± 0.58 cd	6.8 ± 1.42 d	3.1 ± 0.64 b
-	2.0	70.6 ef	8.2 ± 1.04 ef	1.4 ± 0.86 e	4.5 ± 0.86 f	1.9 ± 0.32 f

IAA = Indole -3-acetic acid; IBA = Indole-3-butyric acid; (%) = percentage; cm = centimetre; Values are mean ± standard error (SE). Mean followed by same letters in each column are not significantly different (p = 0.05) using Duncan's multiple range test.

IAA and the rooted plantlets were successfully hardened in tissue culture conditions. *In vitro* flowering protocol succeed in 2.0 mgL^{-1} KIN, 0.5 mgL^{-1} ABA with 5% sucrose and showed 4.7 flowers/shoot followed by rooting and hardened and the characters compared with mother plant. Acclimatization protocols were uniform, healthy and high survival rate after transplantation to field of environment.

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