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Standardization of an Efficient Protocol for Sterilization and Media for Direct Shoot Regeneration from Nodal Segments of Medicinal Herb *Piper longum* Linn

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Abstract

In the present research it was aimed to the development and standardization of an effective sterilization protocol and media for shoot regeneration technique for a medicinal herb *Piper longum* linn. Nodal explants from juvenile plants were cultured on Murashige and Skoog's (MS) media with an array of different combinations of Cytokinins and Auxins. Among the various protocols tested for sterilization, the one containing 200 mg activated charcoal in 250 ml autoclaved water has found to be the best, for nodal segments and cultured in antibiotic containing media. Maximum growth were obtained from the media containing (MSL7) BAP(5 mg/l) NAA(0.5 mg/l) and (MSL10) BAP(3 mg/l)+IAA (0.5 mg/l) along with 15 mg Kanamycin. The findings of this study have to be used for development of an effective protocol for the sterilization and establishment of shoots from nodal explants. Best shoot regeneration was observed from nodal explants was used for further studies and micro shoots were transferred for rooting in IBA containing media and then go for acclimatization and hardening.

Keywords: *Piper longum* linn; Explants; Shoot regeneration; Medicinal herb; Micro shoots

Introduction

Piper longum linn (Piperaceae) is the accepted source of the drugs Pippali and Pippalimulam throughout the country. Pippali is the dried ripe fruits; Pippalimulam is the roots of this plant [1,2]. *Piper longum* is a medicinal plant, it is found almost all over India. It is slender aromatic climber with perennial woody stems. Common name of *Piper longum* is Pippali, Indian long pepper and papal. It is widely used in ayurvedic and unani systems of medicine particularly in diseases of respiratory tract egcough, bronchitis, asthma, etc. The herb has nerve depressant and antagonistic effects on electro-shock and chemo-shock seizures [3].

The plant is a dioecious slender aromatic climber with perennial woody roots, or a perennial creeping under shrub. Branchlets erect, glabrous with swollen nodes; roots clasping at nodes, which help to get attached to the host trees; leaves alternate, ovate, cordate, apex acute to acuminate, margin entire, glabrous. The male and female plants are morphologically very similar till the formation of spikes. Male spikes greenish-yellow, fleshy, cylindrical, with minute flowers, and female spikes, known as long pepper are shorter and thicker than the male spikes. Fruit spikes cylindrical, oblong, berries red or black when ripe, globose with aromatic odour and pungent taste [2,4-6]. It is used to treat flatulence, gout, laryngitis, and paralysis and used to treat abdominal tumors and gastric ulcers, improves the digestive system and also enhances the immune system. It is a native of North East India. It occurs in the hotter parts of India, from central Himmalayas to Assam, Magadhi, Kana, ushana (Sanskrit) [4]. Long pepper is also known and popular in parts of Africa, mainly in the Islamic regions of North and East Africa. It is used to treat flatulence, gout, laryngitis, and paralysis and used to treat abdominal tumors and gastric ulcers, improves the digestive system and also enhances the immune system. The fruits are used as spice and also in pickles. They have a pungent taste and cause salivation and numbness of the mouth [7]. It cures cough, dyspnoea, ascites, leprosy, diabetes, piles, colic indigestion, anemia, thirst and dispels cardiac and spleen disorders, chronic fever and loss of appetite. The drug is used in Ayurvedic treatment for abdorminal tumours and distention, to improve the digestive fire, *kapha* disorders, flatulence, gout, laryngitis, paralysis, rheumatic pain, sciatica, worms, and for the immune system. It is used in manufacturing cold relief balm, pain balm, joint care balm, and in heart and stress care and cough syrups [2].

Plant cell can differentiate into a whole plant, a process called regeneration, through addition of plant hormones in culture medium. This ability is known as totipotency. Regeneration of a whole plant from a single cell allows researchers to recover whole plants that have a copy of the transgene in every cell. The objectives of this study were to investigate the best surface sterilization method and standardize the media for direct shoot regeneration from nodal explants of *Piper longum* linn. And to remove the bacterial contamination produced after culture initiation by using antibiotics. Then root induction was done after shoot formation and finally plants go to lab to land.

Material and Method

Media preparation

MS Murashige and Skoog medium with 10 different combinations of Auxin and Cytokinin were analyzed for shoot formation Table 1. After preparation of the medium, and pH was maintained at 5.8-5.9 then Clarigel was used as a gelling agent. Media was dispensed into small culture bottles; the same were autoclaved for 20 min. at 15 psi and 121°C and stored at 23±1°C for 2 days. MS medium with auxin and cytokinin were analysed for bacterial contamination was prepared

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by adding the antibiotics in the same medium by filter sterilization method under the laminar air flow. And for root induction different rooting media (Table 2) was prepared.

Plant materials

The *Piper longum linn* (Figure 1) nodal explants were collected from the wild habitats and make into herbarium sheets finally identified with the help of recognized scientist of National herbaria.

Explants surface sterilization

Freshly harvested nodal segments were cleaned and dead parts separated, that were washed with the running tap water for 10 minutes to remove the dust or sand particles. These were then surface sterilized by using two different methods. In the first method, all explants were treated with Labolene (10 ml) and Savlon (5 ml) for 15 min. then 1% Bavistin (a brood spectrum systemic fungicide containing 50% WP carbendazim) for 25 mins. And then washed with 70% Ethanol for 1 min. Then inside the laminar airflow treat with 0.15% $HgCl_2$ for 8 min and then finally washed thrice with autoclaved water.

In the second method, first treated the explants with 10 ml Labolene and 5 ml Savlon in 250 mg water for 15 min. and washed with 1% Bavistin (a brood spectrum systemic fungicide containing 50% WP carbendazim) for 25 min. Then inside the laminar air flow treated with 0.15% $HgCl_2$ for 5 min. and 100% Ethanol for 30 seconds only, then treated with 200 mg Activated Charcoal in 250 ml of autoclaved water (chilled) for 25 min with gentle shaking under sterile conditions. After that, the explants were washed three times with autoclaved water. Now the explants were ready for culture initiation.

Initiation of explants

Inside the laminar air flow, the nodal explants were cut at both ends to remove the undesirable/dead portions after surface sterilization and subsequently all explants were then transferred aseptically in the bottle containing media then cultures were stored under controlled environment of growth room, and later were exposed to fluorescent tube lights, with a photoperiod of 16 hour in every 24 hour. Cultures were observed regularly to observe their response to tissue culture. Data were recorded after every 10^{th} day for four weeks.

Shoot induction

Nodal segments are grown on the medium containing different composition of cytokinin and auxin. And after that proliferated shoots were sub cultured for every 15 days for further mass multiplication.

Root induction and acclimatization

After successful shoot induction, when multiple shoots were generated then regenerated shoots were separated and transferred into MS medium containing IBA (In dole 3 butyric acid) for rooting. Acclimatization of rooted plantlets was achieved by transferring them into pots containing garden soil and sand under shade house [8] conditions where approximately 90% seeding survive.

Results and Discussion

Effect of sterilization method

The surface sterilization was optimized that prevented the blackening of tissues. The sterilization with 200 mg activated charcoal proved a little bit successful procedure as maximum explants were undamaged and responded to tissue culture medium which grow well and only 25% of them became infected, because plants produce a huge variety of secondary compounds as natural protection against microbial and insect attack [9] and activated charcoal plays a important role (for sterilization) in plant growth (Figure 1) at the time of tissue culture. On the other hand, treatment with 70% ethanol in 250 ml water failed due to contamination of 90% explants. But after using antibiotics in culture medium by filter sterilization, only 5% contamination occurs in our second sterilization procedure. So 200 activated charcoal proved the most successful sterilization procedure in the medium containing 15 mg Kanamycin (antibiotic), as all the explants safely grow on that medium.

Shoot induction

Auxins and Cytokinins are major growth regulators that have showing great results of cell division, shoot induction and regeneration. And Auxin-Cytokinin interactions are usually considered to be the most important for regulating growth and organized development in plant tissue and organ growth [10]. In the present study maximum growth were obtained from the media containing (MSL7) BAP (5 mg/l) NAA (0.5 mg/l) and (MSL10) BAP (3 mg/l)+IAA (0.5 mg/l) along with 15 mg Kanamycin (Table 3) Overall Plant Growth progress (Table 4).

Root induction and acclimatization

Auxins are major growth regulators that have showing great results of root formation. In the present study maximum rooting (70%) were obtained from the media containing IBA 0.5-1 mg/l in Table 5.

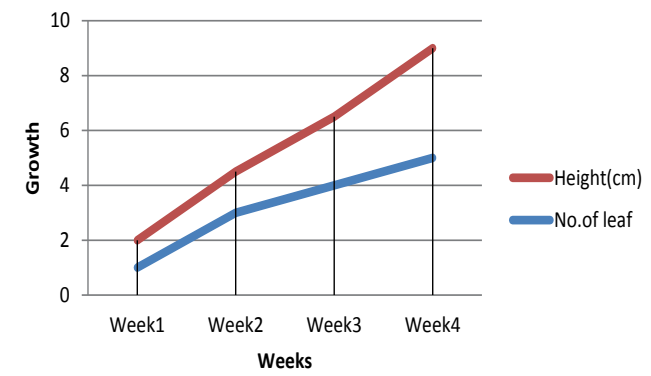

Figure 1: Graphical representation of Plant growth.

MS Media	PGR (mg/l)			
Media code	BAP	AS	NAA	IAA
MSL1	2		0.5	
MSL2	3		1	
MSL3	2		1.5	
MSL4	4		0.5	
MSL5	4		1	
MSL6	4		1.5	
MSL7	5		0.5	
MSL8	5		1	
MSL9	5		1.5	
MSL10	3			0.5

Table 1: Different media composition for shoot regeneration.

Media Code		Sucrose	PGRs (mg/l) ml/l from stock
	MS		IBA
MSR-1a		3%(30 gms/L)	0.25-0.5
MSR-2a		3%(30 gms/L)	0.5-1

Table 2: Different media composition for root formation.

Media code	Media composition	Treatments	Shoot induction percentage	Plant growth size in 1 month
MSL7	MS+BAP(5)+NAA(0.5)+Kanamycin	30	92%	4-5 cm
MSL10	MS+BAP(3)+IAA(0.5)+Kanamycin	30	85%	5 cm

Table 3: Effect of different media composition on Shoot formation or plant growth.

S.No.	Plant	Week-1		Week-2		Week-3		Week-4	
		No. of Leaf	Height	No. of Leaf	Height	No. of Leaf	Height	No. of Leaf	Height
1	1	1	1 cm	3	1.5 cm	4	2.5 cm	5	4 cm
2	2	1	1 cm	3	2 cm	4	3 cm	5	4 cm
3	3	2	1 cm	4	2.5 cm	5	3.5 cm	6	5 cm
4	4	1	1 cm	3	2 cm	3	2.5 cm	4	4 cm
5	5	2	1 cm	4	2 cm	5	3.5 cm	5	4 cm

Table 4: Effect of different media composition on shoots formation or plant growth.

S.No.	Media	No. of cultures	Survivability	Sprouting	Root induction
1	**MSR1a**	15	12	positive	MSR 2a show 70% rooting
2	**MSR2a**	15	13		

Table 5: Rooting effects on plants.

Figure 1: Stepwise Regeneration of nodal explants of *Piper longum* linn.

Conclusions

In vitro regeneration of plants has gained significance during recent years as this technique can be used for the rapid multiplication and ex situ conservation of some plants having threatened aspects. Selected media with different combinations of Auxins and Cytokinins were analyzed to determine the best media composition for shoot induction in *P. longum* Linn. In the present study, *in vitro* regeneration of *P. longum* was reported wherein nodes of in mother plant were utilized as initial explants for multiple shoot formation, sterilization of explants, followed by rooting of individual microshoots and successful transplantation of plantlets. The findings of this study have resulted in development of an effective protocol for sterilization and establishment of shoot formation from nodal explants. When the regenerated shoots become 6-7 cm long, they are transferred to MS medium containing rooting hormones for rooting. And after that rooted plantlets are transferred to greenhouse conditions. This method of micro propagation can be used for the development large scale plantlets, establishment of nurseries of *P. longum* and also be used for the ayurvedic medicinal purposes.

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