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EFFECT OF PLANT GROWTH REGULATORS IN MICROPROPAGATION OF *PIPER LONGUM* LINN. - AN ENDANGERED MEDICINAL PLANT

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India has one of the richest plant based ethno-medicinal traditions in the world. There is need for conservation of all useful plant species, and also cultivation, maintenance and assessment of germplasm for future use. Conservation of endangered medicinal plants such as *Piper longum* Linn are more successful through micropropagation than conventional plant breeding methods. In the present study, direct embryogenesis and multiple organogenensis were induced using petioles and young leaves as explants in MS media. Maximum frequency of direct embryogenesis was observed in petiole in MS media supplemented with hormones IBA and BA (1.5 mg/l IBA and 2.5mg/l BA). Direct organogenesis with shoot induction was well supported in petiole in MS media supplemented with hormones of *BA* and *K* (1.5 mg/l BA and 0.5mg/l K). Maximum frequency of direct organogenesis with root induction was observed in leaf in MS media supplemented with 0.5 mg/l IBA and 1.5 mg/l K. In the indirect organogenesis with shoot induction, maximum frequency was observed in MS media supplemented with IBA and BA (1 mg/l IBA and1.5mg/l BA) in leafy explants. Increase of indirect organogenesis with root induction was more successful in MS media supplemented with 1.5 mg/l IBA and 2.5 mg/l K. The obtained results may be useful for further studies and large scale multiplication for conservation of this very important endangered medicinal plant.

Keywords: Embryogenesis; Multiple shooting; *Piper longum;* Plant growth regulators.

Introduction

Medicinal plants constitute a very important national resource to India. The plant-based drugs, however, have shortened the lifespan of the source of material. There is continuous search for more potent and cheaper raw material to feed the industry. For the conservation and rapid propagation of medicinal plants tissue culture system offers advantages at many levels including very high multiplication rates[1]. Medicinal plant micropropagation can provide plants to be field planted for later chemical extraction of bioactive molecules, conservation of useful plant species, and also maintenance and assessment of germplasm for future use, since among the most vulnerable plant species in India, the most over-exploited are the medicinal plants. *In vitro* culture of plant cells has many applications in the search of bioactive molecules and medicinal plant research[2].

Piper longum Linn. is a slender, aromatic plant cultivated for its fruit, which is usually dried and used as a spice and seasoning. It is commonly called Pippali or Tippali. The compound of medicinal value is mainly present in the female spike (inflorescence). *P. longum* contains alkaloid piperine as one of the active ingredient. Other alkaloids include piperine, piperettine, piperolein A, piperolin B, piperanine and trichostachine[3]. The volatile oil contains large amounts of terpenes. The dry female spike of *P. longum* is widely used in Ayurvedic and Unani systems of medicine particularly for diseases of respiratory tract most of them include cough, bronchitis and asthma[4]. Several compounds of medicinal use were reported to be isolated and used for various studies like antibacterial, antifungal, lavicidal and antiamoebal screening[5-8]. The objectives of present study include *in vitro* clonal propagation of *P. longum* through various tissue culture techniques by finding suitable hormonal combination for maximum callus induction, embryogenesis, direct and indirect organogenesis and mass production of *P. longum* by multiple shooting using leaf and petiole as explants. Effect of plant regulators in micropropagation of *P. longum* is reported in the present paper.

Material and Methods

One-year-old *Piper longum* Linn. plants growing in the botanic garden of University College, Thiruvananthapuram served as the source of explants

Table 1. Hormone combinations in various concentrations that induced callus induction in petiole.

Media	Plant Growth Regulator (PGR)	Concentration (mg/ml)	Duration (days)	Percentage
1	2,4 D BA	0.5 0.1	61	58
2	2,4 D BA	1.0 0.1	61	68
3	BA K	0.5 0.1	82	52
4	BA K	0.5 0.5	40	60
5	BA K	1.5 0.5	40	66
6	BA K	2 0.5	40	66
7	BA K	2.5 0.5	40	68
8	IBA K	0.5 1.5	25	50
9	IBA BA	1 1.5	25	48

(petioles and leaves) for *in vitro* experiments. The explants were surface sterilized with mercuric chloride (0.1%) for 7-8 min and washed in sterile double distilled water before inoculating in Murashige and Skoog basal medium[9] with various hormone combinations. Auxins - IAA, IBA, NAA, 2,4D and cytokinins BA and K at concentrations ranging from 0.1mg/l to 2.5 mg/l were tried alone and in various combinations, in basal MS medium. Effects of various combinations of cytokinins alone were also tried. Only fresh material was used and work was done in triplicates with minimum 10 explants. Callus was subcultured into MS media with different hormone combinations ranging from 0.1mg/l to 2.5 mg/l. Calli with roots were inoculated into the sub culturing media supplemented with various hormone combinations for shoot induction. Observations were done and noted in every second day. Number of days for initiation of callus, embryogenesis, shooting, rooting etc and number of explants producing callus, embryos, shoots and roots were calculated.

Results and Discussion

Callus induction, embryogenesis and organogenesis was observed starting from one week after inoculation up to four months.

Callus induction - When petiole was used as explants, callus was induced in 68 % of them in MS media supplemented with 2,4 D and BA (1.0mg/l and 0.1mg/l, respectively) after 61 days. When MS media with Plant Growth Regulators IBA and K were tried in different concentrations, callus was induced in 40 days with 68% callusing, with IBA 2.5 mg/l and K 0.5 mg/l. But IBA and K, in different concentrations, callus was induced in 50 % in 25 days (IBA 0.5 mg/l and K 1.5 mg/l) and 48 % in 25 days (IBA 1mg/l and BA 1.5 mg/l) (Table 1) [Fig. 1(a)]. In the case of leafy explants callus was induced in 68% in MS media with hormone 2,4-D (0.1mg/l) alone after 61 days. When hormone IBA and BA was used in different concentrations, IBA 1.5 mg/l and BA 1 mg/l and IBA 1.5 and BA 2.5 mg/l callus was in induced in 25 days with 60 % and 62% efficiency. In case of combination IBA and K (1 and 0.5mg/l, 1.5 and 0.5mg/l, 2 and 0.5mg/l) callus was induced in 40 days but third combination induced 55% callusing while other two induced 50% callusing (Table 2) [Fig. 1(b)].

Embryogenesis - In the case of petiole explants somatic embryo were noticed in MS media with different hormonal combinations. The petioles inoculated in MS medium with BA (2mg/l) alone produce direct embryogenesis after 40 days with 40% efficiency. IBA alone (0.5mg/l) in MS

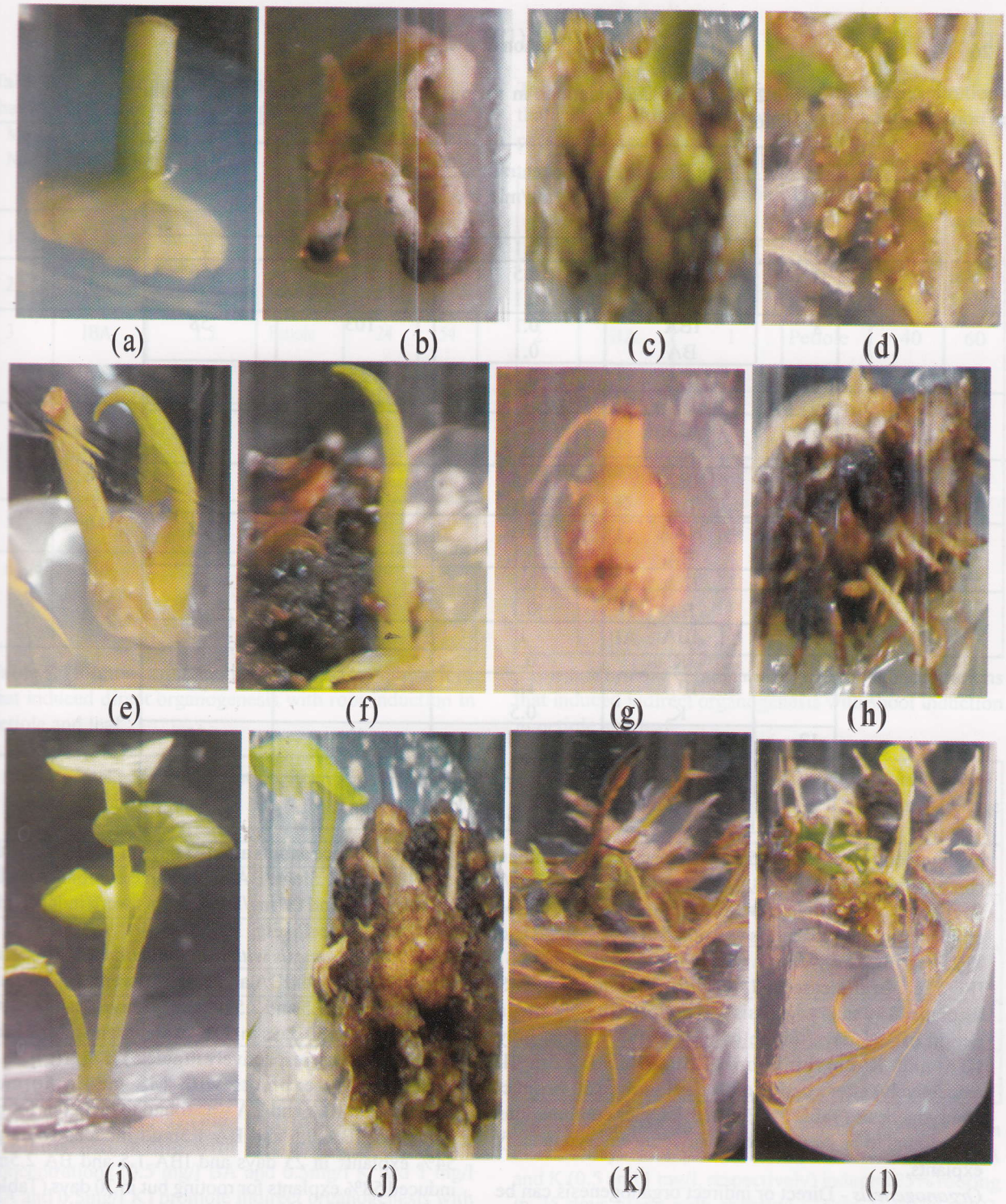

Fig.1. Showing callusing, embryogenesis, shooting and rooting in *Piper longum* L
(a) Callus induction in petiole (b) Callus induction in leaf (c) Embryogenesis in petiole
(d) Embryogenesis in leaf (e) Shoot induction in petiole (f) Shoot induction in leaf
(g) Root induction in petiole (h) Root induction in leaf (i) Multiple shooting in petiole
(j) Multiple shooting in leaf (k) Multiple shooting and rooting in petiole derived callus
(l) Multiple shooting and rooting in leaf derived callus.

Table 2. Hormone combinations in various concentrations that induced callus induction in leaf.

Media	Plant Growth Regulator (PGR)	Concentration (mg/ml)	Duration (days)	Percentage
1	2,4 D	0.1	61	68
2	2,4 D BA	0.5 0.1	61	66
3	IBA BA	0.1 0.1	103	58
4	IBA BA	0.5 1	25	58
5	IBA BA	1.5 1	25	60
6	IBA BA	1.5 2.5	25	62
7	NAA BA	0.2 0.1	112	28
8	IAA BA	0.1 0.1	104	30
9	IBA K	1 0.5	40	50
10	IBA K	1.5 0.5	40	50
12	IBA K	2 0.5	40	50
13	IBA K	2.5 0.5	40	55

medium took 40 days with 50% efficiency while hormone combination of IBA and BA (1.5 mg/l IBA and 2.5mg/l BA) took only 21 days with 54 % efficiency. Same efficiency in embryogenesis was noted in IBA 1.5mg/l and K 2.5mg/l but only after a period of 24 days. (Table 3) [Fig. 1c]. In the case of IAA alone (0.01mg/l) in MS medium 48 % embryogenesis was noticed but only after a period of 104 days. In the case of leafy explants, only IBA and K combination (1.5mg/l and 2.5mg/l, respectively) after a period of 24 days showed 50 % direct embryogenesis (Table 3) [Fig. 1(d)]. Other combinations were found to be ineffective for inducing embryo in leafy explants.

Organogenesis - Direct or indirect organogenesis can be induced in MS medium with different hormone combinations.

Direct organogenesis with shoot induction - In the case of petiole explants, 60 % was induced shooting in BA 1.5 mg/l and K 0.5mg/l and BA 1 mg/l and K 0.5mg/l after 40 days. IBA 0.5 mg/l and K 1.5 mg/l induced shooting in 25 days but with 50% efficiency (Table 4) [Fig. 1(e)]. Leafy explants produce shoots after 25 days in MS media with 1 mg/l IBA and 1.5mg/l BA but in some combinations like 0.5 mg/l IAA and 0.1mg/l BA shoots were induced after 104 days (Table 4) [Fig. 1(f)].

Direct organogenesis with root induction - When petiole is used as explants IBA and K in different concentration (1 and 1.5mg/l, 1.5 and 2.5mg/l, respectively) showed root induction after 30 days in 50% explants and after 25 days in 56% explants, respectively (Table 5) [Fig. 1(g)]. In the case of leafy explants with MS media hormone combination IBA and BA was found to be better for root induction. IBA 0.5 and BA 1.5mg/l induced rooting in 54% explants in 25 days and IBA 1.5 and BA 2.5mg/l induced 58% explants for rooting but in 30 days (Table 5) [Fig. 1(h)].

Indirect organogenesis with shoot induction -Callus from petiole showed indirect organogenesis in MS media with various hormone combinations. IBA and BA at different concentration (0.5 and 1mg/l, 0.5 and 0.1mg/l, respectively) showed indirect organogenesis with 40% shooting after 25 days and 38% shooting 40 days

Table 3. Hormone combinations in various concentrations that induced embryogenesis in petiole and leaf.

Media No.	Plant Growth Regulators	Concentration (mg/l)	Explant	Duration (days)	Per-centage
1	IAA	0.1	Petiole	104	48
2	IBA	0.5	Petiole	40	50
3	IBA BA	1.5 2	Petiole	24	54
4	IBA BA	1.5 2.5	Petiole	21	54
5	BA	2	Petiole	40	40
6	IBA K	1.5 2.5	Leaf	24	50

Table 4. Hormone combinations in various concentrations that induced direct organogenesis with shoot induction in petiole and leaf.

Media No.	Plant Growth Regulators	Concentration (mg/l)	Explant	Duration (days)	Per-centage
1	BA K	0.5 0.5	Petiole	40	58
2	BA K	1 0.5	Petiole	40	60
3	BA K	1.5 0.5	Petiole	40	60
4	IBA K	0.5 1.5	Petiole	25	50
5	IAA BA	0.5 0.1	Leaf	104	28
6	IBA BA	1 1.5	Leaf	25	30

Table 5. Hormone combinations in various combinations that induced direct organogenesis with root induction in petiole and leaf.

Media No.	Plant Growth Regulators	Concentration (mg/l)	Explant	Duration (days)	Per-centage
1	IAA	0.1	Petiole	104	26
2	IBA K	1 1.5	Petiole	30	50
3	IBA K	1.5 2.5	Petiole	25	56
4	IBA	0.5	Leaf	40	50
5	IBA K	0.5 1.5	Leaf	25	58
6	IBA K	1.5 2.5	Leaf	30	58

Table 6. Hormone combinations in various concentrations that induced indirect organogenesis with shoot induction in petiole and leaf.

Media No.	Plant Growth Regulators	Concentration (mg/l)	Explant	Duration (days)	Per-centage
1	IBA BA	0.5 0.1	Petiole	40	38
2	IBA BA	0.5 1	Petiole	25	40
3	IBA K	0.5 0.5	Leaf	40	44
4	IBA K	1 0.5	Leaf	40	46
5	IBA BA	1 1.5	Leaf	24	46

respectively(Table 6) (Fig. 1(i). In the case of leafy explants MS media with BA and K at different concentrations (0.5 mg/l BA and 0.5mg/l K and 1 mg/l BA and 0.5mg/l K) promote indirect organogenesis with shoot induction after 40 days in both concentrations with 44% and 46% efficency. But in 1 mg/l IBA and 1.5 mg/l BA induced 49% indirect organogenesis with shoot induction after 24 days (Table 6)[Fig. 1(j)].

Indirect organogenesis with root induction- In the case of petiole derived callus MS medium supplemented with the hormone IBA (0.5mg/l) alone shows root induction after 26 days with 50% efficiency. Combination of IBA and K (0.5 and 1 mg/l, respectively) induced 25% rooting in 25 days along with shooting (Table 7) [(Fig. 1(k)]. Combinations of 2,4-D and BA and IBA and K were found to be good for rooting in leaf derived callus. 0.5 mg/l 2,4-D and 0.1 mg/l BA performed good in rooting with 32% efficiency in 61 days. And IBA and K (1.5mg/l IBA and 2 mg/l K and 1.5 mg/l IBA and 2.5 mg/l K) induced 32% rooting in 25 days (Table 7) [Fig. 1(l)].

Table 7. Hormone combinations in various concentrations that induced indirect organogenesis with root induction.

Explant	Media No.	Plant Growth Regulators	Concentration (mg/l)	Duration (days)	Percentage
Petiole	1	IBA	0.5	26	50
	2	IAA BA	0.5 0.1	104	20
	3	IBA K	0.5 1	25	25
	4	IBA K	0.5 0.1	68	25
Leaf	5	2,4 D	0.1	61	30
	6	2,4 D BA	0.5 0.1	61	32
	7	IBA K	0.5 0.1	68	36
	8	IBA K	0.5 0.5	40	28
	9	IBA K	0.5 1	25	25
	10	IBA K	1 1	25	30
	11	IBA K	1 1.5	25	30
	12	IBA K	1.5 2	25	30
	13	IBA BA	1.5 2.5	25	32

Influence of plant growth regulators - In the present study MS media with IBA and K was found to be more successful than other combination for callus induction. Callus induction was also observed in MS media in combination with IBA and BA, 2,4 D and BA, IBA and K, IAA and BA, NAA and BA, but produces callus in decreasing order. Competent callus was reported to be initiated around the nodal ring of tissue and few callus was reported to be induced in presence of NAA and BA[10] but results with same hormone combinations were unsatisfactory according to the present study and MS media with BA and K was found to be much better compared to NAA and BA.

Among the different concentration and combination of auxins and cytokinins tried maximum frequency of embryogenesis was observed in MS media supplemented with hormones of IBA and K and IBA and BA in petiole and leafy explant. Minimum response was observed in MS media with IAA and BA and BA only. Bhatt *et al.*[10], reported explants regenerated micro shoots along with few callus and embryoids on the supplemented with 1mg/l BAP and 0.1mg/l NAA[10]. But in the present investigation hormone combination IBA and K was found to be better for embryogenensis compared to NAA.

The influence of plant growth regulators in previous studies about *in vitro* micropropagation of *Piper longum* Linn. is through shoot tip multiplication and direct regeneration. Multiple shoots were reported to be induced from shoot tips on Murashige and Skoog (MS) medium containing NAA and BA[11], and BA and K[12]. Present studies support the use of plant growth regulators BA and K for multiple shoot induction and optimum concentration is different in explants like leaf and petiole but NAA was not found to be beneficial. Among the different hormone combinations tried, maximum frequency of direct organogenesis with shoot induction was observed in MS media supplemented with hormone combination of BA and K and minimum response was observed in IBA and

BA, IAA and BA, IBA and K in the decreasing order.

Elongated shoots were reported to be separated and rooted in MS supplemented with IBA[12]. But in the present study, of the various hormone combinations tried, maximum frequency of direct organogenesis from explants with root induction was observed in MS media supplemented with IBA and K rather than using IBA alone.

In case of indirect organogenesis with shoot induction maximum frequency was observed in MS media supplemented with IBA and BA and minimum response in BA and K. Of the different hormone combinations tried, increase of indirect organogenesis with root induction was more successful in MS media supplemented with IBA and K than other combinations and combinations of IBA and BA, 2,4-D and BA, IAA and BA, BA and K, produces minimum indirect organogenesis with root induction in decreasing order. Indirect organogenesis with numerous roots was not reported earlier in case of *P. longum*. Present study suggests that BA and K induces maximum number of shooting from different explants. This was also supported by other reports[13]. They reported shoot differentiation which occurred directly from the leaf bases without intermediate callus formation.

So PGRs regulate callusing, embryogenesis, shooting and rooting at various concentrations. Hormonal combinations that induces maximum callusing and embryogenesis was not good for rooting and shooting and *vice versa*. And also effect of hormones will be different in different explants. In the present study different hormonal combinations for inducing callus, embryogenesis, multiple shooting and rooting were identified for explants like leaf and petiole instead of shoot tips and this can be used for further studies and conservation of this endangered medicinal plant.

References

1. Hunter D 1996, Light quality influences adventitious shoot production from cotyledon explants of lettuce. *In vitro Cell Developmental Biology* **8** 215-220.
2. George E F 1996, Plant propagation by tissue culture. Part 2: In : Practice. Exgetics Ltd Edington, Wiltshire 1176.
3. Shankaracharya N B Rao *et al.* 1997, Characterisation of chemical constituents of Indian long pepper (*Piper longum* L.). *J. Food Sci. and Tech.* **34**(1) 73-75.
4. Nadkarni 2004, *Indian plants and drugs-with their medical properties and uses.* Asiatic Publishing House, Delhi (India). 302-304.
5. Ali A M, Alam N M, Yeasmin S, Khan A M and M. Sayeed A 2007, Antimicrobial screening of different extracts of *Piper longum* Linn. *Res. J. Agri. and Biol. Sci.* 3(6) 852 - 857.
6. Lokhande P D, Gawal K R, Kodam K M and Kuchekar B S 2007, Antibacterial activity of extracts of *Piper longum. J. Pharma. and Toxicology* **2**(6) 574-579.
7. Yang A C, Lee S G, Lee H K, Kim M K, Lee S H and Lee H S 2002, A Piperidine amide extracted from *Piper longum* L. fruit shows activity against *Aedes aegypti* mosquito larvae. *J. Agric. Food Chem.* **50**(13) 3765-3767.
8. Ghoshal S and Lakshmi V 2002, Potential antiamebic property of the roots of *Piper longum* Linn. *Phytotherapy Res.* **16**(7) 689-691.
9. Murashige T and Skoog F 1962, A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15**(3) 473-497.
10. Bhat S R, Kackar A and Chandek K P S 2004, Plant regeneration from callus cultures of *Piper longum* L. by organogenesis. *Plant cell Reports* **11**(10) 525-528.
11. Philip S, Banerjee N S and Das M R 2000, Genetic variation and micropropagation in three varieties of *Piper longum* L. *Curr. Sci.* Bangalore. Jan **78**(2) 169-173.
12. Soniya E V and Das M R 2002, *In vitro* micropropagation of *Piper longum* - an important medicinal plant, *Plant cell Tissue and Organ Culture* **70** 325-327.
13. Sharon, Madhuri, Maurya, Ghanshyam, Ibrahim, Medinaa, Nissen, Harold J and Siegel L 2008, Method of *in vitro* micropropagation of *Piper longum* plants, United states patent 7402433. Primary class 435/420. Publication date - 07/22/2008. Filing date 04/10/2003.