In vitro regeneration of *Piper longum* L. and comparative RP-HPLC analysis of piperine production of *in vitro* and *in vivo* grown plants

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Abstract

Piper longum L. is a well known spice plant belonging to the family Piperaceae with high pharmacognosy potential, but it is becoming threatened due to overexploitation. Thus, this investigation aims to standardize a cost effective protocol for in vitro propagation of this economically important plant. Internodal segments were used as explant for callogenesis in Murashige and Skoog medium with 3% sucrose and 0.8% agar, with NAA or 2,4 D. Optimum callus induction was observed in MS medium with 5.0 mg/L NAA. Calli were subcultured on shoot regeneration media containing different concentrations of cytokinin (KIN/BAP) along with 0.1mg/L NAA. Best shoot regeneration was obtained on MS media supplemented with 2.0 mg/L KIN and 0.1mg/L NAA. Induced shoots were rooted in either NAA or IBA and highest rooting was induced in MS medium enriched with 0.5 mg/L NAA. Rooted plantlets were acclimatized and 88% of hardened plants survived. Field emission scanning electron microscopic (FE-SEM) showed that regeneration from callus had occurred by somatic embryogenesis. A comparative study on identification and quantification of piperine (the chief alkaloid of the cultivar) were done from root and fruit of both in vitro and in vivo grown plants through Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) method. I n vitro grown fruit was found to have the maximum amount of piperine.

Key Message

For the first time, a comparative study in identification and quantification of piperine was done from both in vitro and in vivo grown Piper longum through RP-HPLC method.

Introduction

Archaeological evidences indicate the use of plants as medicine since five thousand years ago, including Egyptians, Mesopotamians, and ancient Indian Ayurveda. *Piper longum* L., the close relative of black pepper (*Piper nigrum*), commonly known as 'Indian long pepper' (India Biodiversity Portal), belongs to the family Piperaceae. This flowering vine is widely distributed in the tropical and sub tropical regions of the world (Kumar et al. 2016; Catalogue of Life. 2020; Satyavati et al. 1987), where it is cultivated for its fruit which is used as spice from the fifth or sixth century BCE by Greeks and romans (www.gosumitup.com), while the ancient textbook of Ayurveda also refers its medicinal and dietary use. This spice is generally used for seasoning and contains various bioactive compounds with a broad range of medicinal properties (Kumar et al. 2011; Yadav et al. 2020). A number of previous works (Kanaki et al. 2008 ; Kumar et al. 2011) have suggested that Piperine ($C_{17}H_{19}O_3N$) is one of the major alkaloids, which is efficient to exhibit various pharmacological activities and responsible for its pungency too (Kumar et al. 2011).

P. longum can be multiplied naturally via seed germination or by vegetative propagation. However, conventional seed germination cannot be easily accomplished due to the low availability of viable seeds, delayed and prolonged germination period (Pradhan 2015; Rani and Dantu 2012), while scanty rooting is

a great constraint of vegetative propagation of the plant. The plant has been included in IUCN Red list of threatned medicinal plants of India under medicine for human and veterinary group (Gowthami et al. 2021). High economic value, rare commercial availability of long pepper (www.ayushveda.com) and its existential threat (Nair 2000) have created an immediate need for establishing suitable cost effective protocol for micropropagation.

In vitro callogenesis and indirect clonal propagation provide feasible and efficient methods of recovery of secondary metabolites throughout the year without seasonal coercion (Isah et al. 2018). Huge amounts of callus can be induced for quantitative enhancement of potent bioactive compounds and their commercialization, and large numbers of plantlets can be produced in short span of time to meet up the increasing market demand of the plants. There is some available literature on *in vitro* propagation of *P.longum* (Bhat et al. 1992**;** Sarasan et al. 1993**;** Soniya and Das 2002**;** Parida and Dhal 2011**;** Rani and Dantu 2012; Pradhan 2015; Ravindaran et al. 2016 ; Sathelly 2016 ; Saravanan 2019 ; Prajapati et al. 2019**).** However, the indirect regeneration of the plant with a study of callus morphology was seldom documented, in particular with confirmation of the regeneration pathway through FE-SEM, a standardized efficient and cost effective protocol for indirect regeneration from callus has not been provided much. Moreover, there are no reports on the comparative profiling as well as quantification of the principal bioactive alkaloid Piperine, through RP-HPLC from dry roots and fruits,callus of *in vitro* and *in vivo* grown plants. This research sets out to fill in those gaps.

Materials And Methods

Collection of explants

The plant material (monoecious female) was collected in June 2019 from the Haripal (Located: 22^{o}49’53”N88^{o}7’7”E; altitude: 16m {52ft}) Hooghly District, W.B, India. Fresh rooted shoots of *P.longum* were collected and maintained in the garden of Dept. of Biotechnology, The University of Burdwan, WB, India. The material was confirmed by Botanical Survey of India,Kolkata (**Supplementary certificate 1:**Voucher specimen no MC-01) and used as source of explants for the *in vitro* regeneration.

Sterilization of explant and culture establishment

Tender twigs with 4-5 nodes were excised from stock plants in the month of March to avoid mealy bug attack, and were washed thoroughly in running tap water for 10 minutes. Twigs were then treated with 70% (v/v) ethyl alcohol for 30 sec, followed by immersing in 0.01% mild detergent, Tween-20 (Merck, USA) for 6 min prior to explant treatment with 0.1% (w/v) $HgCl_2$ (Merck, USA) for 2 min. Finally, explants were rinsed with sterile distilled water (Milli-Q water system, Merck Millipore, USA) thrice to wash out all used chemicals and were blotted dry on sterile filter paper. All procedures were done in sterile conditions (Biosafety Cabinet A2, Biobase Inc., China). Internodal segments (approx. 1 cm long) were excised and inoculated in MS (Murashige and Skoog 1962) basal medium (Himedia, India), fortified with NAA (0.1 mg/L- 5.0 mg/L) and 2,4-D (0.1 mg/L-5.0 mg/L). Cultures were incubated in a growth chamber (Thermo

Fisher Scientific, USA) at 25 ± 2^0C with a 16 hrs photoperiod (2000 lux intensity) and 65% relative humidity. All media were done in ten replicates and the experiment was repeated thrice under the same cultural conditions. Data of callus induction frequency (%) and fresh weight were recorded after 28 days of culture.

Shoot and root regeneration

Induced calli with shoot buds were transferred in shoot regeneration medium containing 0.5 mg/L - 5.0 mg/L BAP or 0.5 mg/L - 5.0 mg/L KIN in combination with 0.1mg/L NAA. After 25 days, data were recorded for statistical analysis and further, regenerated shootlets were transferred onto rooting media supplemented with 0.1 mg/L - 1.0 mg/L NAA or 0.1 mg/L - 1.0 mg/L IBA to study the effect of different auxins in rhizogenesis. After 10 days of culture in rooting media, data were collected for statistical analysis. Ten replicates were studied for each culture condition and experiments were repeated thrice under the same physical and chemical environment.

Hardening of *in vitro* grown plantlets

Complete plantlets were hardened in plastic bags with sterilized sand:soil (1:1) for the first 18 days, with supplementation of half strength MS nutrient broth and watering done with sterile distilled water. Thenceforth, acclimatized plantlets were transferred into a second plastic bag containing garden soil:sand:organic manure (1:1:1) and maintained for 10 more days. Finally, acclimatized plantlets were transferred into earthen pots and shifted to the polyhouse (Dept. of Biotechnology, BU, Pin:713104, WB, India).

Sample preparation for FE-SEM

Regenerated callus (28 days old) was fixed in 2.5% solution of glutaraldehyde with Phosphate buffer 0.2M (pH-7.0) at 4ºC for 4 hours. The specimens were washed in the same buffer (20min) and then dehydrated in increasing alcohol concentrations (20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%), followed by absolute ethanol, for 15 min in each solution. After the alcoholic dehydration, specimens were subjected to a series of absolute ethanol and pure iso-amyl acetate mixture (1:3, 2:2, 3:1 and then pure iso-amyl acetate) before CPD (Critical point drying) and then dried to critical point with liquid CO2. Then, stub was prepared by coating with gold sputter for 40 seconds and observed under FE-SEM (Carl Zeiss Gemini 300).

Statistical Analysis

All experiments included ten replicates and were repeated thrice. All collected data like fresh callus weight, number of shoots per explant, shoot length, number of leaves per plantlets and number of roots

per plantlets were recorded after regular intervals as outlined above. The mean±SE of all data were statistically evaluated by one way analysis of variance (ANOVA) followed by Duncan's multiple range test at $p<0.05$, performed by the IBM SPSS software.

Sample preparation and analysis of Piperine through RP-HPLC

Piperine was extracted and samples were prepared with some modification as reported by Santosh et al. (2005). Fresh *in vitro* cultured callus, roots and matured dry fruits were collected from both *in vitro* and *in vivo* grown plants, washed thoroughly under tap water and shed dried for 72 Hrs. Dried samples were then crushed and ground into fine powder by using porcelain mortar and pestle. Each powdered sample (100 gm) was refluxed with 500 ml of HPLC grade methanol (Merck, USA) for 5 Hrs in Soxhlet apparatus. The sample-solvent mixture was then allowed to cool and filtered to remove solvent. Reflux was performed again by using fresh methanol (500 ml) for another 4 Hrs. The methanolic extract was then concentrated in a rotary evaporator (Biobase Inc, China) at 70^0C followed by lyophilizer drying (Eyela Inc, Japan). Each sample (1 gm) was resuspended in 20ml of 100% HPLC grade methanol (Merck, USA), homogenized for 10 mins and subjected to ultrasonication for 45 mins. Extracts were then filtered subsequently by using Whatman No1 filter paper (Merck,USA) and membrane polytetrafluroethylene syringe filtre (PTFE with 0.22 µm porocity; Himedia, India). Chromatographic analysis were done by using a RP-HPLC system (equiped with 5160 quartenary gradient pump and 5420 UV-Vis detector, Model: Chromster, Make: Hitachi Corporation, Japan). The isocratic solvent system of methanol and water (80:20) was used at a flow rate of 1 ml/min. The detecting wave length and total run time were set at 343 nm and 20 minutes respectively. The sample was injected manually in a manual injector port (20 µl for each sample). The reverse phase C_{18} packed column (5C18-MS-II, 4.6ID x 250 mm, COSMOSIL) was used for the HPLC analysis. Chromatograms were obtained from individual running of prepared samples (*in vivo* and *in vitro* root and fruit) and standard piperine (Sigma Aldrich, USA, Catalogue No. 75047-50mg) were analyzed. The following fomula was used (Siva et al. 2015) for quantification of piperine.

Sample concentration =

$$\frac{\text{Sample area}}{\text{Mean standard area}} \times \frac{\text{Standard weight}}{\text{Standard dilution}} \times \frac{\text{Sample dilution}}{\text{Sample weight}}$$

Results And Discussion

Induction of callus

Callus induction was obtained in MS media fortified with variable concentrations of NAA (0.1 mg/L, 0.5 mg/L, 1.0 mg/L, 2.0 mg/L, 3.0 mg/L, 4.0 mg/L, 5.0 mg/L) or 2,4D (0.1 mg/L, 0.5 mg/L, 1.0 mg/L, 2.0 mg/L, 3.0 mg/L, 4.0 mg/L, 5.0 mg/L). After 28 days, three different types of callus were observed: compact, loosely clustered friable, transparent (**Supplementary table 1).**

Callus induction frequency was better in both the ranges of higher (3.0 mg/L-5.0 mg/L) and lower (0.1 mg/L-2.0 mg/L) concentrations of NAA rather than with 2,4-D. Callus induction frequency was maximum (90-98%) in 0.1 mg/L-5.0 mg/L NAA with green, regular, compact, organogenic callus **(Fig. 1a)**. This type of calli were called embyogenic calli as in a previous report (Haeyoung et al. 2007). Conversely, callus induction was reduced (25-38%) to moderate (45-50%) in media with of 2,4-D, and such calli were brownish, often transparent **(Supplementary Fig. 1)**, and non embryogenic (Haeyoung et al. 2007). Hence, NAA was more efficient for induction of embryogenic callus in comparison to 2,4D in *P.longum.*

During data recording of callogenesis, it had been observed that fresh weight of callus was directly proportional with PGR concentration. Highest mass of callus was obtained in MS media supplemented with 5.0 mg/L of NAA **(Supplementary table 1 and Fig. 2)** but mean difference was significantly different in MS medium containing 2,4 D when tested $p<0.05$ level during one way ANOVA followed by Duncan's multiple range test.

In previous report (Sarasan et al. 1993), profuse nodular callusing was found at 2.0mg/L 2,4-D + 1.0 mg/L BA. Sathelly et al. (2016) observed apperence of globular compact callus in MS medium with a combination of auxin and cytokinin(1.0 mg/L IAA + 1.0 mg/L BAP) but in current study, callus was induced only in presence of auxin (2,4-D /NAA). Among those two used auxin, NAA proved to be potent one. 2,4-D showed ill response **(Supplementary Fig. 1)** in context to callusing and it strongly contradicated with the response obtained by Sarasan et al. (1993).

In vitro Shoot regeneration

Shoot regeneration were induced in both the variable concentrations of BAP (0.5 mg/L - 5.0 mg/L) as well as Kinetin (0.5 mg/L- 5.0 mg/L) with fixed concentration of NAA (0.1 mg/L). Ten replicates were cultured for each concentration. Observations were done for evaluating the growth of shoot number, shoot length, leaf number. The best result was observed at 2.0mg/L Kinetin + 0.1mg/L NAA concentrations in MS media **(Table 1, Fig. 1b and Supplementary Fig. 2b)** which was found to elicit optimal response with an average 18.46 ± 0.51 shoot bud induction, 8.06 ± 0.24cm shoot length and 33.06 ± 0.38 leaf number **(Table 1)**. It was also observed that ascending PGR concentration induced yellowish green colored succulent leaf with stunted shoot growth that seemed phenotypically different from *in vivo* grown plant.

In previous report (Sathelly et al. 2016), it was stated that media with individual kinetin showed lowest response and the combination of two cytokinin (BAP and Kinetin) showed highest percentage of shoot induction. But in this study, it was proved that only one cytokinin (KIN) is efficient enough to induce maximum number of shoot per explant which was also cost effective.

In vitro root induction

After a sub culturing, elongated shoots were excised and cultured in MS media having different concentrations of NAA (0.1mg/L, 0.5mg/L, 1.0mg/L) and IBA (0.1mg/L, 0.5mg/L, 1.0mg/L). Experiments were repeated thrice and a subculture was done in MS medium without growth hormone as control.

Initiation of rooting was started after 10 days on medium containing NAA. Control culture showed delayed rooting. Response of root inducing growth regulators were tabulated **(Table 2)**. Though all cultures induced root in presence of both the auxin but best root induction was observed at 0.5mg/L NAA **(Fig. 1c and Supplementary Fig. 3)** with an effective mean root number 33.33 ± 0.42. The control culture was also induced root but took prolonged time nearly 45 days.

All the data was recorded in **Table 2** do not imply similarity with any one of those previously reported works (Sathelly et al. 2016 ; Prajapati et al. 2019) on root induction. Those literature showed root induction on MS media fortified with 1 mg/L NAA and 1.0 mg/L IBA respectively but in this present study, maximum number of roots were generated in MS media enriched with 0.5mg/L NAA that was again more competent in the sence of cost effectivity.

Hardening of plantlets

Primary hardening**,** followed by secondary hardening **(Fig. 1d)** was accomplished with 88% of success rate. After proper maintenance, growth and development of the plants were monitored carefully. Flowering of the *in vitro* grown plant was observed in the month of July and August and fruiting started in the month of September(**Fig. 1e**). So, no changes were found in the reproductive and harvasting season (India Biodiversity Portal ; Vikaspedia) of *in vitro* propagated plants.

Few literatures of clonal propagation of this cultivar was reported previously, but all studies were documented only upto the direct or indirect propagation and no one had even tried to observe the season of flowering and fruiting of the *in vitro* grown plants. From this study it had been demonstrated that profuse amount of *P.longum* plantlets can be obtained by following this cost effective process.

SEM Analysis

Under the SEM, embryogenic calli were showing various stages of somatic embryos such as heart (HS) and globular (GS) shaped structures **(Fig. 3a)** and scutellum (SC) associated with coleoptile (CO) **(Fig. 3b)** over the surface of 28 days old regenerated callus tissue .

Very few studies on callus induction of *P.longum* were documented previously but morphology was not well studied. In this attempt, the morphology of embryogenic callus was observed under Scanning Electron Microscope (Carl Zeiss Gemini 300). This study was confirmed through literature of SEM analysis in different plants (Chaudhury and QU 2000; Cabral et al. 2011, Sivanesan et al. 2015).

HPLC Analysis of Piperine

Chromatograms **(Fig. 4)** obtained from HPLC analysis clearly indicated the noteworthy differences in piperine content among different plant parts grown under *in vitro and in vivo* conditions. Piperine was

identified by compairing retention times in chromatograms of test samples with standard piperine (R_t -5.963 min). Retention times of *in vitro* grown dry fruit (R_t -5.964 min), *in vivo* grown dry fruit (R_t -5.968 min), *in vitro* grown plant's root (R_t - 5.957 min), *in vivo* grown plant's root (R_t -5.972 min) and *in vitro* grown callus tissue extract (R_t - 5.967 min) were used to calculate quantity of piperine following the formula proposed by Shiva et al. (2015) and obtained results of piperine content were 8.802 µg/ml, 0.650 µg/ml, 0.639 µg/ml, 0.038 µg/ml, 0.476 µg/ml respectively. Among all those samples, highest quantity of piperine was found in dry fruits of *in vitro* grown *P. longum* plant.

Such quantitative enhancement of chief alkaloid piperine in fruit of *in vitro* grown *P.longum* might be due to a cumulative effect of suitable plant growth promoters like kinetin and NAA on enzymetic activities involving in the biosynthetic pathway of piperine. The stress effect of cultural temperature (25 ± 2^0C), which is quite less in comparison to the required temperature (30 ± 2^0C) for *in vivo* growth of the cultivar (agritech.tnau.ac.in) might be one of the reason behind the quantitative enhancement of piperine.

Few existing literatures (Rajopadhye et al. 2012, Khound et al. 2017) were confirmed the presence of piperine in different parts, predominantly in root and fruit of naturally grown *P.longum* plant with different genotype. But no one had tried to establish the effect of *in vitro* culture on piperine content.

In this endeavor, quantitative enhancement of pharmacologically important piperine in *in vitro* cultured plant may be proved to be significant enough for standardizing the cost effective protocol for *in vitro* biomass culture of *P. longum* L. that may lead to commercial exploitation.

Conclusion

To sum up, the novel finding of this study was enhancement of piperine content in the plant part of *P.longum* grown under *in vitro* condition. It was clearly proved that *in vitro* grown fruits found to produce much more quantity of piperine in comparison to fruits of *in vivo* grown plant. Hence, isolation and enhancement of secondary metabolite like piperine, in culture condition, will lead to commercialization of highly valuable micropropagated plantlet and that may be considered as source of plant based natural drug for many pharmacological formulation. Overall, the study revealed a good synergistic effect with best significant response for standardization of cost effective protocol for micropropagation of *P.longum*. and its conservation. Thus, our finding may encourage in mass cultivation of this economically important plant and provide a promising option for socio-economic development of Indian farmers.

Abbreviations

MS : Murashige and Skoog

NAA: 1-Napthaleneaceticacid

2,4-D: 2,4-Dichlorophenoxyacetic acid

KIN: Kinetin

BAP: N^6-Benzylaminopurine

IBA: Indole-3-butyric acid

mg/L: Milligram/Litre.

FE-SEM: Field emission scanning electron microscope

RP- HPLC: Reverse Phase- High Performance Liquid Chromatography

R_t: Retention time

ANOVA: Analysis of variance

Declarations

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Declarations Authors certify that the work contained in this paper is original, and has not been submitted to any other journal for publication.

Conflicts of interest All authors declare that they have no conflict of interest.

Author Contributions The experiment was designed by Mousumi Chatterjee and Dr. Indrani Chandra. Collection of plant, sample preparation and whole research work was executed and statistically analyzed by Mousumi Chatterjee. Dr.Sabyasachi Chatterjee monitored the research significantly. This experimental work is a part of Ph.D thesis of Mousumi Chatterjee.All authors read the manuscript, revised critically and approved the final version.

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Tables

Table 1 Response of PGRs for shoot regeneration after 25 days

NAA (mg/l)	BAP (mg/L)	KIN (mg/L)	Number of shoots/explant (Mean±SE)	Shoot length(cm) (Mean±SE)	Number of leaf (Mean±SE)
0.1	0.5	-	09.40±0.42^{ef}	3.33±0.10^{fg}	15.93±0.94^{ij}
0.1	1.0	-	15.00±0.04^{ab}	4.79±0.17^{ef}	25.53±0.23^{cd}
0.1	2.0	-	12.73±0.37^{cd}	3.20±0.14^{fg}	19.13±0.43^{ef}
0.1	3.0	-	10.46±0.24 ^{de}	3.03±0.14^{gh}	17.80±0.30^{gh}
0.1	4.0	-	09.13±0.31 ^{ef}	2.72±0.15^{gh}	16.26±0.85^{gh}
0.1	5.0	-	08.60±0.50^{ef}	2.35±0.13^{gh}	15.26±0.77ij
0.1	-	0.5	10.06±0.31^{de}	4.40±0.26^{ef}	18.53±0.83^{ef}
0.1	-	1.0	12.26±0.37^{cd}	5.46±0.39^{cd}	22.80±0.63^{e}
0.1	-	2.0	18.46±0.51^{a}	8.06±0.24^{a}	33.06±0.38^{a}
0.1	-	3.0	15.60±0.50^{ab}	6.46±0.15^{b}	29.33±0.22^{b}
0.1	-	4.0	14.06±0.44^{c}	5.53±0.14^{cd}	27.80±0.92^{c}
0.1	-	5.0	11.06±0.37^{cd}	4.80±0.21^{e}	24.60±0.51^{cd}

(Values were represented by (Mean±SE) of 10 Replicates per treatment with three times repeatation.Values denoted by same letter were not significantly different (P<0.05) using Duncan's multiple range test. ,here S.E represents Standard error of mean).

Table 2 Response of root induction at various concentrations of NAA and IBA after 10 days.

NAA(mg/L)	IBA(mg/L)	Number of roots(Mean±SE)
0.1	-	14.20 ± 0.37^{c}
0.5	-	33.33 ± 0.42^{a}
1.0	-	25.00 ± 0.67^{b}
-	0.1	09.40 ± 0.40^{ef}
-	0.5	11.20 ± 0.37^{de}
-	1.0	08.46 ± 0.24^{ef}

(Values were represented by (Mean±SE) of 10 replicates per treatment with three times repeatation. Values denoted by same letter were not significantly different (P<0.05) using Duncan's multiple range test., here S.E represents standard error of mean).

Figures

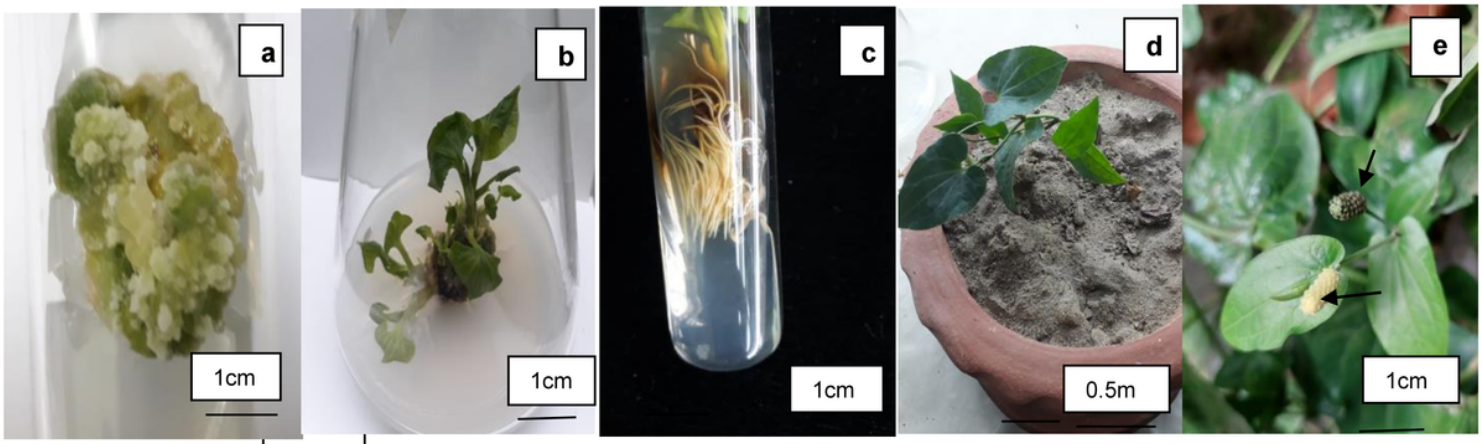

Fig.1 Stereomicrograph of induced callus, organogenesis, hardened plantlet,flowering and fruiting of *Piper longum* L. **a** Embryogenic callus in MS media containing NAA (5.0 mg/L), **b** Shoot regeneration in MS media containing 2 mg/L Kinetin +0.1mg/L NAA, **c** root induction(0.5 mg/L), **d** Hardened plantlets, **e** Flowering and fruiting in *in vitro* grown plant.

Figure 1

See image above for figure legend.

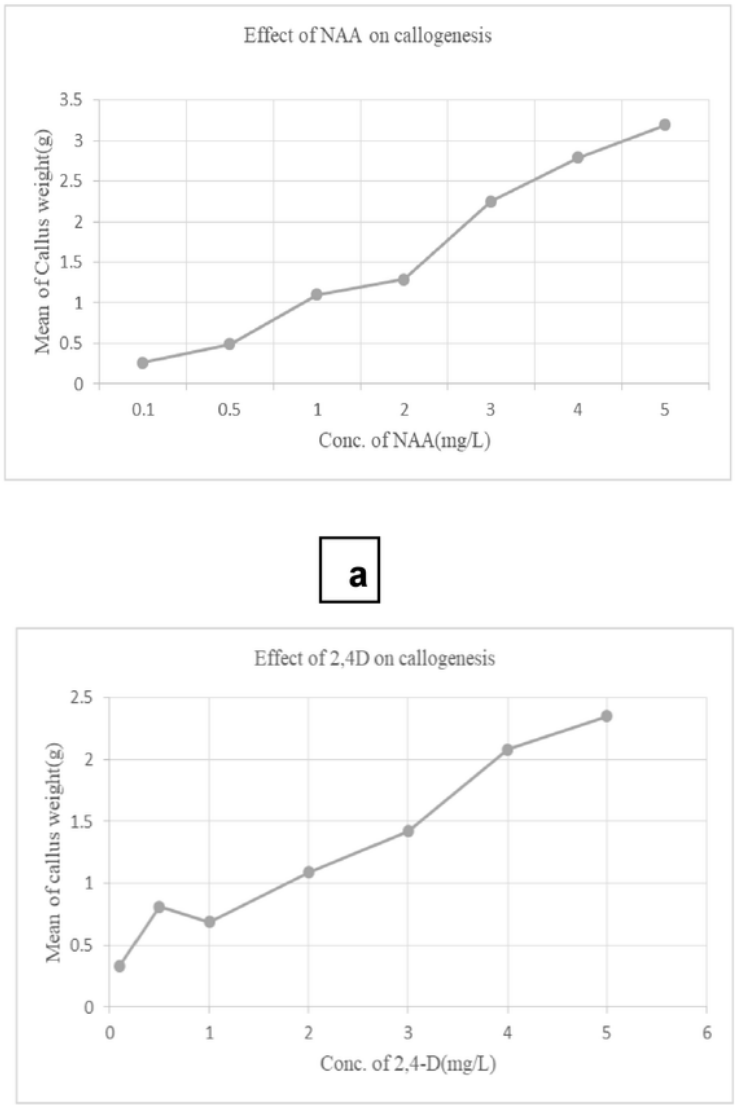

Fig.2 Callus weight (gm) atdifferent concentration of **a** NAA and **b**2,4-D

Figure 2

See image above for figure legend.

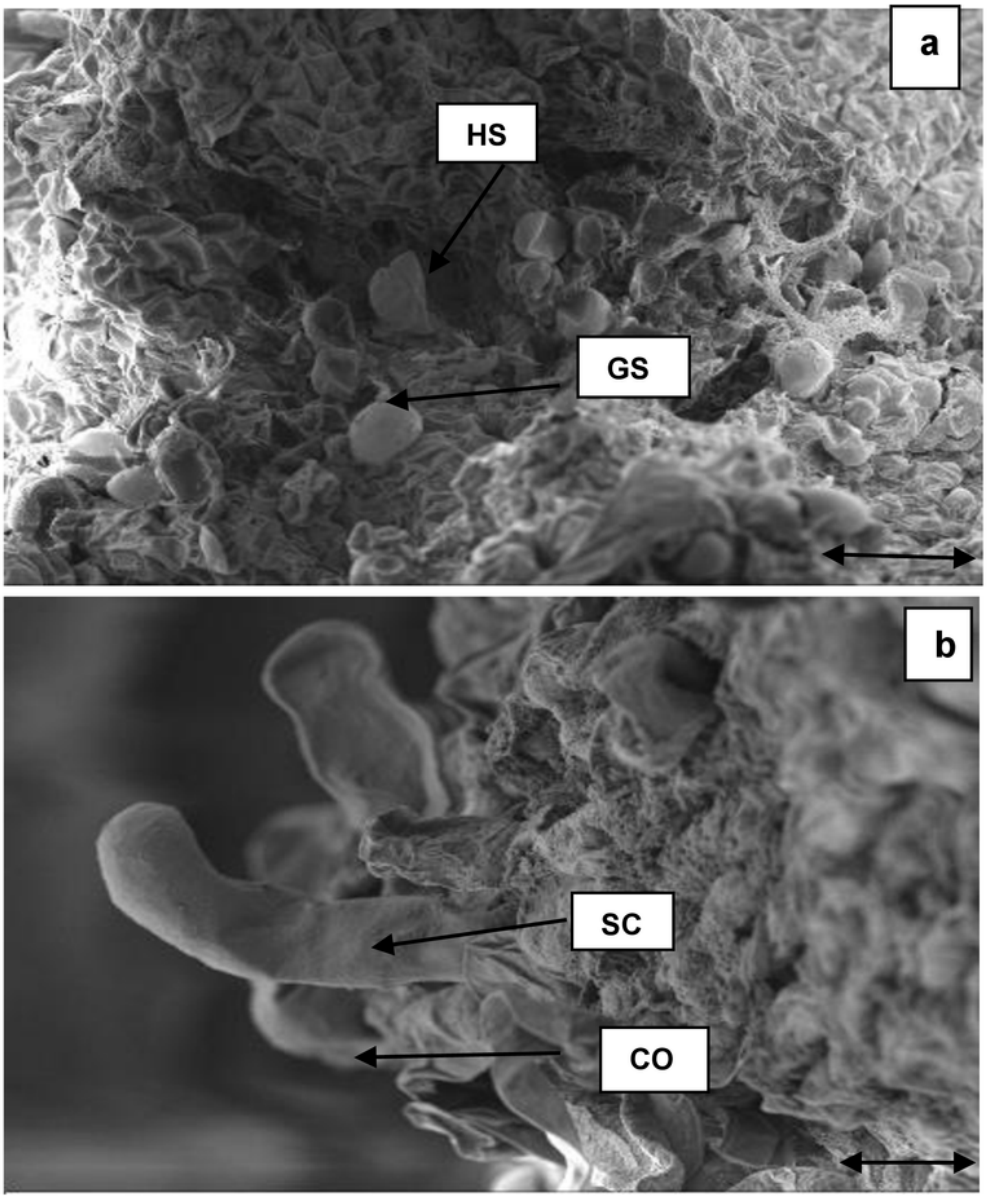

Fig.3 Micrograph of Scanning Electron Microscope (Carl Zeiss) of **a** embryogenic callus(Magnified at 400X with 20µm aperture) showed various stages of somatic embryos such as **a** globular (GS), heart shaped (HS) structure (marked by arrow), **b** scutellum (SC) associated with coleoptile (CO) with 300KX with 3µm aperture size

Figure 3

See image above for figure legend.

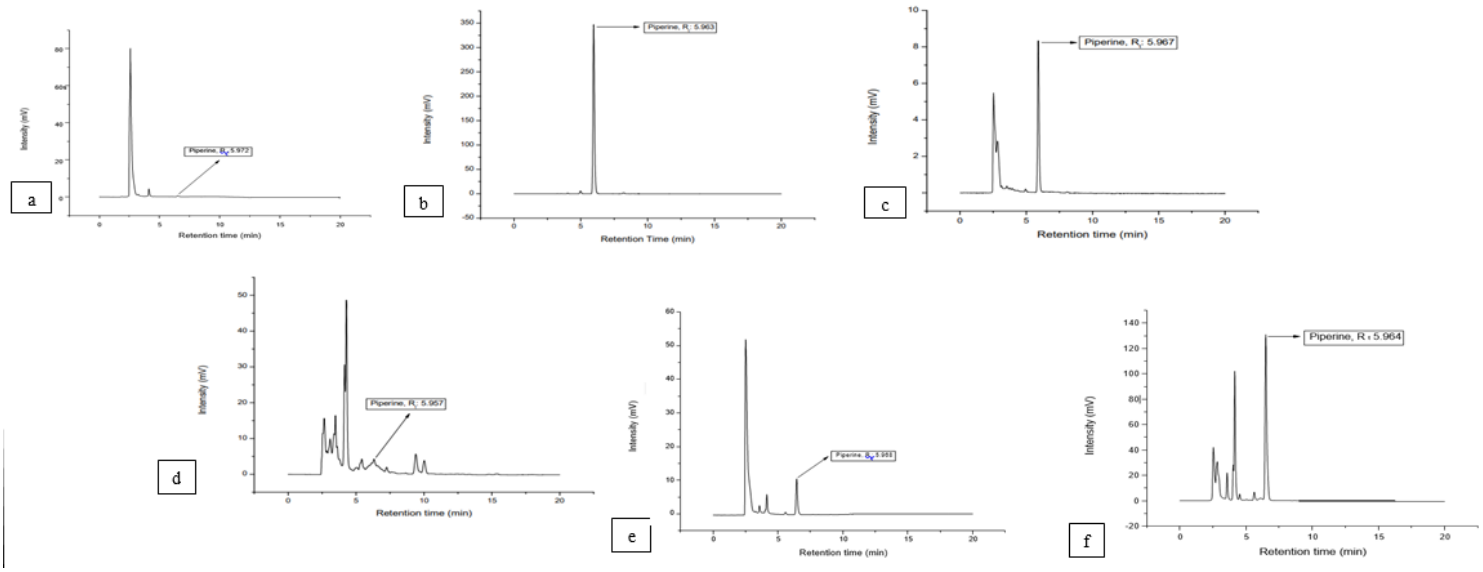

Figure 4

HPLC chromatograms of standard Piperine and piperine present in various in vitro and in vivo grown plant extracts of Piper longum L. a Chromatogram of standard piperine, b Chromatogram of callus extract, c Chromatogram of in vitro root extract, d Chromatogram of in vivo root, e Chromatogram of in vitro fruit extract, f Chromatogram of in vivo fruit extract.

Supplementary Files

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- SupplementarymaterialforPCTOC.docx