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RESEARCH ARTICLE

Improved Micropropagation Protocol and Molecular Marker Based Genetic Stability Assessment of Black Pepper (*Piper nigrum* L.)

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Black pepper (*Piper nigrum* L.) is a highly commercial and valuable spice crop in the world, plants of which have originated from Western Ghats region of southern India. An efficient and reliable micropropagation protocol is reported in this paper, which can be utilized for multiplication of elite genotypes of black pepper. Shoot regeneration was induced on Murashige and Skoog (MS) and Woody Plant Media (WPM) media in 24 combinations with plant growth regulators. Explants cultured in MS media supplemented with 0.5 mg L^{-1} BAP formed 6.5 nodes per plant with longest shoots (10.42 cm) after 12 wks. Regenerated shoots were rooted on MS media supplemented with 2.0 mg L^{-1} IBA, yielding about 15 roots per plant with an average root length of 3.40 cm within 4 wks. The hardening of the shoots under a mist chamber conditions (25±2 °C, 70-80% RH) yielded 100% survival after acclimatization. Genetic stability analysis of the micropropagated and mother plants was done using 48 ISSR markers. The plants found to be genetically stable when compared with mother plants. A ratio of 1:6.5 numbers of nodes is obtained on the MS medium supplemented with 0.5 mgL^{-1} of BAP within 3 months. This research presents an improved and efficient micropropagation protocol with good rate of multiplication of the genetically stable plants that are easily acclimatized to the field conditions.

Key Words: ***In vitro* clonal propagation, *Piper nigrum*, Black pepper, Molecular markers, Field acclimatization**

Introduction

Black pepper (*Piper nigrum* L., Family Piperaceae) is one of the world's most important and commonly used spice. Due to its enormous usage, volume of trade and commerce among spices in the world market, the crop is known as the "king of spices" (Srinivasan, 2007). It is found growing in a wide range of altitudes and has been shown to be tolerant to a variety of environmental conditions (Ravindran and Kallupackaral, 2012). Due to the color of the peppercorn seed, the economically important portion of black pepper, it was given the name "black pepper". It is native to India, particularly the Western Ghats region. Vietnam is the world leader in terms of total production (0.267 million tons). It is grown on 0.259 million ha in India, with a production of 61 thousand tons, of which 16,250 tons were exported, earning INR 5.51 billion worth in foreign currency (Spice Board of India, 2020). Karnataka is the major producer of black pepper in India.

Black pepper is a woody climbing vine, with aerial roots trailed over the support of columns and can grow up to a height of 5-6 m. It features bright, lustrous leaves that are placed alternately. The fruits, which are drupes (~5 mm dia), are the economically important part. Black pepper is a mature fruit that has been dried (peppercorns). Pepper is used in a multitude of ways, mostly as a spice, a condiment, a preservative, an insecticide and as herbal medicine (Wang *et al*., 2017). The component piperine, a pungent alkaloid, is responsible for the black pepper's spicy flavor (De Almeida, 2020). It improves the bioavailability of a wide range of other structural and therapeutic medicines (Khajuria, 2002). It is used in a diverse variety of cuisines around the world.

Piper nigrum plant is a climber, propagated *via* seeds, cuttings (2-6 nodes per cutting), layering and grafting. Due to the heterozygous nature of the seeds, propagation *via* cuttings is commonly used and other techniques of propagation are not used because they are slow and time demanding (Ramakrishna Nair and Datta Gupta, 2003; Hussain *et al*., 2011). Vegetative cultivation of black pepper is hindered by diseases such as foot rot and anthracnose and also insect such as pollu beetle

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(*Longitarsus nigripennis)*. Cucumber mosaic virus and piper yellow mosaic virus are examples of viruses that can be transmitted to progenies (Bhat *et al*., 2018).

The germplasm of black pepper that is free of pest and diseases can be easily maintained and mass produced using plant tissue culture techniques. The exploitation of *in vitro* culture techniques has allowed researchers the unique opportunity to micropropagate plants from somatic tissues, overcoming sexual barrier (Abbasi *et al*., 2010). This method results in disease-free, pest-free and virus-free plants, which are genetically stable and produce identical progenies. Although, the black pepper crop can be grown in tissue culture, there are just a few reports of it being propagated using large-scale micropropagation technique. Explants employed include callus, somatic embryos (Ramakrishna Nair and Dutta Gupta, 2003), shoot tips (Nazeem *et al*., 1992; Philip *et al*., 1992; Babu *et al*., 1993; Joseph *et al*., 1996), nodal explants (Bhat *et al*., 1995) and leaf explants (Sujatha *et al*., 2003). The existence of endogenous bacterial infection is known to impair establishment of fresh *in vitro* cultures (Fitchet, 1990; Philip *et al*., 1992; Abbasi *et al*., 2010).

By analyzing the importance of black pepper crop and the lack of sufficient reports on efficient direct shoot generation and shoot multiplication protocol, in the present study, 24 permutations and combinations of media which included two types of basal media and two types of plant growth regulators were tested for micropropagation protocol development. Further, regenerated plants were tested for genetic stability using molecular markers.

Materials and Methods

Plant material and culture conditions

Piper nigrum accession TCR59 (IC 85371), maintained in the *In Vitro* Gene Bank (IVGB) of ICAR-National Bureau of Plant Genetic Resources (NBPGR), New Delhi, India, was utilized as mother culture (free from endogenous bacteria) to establish fresh cultures. Nodal segments (1-1.5 cm) were excised from 4-wks-old cultures and were further used as explants in the experiments (Fig. 4A).

The experimental media consisted of two basal media combinations *viz*., Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) and Woody Plant Medium (WPM) supplemented with 3% (w/v) sucrose and agar (0.8%) (Himedia Laboratories, Mumbai, India) and plant growth regulators (PGRs) (Sigma- Aldrich, Saint Louis, MO, USA) as shown in Table 1. The pH of media was adjusted at 5.8 using 1N NaOH and HCl prior to autoclaving (121°C at 15 psi pressure for 17 min). All the cultures were incubated under a standard culture room conditions maintaining 25±2°C, 16/8 h photoperiod and a light intensity of 40 $\mu Em^{-2} s^{-1}$ using white fluorescent light (Philips, India).

Shoot proliferation and elongation

Experiments were undertaken to evaluate the shoot regeneration and elongation on MS and WPM media supplemented with 24 permutations and combinations of PGRs comprising kinetin (Kn) and 6-BenzylAmino-Purine (BAP) ranging from 0.25 mg L^{-1} to 2.5 mg L^{-1} (Table 1). Approximately 20 ml of the medium was dispensed into culture tubes (25×150 mm; Borosil, Mumbai, India). Data on shoot length (cm), number of nodes and number of leaves per plantlet were recorded biweekly, up to 12 wks. Based on the data on number of nodes per plantlet and shoot length obtained after 12 wks of culturing, the best medium for shoot proliferation and shoot elongation was identified.

In vitro rooting

For root induction, well-developed and healthy *in vitro* grown shoots (with 3 nodes) were transferred to MS basal salts (3% sucrose, 0.8% agar) supplemented with different concentrations of Indole-3-Butyric Acid (IBA) ranging from 0.5 mg L^{-1} to 2.0 mg L^{-1} and full- and half-strength MS medium (Table 2). Approximately 20 ml of the medium was dispensed into culture tubes (25×150 mm; Borosil, Mumbai, India). Data on number of shoots forming root, root length (cm) and number of roots per shoot were recorded after four wks. Average root number and root length per shoot were computed.

Acclimatization to ex vitro conditions

Plantlets with well-developed roots with approximately 4-5 nodes measuring 7-9 cm were removed from culture tubes and rinsed under running tap water to remove any adhering medium. The washed plantlets were planted separately in protrays filled with a mixture of autoclaved horticultural grade perlite: Irish peat moss mixture in the ratio of 25:75 (Glasil Scientific, New Delhi, India). The plantlets were watered with MS basal salt solution during first week. The tray was covered with transparent polythene bags in order to maintain the high relative humidity (70-80 %) in a mist chamber under normal

Table 1. Effects of different concentrations of plant growth regulators on shoot growth of black pepper (*Piper nigrum* L.) on Murashige and Skoog (MS) medium and Woody Plant Medium (WPM)

Serial no.	Basal medium	Medium code	Plant growth regulator (mg L^{-1})		Shoot length (cm)	Number of nodes/shoot	Number of leaves/ shoot	Callus response
			Kn	BAP				
1	MS	K1	0.25	-	6.60 ± 0.25^{defg}	3.58 ± 0.37^{cde}	3.66 ± 0.14^{efgh}	
2		K2	0.5	-	7.43 ± 0.36^{cde}	3.25 ± 0.25^{cdef}	3.75 ± 0.13^{defg}	
3		K3	1.0	-	8.32 ± 0.44^{bcd}	2.33 ± 0.14^{efg}	3.25 ± 0.13^{ghij}	+
4		K4	1.5	-	8.42 ± 0.66^{bc}	2.58 ± 0.28^{efg}	3.08 ± 0.31^{ghij}	+
5		K5	2.0	-	9.45 ± 0.35^{ab}	2.75 ± 0.21^{defg}	3.50 ± 0.19^{fghi}	+
6		K6	2.5	-	7.78 ± 0.60^{bcde}	6.08 ± 0.89^{b}	3.58 ± 0.19^{efghi}	+
7		B1	-	0.25	10.29 ± 0.94^{a}	4.08 ± 0.48^{cd}	4.58 ± 0.41^{bcd}	+++
8		**B2**	**-**	**0.5**	$\mathbf{10.42 \pm 0.78^{a}}$	$\mathbf{6.58 \pm 0.65^{b}}$	$\mathbf{5.58 \pm 0.35^{a}}$	+++
9		B3	-	1.0	8.30 ± 0.64^{bcd}	5.83 ± 0.50^{b}	4.41 ± 0.28^{bcde}	+++
10		B4	-	1.5	6.31 ± 1.25^{efg}	4.25 ± 1.03^{c}	3.66 ± 0.67^{efgh}	+++
11		B5	-	2.0	8.26 ± 0.63^{bcd}	8.33 ± 0.80^{a}	5.25 ± 0.27^{ab}	+++
12		B6	-	2.5	2.63 ± 0.72^{g}	1.66 ± 0.78^{g}	1.75 ± 0.53^{k}	+++
13	WPM	WK1	0.25	-	7.03 ± 0.24^{cdef}	2.16 ± 0.11^{efg}	3.16 ± 0.11^{ghij}	+
14		WK2	0.5	-	6.90 ± 0.17^{cdefg}	2.16 ± 0.11^{efg}	2.91 ± 0.08^{ghij}	+
15		WK3	1.0	-	6.83 ± 0.36^{cdefg}	2.00 ± 0.00^{fg}	2.75 ± 0.13^{hij}	++
16		WK4	1.5	-	6.76 ± 0.29^{cdefg}	1.83 ± 0.11^{fg}	2.50 ± 0.15^{jk}	++
17		WK5	2.0	-	6.62 ± 0.27^{cdefg}	2.16 ± 0.16^{efg}	2.66 ± 0.14^{ij}	++
18		WK6	2.5	-	6.90 ± 0.17^{cdefg}	2.16 ± 0.16^{efg}	2.91 ± 0.14^{ghij}	++
19		WB1	-	0.25	6.78 ± 0.26^{cdefg}	4.16 ± 0.27^{cd}	4.66 ± 0.18^{bc}	++
20		WB2	-	0.5	6.90 ± 0.21^{cdefg}	4.08 ± 0.19^{cd}	4.83 ± 0.11^{abc}	++
21		WB3	-	1.0	6.92 ± 0.21^{cdefg}	4.41 ± 0.19^{c}	4.83 ± 0.24^{abc}	++
22		WB4	-	1.5	5.19 ± 0.34^{g}	4.25 ± 0.44^{c}	4.25 ± 0.35^{cdef}	+++
23		WB5	-	2.0	6.05 ± 0.23^{efg}	4.50 ± 0.15^{c}	4.83 ± 0.16^{abc}	+++
24		WB6	-	2.5	5.63 ± 0.46^{fg}	4.25 ± 0.25^{c}	4.33 ± 0.30^{cdef}	++

Means ± SE within a column superscripted by the same letter are not significantly different at P≤ 0.05
BAP: 6-benzylaminopurine; Kn: kinetin

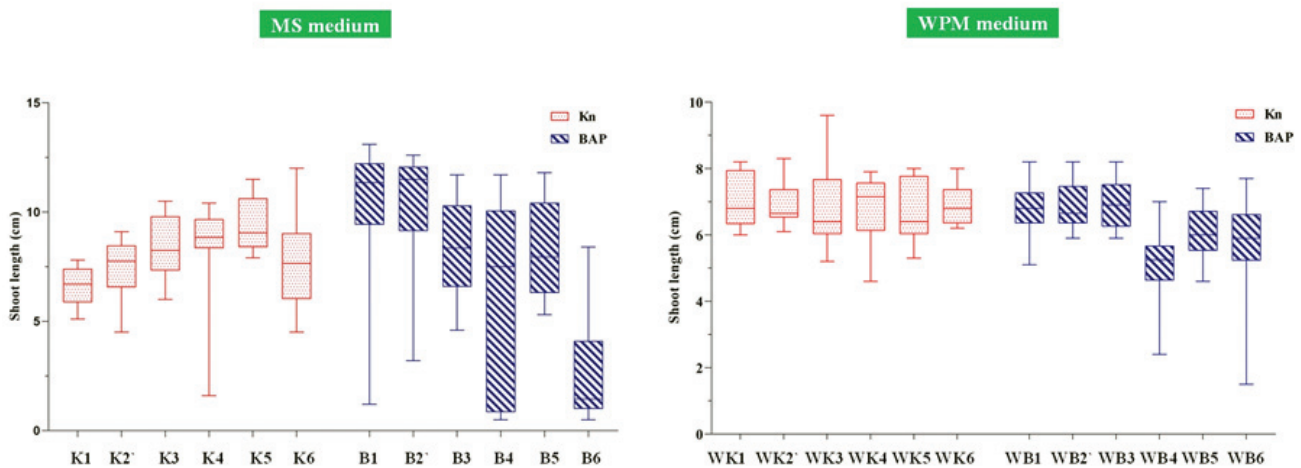

Fig. 1. Box plot representation of shoot length in cultures of *Piper nigrum* L. in MS and WPM media supplemented with Kn and BAP (for media codes refer to Table 1).

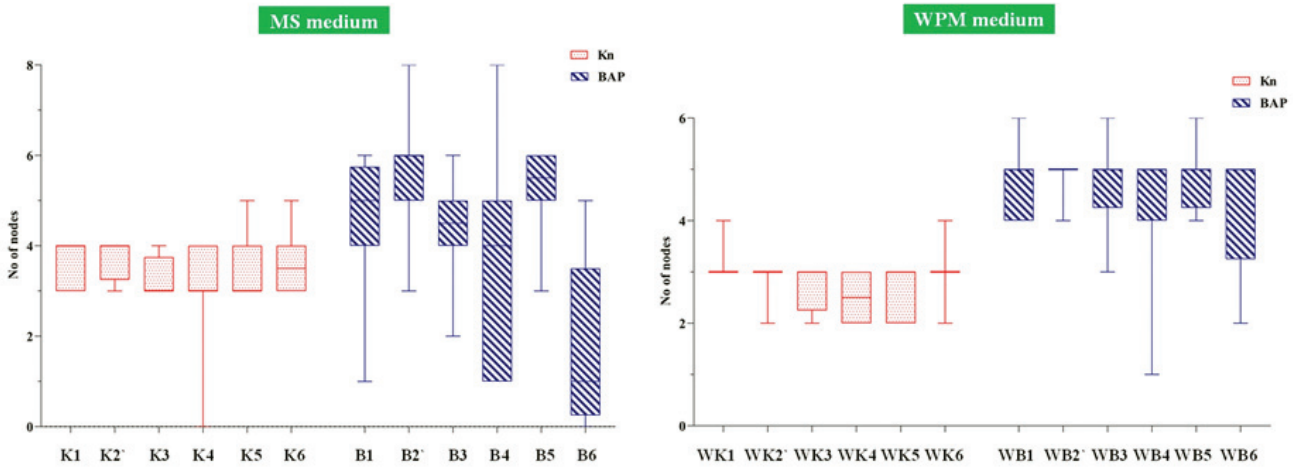

Fig. 2. Box plot representation of number of nodes in cultures of *Piper nigrum* L. in MS and WPM media supplemented with Kn and BAP (for media codes refer to Table 1).

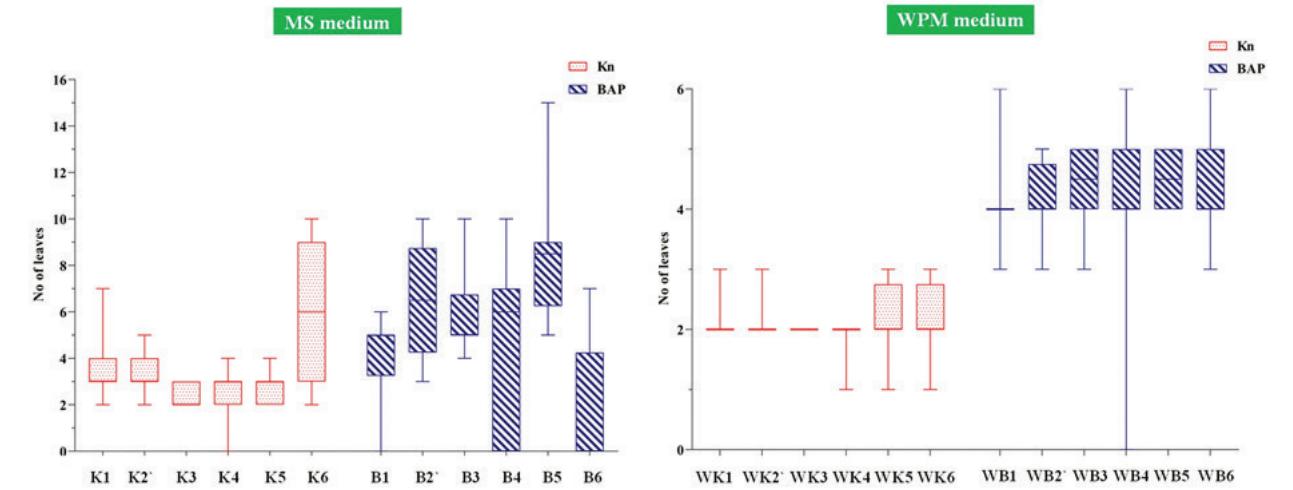

Fig. 3. Box plot representation of number of leaves in cultures of *Piper nigrum* in MS and WPM media supplemented with Kn and BAP (for media codes refer to Table 1).

growth conditions (16/8 h of photoperiod, 25±2°C). After 2-3 days small holes (4-5) were made for aeration in the polythene bags. After first week, once in every five days plants were irrigated using Hoagland solution (HiMedia laboratories, Mumbai, India). The seedlings (hardened plants) were transferred to earthen pots (10'' dia) with soil and farmyard manure (1:1) after 4 wks and kept under the shade net conditions. The survival of the rooted plants was documented after 3 wks of hardening.

Statistical analysis

All the experiments were carried out in 12 replications by taking one culture as one replicate and repeated twice using completely randomized design (CRD). Data on shoot length, number of nodes, leaves and root, rooting percentage and root length (cm) are represented as mean ± standard error. Analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) were used for comparison and for significant difference among means (Duncan, 1955) ($P \leq 0.05$) using SPSS statistics version 22.0 software package. Two-way ANOVA data were subjected to post-hoc Sidak's multiple comparison test ($P \leq 0.05$) utilizing GraphPad Prism 9.1.0 software.

Assessment of genetic stability

Genetic stability analysis was carried out by comparing mother stock cultures (12 replicates) with regenerants from the two best micropropagation media, namely K2

Fig. 4. *In vitro* propagation of *Piper nigrum* L. using nodal segment as explant: (A) Nodal segment explants (B) Shoot development on MS media supplemented with 0.5 mg/L of BAP (C) Root induction on MS media supplemented with different doses of IBA (0.5-2.0 mg L^{-1}), full-and half-strength MS medium (D) Rooted plant before transplanting into seedling trays (E) Plants 3 week after transplanting (F) Plants transplanted to pots

(MS+ 0.5 mg L^{-1} Kn) and B2 (MS + 0.5 mg L^{-1} BAP). The DNA from young leaves (from 3-month-old culture) were isolated using Cetyl Trimethyl Ammonium Bromide (CTAB) method (Doyle and Doyle, 1987) with slight modifications. The quality and the quantity of DNA were estimated using Nanodrop 1000 (Thermo Fisher Scientific, Wilmington, DE, USA).

A total of 48 ISSR primers (Eurofins Genomics India Pvt. Ltd., Bengaluru, India) were tested for PCR amplification (Table 4). The reaction mixture comprised DNA template (3 µl), 5 µl master mixture (1x), 0.5 µl primer and 1.5 µl water in 10 µl reaction volumes. Primers yielding reproducible bands were used for the analysis. Reaction was carried out in a thermal cycler (Gene Pro, Hangzhou Bioer Technology Co., Hangzhou, China). The thermal cycler was programmed for (i) Initial denaturation (94°C for 2 min); (ii) Denaturation (94°C for 10 sec); (iii) Primer annealing temperature (T_m) based on primer for 30 sec (iv) Primer extension (72°C for 65 sec); (v) Final extension (72°C for 10 min) and 4°C as holding temperature. The amplified PCR products were stored at 4°C for further analysis.

The ISSR-PCR amplification products were analyzed by gel electrophoresis in 2% agarose gel immersed in 1xTAE buffer (G-Biosciences, Saint Louis, MO) which stained with Ethidium Bromide (Etbr) and was run at a constant voltage of 5 V cm^{-1}. All the amplified products were electrophoresed and gel was imaged by Gel Documentation System (GenoSens 2100, Clinx Science Instruments Co., Shanghai, China). The banding pattern of the mother plant and the micropropagated plant were compared to record for any variation existing at the molecular level.

Results and Discussion

Shoot proliferation

An optimal selection process for an efficient micropropagation protocol involves the selection of a suitable explant, as well as the proper combinations of the PGRs in optimum ratio, as these factors have a direct impact on the shoot and root proliferation of the plantlets. In this study, various characters like the shoot length, number of nodes and number of leaves were influenced by two types of the basal media (MS and WPM) and to varying concentrations of the Kn and BAP tested.

In the present study, among the 24 combinations of cytokinins tested, BAP alone in MS medium was found better with respect to shoot growth and multiplication (Table 1). On MS medium mean shoot length ranged from 2.63 cm (2.5 mg L^{-1} BAP) to 10.42 cm (0.25 mg L^{-1} BAP) (Fig. 1a); number of nodes ranged from 1.66 (2.5 mg L^{-1} BAP) to 8.33 (2.0 mg L^{-1} BAP) (Fig. 2a); leaves from 1.75 (2.5 mg L^{-1} BAP) to 5.58 (0.5 mg L^{-1} BAP) (Fig. 3a). Contrarily on WPM media, the average shoot length ranged from 5.19 cm (1.5 mg L^{-1} BAP) to 7.03 cm (0.25 mg L^{-1} Kn) (Fig. 1b); number of nodes ranged from 1.83 (1.5 mg L^{-1} Kn) to 4.50 (2.0 mg L^{-1} BAP) (Fig. 2b); number of leaves ranged from 2.50 (1.5 mg L^{-1} Kn)to 4.83 (0.5 mg L^{-1} BAP) (Fig. 3b). A cream colored hard nodular callus formed at the cut end of the explants, which did proliferate significantly (Table 1). ANOVA analysis of the micropropagated plantlets revealed that treatment effect (both basal media, type of PGR, concentration of PGR) was highly significant for the parameters recorded as well as their interaction ($P \leq 0.0001$) (Table 3).

The Sidak's post-hoc test ($P \leq 0.05$) was applied to explore the paired difference between the treatment means in MS media supplemented with each PGR (Kn, BAP) and basal medium (MS, WPM) while controlling the experiment-wise (family) error rate for all parameters and are depicted in Supplementary Fig. 1. It is clear from this analysis that shoot regeneration in MS was statistically comparable ($P \leq 0.05$) in 8 combinations of Kn and BAP while in the other five treatments Kn and BAP gave significant differences. Contrarily in WPM media, the analysis of shoot regeneration was statistically comparable in 12 treatments of Kn and BAP while in three treatments Kn and BAP gave significant differences.

Similarly for number of nodes developed on MS media supplemented with Kn and BAP, 10 treatments gave statistically comparable results while in other five Kn and BAP gave significantly better results (Supplementary Fig. 2). Whereas in WPM media supplemented with Kn and BAP the analysis of number of nodes was statistically comparable among all the treatments and no significant difference was observed (Supplementary Fig. 2). For number of leaves all the treatments gave statistically comparable results among MS and WPM media supplemented with Kn and BAP respectively, except one treatment (K4B4-K5B5) was significant in MS supplemented medium (Supplementary Fig. 3).

The fundamental goal of the micropropagation is to replicate the plant as quickly as possible with more number of nodal explants, since each nodal explant will develop into a plant when re-cultured. In the present work, MS medium supplemented with 0.5 mg L^{-1} BAP yielded the highest shoot length (10.42±0.78 cm) and number of nodes (6.58±0.65) (Fig. 4B).

In the present investigation, all explants cultured on BAP-supplemented media (both MS and WPM) exhibited callus formation at varying rates at the cut end (Table 1). Similar to the present study Bhat *et al.*(1995) observed a fast growing yellowish callus in *Piper nigrum* when cultured on medium containing 0.2 mg L^{-1} of BAP along with 1 mg L^{-1} of NAA. Hussain *et al*. (2011) obtained the best callus on MS media supplemented with 1.5 mg L^{-1} of BAP and the shoot regeneration in 0.5 mg L^{-1} of BAP. Kadam *et al.* (2020) observed maximum shoot induction (93.33 %) using nodal explants in MS media comprising of 4.0 mg L^{-1} BAP + 1.0 mg L^{-1} IAA.

A ratio of 1:6.5 numbers of nodes is obtained on the MS medium supplemented with 0.5 mg L^{-1} of BAP within three months. If this multiplication rate is maintained, 1,300 plants can be raised in a year from a single nodal explant. There are previous reports on *Piper in vitro* multiplication but none of the experiments tested for more than one basal media along with different concentration of PGRs as analyzed in the present study. Also, rate of shoot multiplication in the present work is better than the previously published reports on *Piper* species. Callus mediated shoot regeneration was described in *Piper colubrinum* by Kelkar *et al*. (1996), shoot buds were induced (7.6 shoots/explant) and elongated on half-strength MS supplemented with 2.0 mg L^{-1} BA and 0.5 mg L^{-1} NAA using leaf explants. Zhang *et al*. (2008) induced aseptic cluster shoots (6-8 shoots) on

Table 2. Effect of Murashige and Skoog (MS) medium in different strengths along with different concentration of Indole-3-butyric acid (IBA) on *in vitro* rooting of *Piper nigrum* L. shoots

Sl. No.	Composition	Rooting (%)	Number of roots/shoot	Root length (cm)
1	Full strength MS+ IBA (0.5 mg L^{-1})	100	3.25 ± 0.70^{d}	2.99 ± 0.58^{a}
2	Full strength MS+ IBA (1.0 mg L^{-1})	100	10.08 ± 1.33^{b}	3.06 ± 0.86^{a}
3	Full strength MS+ IBA (1.5 mg L^{-1})	100	6.83 ± 0.82^{c}	3.40 ± 0.30^{a}
4	**Full strength MS + IBA (2.0 mg L^{-1})**	**100**	**15.00 ± 2.04^{a}**	**3.40 ± 0.28^{a}**
5	Full strength MS	25	0.50 ± 0.26^{d}	0.96 ± 0.52^{b}
6	Half strength MS	8.33	0.08 ± 0.08^{d}	0.25 ± 0.25^{b}

Means ± SE within a column followed by the same letter are not significantly different at $p \leq 0.05$

Table 3. ANOVA of data presented in Table 1

Factors	SS	DF	MS	F (DFn, DFd)	P value
Row Factor	1164	23	50.60	F (23, 792) = 22.31****	P<0.0001
Column Factor	2274	2	1137	F (2, 792) = 501.2****	P<0.0001
Interaction	640.4	46	13.92	F (46, 792) = 6.137****	P<0.0001
Residual	1797	792	2.268		

MS medium supplemented with 0.5 mg dm^{-3} IAA and 0.5 mg dm^{-3} BA of *Piper methysticum* using auxiliary buds as explants.

Induction of in vitro *rooting*

Rooting of black pepper shoots on the rooting media combinations is presented in Table 2. The rooting of the shoots is a prerequisite for their further establishment in the field conditions. Rhizogenesis was observed in 100% shoots on all the media combinations having IBA but their length and number varied across different concentrations. Absence of IBA in MS full- and half-strength media had lower rooting (8.33 to 25%), root length and the number of roots compared to the MS medium supplemented with IBA. Highest number of roots (15.00±2.04) and highest root length (3.40±0.28) were observed on MS supplemented with 2.0 mg L^{-1} of IBA (Fig. 4C & 4D). Earlier reports suggested development of 8 to 10 roots/ shoot when it was cultured on half-strength MS media (Philip *et al*., 1992) and 2-4 roots were observed when transferred to media containing 1µm IAA (Bhat *et al*., 1995). Rajasekaran and Mohankumar (1997) observed 3-5 roots in *Piper nigrum* on full strength white's media without any growth regulators after 14 days of inoculation. Ahmad *et al.* (2014) observed higher rooting percentage (90%) on IBA (1.5 mg L^{-1}) in black pepper. Salim *et al.* (2017) obtained 80 % rooting of *P. nigrum* when cultured on half-strength MS medium containing 1.5 mg L^{-1} IBA. Ramos *et al.* (2020) observed number of roots (6.40) in "Clonada" genotype in after 8 wks of inoculation when cultured on MS medium with 0.05 mg L^{-1} NAA in black pepper.

The present study provides an evident protocol with 100% rooting, highest number roots/shoot (15.00±2.04) and root length (3.40±0.28) when compared to the earlier studies establishing the superiority of present protocol to develop robust plantlets of black pepper.

Acclimatization to ex vitro *conditions*

The ultimate success of *in vitro* propagation lies in the successful establishment of plantlets in the field conditions. In the present study plantlets showed 100 % survival in mist chamber at the primary hardening stage. The plantlets resumed the growth within 3 wks (Fig. 4E). After 4 wks the hardened plants were planted in earthen pots and were placed under shade net condition (Fig. 4F). The different stages of micropropagation protocol standardized in the present work is depicted in Fig. 5.

Genetic fidelity studies using ISSR marker

Many researchers have demonstrated the value of molecular analysis of *in vitro* regenerated plants (Bhatia *et al*., 2011; Saha *et al*., 2016). The genetic stability of micropropagated plants has enormous practical utility and commercial value since it provides information about the structural and functional stability of the plants that have been regenerated. With this context in mind, the molecular analysis was undertaken using the ISSR markers, by comparing the mother plants with the regenerated plants.

Table 4. Primer sequence and number of scorable bands produced by ISSR primers in *Piper nigrum* L. in mother plant and micropropagated plantlets

Sl. No.	Primer name	Tm(°C)	Primer sequence (5^1-3^1)	Total number of scorable bands
1	UBC 801	37	$(AT)_8T$	0
2	UBC 802	37	$(AT)_8G$	0
3	UBC831	37	$(AT)_8TA$	0
4	UBC 832	37	$(AT)_8TC$	0
5	UBC 833	37	$(AT)_8TG$	0
6	IS10	49.2	$C(GA)_8$	4
7	UBC 860	49.2	$(TG)_8GA$	0
8	UBC 814	50	$(CT)_8A$	1
9	UBC 819	50	$(GT)_8A$	0
10	UBC 820	50	$(GT)_8C$	0
11	UBC 836	50	$(AG)_8TA$	3
12	UBC 843	50	$(CT)_8GA$	0
13	UBC 829	50	$(TG)_8C$	3
14	UBC 840	50	$(GA)_8TT$	0
15	UBC 824	50	$(TC)_8G$	0
16	UBC 834	50.6	$(AG)_8TT$	3
17	UBC 835	50.6	$(AG)_8TC$	2
18	IS7	51	$(GT)_8A$	0
19	UBC 858	51	$(TG)_8GT$	5
20	IS8	51	$(AG)_8C$	4
21	UBC 870	51	$(TGC)_6$	4
22	UBC 871	51	$(TAT)_5$	0
23	UBC 872	51	$(GATA)_3GAT$	0
24	UBC 859	51	$(TG)_8GC$	0
25	UBC 842	51	$(GA)_8TG$	4
26	UBC 813	52	$(CT)_8T$	0
27	IS11	52	$(CA)_7G$	5
28	IS65	52	$(AG)_8T$	3
29	UBC 826	52.7	$(AC)_8C$	4
30	UBC 825	52.7	$(AC)_8T$	2
31	IS 12	52.7	$(GT)_8C$	0
32	IS 9	53.5	$(TG)_8A$	2
33	UBC 873	53.5	$(GACA)_4$	0
34	UBC 841	53.5	$(GAGA)_4TC$	4
35	IS 53	54	$(GA)_8C$	2
36	IS 61	54	$(GA)_8T$	3
37	UBC 861	56.6	$(ACC)_6$	4
38	UBC 856	50	$(AC)_8CA$	3
39	UBC 846	50	$(CA)_8AT$	3
40	UBC 847	50	$(CA)_8AC$	3
41	UBC 848	50	$(CA)_8AG$	5
42	UBC 849	50	$(GT)_8CA$	2
43	UBC 850	50	$(GT)_8CC$	2
44	UBC 851	50	$(GT)_8CG$	3
45	UBC 852	50	$(TC)_8GA$	0
46	UBC 853	50	$(TC)_8AT$	0
47	UBC 854	50	$(TC)_8$ AG	0
48	UBC 855	50	$(AC)_8$ CT	3
			TOTAL	86

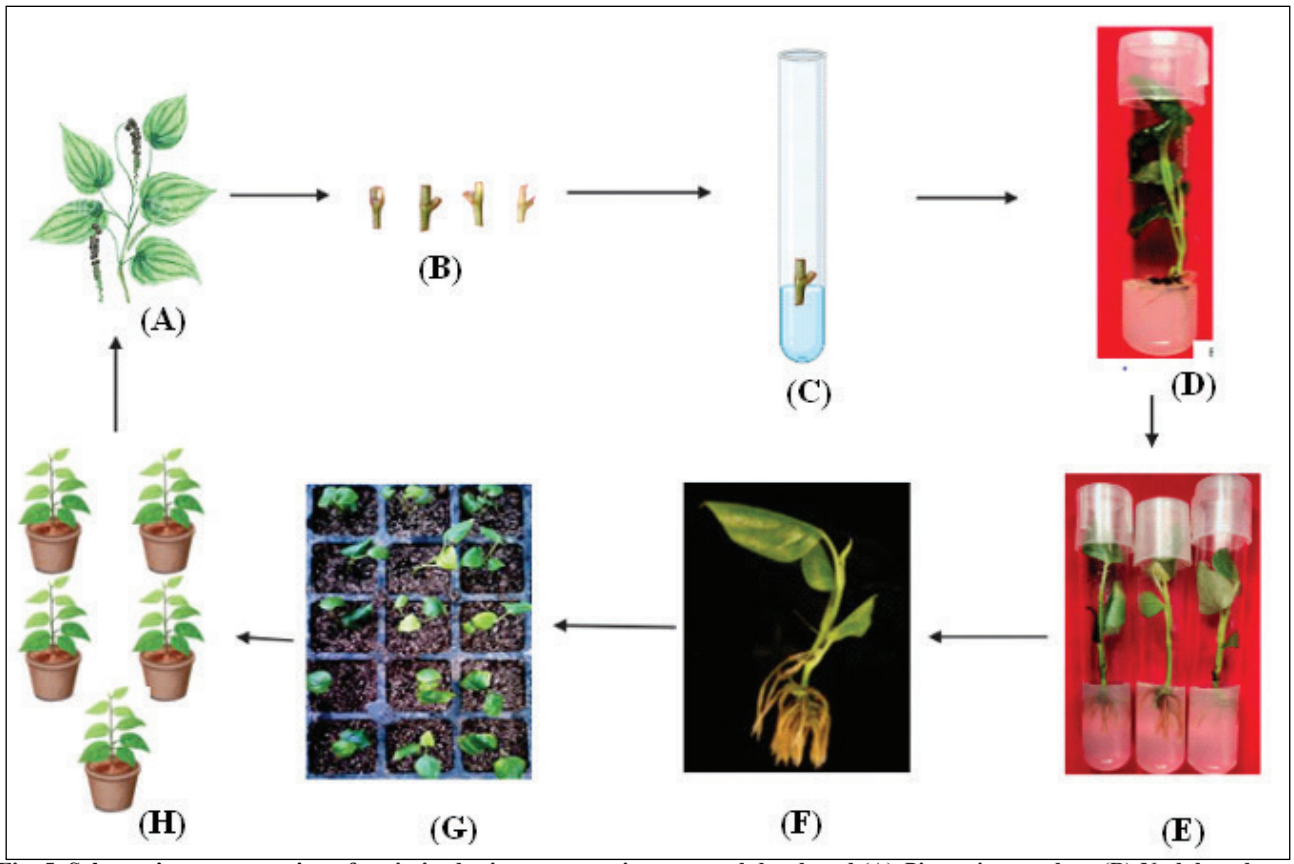

Fig. 5. Schematic representation of optimized micropropagation protocol developed (A) *Piper nigrum* plant (B) Nodal explants used (C) Culturing of the nodal explants on different media combinations (D) Cultured plants on MS media supplemented with 0.5 mg L^{-1} BAP (E & F) Plants rooted on MS media supplemented with 2.0 mg $L^{-1}$of IBA (G) Hardening of plants in mist chamber (H) Transferring of hardened plants to pots containing 1:1 ratio of soil and farm yard manure (FYM)

Genetic stability was assessed by comparing DNA isolated from mother stock cultures, plantlets regenerated on K2 medium (MS + 0.5 mg L^{-1} Kn) and B2 medium (0.5 mg L^{-1} BAP). Higher callus was recorded at the cut ends of the shoot in B2 medium as compared to K2, hence the comparison for genetic stability between the two media was considered prudent. Only 29 out of 49 markers examined yielded clear and reproducible bands. The optimal annealing temperature was found to be between 37 °C to 56.6 °C (Table 4). The 29 ISSR markers resulted in 86 distinct and scorable bands with sizes ranging from 120 (UBC842) to 1,200 (UBC841). The number of scorable loci ranged from 1 (UBC 814) to 5 (UBC 858 and IS11), with an average of 3.18 loci per primer. The maximum number of loci was restricted to the ladder range of 300 to 1000 base pair. Among the 29 markers examined, all the generated loci were found to be monomorphic. Furthermore, no difference in banding pattern was observed between the micropropagated and mother plants (Fig. 6). This genetic stability, in turn, indicates the suitability of this protocol for large-scale commercial propagation within three months. Similar results have been reported by Malhotra *et al*. (2020) in cardamom plants.

Conclusion

A two-step improved and a reproducible micropropagation protocol of black pepper is presented in this study. The medium comprising full-strength MS basal media supplemented with 0.50 mg L^{-1} of BAP was the most appropriate to produce highest number of nodes and shoot length in unit time. The developed shoots were vigorous and rooted well on MS media supplemented with 2.0 mg L^{-1} of IBA. This protocol regenerated plantlets which are genetically stable as assessed by ISSR markers. Because of its high economic value and its trade across the countries, a tissue culture protocol with good rate of multiplication and maintenance of genetic stability will be useful for commercial application of the developed protocol.

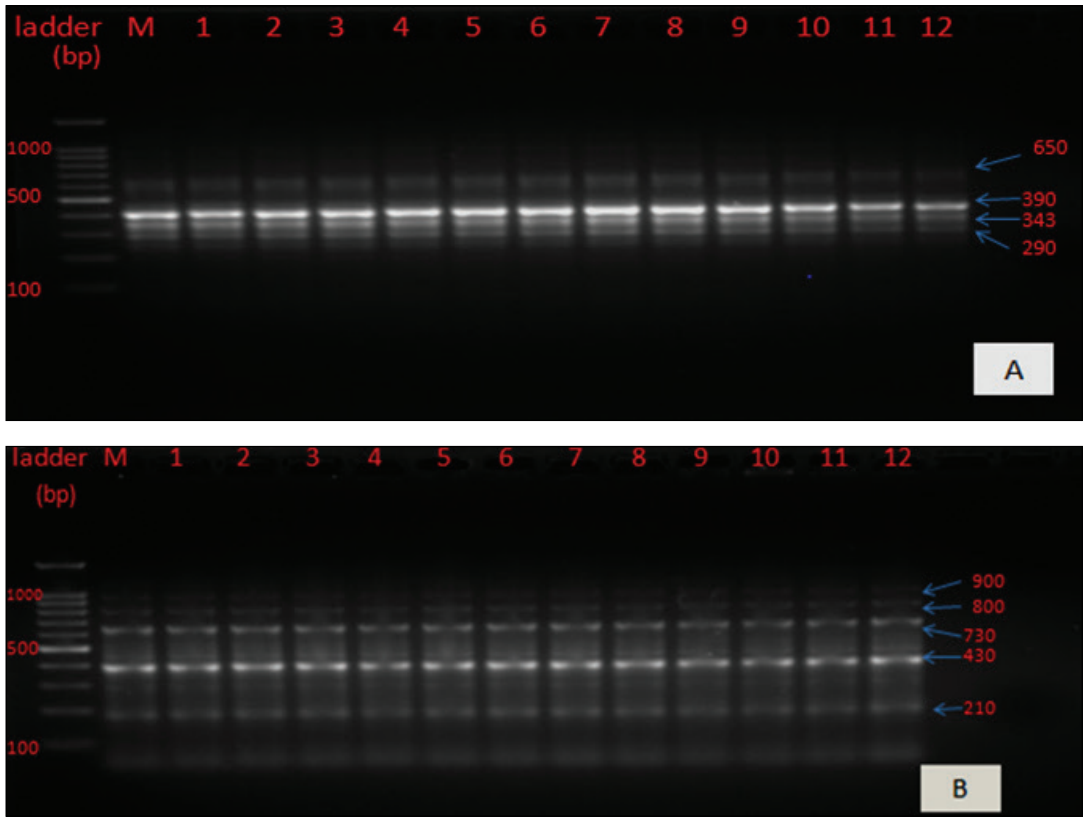

Fig. 6. ISSR banding profiles of obtained with ISSPR primer IS 8(A) and UBC 848 (B) of the mother plant and micropropagated plants from B2 medium (Ladder lane: 100 bp DNA marker, M- mother plant, 1-12: micropropagated plants)

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Author Contributions

DDA carried out all the experiments and wrote the draft manuscript. AA provided overall supervision for the work and critically edited the manuscript. EVM designed the experiments and provided guidance. DDA and MS carried out statistical analysis. All authors have read and approved the manuscript.

*Supplementary Table or Figure mentioned in the article are available in the online version.

References

Abbasi BH, N Ahmad, H Fazal and T Mahmood (2010) Conventional and modern propagation techniques in *Piper nigrum*. *J. Med. Plants Res.* **4(1)**: 7-12.

Ahmad N, BH Abbasi, H Fazal, MA Khan and MS Afridi (2014) Effect of reverse photoperiod on *in vitro* regeneration and piperine production in *Piper nigrum* L. *Comptes Rendus Biologies*. **337(1)**: 19-28.

Babu KN, L Regy and PN Ravindran (1993) Tissue culture of tropical spices. In: P Vidyasekharan (Ed) *Genetic Engineering Molecular Biology and Tissue Culture for Crop, Pest and Disease Management.* Daya Publishing House, New Delhi, pp 257-267.

Bhat SR, KPS Chandel and SK Malik (1995) Plant regeneration from various explants of cultivated *Piper* species. *Plant Cell Rep.* **14(6)**: 398-402.

Bhat AI, CN Biju, V Srinivasan and SAK Krishnamurthy (2018) Current status of viral diseases affecting black pepper and cardamom. *J. Spices Aromatic Crops* **27(1)**: 1-16.

Bhatia R, KP Singh, TR Sharma and T Jhang (2011) Evaluation of the genetic fidelity of *in vitro*-propagated gerbera (*Gerbera jamesonii* Bolus) using DNA-based markers. *Plant Cell Tiss. Org. Cult.* **104(1):** 131-135.

De Almeida GC, LF Oliveira, D Predes, HH Fokoue, RM Kuster, FL Oliveira, FA Mendes and JG Abreu (2020) Piperine suppresses the Wnt/β-catenin pathway and has anti-cancer effects on colorectal cancer cells. *Sci. Rep*. **10(1)**: 1-12.

Doyle JJ and JL Doyle (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull*. **19**: 11-15.

Duncan DB (1955) Multiple range and multiple F tests. *Biometrics* **11**: 1-42.

Fitchet M (1990) Establishment of *P. nigrum in vitro*. *Acta Hortic*. **257:** 285-291.

Hussain A, S Naz, H Nazir and ZK Shinwari (2011) Tissue culture of black pepper (*Piper nigrum* L.) in Pakistan. *Pak. J. Bot*. **43(2)**: 1069-1078.

Joseph L, PA Nazeem, MS Thampi, S Philip and M Balachandran (1996) *In vitro* techniques for mass multiplication of black pepper (*Piper nigrum* L.) and *ex vitro* performance of the plantlets. *J. Plant. Crops* **24** (suppl.): 511-516.

Kadam SS, DV Rasam, KH Joshi and AD Jadhav (2020) Micropropagation of black pepper, cv. Panniyur-1: standardization of sterilization protocol and media composition. *Global J. Biosci. Biotechnol.* **9(2)**: 45-49.

Kelkar SM, GB Deboo and KV Krishnamurthy (1996) *In vitro* plant regeneration from leaf callus in *Piper colubrinum* L. *Plant Cell Rep.* **16(3)**: 215-218.

Khajuria A, N Thusu and U Zutshi (2002) Piperine modulates permeability characteristics of intestine by inducing alterations in membrane dynamics: influence on brush border membrane fluidity, ultra-structure and enzyme kinetics. *Phytomedicine* **9(3)**: 224-231.

Malhotra EV, M Kamalapriya, S. Bansal, DPS Meena and A Agrawal (2020) Improved protocol for micropropagation of genetically uniform plants of commercially important cardamom (*Elettaria cardamomum* Maton). *In Vitro Cell. Dev. Biol.-Plant* **57(3)**: 407-413.

Murashige T and F Skoog (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant*. **15(3)**: 473-497.

Nazeem PA, L Joseph, CK Geetha and G Sreekandannair (1992) *In vitro* techniques for cloning of black pepper, *Piper nigrum* L. *J. Plantation Crops* **20***:* 257-257.

Philip VJ, D Joseph, GS Triggs and NM Dickinson (1992) Micropropagation of black pepper (*Piper nigrum* Linn.) through shoot tip cultures. *Plant Cell Rep*. **12(1)**: 41-44.

Rajasekharan P and P Mohankumar (1997) In vitro propagation of black pepper (*Piper nigrum* L.). In: S Edison, KV Ramana, B Sasikumar, K Nirmal Babu and SJ Eapen (Eds) *Biotechnology of Spices, Medicinal and Aromatic Plants,* Indian Society for Spices, Calicut, Kerala, pp.13-15.

Ramakrishnan Nair R and S Dutta Gupta (2003) Somatic embryogenesis and plant regeneration in black pepper (*Piper nigrum* L.): I. Direct somatic embryogenesis from tissues of germinating seeds and ontogeny of somatic embryos. *J. Horticultural Sci. Biotechnol*. **78(3)**: 416-421.

Ramos GDS, OF DeLemos, EFM Cunha, ADJ Boari, DP Mendonça, LRR dos Santos, SDM Rodrigues and IC DeMenezes (2020) Identification and micropropagation of virus-free black pepper genotypes (*Piper nigrum* L.). *Revista Ciência Agrícola* **18(1)**: 57-64.

Ravindran PN and JA Kallupurackal (2012) Black pepper. In : *Handbook of Herbs and Spices, 2nd ed.*, *Vol. 1,* Woodhead, Cambridge, UK, pp. 86-115.

Saha S, S Roy, C Sengupta and P Ghosh (2014) Micropropagation and analysis of genetic stability in regenerated plantlets of *Ocimum canum* Sims. *Indian J. Plant Physiol*. **19(2)**: 174-183.

Salim, K, TA Banu, I Mousona, H Ahashan, F Aleya, D Nilima and A Shahina (2017) *In vitro* regeneration of *Piper nigrum* L. *Bangladesh J. Bot.* **46(2)**: 789-793.

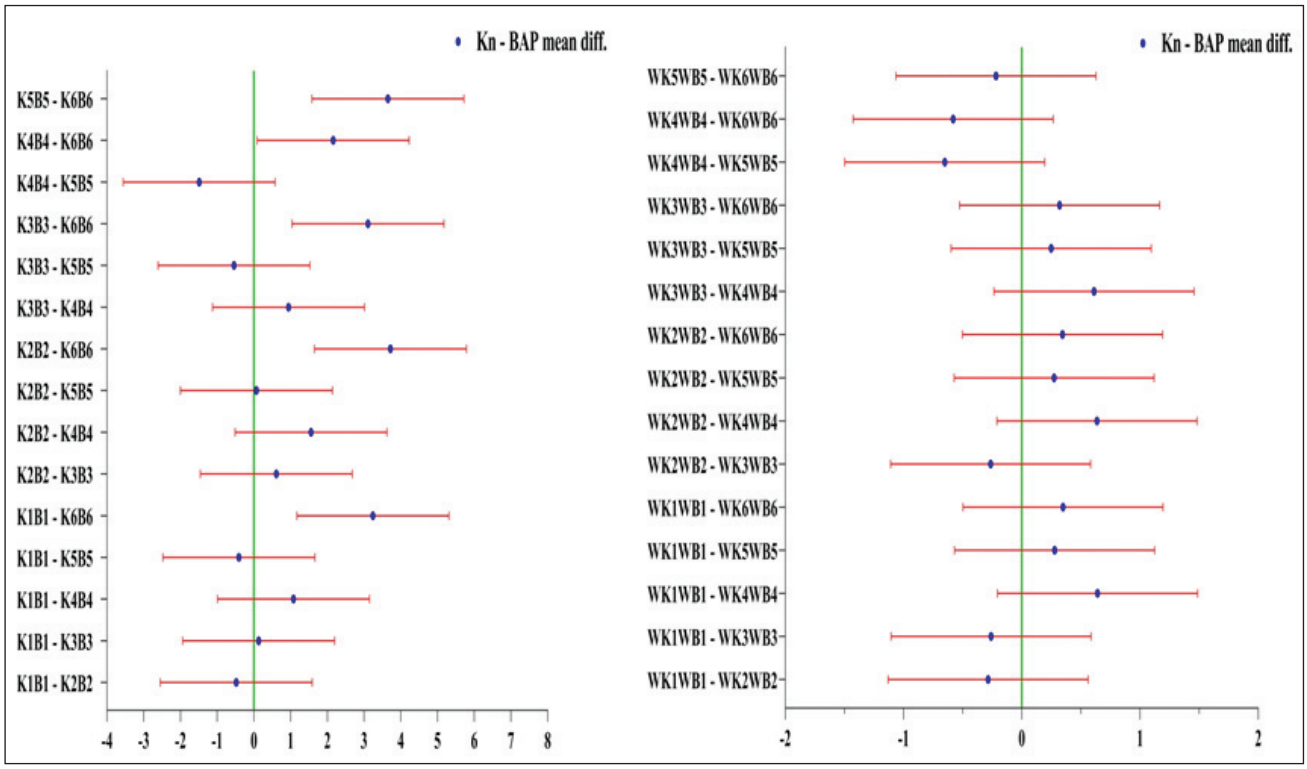
Spice board of India (2020) http://www.indianspices.con/sites/default/files/majorspicewise2021.pdf

Srinivasan K (2007) Black pepper and its pungent principle-piperine: A review of diverse physiological effects. *Critical Rev. Food Nut*. **47**: 735-748.

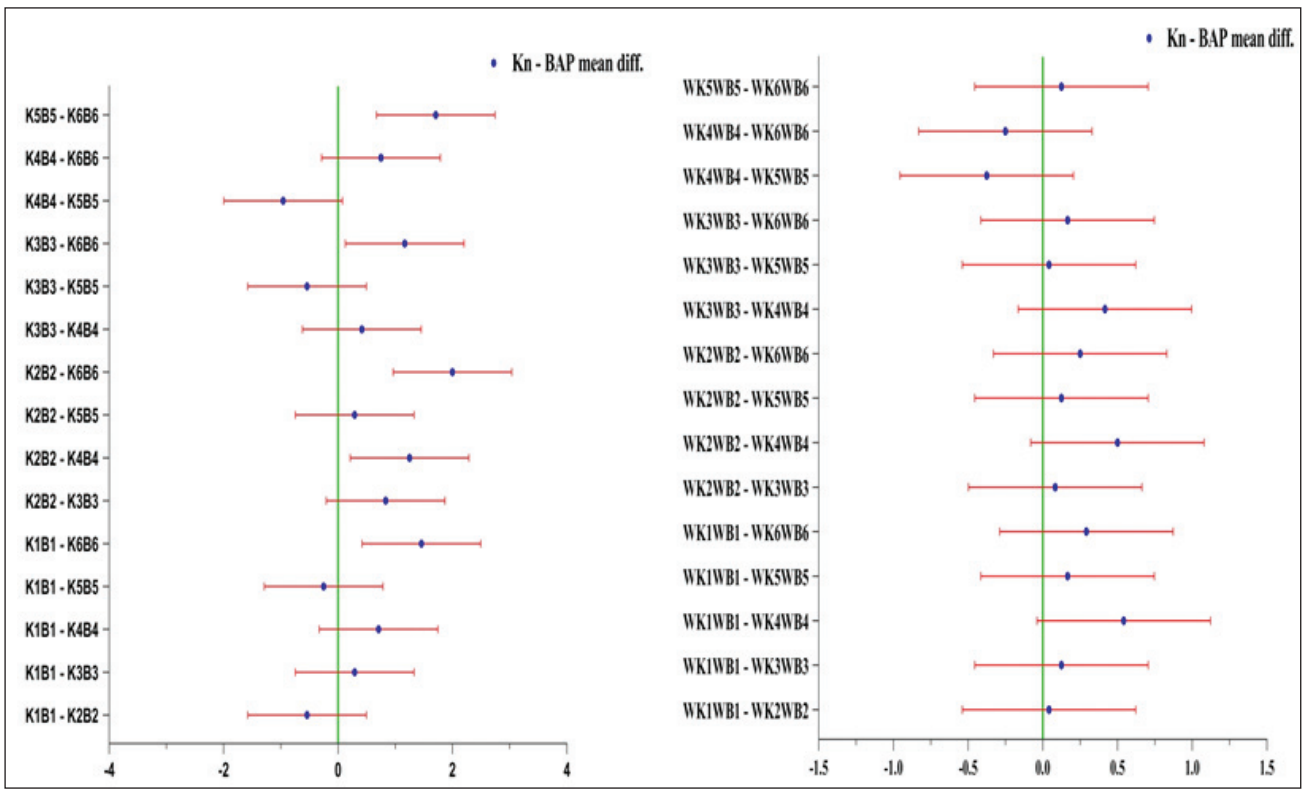
Sujatha R, LC Babu and PA Nazeem (2003) Histology of organogenesis from callus cultures of black pepper (*Piper nigrum* L.). *J. Trop. Agric*. **41**: 16-19.

Wang B, Y Zhang, J Huang, L Dong, T Li and X Fu (2017) Anti-inflammatory activity and chemical composition of dichloromethane extract from *Piper nigrum* and *P. longum* on permanent focal cerebral ischemia injury in rats. *Revista Brasileira de Farmacognosia* **27:** 369-374.

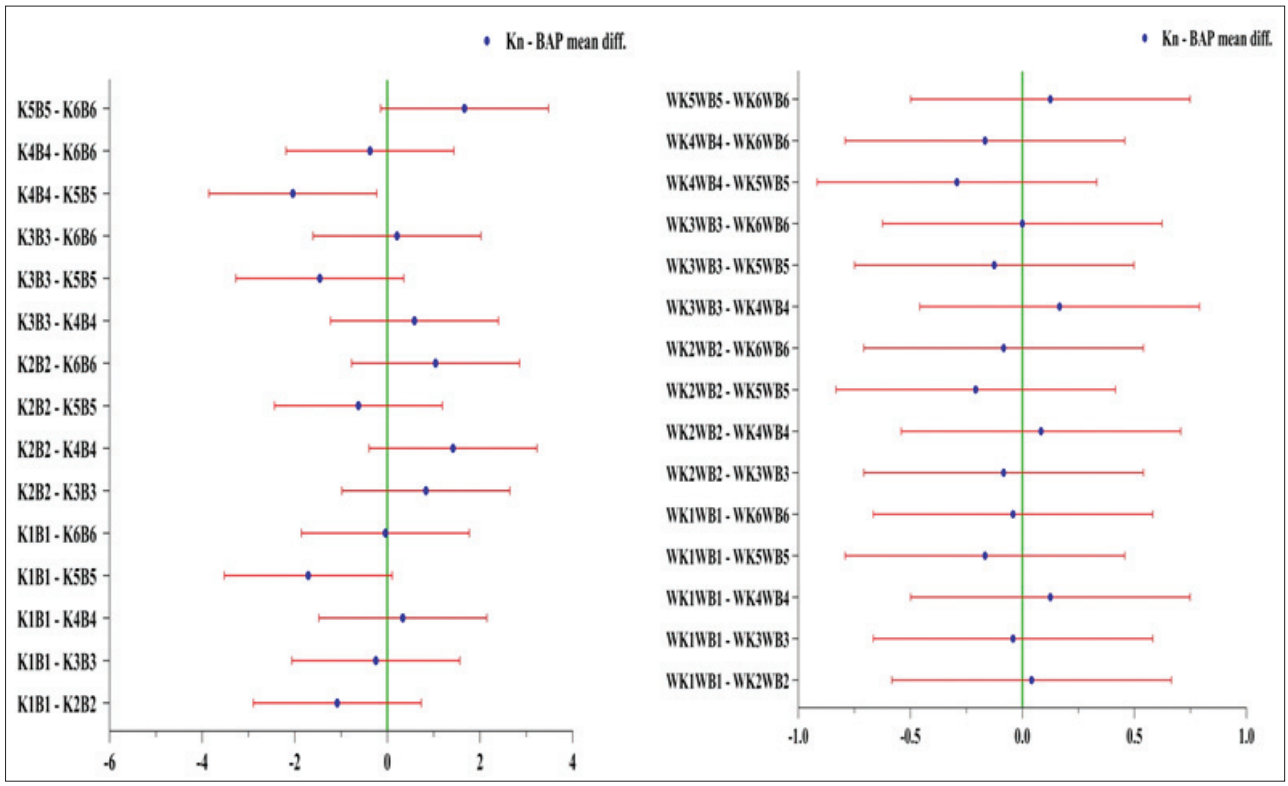
Zhang Z, L Zhao, X Chen and X Zheng (2008) Successful micropropagation protocol of *Piper methysticum*. *Biol. Plant*. **52(1)**:110-112.



Supplementary Fig. 1. Paired comparison of *Piper nigrum* L. shoot length for Kn and BAP in MS and WPM media using post-hoc Sidak's multiple comparison test (for media codes refer to Table 1).



Supplementary Fig. 2. Paired comparison of *Piper nigrum* L. number of nodes for Kn and BAP in MS and WPM media using post-hoc Sidak's multiple comparison test (for media codes refer to Table 1).



Supplementary Fig. 3. Paired comparison of *Piper nigrum* L. number of leaves for Kn and BAP in MS and WPM media using post-hoc Sidak's multiple comparison test (for media codes refer to Table 1).