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RESEARCH ARTICLE

SURFACE STERILIZATION PROTOCOLS OF LEAF AND BUD EXPLANTS FOR INITIATING *IN VITRO* CULTURES OF *Piper nigrum* L. (PEPPER)

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ABSTRACT

Piper nigrum* L. is traditionally propagated by stem cuttings and seeds, but seeds tend to produce progenies with higher variations due to cross-pollination habits. Plant tissue culture technique is the most efficient and reliable method for rapid clonal multiplication, however, endophyte microbial contamination limits the success. Twelve surface sterilization protocols were tested on two bud types (apical and axillary buds) and three different maturity stages of the leaf (first, third and fifth leaves) to optimize the conditions for culture initiation. The Completely Randomized Design was used with 60 explants. Maximum likelihood analysis of variance was conducted using the Proc CatMod procedures of PC-SAS to analyse the count data. The continuous data were analysed using Analysis of Variance and the mean separation was done using Least Significant Difference. Results revealed that the optimal sterilization protocol was specific to the explant type. The third leaf from the top of the plant and the apical bud was the best explants giving minimum tissue contamination and browning. Fungal contamination was frequent in leaf explants whereas bacteria in bud explants. The protocols containing 70% ethanol (30s), 0.1% $HgCl_2$ (5 min) and sterile distilled water with activated charcoal (1 gL^{-1}; 25 min), and 20% sodium hypochlorite (NaOCl) (15 min) with 70% ethanol (1 min) were comparable for the third leaf. In apical buds, the protocols of 0.1% $HgCl_2$ (10 min) and 70% ethanol (1 min), and 10% NaOCl (15 min) with 70% ethanol (1 min) provided comparable performances with the highest survival and least contamination rates. The potential of replacing hazardous $HgCl_2$ with non-toxic NaOCl by manipulating the concentration and the exposure time in combination with 70% ethanol was highlighted.

Keywords: Browning, Contamination, Micropropagation, *Piper nigrum*, Sterilization

INTRODUCTION

Black pepper (*Piper nigrum* L.) is a perennial woody vine that belongs to the family Piperaceae. Western Ghats of South India is considered the motherland of black pepper (Abbasi *et al*. 2010) and at present, it has been popularized throughout the tropical region due to its ability to survive under a wide range of environmental conditions (Wankhade 2014). Vietnam is the leading country in the global production of black pepper contributing 34% of the world's production (Sawe 2017) whereas Sri Lanka falls in the seventh place (Factfish 2018). Department of Census and Statistics of Sri Lanka (2020) reported that the total extent of land area under black pepper cultivation was 45 267 hectares with the production of 41 429 metric tons in 2019. Black pepper is mainly cultivated in the low country and mid-country wet and intermediate agro-climatic zones in Sri Lanka (Anon 2010). Among spices, black pepper is the most commonly used condiment in the food industry whereas it is also used in traditional medicine due to its properties such as inhibiting parasites and curing several illnesses (Ahmad *et al.* 2012). Berries and leaves contain a lot of beneficial constituents such as terpenes, alkaloids, flavonoids, sterols

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and methanolic extract (Wankhade 2014). Piperine is a pungent alkaloid which gives the spicy taste to black pepper.

The plant can be propagated through seeds and vegetative parts like cuttings, layering and grafting (Hussain *et al.* 2011). Obtaining genetically identical plants is difficult through seed propagation due to its cross-pollination and thereby high heterozygous nature which leads to genetic variations among the progeny. The application of conventional vegetative propagation methods is also not effective since the process is slow and time-consuming (Kadam *et al.* 2011). About 50 cuttings can be obtained per year as planting materials from the mother plants grown under field conditions. However, the traditional propagation methods are insufficient to meet the demand for high quality and healthy planting materials specially for the new varieties developed through breeding programmes (Bhat *et al.* 1995). Thus, an efficient technique is necessary for mass-scale production of high-quality planting materials. Plant tissue culture is the best option to overcome those constraints in traditional propagation methods (Hussain *et al*. 2011) aiming at increasing the production and productivity of black pepper. Furthermore, piperine, the major bioactive component of black pepper, that is pungent with numerous beneficial therapeutic properties (Gorgani *et al*. 2016) can be produced on the mass scale if an efficient *in vitro* protocol is available. Culture initiation with viable explants free from contaminants is the first step to be optimized in establishing an effective and efficient protocol. The presence of systemic microorganisms in the explants has limited the frequent use of *in vitro* protocols (Rajmohan *et al.* 2010). The systemic microbes or endophytes may either become localized at the point of entry, vascular system or throughout the plant. From the internal tissues of the root and stem of black pepper, 74 endophytic bacteria which belong to six genera and eight unidentified strains were isolated and characterized (Aravind *et al.* 2008). When the explants are inoculated into the tissue culture media, the endophytic bacteria multiply faster and contaminate the cultures. Therefore, a viable sterilization protocol is a primary requirement for establishing the technique with a higher success rate.

Mercuric chloride ($HgCl_2$), sodium hypochlorite (NaOCl) and ethyl alcohol (EtOH) are some of the widely used disinfectants to control microbial contaminations, though they are phytotoxic in nature (Oyebanji *et al.* 2009). Even though $HgCl_2$ is effective in controlling the systemic microorganisms in *Piper* (Subasinghe *et al*. 2003), it is important to find alternative protocols devoid of this chemical due to its hazardousness for humans and the environment. Furthermore, browning of the explants and the culture medium is another common problem in plant tissue culture in the species like *Piper* which produce toxic polyphenols or quinones that inhibit the growth of explants by oxidation (Madhusudhanan and Rahiman 2000). The use of non-specific absorbents like activated charcoal as surface sterilization treatments or as an ingredient of the culture media (Thomas 2008) and the application of heat shocks (Velarde and Saltveit 2001) have been used to control the occurrence of browning in the explant tissues. Thus, in the present study, the effectiveness of different sterilization protocols containing a single or combined sterilizing agents for initiating contaminant-free viable *in vitro* black pepper cultures with minimal browning effect was investigated using two bud types (apical and axillary buds) and three different maturity stages of the leaf (first, third and fifth leaves).

MATERIALS AND METHODS

Plant materials

Leaves and buds collected freshly from a mother vine collection of black pepper cultivar Panniyur-1 at the Department of Export Agriculture, Plant Production and Tissue Culture Research Centre, Walpita were used for the study. Three leaf stages, first (L_1), third (L_2) and fifth (L_3) leaf and two bud types, the apical buds and the axillary buds collected from the second node from the apex were used in the study.

Explant preparation, surface sterilization and culture initiation

Collected leaves and buds were washed under slow running tap water for five minutes to remove the attached inert particles from the surface of the explant. For pre-surface sterilization, explants were washed by shaking in distilled water containing detergent (Teepol) and a fungicide {Mancozeb 800 mg/ L (w/v)} for 30 minutes. Then, the explants were rinsed four to five times with sterile distilled water.

To select the most effective surface sterilization method to control browning and contamination with the highest survival rate of explants, twelve different surface sterilization protocols (Table 01) were tested under a sterile environment in the laminar flow cabinet. After applying each disinfectant, the explants were thoroughly washed four times with autoclaved distilled water, until free from the disinfectant residues. The leaves were trimmed to approx. 1 cm^2 piece containing the mid-rib and the leaf laminar. Nodal explants containing axillary buds were cut into 1.5-2.0 cm long pieces**.** The 1.0-1.5 cm stem pieces containing the apical buds were prepared after removing the leaf sheaths carefully.

Sterilized explants were aseptically cultured in the test tubes with Woody Plant Medium (WPM) (McCown and Lloyd 1981) supplemented with 20 g/L sucrose, 2 g/L activated charcoal and solidified with 8 g/L agar without hormone. The pH of the culture medium was adjusted to 5.8 before autoclaving at 121^0C and 1.5 kg cm^{-3} for 20 minutes. The test tubes were prepared with a slanted culture surface for maximum light exposure for the leaf explants while nodal explants were cultured in test tubes without any slant. Each test tube contained one explant. The cultures were incubated at 26 °C of temperature, 60% - 70% of relative humidity and 3000 Lux of light intensity for a 16 h photoperiod. Twenty explants were used for each treatment and the experiment was repeated three times.

Observations were made after 14 days of culture initiation by careful visual observations. The contamination rate was recorded together with the source of contamination by general identification of the presence of bacteria, fungi or both bacteria and fungi. Cultures containing a jelly-like milky spread on the media were identified as bacterial contaminations while velvet growth was identified as the fungal contaminations.

Table 1 : Combination of disinfectants and the time duration of the treatments (min) applied in different sterilization protocols tested for black pepper explants

Tested pro-tocols	Treated time duration (min)					
	0.1% $HgCl_2$	70% EtOH	Charcoal	50 ^{0}C	10% NaOCl	20% NaOCl
P_1	10	-	-	-	-	-
P_2	10	1	-	-	-	-
P_3	5	0.5	25	-	-	-
P_4	10	-	-	1.5	-	-
P_5	-	-	-	-	15	-
P_6	-	1	-	-	15	-
P_7	-	0.5	25	-	5	-
P_8	-	-	-	1.5	15	-
P_9	-	-	-	-	-	15
P_{10}	-	1	-	-	-	15
P_{11}	-	0.5	25	-	-	5
P_{12}	-			1.5	-	15

$HgCl_2$- Mercuric Chloride, NaOCl- Sodium hypochlorite, EtOH- Ethyl alcohol, Charcoal - Sterile distilled water with activated charcoal, min- minutes

The explants that remained green in colour were recorded as the survived explants. The rest of the cultures with dried or dead explants (without any contamination and lost their green colour) were counted as dead cultures and considered as the mortality rate.

Validation of the sterilization protocol and the explant type for callus initiation

The selected leaf maturity stage was sterilized by the optimized sterilization protocol and cultured onto the WPM supplemented with 2, 4-Dichlorophenoxyacetic Acid (2,4-D; 0, 4, 6 mg L^{-1}) in combination with Benzylaminopurine (BAP; 0, 3, 6 mg L^{-1}). The effect of light was also tested by keeping half of the cultures of each experiment in the light and the other half in the dark. Each treatment was replicated 10 times; four explants were used in each jar (60 x 90 mm). The experiment was repeated three times. Observations were made on explant viability, browning and callus induction after 50 days from culture initiation.

Estimation of explant browning and viability

The browning or blacking intensity of the explants was recorded using a 10 mm^2 graph paper at the time of data recording. The number of browned squares were recorded by careful observation under a dissecting microscope (Euromax, Arnhem, The Netherlands) and recorded as the percentage. The number of explants with less than 20% browned area was considered as an absence of browning in the first experiment conducted to test the effect of sterilization protocol. In the experiment conducted to validate the selected protocol, the browned area was recorded in each treatment containing plant growth regulator combinations.

The explants used for the study were moderately fragile, thus the tissue damage caused by the conditions used for sterilization protocol and the concentrations and combinations of growth regulators could damage the live tissue that affect the viability of the leaf explant. Thus, the inoculated samples into all tested culture media were subjected to testing viability using Evans Blue. The viability loss was calculated as a percentage using the same protocol for determining the browning. Area stained with Evans Blue was determined by counting the number of stained squares under a dissecting microscope. The viability loss was assessed by calculating the percentage area with stain.

Data recording and statistical analysis

The Completely Randomized Design (CRD) was used for the study. Maximum Likelihood Analysis of Variance was conducted using the Proc CatMod procedures of PC-SAS (SAS version 9.2) to analyse the count data and the continuous data were analysed using Analysis of variance (ANOVA) where the mean separation was done using the least significant difference at α=0.5 level.

RESULTS AND DISCUSSION

Sterilization of Leaf Explants

Leaf Maturity Level 1 *(First leaf; L_1):* The tested sterilization protocols significantly affected the contamination rate ($p<0.0001$; X^2 -40.12). The survival rate of the tissues was comparable in all the sterilization protocols. Protocols 1, 2, 3, 4 and 10 showed the lowest contamination rate (Table 02). It indicates that the effect of 1% $HgCl_2$ and the 20% NaOCl are equally effective for controlling the contamination. Protocol 5 with 10% NaOCl for 15 minutes recorded the highest contamination rate (46.7%), 50% survival rate and 1.7% mortality rate. Protocols 1, 2, 4, 5, 8, 9 and 10 showed less than 10% mortality rate whereas the other treatments did not show any tissue death indicating that the treatments are not detrimental to live cells. Fungal contamination was prominent in all protocols except in 4, 7 and 11 where the bacterial contamination was higher, probably due to inadequate exposure time to the disinfectant (Table 03). The highest frequency of explants with the absence of any tissue browning was recorded in protocols 7 and 11 where a minimum exposure time is given for the disinfectants ($p<0.0001$; X^2-65.98; Table 05).

Leaf Maturity Level 2 *(Third leaf; L_2):* The sterilization protocols affected significantly for tissue contamination ($p<0.0001$; X^2-65.98)

Table 2. Effect of the sterilization protocol on survival and contamination rates of the explants obtained from three leaf stages, first (L_1), third (L_2) and fifth (L_3) leaves and two bud types, apical (S_1) and axillary (S_2) buds

	Survival rate (%)					Contamination rate (%)				
SP	L_1	L_2	L_3	S_1	S_2	L_1	L_2	L_3	S_1	S_2
P_1	85.0^{a}	75.0^{ab}	65.0^{a}	80.0^{a}	40.0^{a}	13.3^{a}	25.0^{a}	35.0^{a}	20.0^{a}	60.0^{b}
P_2	80.0^{a}	80.0^{ab}	75.0^{a}	75.0^{a}	65.0^{a}	15.0^{a}	20.0^{a}	25.0^{a}	23.3^{a}	35.0^{a}
P_3	95.0^{a}	81.7^{ab}	75.0^{a}	40.0^{b}	55.0^{a}	5.0^{a}	16.7^{a}	25.0^{a}	60.0^{bc}	45.0^{a}
P_4	85.0^{a}	80.0^{ab}	60.0^{a}	25.0^{bc}	28.3^{b}	11.7^{a}	20.0^{a}	40.0^{a}	75.0^{cd}	71.7^{b}
P_5	50.0^{a}	38.3^{de}	36.7^{b}	53.3^{a}	13.3^{cd}	46.7^{d}	61.7^{c}	63.3^{b}	46.7^{b}	86.7^{bc}
P_6	68.3^{a}	58.3^{b}	25.0^{c}	65.0^{a}	25.0^{bc}	31.7^{bcd}	41.7^{c}	75.0^{c}	35.0^{a}	75.0^{b}
P_7	81.7^{a}	36.7^{de}	20.0^{c}	10.0^{cd}	15.0^{cd}	17.3^{b}	63.3^{c}	80.0^{c}	90.0^{de}	85.0^{bc}
P_8	68.3^{a}	61.7^{ab}	70.0^{a}	0.0	0.0	23.3^{bc}	38.3^{b}	30.0^{a}	100.0^{e}	100.0^{d}
P_9	63.3^{a}	40.0^{cde}	41.7^{b}	23.3^{bc}	6.7^{de}	35.0^{bcd}	60.0^{bc}	58.3^{b}	76.7^{cd}	93.3^{cd}
P_{10}	81.7^{a}	80.0^{ab}	60.0^{a}	20.0^{c}	31.7^{b}	13.3^{a}	20.0^{a}	40.0^{a}	80.0^{d}	68.3^{b}
P_{11}	81.7^{a}	88.3^{a}	40.0^{b}	15.0^{cd}	5.0^{de}	18.3^{b}	11.7^{a}	60.0^{b}	85.0^{de}	95.0^{cd}
P_{12}	63.3^{a}	35.0^{e}	16.7^{c}	20.0^{c}	20.0^{c}	36.7^{cd}	61.7^{c}	80.0^{c}	80.0^{d}	80.0^{bc}
P	*0.2496*	*0.0001*	*0.0001*	*0.0001*	*0.0001*	*0.0001*	*0.0001*	*0.0001*	*0.0001*	*0.0004*
X^2	*13.71*	*42.35*	*56.38*	*96.24*	*78.88*	*40.12*	*65.98*	*53.37*	*66.53*	*33.9*

Sterilization protocol (SP); Means in a column with the same letters are not significantly different at p=0.05 at 95% confident level; n=60; P_1-P_{12} See Table 1; p- p value; X^2- Chi square value

Table 3. Effect of the sterilization protocol on contamination (%) by different contaminants in the explants obtained from three leaf stages, first (L_1), third (L_2) and fifth (L_3) leaves

	L_1			L_2			L_3		
SP	Fungi	Bacteria	Fungi+ Bacteria	Fungi	Bacteria	Fungi+ Bacteria	Fungi	Bacteria	Fungi+ Bacteria
P_1	91.7^{a}	0.0	8.3^{a}	76.7^{ab}	4.2^{b}	19.2^{a}	79.4^{a}	5.6^{a}	15.1^{a}
P_2	72.2^{a}	0.0	27.8^{a}	85.0^{ab}	15.0^{ab}	0.0	86.7^{a}	0.0	13.3^{a}
P_3	50.0^{a}	0.0	16.7^{a}	77.8^{ab}	22.2^{ab}	0.0	73.3^{a}	6.7^{a}	20.0^{a}
P_4	22.2^{a}	33.3^{abc}	44.4^{a}	55.6^{ab}	30.6^{ab}	13.9^{a}	88.4^{a}	0.0	11.5^{a}
P_5	74.1^{a}	3.7^{c}	22.2^{a}	81.2^{ab}	5.3^{ab}	13.5^{a}	81.6^{a}	5.3^{a}	13.0^{a}
P_6	85.7^{a}	4.8^{c}	9.5^{a}	93.3^{ab}	3.3^{b}	3.3^{a}	93.3^{a}	0.0	6.7^{a}
P_7	30.0^{a}	70.0^{a}	0.0	48.4^{ab}	35.3^{a}	16.3^{a}	70.8^{a}	10.4^{a}	18.4^{a}
P_8	83.3^{a}	0.0	16.7^{a}	34.5^{ab}	52.4^{ab}	13.1^{a}	77.8^{a}	22.2^{a}	0.0
P_9	83.3^{a}	4.2^{c}	12.5^{a}	86.1^{ab}	8.3^{ab}	5.6^{a}	85.4^{a}	3.0^{a}	11.6^{a}
P_{10}	83.3^{a}	16.7^{bc}	0.0	33.3^{b}	41.7^{ab}	25.0^{a}	62.5^{a}	33.3^{a}	4.2^{a}
P_{11}	42.2^{a}	57.8^{ab}	0.0	44.4^{ab}	55.6^{ab}	0.0	63.9^{a}	33.3^{a}	2.8^{a}
P_{12}	86.9^{a}	4.8^{c}	8.3^{a}	94.8^{a}	5.1^{ab}	0.0	95.8^{a}	2.1^{a}	2.1^{a}
P	0.0001	0.0563	0.5872	0.0001	0.0005	0.6154	0.0001	0.0041	0.1787
X^2	44.12	13.75	6.54	74.26	33.31	5.37	56.52	22.46	13.88

Sterilization protocol (SP); Means in a column with the same letters are not significantly different at p=0.05 at 95% confident level; n=60; P_1-P_{12} See Table 1; p- p value; X^2- Chi square value

and survival ($p< 0.0001$; X^2-42.35) rates. The protocols of 1, 2, 3, 4, 10 and 11 showed similar effect on sterilization giving the lowest contamination rate and the highest tissue survival rate (Table 02). Protocol 7 with 70% EtOH (30 s), 10% NaOCl (5 min) and Charcoal (25 min) recorded the highest contamination rate (63.3%) indicating the insufficiency of exposure time to the disinfectants. The protocols 3 and 12 recorded 1.7% and 3.3% of mortality rates respectively whereas the others did not show any tissue death. Fungal contamination was prominent however; a higher occurrence of bacterial contamination was observed compared to the L_1 (Table 03). A significant difference was not observed in the tissues with the absence of browning (Table 05).

Leaf Maturity Level 3 *(Fifth leaf; L_3):* The tissue contamination ($p<0.0001$; X^2-53.37) and survival ($p<0.0001$; X^2- 56.38) rates were significantly affected by the tested sterilization protocol. Protocols 1, 2, 3, 4, 8 and 10 (Table 02) were effective in controlling contamination and maintaining tissue survival. Protocols 2 and 3, {0.1% $HgCl_2$ (10 min) + 70% EtOH (1 min); with and without charcoal} recorded the highest survival rate (75%). The mortality rate was recorded as 3.3% only in protocol 12 with distilled water at 50 ± 1°C for 90 s followed by 20% NaOCl (15 min). Protocol 7 with 70% EtOH (30 s), 10% NaOCl (5 min) and Charcoal (25 min) recorded the highest contamination rate (80%). Fungi contamination was prominent over bacterial contamination (Table 03). Sterilization protocol from any tested treatments did not show a significant effect on maintaining tissue viability with the absence of tissue browning (Table 05).

In all three-leaf stages, the major contaminant was fungi. Results indicated that 70% ethanol in combination with 20% NaOCl is effective in minimising fungal contamination. The occurrence of bacterial contamination is at a lower frequency except in a few cases. Considering the contamination rate of three different leaf explants (L_1, L_2 and L_3) protocols 1, 2, 3, 4 and 10 were effective where the first four consist of $HgCl_2$ and the latter contains non-toxic EtOH and NaOCl. Protocol 3 {70% EtOH (30 s); 0.1% $HgCl_2$ (5 min); Charcoal (25 min)} can be selected as the most effective protocol for disinfection of leaf explants of all three stages. However, minimum usage of heavy metal disinfectants is beneficial for sustainable production systems; thus, protocol 10 containing 70% EtOH for 1 min and 20% NaOCl for 15 min is recommended for sterilization of more mature leaves. Overall contamination is lower in the explants at L_1 over the other two stages.

Sterilization procedures should not interfere with the viability of explants. The highest mortality rate recorded in the L_1 could be attributed to rapid loss of biological activity under physical and chemical stresses for the soft immature tissues. Treating immature explants with activated charcoal dissolved water can damage tissues by abrasion. Mature tissues can survive physical damage than juvenile tissues. However, the contamination rate of the L_3 is higher than the other two stages. Expanded and well-exposed leaf blades may provide a better habitat for the air-born contaminants. Therefore, the leaf maturity level 2, (third leaf; L_2) can be selected as the most suitable leaf explant for initiating the *in vitro* cultures of black pepper.

Sterilization Protocol of Bud explants

Apical bud (S_1): The contamination ($p< 0.0001$; X^2-66.53) and survival ($p<0.0001$; X^2 - 96.24) rates of the apical buds were significantly affected by the sterilization protocol. Protocols 1, 2 and 6 recorded significantly lower contamination rates ranging from 20% to 35% (Table 02). The highest contamination rate (100%) was recorded in protocol 8. Only protocol 2 caused tissue mortality with a rate of 1.7%. Protocols 1, 2, 5 and 6 recorded the highest survival rate whereas protocol 1 gave an 80% survival rate. Bacterial contamination is common whereas Protocols 2 and 5 were effective in controlling their contamination (Table 04). Protocols did not show any difference in maintaining the fresh appearance of the tissues with the absence of tissue

browning (Table 05).

Axillary bud (S_2): The sterilization protocols were significantly affected the tissue contamination ($p<0.0004$; X^2-33.9) and survival ($p<0.0001$; X^2- 77.87) rates of the axillary buds as well. Protocols 2 and 3 gave the lowest contamination rate with 35 and 45 % respectively (Table 02). Similar to the apical bud, the highest contamination rate (100%) was recorded in protocol 8. Bacterial contamination is prominent over fungi (Table 04). None of the protocols caused tissue mortality indicating the tolerance of the explants for the toxicity created by the sterilization agents. The survival rate was higher in protocols 1, 2 and 3. Protocols significantly affect on tissue browning ($p<0.01$; X^2-27.82) where protocol 12 was effective in minimising the frequency of tissue browning (Table 05).

The major contaminant in bud explants was bacteria. It has been reported that the endophytic bacteria are present in stem and roots in black pepper plants but absent in leaves (Aravind *et al*. 2008) revealing that endophytic bacteria is not a serious problem in leaf culture but for the bud culture that should be importantly considered in mass propagation of black pepper.

Considering minimum contamination and maximum survival rates, the protocols 1 or 2 with 0.1% $HgCl_2$ (10 min); 70% EtOH (1 min) can be selected as the best sterilization protocol for both bud types. However, the sterilization protocol 6 with 10% NaOCl (15 min) and EtOH (1 min) was effective for the apical bud. The contamination rate of apical bud is less than the axillary bud. The apical bud is fully covered by leaf sheaths forming a barrier for reaching the fungi spores to the bud area. Axillary buds are open to the outer environment thus the spores can easily land on the surface of the bud which is difficult to control due to the hidden habitats. Therefore, the apical bud can be selected as the best explant among the two bud types for initiating the *in vitro* culture of black pepper.

The results demonstrated that browning of the third leaf, fifth leaf and apical bud (Table 05) was statistically significant with the sterilization procedures. Based on the results, a combination of either thermotherapy, exposure to 70% ethyl alcohol or treatment with activated charcoal dissolved water was

Table 4. Effect of the sterilization protocol on contamination (%) by different contaminants in the explants obtained from bud types, apical (S_1) and axillary (S_2) buds

SP	S_1			S_2		
	Fungi	Bacteria	Fungi+ Bacteria	Fungi	Bacteria	Fungi+ Bacteria
P_1	8.3^{b}	75.0^{a}	16.7^{a}	8.6^{bc}	80.7^{a}	10.7^{a}
P_2	88.6^{a}	0.0	11.4^{a}	19.8^{b}	70.4^{a}	9.7^{a}
P_3	6.7^{b}	76.2^{a}	17.1^{a}	33.3^{ab}	33.3^{c}	33.3^{a}
P_4	2.2^{b}	95.6^{a}	2.2^{a}	13.6^{bc}	67.8^{a}	18.6^{a}
P_5	84.5^{a}	8.3^{b}	7.2^{a}	7.9^{bc}	82.9^{c}	12.9^{a}
P_6	14.3^{b}	65.1^{a}	20.6^{a}	6.5^{c}	84.5^{a}	8.9^{a}
P_7	0.0	92.4^{a}	7.6^{a}	11.7^{bc}	66.7^{ab}	21.6^{a}
P_8	3.3^{b}	86.7^{a}	10.0^{a}	41.7^{a}	41.7^{bc}	16.7^{a}
P_9	0.0	84.7^{a}	15.3^{a}	43.2^{a}	44.8^{b}	12.0^{a}
P_{10}	6.3^{b}	89.7^{a}	4.0^{a}	4.2^{c}	79.1^{a}	12.9^{a}
P_{11}	0.0	92.0^{a}	8.0^{a}	17.5^{b}	71.9^{a}	10.5^{a}
P_{12}	0.0	91.7^{a}	8.3^{a}	43.2^{a}	33.8^{c}	23.0^{a}
P	0.0001	0.0001	0.5437	0.0001	0.0001	0.3485
X^2	47.95	65.14	9.85	64.35	39.97	12.20

Sterilization protocol (SP); Means in a column with the same letters are not significantly different at p=0.05 at 95% confident level; n=60; P_1-P_{12} See Table 1; p- p value; X^2- Chi square value

Table 5. Effect of the sterilization protocol on browning percentage of the explants obtained from three leaf stages, first (L_1), third (L_2) and fifth (L_3) leaves, and two bud types, apical (S_1) and axillary (S_2) buds

SP	Leaf stages			Bud types	
	L_1	L_2	L_3	S_1	S_2
P_1	43.3^{c}	63.3^{a}	75.0^{a}	81.7^{a}	63.3^{b}
P_2	53.3^{c}	76.7^{a}	58.3^{a}	58.3^{a}	73.30
P_3	81.7^{ab}	83.3^{a}	93.3^{a}	81.7^{a}	85.0^{a}
P_4	81.7^{ab}	96.7^{a}	93.3^{a}	76.7^{a}	95.0^{a}
P_5	60.0^{c}	85.0^{a}	71.7^{a}	80.0^{a}	36.7^{b}
P_6	78.3^{ab}	86.7^{a}	90.0^{a}	63.3^{a}	95.0^{a}
P_7	96.7^{a}	73.3^{a}	68.3^{a}	60.0^{a}	86.7^{a}
P_8	66.7^{ab}	81.7^{a}	86.7^{a}	76.7^{a}	66.7^{b}
P_9	56.7^{c}	83.3^{a}	60.0^{a}	65.0^{a}	61.7^{b}
P_{10}	85.0^{ab}	91.7^{a}	78.3^{a}	90.0^{a}	100.0^{a}
P_{11}	96.7^{a}	90.0^{a}	85.0^{a}	83.3^{a}	91.7^{a}
P_{12}	86.7^{ab}	41.7^{a}	71.7^{a}	83.3^{a}	86.7^{a}
P	0.0052	0.0909	0.3287	0.4876	0.0035
X^2	26.64	17.62	12.48	10.48	27.82

Sterilization protocol (SP); Means in a column with the same letters are not significantly different at p=0.05 at 95% confident level; n=60; P_1-P_{12} See Table 1; p- p value; X^2- Chi square value

effective in controlling browning other than the use of disinfectants alone.

Mercuric chloride is a heavy toxic compound for living organisms in the environment and sodium hypochlorite is commercially available laundry bleach which is a cheap and the most frequent choice for surface sterilization over $HgCl_2$ in tissue culture (Oyebanji *et al.* 2009). All disinfectants have a possibility of phytotoxicity to living explants. Therefore, a proper balance between concentration and time of exposure is important.

Callus induction using the optimized sterilization protocol and explant type

As reported above, a very low contamination rate was observed in the cultured leaf explants excised from the leaf stage 2 (third leaf; L_2) sterilized by the selected protocols validating the aforementioned results. A certain level of tissue damage can occur during the preparation and sterilization of the explants which may cause tissue browning and loss of

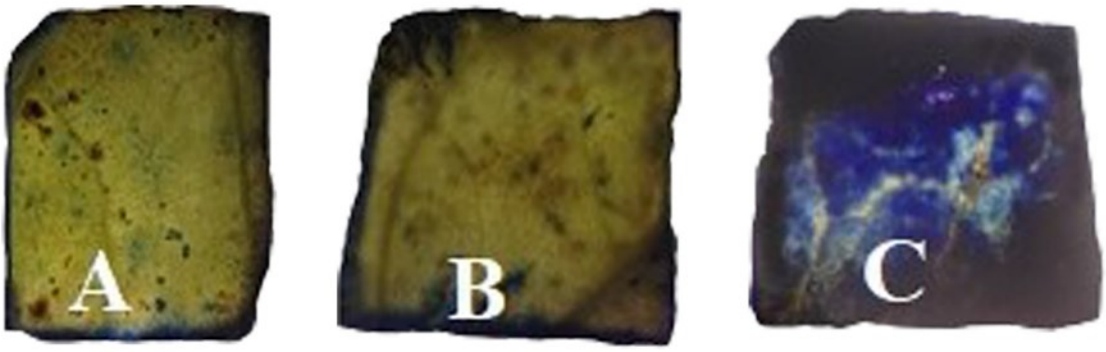

Figure 1 Testing the tissue viability by staining with Evan's Blue A. Fresh explant before sterilization (positive control). B. Leaf explant sterilized using the selected protocol C. Dead leaf explant after dipping in boiling water (negative control)

Table 6. Validation of the selected sterilization protocol on response of the explants cultured onto the callus induction media supplemented with BAP and 2, 4-D

Concentration of 2, 4-D + BAP (mgL^{-1})	Light regime					
	Dark			Light		
	Browning %	Viability loss %	Callus induction	Browning %	Viability loss %	Callus induction
0, 0	5.88^{ef}	11.22^{d}	-	17.44^{fe}	26.77^{dc}	-
0, 3	9.88^{ed}	25.77^{bc}	-	19.44^{de}	27.66^{dc}	-
0, 6	11.22^{cd}	20.11^{c}	-	21.22^{dc}	36.55^{dc}	-
4, 0	14.88^{cb}	28.88^{ba}	-	28.66^{b}	36.55^{ba}	-
4, 3	7.33^{ed}	19.66^{c}	+	19.55^{de}	28.00^{c}	-
4, 6	16.88^{b}	27.33^{ba}	-	24.33^{c}	31.66^{bc}	-
6, 0	26^{a}	34.11^{a}	-	32.66^{a}	39.11^{a}	-
6, 3	4.88^{f}	9.11^{d}	++	14.77^{f}	21.88^{d}	-
6, 6	9.66^{ed}	12.55^{d}	+	18.77^{de}	26.66^{dc}	-

Note: Means in a column with the same letters are not significantly different at p=0.05 at 95% confident level; n=120; 2,4-D =2,4-Dichlorophenoxyacetic Acid; BAP = Benzyl Amino Purine

cell viability. However, tissues stained with Evans Blue after sterilization revealed that the sterilization protocol did not affect the cell viability compared to the live and dead samples used as the positive and negative controls (Figure 1). It indicated that the tissue damage caused by the sterilization protocol was minimum. However, the incorporation of PGR (Plant Growth Regulators) into the culture media could damage the tissue by enhancing the tissue browning and loss of tissue viability. Newly produced excretory substances by cultured explants may also create a toxic environment for the explants which may cause to increase the tissue browning.

The highest browning and viability loss percentage was recorded in the presence of the highest concentration of 2 4-D devoid BAP in the medium maintained either in the dark or light conditions (Table 6). The lowest rates were observed either in the control without any PGR or in the combination of 6 mg L^{-1} 2, 4-D and 3 mg L^{-1} BAP. Callus initiation was observed in the cultures maintained in the dark for 50 days after explant inoculation. The leaf explants cultured on to the media containing 4/3, 6/3, 6/6, mg L^{-1} 2, 4-D and BAP, showed the least browning and viability loss was effective in inducing callus initiation. Higher the concentration of plant growth regulators increased tissue browning and viability loss was observed. Comparatively, explants maintained in the light showed higher browning and viability loss that was not favourable for callus induction. Improved callus induction protocol is essential for the research on biologically active molecules in medicinal plant species (Ahmad *et al*. 2010).

The optimized conditions can be used for developing the protocol for mass-scale production of clonal plants through somatic embryogenesis or organogenesis to fulfil the demand for the plating materials. Moreover, the protocol can also be used for initiating the cell cultures to produce piperine, a value-added product of black pepper, on mass scale using the bioreactors.

CONCLUSIONS

The common contaminant was specific to the explant type. To initiate leaf cultures, the third leaf is the best whereas the apical bud is the most suitable bud for establishing *in vitro* cultures for mass propagation of black pepper. The protocols with 70% EtOH (30s); 0.1% $HgCl_2$ (5 min) and sterile distilled water with activated charcoal (200 mg/200 ml) for 25 min, are comparable with the protocol of 20%

sodium hypochlorite (NaOCl) (15 min) with 70% ethanol (1 min) to control the contamination in the third leaf explants. The protocol with 0.1% $HgCl_2$ for 10 min + 70% EtOH for 1 min was comparable with the protocol of 10% NaOCl (15 min) with 70% ethanol (1 min) for controlling the contamination in the apical buds. Browning of black pepper explants can be controlled using a combination of ethyl alcohol, activated charcoal and thermotherapy with mercuric chloride or sodium hypochlorite. The optimized sterilization protocol and the leaf stage were validated through callus induction in the WPM supplemented with 2, 4-D and BAP.

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AUTHOR CONTRIBUTION

DBRS, KDNP and PIPP designed the experiments; WPDSG and HMAPM conducted the experiments, PIPP handled and interpreted the data, PIPP and WPDSG wrote the manuscript

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