

Research Article

Investigation on callus formation in *Piper nigrum* L

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ABSTRACT

The *Piper nigrum* L. (black pepper) is generally cultivated by seed because other vegetative propagation methods are slow and time consuming. Therefore the tissue culture technique is considered more efficient and reliable method for rapid and mass propagation of this economically important plant. The present study was initiated to develop protocol for callus formation of black pepper. The leaf explant from mature plant was cultured on MS medium supplemented with different concentrations of plant growth regulators (2, 4-D, BAP, IAA, Kinetin). Best callus was produced on MS medium with 4.0+3.0 mg/l 2, 4, D+ BAP by leaf explant.

Keywords: Murashige & Skoog Medium, 2, 4-D: 2, 4-diphenylphenoxyacetic acid.

INTRODUCTION

Plant tissue culture mainly encompasses the collection of experimental method of growing large number of isolated cells or tissue under sterile and controlled conditions. The callus is the undifferentiated and unorganized mass of plant cells. It is basically a tumor tissue which usually forms on wounds of differentiated tissue or organ. The induction of callus growth and subsequent differentiation and organogenesis is accomplished by the differential application of growth regulators and the control conditions in the culture medium. With the stimulus of endogenous growth substances or by addition of exogenous growth regulators to the nutrient medium cell division, cell growth and tissue differentiation are induced. A balance of both auxin and cytokinin will often produce an unorganized growth of cells, or callus, but the morphology of the outgrowth will depend on the plant species as well as the medium composition. As cultures grow, pieces are typically sliced off and transferred to new media (subcultured) to allow for growth or to alter the morphology of the culture. The skill and experience of the tissue culturist are important in judging which pieces to culture and which to discard (Bhojwani *et al.*, 1996).

Over three - quarters of the world population relies mainly on plants and plant extracts for health care. More than 30% of the entire plant species, at one time or the other were used for medicinal purposes. Medicinal plants are of great interest to the researchers in the field of biotechnology as most of the drug industries

depend, in part, on plants for the production of pharmaceutical compounds (Chand *et al.*, 1997). The Ayurveda system of medicine uses about 70 species, Unani 700, Siddha 600 and modern medicine around 30 species. The drugs are derived either from the whole plant or from different organs, like leaves, stem, bark, root, flower seed etc. The scientific study of traditional medicines, derivation of drugs through bioprospecting and systematic conservation of the concerned medicinal plants is of great significance. (Joy *et al.*, 1998). In this regard attempt have been need for regeneration of the medicinal plant either through traditional horticulture methods or through modern approaches like Plant tissue culture.

Black pepper (*Piper nigrum* L.) is a flowering vine in the family Piperaceae, cultivated for its fruit, which is usually dried and used as a spice and seasoning. The fruit, known as a peppercorn when dried, is approximately 5 millimetres (0.20 in) in diameter, dark red when fully mature, and, like all drupes, contains a single seed. Peppercorns, and the powdered pepper derived from grinding them, may be described simply as pepper or more precisely as black pepper (cooked and dried unripe fruit), green pepper (dried unripe fruit) and white pepper (dried ripe seeds). Black pepper is native to south India, and is extensively cultivated there and elsewhere in tropical regions. Currently Vietnam is the world's largest producer and exporter of pepper, producing 34% of the world's *Piper nigrum* L. crop as of 2008. Dried

ground pepper has been used since antiquity for both its flavour and as a medicine. Black pepper is the world's most traded spice. It is one of the most common spices added to European cuisine and its descendants. The spiciness of black pepper is due to the chemical piperine. It is ubiquitous in the industrialized world, often paired with table salt.

Medical Uses of Black Pepper

Berries and leaves from *Piper nigrum* L. contain many compounds that exhibit beneficial properties for humans. Methanolic extracts, terpenes, alkaloids, flavonoids, and sterols are just some of the compounds that have been extensively studied by researchers in pursuit of medicinal discoveries. Studies have shown that extracts prepared from *Piper nigrum* L. exhibit strong parasite inhibiting qualities as well as the promotion of tissue health through a handful of pathways.

Testing has yielded extensive evidence that black pepper is a fierce weapon against many notable parasites. Extracts prepared from *P. nigrum* L. are capable of reducing or eliminating infection from such notable parasites as malaria (genus *Plasmodium*), *Leishmania*, and sleeping sickness (genus *Trypanosoma*). Other evidence has been shown that reinforces some long standing historical claims to the medicinal value of black pepper. Evidence of use as an anti-inflammatory, analgesic (painkiller), and antioxidant dates back as early as ancient Indian and Egyptian cultures more than 3,000 years ago. Extracts from *P. nigrum* L. have also shown anti-cancerous / cytotoxic facilitation in recent studies. Piperene is a potent chemical which in addition to the aforementioned properties also promotes thermogenesis in humans. Thermogenesis is the heat producing catabolism (breakdown) of fats in the body. Like many eastern spices, pepper was historically both a seasoning and a medicine. Long pepper, being stronger, was often the preferred medication, but both were used. Black Pepper (or perhaps long pepper) was believed to cure illness such as constipation, diarrhoea, earache, gangrene, heart

disease, hernia, hoarseness, indigestion, insect

bites, insomnia, joint pain, liver problems, lung disease, oral abscesses, sunburn, tooth decay, and tooth-aches (Turner, 1999). Various sources from the 5th century onward also recommend pepper to treat eye problems, often by applying salves or poultices made with pepper directly to the eye. There is no current medical

evidence that any of these treatments has any benefit; pepper applied directly to the eye would be quite uncomfortable and possibly damaging. Nevertheless, Black pepper, either powdered or its decoction, is widely used in traditional Indian medicine and as a home remedy for relief from sore throat, throat congestion, cough etc.

Pepper is known to cause sneezing. Some sources say that piperine, a substance present in black pepper, irritates the nostrils, causing the sneezing (U.S. Library of Congress Science Reference Services "Everyday Mysteries", 2005.) Few, if any, controlled studies have been carried out to answer the question. It has been shown that piperine can dramatically increase absorption of selenium, vitamin B, beta-carotene and curcumin as well as other nutrients (Dudhatra, et al. 2012). Pepper contains small amounts of safrole, a mildly carcinogenic compound. (James A. Duke 1993) so, it is eliminated from the diet of patients having abdominal surgery and ulcers because of its irritating effect upon the intestines, (Lichtenberger, 1998) being replaced by what is referred to as a bland diet. However, extracts from black pepper have been found to have antioxidant properties and anti-carcinogenic effects, especially when compared to chili. (Nalini N et al. 2006) Piperine present in black pepper acts as a (Malin T, 1999) thermogenic compound. Piperine enhances the thermogenesis of lipid and accelerates energy metabolism in the body and also increases the serotonin and beta-endorphin production in the brain. Piperine and other components from black pepper may also be helpful in treating vitiligo although when combined with UV radiation should be staggered due to the effect of light on the compound. (Soumyanath A, 2006).

Initiation and establishment of aseptic culture - Vegetative parts or reproductive parts are used for the propagation and shoot tip and auxiliary buds are often used for this. In this process explants are surface sterilized by treating it with disinfectant solution such as Ethyl alcohol, bromine water, mercuric chloride etc. Establishment of explant on appropriate medium - There is not any universal culture medium; however modifications of Murashige and Skoog basal medium are most frequently used. Formation of Callus - Formation of callus from inoculating explants on Murashige and Skoog basal medium with suitable growth regulators like cytokinin and auxin.

MATERIAL AND METHODS

Surface sterilization of explant

All aseptic operations were performed in laminar airflow cabinet in order to avoid contamination. Explants that were used are shoot tips, nodal explants, leaf bits selected from healthy and disease free plants. The explants were subjected to washing thoroughly with running tap water and then with sterile double distilled water. The explants were then subjected to washing with soap solution (Labolin) followed by washing with sterile distilled water. Explants were surface sterilized with 0.1% mercuric chloride (HgCl_2) for 2-3 minutes followed by rinsing with sterile double distilled water for 3-4 times. Then the explants were surface sterilized with 70% alcohol for nearly 2- 3 minutes, followed washing with sterile double distilled water to remove the traces of surface sterilants, all this procedure was carried out in laminar air flow cabinet. Then explants were soaked by placing them on sterile tissue paper and edges of the explants were cut with sterile scalpel near to the blower. After completion of sterilization, the explants were inoculated on Murashige and Skoog's Medium with different concentrations and combinations of auxins and cytokinins.

Culture conditions

All the standard physical conditions were provided to culture *in-vitro*. The photoperiod was adjusted as 16 hours light and 8 hours dark, as per the requirement. The culture was kept at $21 \pm 2^\circ\text{C}$ temperature and 70% humidity. Observations were recorded for the initiation of callus induction after 30 days of inoculation and observation was made to counter check contamination and progress in plant development. The idea of culturing plant cells *in-vitro* was put forth by the German botanist Gottlieb Haberlandt (1834-1945) who was the first person to culture isolated fully differentiated cell. Haberlandt (1902) chose single isolated cell from leaves. He used tissue of *Lamium purpurum* and *Eichornia crassipes* and inoculated them on Knop's (1865) salt solution with sucrose and observed obvious growth in the palisade cells. The first viable callus culture was reported by White (1939) in tobacco and carrot. Since that time the technique of tissue culture became an advanced and rapidly growing branch of plant biotechnology and it proved to be an important tool for studying the basic and applied aspects of plant science (Bhojwani and Razdan 1996). Murashige and Skoog in 1962 stated that, tissues from different parts of plant may have different requirements for satisfactory growth. According to the recommendations of the

International Association for Plant Physiology the elements required by plants in concentrations greater than 0.5 mmol^{-1} are referred to as macroelements and those in concentration less than 0.5 mmol^{-1} are microelements (De Fossard 1976) (Bhojwani and Razdan 1996).

In the present investigation the plant of *Piper nigrum* L. were collected from Dr. Panjabrao Deshamukh Agriculture University, Akola Dist. Akola, Maharashtra and were grown in the garden of Department of Botany, Sant Gadge Baba Amravati University, Amravati and explants such as leaves from plantlets of *Piper nigrum* L. was used. The complete experiment was performed under aseptic and controlled conditions in plant tissue culture laboratory of Department of Botany, Sant Gadge Baba Amravati University, and Amravati.

Chemical

Murashige and Skoog's basal medium (Hi-Media), Agar (Hi-Media), Calcium chloride (Hi-Media), Sodium hydroxide (Loba), Hydrochloric acid (Loba), Cytokinin i.e. Kinetin, 6- Benzyl-amino-purine (Hi-Media), Auxin i.e. 2,4-Dichlorophenoxy acetic acid, α - Naphthalene acetic acid (Hi-Media), Indol acetic acid (IAA).

Sterilization of Glasswares

The sterilization is an important step in plant tissue culture. Prior to go for culture there must be sterilization of all accessories. Test tubes, flasks, petriplates, etc. were dried in oven at 80°C to 100°C for 3 hours or by keeping them in the oven for overnight at 50°C to 60°C . The dried glasswares were then wrapped in polyethylene bags with the help of rubber bands in order to avoid water entry in glasswares and were placed in the autoclave. The glasswares were autoclaved at 15 lb pressure at approximately 121°C temperature for 15 minutes.

Preparation of stock solution

Stock solution for Auxins

2,4Diphenylphenoxy acetic acid (2,4-D)

20 mg of 2,4-D was weighed and dissolved in 1 ml alcohol of by making a volume up to 20 ml with sterile double distilled water.

Naphthalene Acetic Acid (NAA)

20 mg of NAA was weighed and dissolved in 1 ml of 1N NaOH by making a volume up to 20 ml with sterile double distilled water.

Indole-3-Acetic Acid (IAA)

20 mg of NAA was weighed and dissolved in 1 ml of 1N NaOH by making a volume up to 20 ml with sterile double distilled water.

Stock solution for Cytokinin Benzyle Amino Purine (BAP)

20 mg of BAP was weighed and dissolved in 1 ml of 1N NaOH by making a volume up to 20 ml with sterile double distilled water.

Kinetin

20 mg of kinetin was weighed and dissolved in 1 ml of (1N) HCl by making a volume up to 20 ml with sterile double distilled water.

Media Preparation (MS Media)

The MS media (34.8 gm/lit) and CaCl₂ (440 mg/lit) was dissolved in 1000 ml of sterile double distilled water and subjected to adjust the pH 5.8. Thereafter, there was addition of agar powder (8 gm/lit) dissolved in the prepared media solution. However, after dissolution of agar, 20 ml media was poured into sterile tissue culture tubes and bottles. The tubes and bottles containing media were subjected to autoclaving at 15 lb pressure, at 121^oC temperature for 15 min. After autoclaving the test tubes were placed in slanting position in a slant stand to obtain the slant and the media was allowed to solidify for about 60 minutes, contamination was checked for 2 days and then inoculated by proper

explants. Observations were recorded time to time and all the culture tubes and bottles were kept under observation. Moreover, after consumption of the nutrients by growing explant after some period of time, the explant was subjected to sub-culturing.

OBSERVATION AND RESULTS

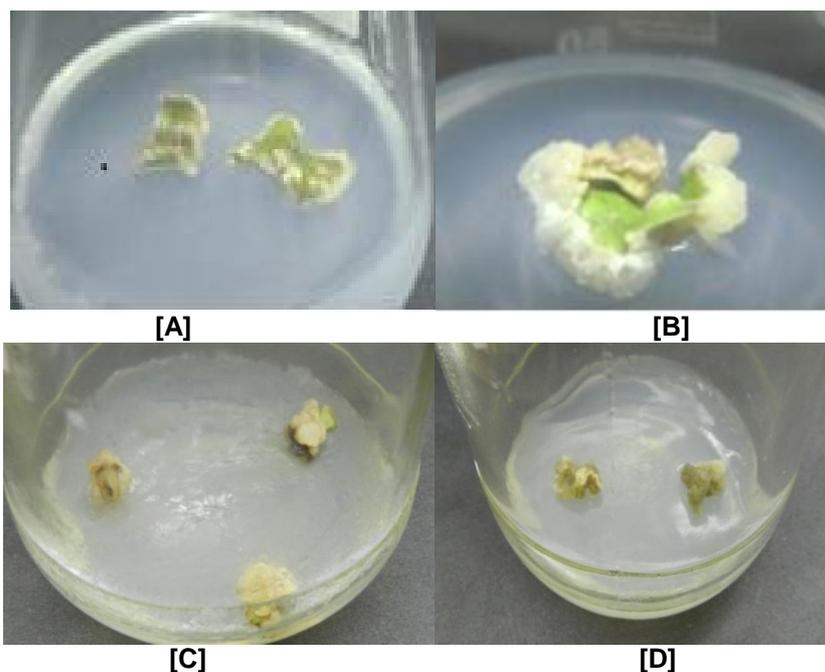
In the present investigation, explants such as leaves, was surface sterilized and inoculated in MS Media with various concentrations and combination of Growth regulators. Growth regulators used were 2,4-D, IBA, IAA, BAP, and Kinetin, and were kept in controlled aseptic conditions. Observations were recorded, by checking culture vessels at regular time interval of 10 days. After every 10th day the observations were noted till 3 month, and observation table was made by such recorded values from 10th day to 70th day. Also the average values were obtained where ever it was necessary. The callus was obtained by inoculating various type of explants of *Piper nigrum* L. in MS media with varied combination and concentration of growth regulators like 2,4-D and Kinetin. Leaf explants gives good results in 2, 4-D+ BAP in concentration 4.0+3.0 mg/lit and 4.5+3.0 mg/lit respectively. Negative effect also observed in the different concentration or combination of the growth regulators such as 2, 4-D + BAP and having concentration 4.0+3.0 mg/lit by inoculating leaf part.

Table 1: Response of Leaf explants of *Piper nigrum* L. for callus induction to different concentrations of Plant growth regulator, 2,4-D and BAP

S. No	Growth hormone	Explants used	Time required to obtain callus (days)	Callus morphology		Amount of Callus
	2,4D+BAP			Colour	Texture	
1.	0.2+1.0	Leaf	20	No callus	-	-
2.	3.0+2.0	Leaf	30	Light brown	Soft	+
3.	4.0+3.0	Leaf	40	Light brown	Soft	++
4.	3.0+3.5	Leaf	50	No callus	-	-

- : No callus , + : Average amount of callus , ++ : Large amount of callus

The Callus initiation was obtained by inoculating Leaf part as explants of *Piper nigrum* L. in MS Media with various combination and concentration of Growth regulators such as 2,4-D and BAP in combination, 3.0+2.0 mg/lit., (Table 1).

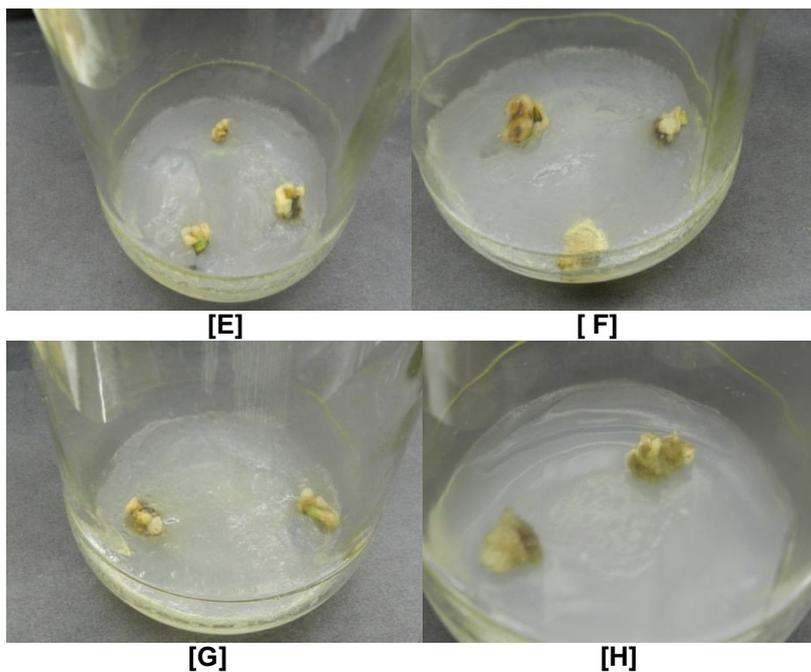


[A] = Initiation of callus from leaf explants after 30-40 days. [B] = Callus obtained from leaf explants after 40-50 days [C] = Callus obtained from Leaf explants after 30-40 days . [D]= Callus obtained from Leaf explants after 30-40 days

Table 2: Response of leaf explants of *Piper nigrum* L. for callus induction to different concentrations of Plant growth regulator, 2,4-D and kinetin

Sr. No	Growth hormone	Explants used	Time required to obtain callus (days)	Callus morphology		Amount of Callus
	2,4-D+Kinetin			Colour	Texture	
1.	0.5+1.5	Leaf	20	No callus	-	-
2.	0.5+2.0	Leaf	30	Light brown	Soft	+
3.	0.5+3.0	Leaf	40	Light brown	Soft	+
4.	0.5+3.5	Leaf	50	Greenish yellow	Hard	+

The Callus initiation was obtained by inoculating leaf part as explants of *Piper nigrum* L. in MS Media with various combination and concentration of Growth regulators such as 2,4-D and Kinetin in combination, 0.5+1.5mg/lit, 0.5+ 2.00 mg/lit, and 0.5+3.00mg/lit, 0.5+3.5mg/lit respectively (Table 2).

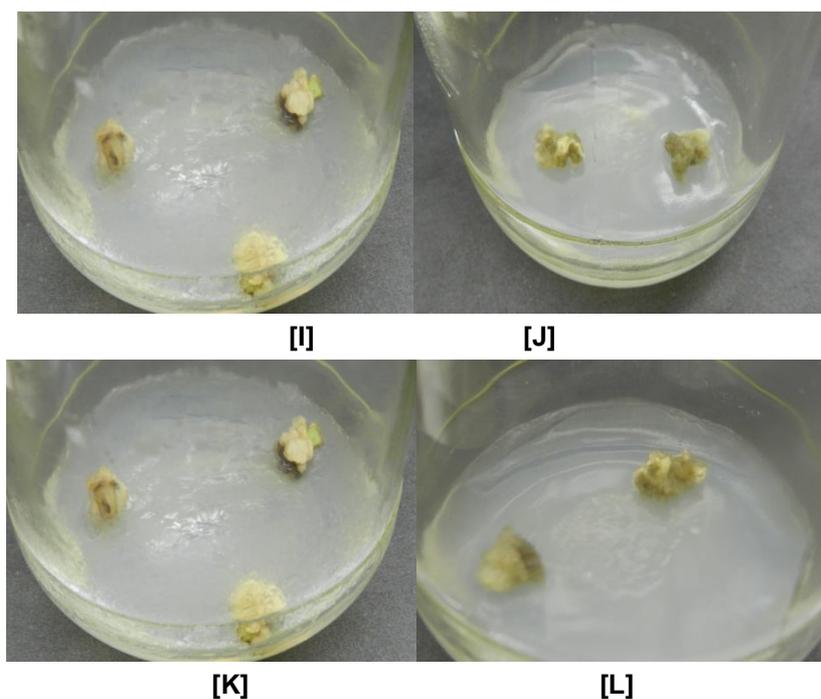


[EFGH] = Callus obtained from Leaf explants after 35 - 45 day

Table 3: Response of leaf explants of *Piper nigrum* L. for callus induction to different concentrations of Plant growth regulator, 2,4-D

S. No	Growth hormone	Explants used	Time required to obtain callus (days)	Callus morphology		Amount of Callus
	2,4-D			Colour	Texture	
1.	1.5	Leaf	20	No callus	-	-
2.	2.0	Leaf	30	Light brown	Soft	+++
3.	2.5	Leaf	40	Light brown	Soft	++
4.	3.0	Leaf	50	Greenish yellow	Hard	+
5.	3.5	Leaf	60	Greenish yellow	Hard	+

Initiation of callus obtained by inoculating inter nodal part as explants of *Piper nigrum* L. in MS Media with various combination and concentration of Growth regulators such as 2,4-D in combination as 2.0 mg/lit (Table 3).



[IJKL] = Callus obtained from Leaf explants after 35 - 50 days

DISCUSSION

Piper nigrum L. commonly known as "Black pepper", has gained a global consideration because of its volume in the spice industry. This plant has shown great potential for the discovery of novel biologically active compounds and need for techniques to enhance the production of high quality consistent plant material for feasible accumulation of metabolites. Tissue culture of *P. nigrum* can play a vital role in germplasm conservation, enhanced multiplication and genetic engineering for feasible production of biologically active compounds. Liquid culture is yet to be established and reserves corner for industrial production of these active components. This review provides the developments in the propagation practices and challenges that remain in *P. nigrum* L. biotechnology. (Bilal Haidar Abbasi *et al.*, 2009) Black pepper (*Piper nigrum* L.) is a flowering vine in the family Piperaceae, cultivated for its fruit, which is usually dried and used as a spice and seasoning. The fruit, known as a peppercorn when dried, is approximately 5 millimetres (0.20 in) in diameter, dark red when fully mature, and, like all drupes, contains a single seed. Peppercorns, and the powdered pepper derived from grinding them, may be described simply as pepper, or more precisely as black pepper (cooked and dried unripe fruit), green pepper (dried unripe fruit) and white pepper (dried ripe seeds).

In present investigation for in-vitro propagation of *Piper nigrum* L. investigator used different parts like nodal segments, leaf, and as explants. All type of explants was significant for *in-vitro* propagation but only leaf explants gave satisfactory response in different combination of media in present investigation. In present investigation, in *Piper nigrum* L. the induction of callus was achieved when MS media supplemented with combination of 2, 4-D (2.5mg/lit, 3.5mg/lit). BAP at a higher concentration 3.0 mg /lit and 3.50mg/lit induces callus formation was observed from leaf explant. It was observed that callus initiation after 40 to 50 days. In present investigation, *In-vitro* propagation of *Piper nigrum* L. Callus induction was achieved in most of the combinations and concentration of growth regulators such as BAP and IBA in combination, BAP and IAA in combination, BAP and Kinetin in combination.

CONCLUSION

The present study can be used as a protocol for the tissue culture of black pepper under *In-vitro* conditions *via* callus culture. Tissue cultured plants should be acclimatized before introduction to field and must be planted in the fields with minimum frost areas or else inside green house. The period for Callus induction required more than 30 days time period. In the present investigation attempts were made to standardize the protocol for callus formation of *Piper nigrum* L. supplemented with various concentrations of growth regulators with leaf

explants. It has been observed that leaf explants flourished well with callus induction on or after 30 day on the media supplemented with 2,4-D, BAP, Kinetin, IAA. The protocol can be satisfactorily used for commercial and experimental demonstration of *Piper nigrum* L.

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