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Influence of different media on shoot regulation, shoot multiplication and callus induction in long pepper (*Piper longum* L.)

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ABSTRACT : An experiment on tissue culture studies in long pepper (*Piper longum* L.) was carried out in the year 1999-2001, to develop suitable protocol for plant multiplication technique at Green Earth Biotechnologies Ltd., Jigani Industrial Area, Bengaluru in collaboration with UAS, GKVK, Bengaluru. The concentration of 0.1 per cent mercuric chloride ($HgCl_2$) for 60 seconds to achieve disinfection of leaf segment explants was found effective, giving maximum survival (10.02 %) and minimum contamination (0.70 %). MS media containing BAP (1.5 mg/lit.) + 2,4-D (1.0 mg/lit.) from leaf explants gave higher (9.72 %) response for calli induction, 1.54 mg amount of callus with green colour and very good score of callus. BAP 3.0 mg /lit. was ideal for the better proliferation and regeneration of shoot (40 %).The Highest number of multiple shoots (2.00), Length of shoot (1.04 cm) and number of leaves per shoot (3.00), was produced using BAP (1.0 mg/lit.) + NAA(0.1 mg/lit.). Best results for shoot were observed with the MS media combination containing BAP at 1.5 mg/lit., Kinetin at 1.0 mg/lit. and IAA at 0.5 mg /lit., gave the maximum number of buds per calli (36.00) and number of shoots (5.20) and length of shoots (1.62 cm). The *in vitro* rooting was achieved with the application of NAA at 1.0 mg/l 70 per cent rooting, number of primary roots (2.40) length of primary root (2.00) and number of secondary roots (2.00).

KEY WORDS : Long pepper, *In vitro*, Micropropagation, Tissue culture, MS media

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Long pepper (*Piper longum* Linn.) is an important medicinal plant belonging to the family Piperaceae. It is also called by other names like "Peper" (Greek), "Piper" (Latin) and "Pipali" or "Peppali" (Sanskrit) in various parts of the world. Indian Long pepper of commerce is an unripe spike of either *Piper longum* or *Piper pepuloides*. The Pipali mature dry spikes of female types of commerce is widely used in ayurvedic and unani systems of medicine, particularly for the diseases of respiratory tract. The industrial demand for female spike is continuously increasing, as it is used in herbal medical formulations (Kamboj, 2000). Other than the spikes, the roots and thicker parts of stem are cut, dried and used as Piplamul. It shows the presence of alkaloids, piperine (4.5%), piplatin and alkaloid-A. India is not self –sufficient in its requirement and in order to meet its local requirements, huge quantities of the produce is being imported from Indonesia, Malaysia and Sri Lanka. Hence, there is a need for commercial cultivation of this crop with view of import substitution. In order to

grow this crop on large scale, particularly using elite variety, requires large plant population of 27,777 per hectare. The requirement of such huge plant population cannot be easily met by conventional method of propagation (vine cuttings). Hence, there is a need to propagate it through tissue culture techniques, which can serve as a means to propagate plants rapidly on a large scale and increase the availability of planting material. Tissue culture technology requires limited planting material as the starting and can also use to produce disease free plants. Keeping the above factors in the background, the present investigations were conceded out to identify the medium with suitable explants, for inducing callus, shoot regeneration and proliferation from callus and *in vitro* rooting.

RESEARCH METHODS

The present study was conducted in the Green Earth Biotechnologies Limited, Jigani Industrial Area, Bengaluru. The experiment was laid out in Completely Randomized Design. In this study long pepper (*Piper longum* L.) variety "Viswam" was collected from Medicinal and Aromatic plants section, Division of Horticulture, GKVK, Bangalore. The nursery raised seedlings were planted in pots containing soil, sand and peat in 1:1:2 ratio of pot mixture and maintained in green house. The pots were watered once in two days. One day before the collection of explants, the mother plant are treated with bavistin (0.2%). The explants excised from the mother plant were thoroughly washed with tap water for 5 minutes and swabbed with alcohol. Then, the explants were kept under tap water for 30 minutes. Later the explants treated with topsin at 0.1 per cent for 30 minutes were washed thoroughly with double distilled water. Under aseptic conditions of a laminar air flow chamber, again the explants were washed with teepol for 10 minutes. The surface disinfected explants were washed thoroughly with sterile distilled water for 5 - 6 times. The cut ends of the surface sterilized explants were further cut using scalpel blades and carefully inoculated onto the MS (Murashige and Skoog) media. The cultured vessels were closed immediately, labeled and shifted into the growth room. The standard procedure for sterile techniques was suggested by Street (1977) and Razadan (1993) were followed.

The culture media used in the experiment was as per the composition given by Murashige and Skoog (1962), the inorganic constituents grouped into three and organics into the fourth categories (Table 1). The cultures were incubated in an air conditioned room at a temperature of 25±2°C under 16:8 hour (light: dark) photoperiods. The light was provided by cool white fluorescent tubes (Mysore Lamps-20w) for giving an illumination of 22° µEM-2S-1.

Details of experiments :

The treatments tried with the following experiments were based on preliminary studies conducted.

Experiment-I: Optimizing explant and surface sterilization for obtaining aseptic culture

– Mercuric chloride ($HgCl_2$) : 0.1 and 0.12 per cent

Duration: 30, 60 and 120 sec

Explant: Leaf segments

– Mercuric chloride ($HgCl_2$) : 0.1 and 0.12 per cent

Duration: 30 and 60 sec

Explant: Leaf segments

– Mercuric chloride ($HgCl_2$) : 0.05, 0.1, 0.12 and 0.2 per cent

Duration: 30, 60, 120, 180, 300, 420 and 480 sec

Explant: Apical buds, modal stem cuttings, stem internodes and female spikes.

With respective contamination (%) and survival (%) each treatment was replicated as ten bottles with four explants in each bottle.

Experiment-II: Optimizing growth regulators and their concentrations on callus studies.

Callus induction :

T_1: BAP 1.0 mg/lit.+2, 4-D 0.5 mg/lit.+NAA 0.5 mg/lit.

T_2: BAP 0.5 mg/lit. + 2, 4-D 1.0 mg/lit.

T_3: BAP 1.5 mg/lit. + 2, 4-D 1.0 mg/lit.

T_4: 2, 4-D 1.0 mg/lit. + NAA 0.1 mg/lit.

T_5: BAP 0.5 mg/lit.+2, 4-D 2.0 mg/lit. +NAA 0.5 mg/lit.

Explant : Leaf segments :

Each treatment was replicated as five bottles with four explants in each bottle.

Callus proliferation and regeneration :

Kinetin: 1.0, 2.0, 3.0, 4.0 and 5.0 mg /lit.

BAP : 1.0, 2.0, 3.0, 4.0 and 5.0 mg/lit.

Explant: Callus

Regeneration (%) :

Each treatment was replicated as five bottles with four explants in each bottle.

Experiment-III: Optimizing growth regulators and their concentrations for multiple shoot induction

Shoot regeneration :

T_1: BAP 1.0 mg/lit. + NAA 0.1 mg/lit.

T_2: BAP 1.5 mg/lit. + NAA 0.1 mg/lit.

T_3: BAP 2.0 mg/lit. + NAA 0.1 mg/lit.

Explant: Callus

Each treatment was replicated as five bottles with four explants in each bottle.

Shoot multiplication :

T_1: BAP 1.0 mg/lit. + Kinetin 0.5 mg/lit.+ IAA 0.5mg/lit.

T_2: BAP 1.0 mg/lit. + Kinetin 1.0 mg/lit. + IAA 0.5mg/lit.

T_3: BAP 1.5 mg/lit. + Kinetin 1.0 mg/lit.+ IAA 0.5mg/lit.

T_4: BAP 2.0 mg/lit. + Kinetin 1.0 mg/lit. + IAA 0.5mg/lit.

T_5: BAP 2.5 mg/lit. + Kinetin 0.5 mg/lit.+ IAA 0.5mg/lit.

Explant: Nodes from callus :

Each treatment was replicated as five bottles with four explants in each bottle.

Experiment-IV: Optimization of growth regulators and their concentrations for rooting *in vitro*. NAA with control and IBA with control were tried separately to measure the parameter.

NAA: 1.0, 2.0, 3.0 and 4.0 mg /lit.

Explant : Microcutting :

Each treatment was replicated as five bottles with four explants in each bottle.

Table 1 : Composition of Murashige and Skoog's medium

Sr. No.	Particulars	Quantity (mg/lit.)
Solution -A (Macronutrients)		
1.	KNO_3	1900.00
2.	NH_4NO_3	1650.00
3.	KH_2PO_4	170.00
4.	$MgSO_4.7H_2O$	370.00
5.	$CaCl_2.2H_2O$	440.00
Solution -B (Micronutrients)		
1.	$Na_2SO_4.7H_2O$	22.300
2.	$ZnSO_4.7H_2O$	8.600
3.	H_3Bo_3	6.200
4.	KI	0.830
5.	$Na_2NO_2.H_2O$	0.250
6.	$CoCl_2.6H_2O$	0.025
7.	$CuSO_4.3H_2O$	0.025
Solution -C (Micronutrients)		
1.	$FeSO_4.7H_2O$	27.800
2.	Na2EDTA.$2H_2O$	37.300
Solution -D (Vitamins)		
1.	Myoinsitol	100.00
2.	Thiamine hydrochloride	0.10
3.	Nicotinic acid	0.50
4.	Pyridoxin hydrochloride	0.50
5.	Glycine	2.00
	Agar	8(g/lit.)
	Sucrose	30(g/lit.)
	pH	5.7 to 5.8

RESEARCH FINDINGS AND DISCUSSION

The findings of the present study as well as relevant discussion have been presented under following heads :

Surface sterilization:

Standardization of sterilization procedures for the aseptic cultures of Long Pepper explants was done using explants from the green house conditions. The sterilization procedures were carried out for apical buds, stem node cuttings, female spike, stem internode cuttings and leaf segments. The explants were washed with two drops of teepol for 15 mins and were treated with different concentrations of mercuric chloride ($HgCl_2$) (0.05, 0.1, 0.12 and 0.2 %) for different treatment, period 30, 60, 120, 180, 300, 360, 420 and 480 seconds in combinations. The percentage of contamination was recorded, seven days after treatment, the result of leaf explants is presented (Table 2). The treatment differed significantly with respect to contamination and survival of explants. Mercuric chloride at 0.1 per cent treated for 60 seconds gave the lower contamination (0.70%) with maximum survival of 10.02 per cent. This was followed by (3.99%) contamination and 8.81 per cent survival with mercuric chloride at 0.12 per cent treated for 30 seconds. These two treatments were significantly superior when compared with other treatments, even though, the difference between these two treatments were significant. The other explants used, resulted in higher levels of bacterial and fungal contaminations that were

Table 2 : Efficacy of different concentrations of mercuric chloride ($HgCl_2$) on surface sterilization of leaf explants

Treatment dose (%)	Treatment duration (sec)	Per cent	
		Contamination	Survival
0.1	30	8.70	4.11
0.1	60	0.70	10.02
0.1	120	7.11	6.20
0.12	30	3.99	8.81
0.12	60	7.55	6.43
F-test		*	*
S.E.±		0.718	0.839
C.D. (P=0.05)		2.092	2.441

Number of replications / treatment = 10; * indicates significance of value at P= 0.05

Table 3 : Efficacy of different growth regulators on the callus induction from leaf explants

BAP (mg/l)	2,4-D (mg/lit.)	NAA (mg/lit.)	Explants response (%)	Amount of callus (g)	Kind of callus	Colour of callus	Score of callus
1.0	0.5	0.1	7.04	1.21	Compact	Light green	++
0.5	1.0	0.0	0.70	0.71	Compact	Greenish yellow	+
1.5	1.0	0.0	9.72	1.54	Compact to friable	Green	+++
0.0	1.0	0.1	7.29	1.13	Compact	Light green	++
0.5	2.0	0.5	8.77	1.00	Compact	Brownish green	++
F-test			*	*	-	-	-
S.E.±			0.383	0.017	-	-	-
C.D. (P=0.05)			1.116	0.051	-	-	-

Observations were recorded at 30 days after transfer; Number of replications/treatment =5; * indicates significance of value at P=0.05
Scoring: + Poor callusing, ++ Good callusing and +++ Very Good callusing; BAP – 6-Benzyl Amino Purine;
NAA- Napthalene Acetic Acid;

maximum between eighth and tenth day of culture and died due to higher mercuric chloride concentration or treatment period. It was difficult to eliminate micro-organisms, both, outside and endophytically in other explants. Hence, the contamination reappeared in the cultures. Similar observations were also made by Raj Mohan (1985) and Fitchet (1988) in *Piper nigrum*. Of these explants, surface sterilization could be better achieved with leaf explants than with other. Similar findings have been reported in medicinal crop such as *Emblica sonchifolia* (Shylesh and Padikkala, 1996).

Callus induction:

The effect of BAP, 2,4-D and NAA used in combinations at different concentrations on leaf explants of long pepper for callus induction on MS medium were investigated and data obtained are presented in the Table 3. The mean percentage of explants responding to callus induction was recorded maximum (9.72), amount of callus induced (1.54 g), texture of callus was compact to friable, colour of callus (green) and score (very good) which were significant with MS media containing BAP (1.5 mg/lit.) + 2,4-D (1.0 mg/lit.), when compared to other treatments. Leaf has always been a preferred explants source, either to obtain cell culture or to induce callus. The response of leaf for callusing has always been superior to any other vegetative material (George, 1993). The present investigation, resulted high rate of cytokinin to auxins for callus. Similar effect of BAP and 2, 4-D combination has been reported in other medicinal crops *Panax ginseng* (Ahn and Ahn, 1996) and *Heracleum candicans* (Wakhu and Sharma, 1998).

Callus proliferation and regeneration:

The callus obtained from leaf explants of long pepper were transferred to MS media containing BAP and kinetin at different concentrations. Among the sources of cytokinins, BAP and kinetin treatments used, BAP showed better response to callus proliferation and regeneration. BAP at 3 mg/lit., produced compact to friable callus resulted in the increased quantity of very good type of callus regeneration to form shoots was (40%). The superiority of cytokinin, BAP over kinetin and other compounds has already been established in

Piperis spp. (Shaji *et al*., 2000).

Multiple shoots:

In the present study, multiple shoots were regenerated from calli by transferring them to medium containing cytokinins (BAP and Kinetin) and auxins (NAA and IAA) in various concentrations and combinations. The concentrations with high cytokinins to auxins gave the best results for shoot multiplication (Table 4). The treatments varied significantly for the production of number of shoots, shoot length and number of leaves. In the presence of BAP at 1mg/lit. with NAA at 0.1 mg /lit. in combination, was observed to be the best for inducing shoots (2.00), length of shoot (1.04 cm) and number of leaves per shoot (3.00). These findings are similar to the findings with *Solanum nigrum* (Anwar *et al*., 1999). The other combinations of BAP + NAA failed to cause shoot induction.

While the various combinations of BAP, kinetin and IAA were able to induce multiple shoots. The number of shoot buds per callus varied significantly between the treatments (Table 5). It was highest in the case of treatment BAP+kinetin+ IAA at 1.0+1.0+ 0.5 mg/lit., recording 36 number of shoot buds per calli, number of shoots (5.20) and length of the shoots (1.62 cm). These results are in conformity with results obtained earlier by Shaji *et al*. (2000) in *Piper longum* using BAP (2.0mg/lit.), Kinetin (0.1mg/lit.) and IAA (1.0mg/lit.) leading highest percentage (82±5.7) of calli giving 15-20 shoots.

***In vitro* rooting:**

Rhizogensis usually follows treatment with either auxin or with media containing higher auxin then cytokinin. NAA at 1.0 mg/lit. was the first for *in vitro* root production, which induced maximum rooting (70%), number of primary roots (2.40), length of primary root (2.00 cm) and number of secondary roots (2.00 cm). However, the increase in concentration of NAA (2, 3 and 4 mg/l) led to decrease in the rooting per cent, number of primary roots and length of primary root containing no secondary roots. Similar results on the *in vitro* rooting were reported by Philip *et al.* (1992) in *Piper nigrum.*

Conclusion :

To conclude, long pepper (*Piper longum*) variety "Viswam" can be propagated by tissue culture using the above protocol to obtain plants, through indirect organogenesis. Hence, in future, studies on the following

Table 4 : Effect of auxin and cytokinin on shoot regeneration from callus cultures

Treatments (mg/lit.)		Shoot regeneration		
BAP	NAA	Number	Length (cm)	Number of leaves
1.0	0.1	2.00	1.04	3.00
1.5	0.1	--	--	--
2.0	0.1	--	--	--
F-test		*	*	*
S.E.±		0.132	0.030	0.211
C.D. (P=0.05)		0.397	0.090	0.637

Observations were recorded at 4 weeks after transfer; Number of replications/treatment =5;
* indicates significance of value at P=0.05; -- = Resulted in callusing; BAP – 6-Benzyl Amino Purine; NAA- Napthalene Acetic Acid

Table 5 : Effect of auxin and cytokinin on shoot multiplication

Treatments (mg/lit.)			Shoot multiplication			
BAP	Kinetin	IAA	Buds / Callus	No. of shoots	Length of shoot (cm)	No. of leaves
1.0	0.5	0.5	28.80	2.20	0.60	2.60
1.0	1.0	0.5	36.00	5.20	1.62	4.40
1.5	1.0	1.0	20.23	1.80	1.02	2.20
2.0	1.0	1.0	15.60	0.60	0.50	1.20
2.5	1.0	1.0	12.00	1.80	1.16	3.20
F-test			*	*	*	NS
S.E.±			2.471	0.815	0.240	1.135
C.D. (P=0.05)			6.029	1.988	0.585	NS

Observations were recorded at 30 days after transfer; Number of replications/treatment =5;
*indicates significance of value at P=0.05; NS = Non-significant; BAP – 6-Benzyl Amino Purine; IAA- Indole 3-Acetic Acid

aspects may be taken up. Studies oriented towards overcoming entophytic bacterial contamination, for the establishment of explants like apical buds, nodal stem cuttings and stem internodes should be conducted as these explants failed to survive.

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