Effect of different concentration of copper sulphate for sterilization procedure for in Vitro Propagation of Black Pepper (Piper nigrum L.) from Nodal Culture in Ethiopia

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Abstract
Black pepper belongs to the family Piperaceae. It is a perennial woody climbing tree.In Ethiopia, black pepper, known as ‘‘Kundo berbere’ or ‘Yebahir kimem’. Lack of efficient sterilization methods of the explants is one of the bottlenecks for the exploitation of the export potential of black pepper. Thus use of copper sulphate is suggested to alleviate the problem of contamination of planting materials. So far there is no available efficient sterilization protocol on in vitro propagation of black pepper in Ethiopia. Therefore, the objectives of the present study were to develop an efficient sterilization protocol for in vitro propagation of black pepper from nodal culture. The experiments were carried out using CRD design in factorial arrangement at jimma Agricultural Biotechnology Laboratory. Copper sulphate with the concentration of 50, 75 100, & 125 mg/l was used for surface sterilization and each concentration was tested against three different exposure time (20, 25, and 30 min). Data were subjected to ANOVA and significant different means were separated using LSD test. Seeds disinfected in 100mg/l copper sulphate for 20minutes gave 95.62% clean survived and 14.40% germinated explants.

Keyword: copper sulphate, sterilization, explants.

1. Introduction

Black pepper (*Piper nigrum* L.) belongs to the family Piperaceae. It is a perennialwoody climbing tree. It is native to India, Indonesia, Malaysia, South America and West Indies but is also widely cultivated in the tropical regions. Black pepper is a universal table condiment used to flavor all types of cuisines worldwide. It is christened as the 'King of Spices' (Mathew *et al*., 2001; Srinivasan, 2007).

In Ethiopia, black pepper is known as ''*Kundo berbere'* or '*Yebahir kimem'*, it used all over the country as a seasoning during food preparation. Ethiopia had exclusively been importing black pepper to cover the national demand; this in turn has made the spice unaffordable to the vast majority that dried fruits of *Schinus molle* were widely used as its cheaper substitute (Edossa, 1998).

Surface sterilization of starting materials (explants) in chemical solutions (disinfectants) such as copper sulphate, Ethanol, Sodium hypochlorite (NaOCl), Mercuric chloride ($HgCl_2$) and Tween-20 is required (Mineo, 1990; Aishwarya and Robinson, 2013) to control the growth of fungi and bacteria on the growth media (Singh *et al*., 2011).

Most tissue culture procedures are conducted in sterile operations, such as laminar flow cabinet. Besides the special design of gentle flow of sterile air in cabinet, aseptic cabinet is also equipped with germicidal lamp emitting ultraviolet light. This type of radiation is useful in eliminating airborne contaminants and for surface disinfection. Glassware and all the tools used for tissue culture process can also cause contamination. It is extremely necessary to autoclave all the material before using it, so that all the microbial contaminants are destroyed (Reed and Tanprasert, 1995).

Successful *in vitro* techniques for micro propagation of black pepper have been limited due to the systemic presence of microorganisms in the explants. (Girija *et al*, 2002) reported that he observed the association of an endophytic diazotrophic bacterium *Beijerinckia indica* in the *in vitro* cultures of black pepper, Attempts to control the bacterial interference through pre-

sterilization or pre-treatment with mercuric chloride were unsuccessful. Use of antibiotics like streptopenicillin has been prescribed in the protocols developed for black pepper.
However, in most of the cases the *in vitro* response of the explants is drastically affected. This necessitates strategies, avoiding the use of antibiotics.

Copper sulphate has long been recognized to have fungicidal, algicidal, molluscicidal, bactericidal and herbicidal properties .Copper compounds are extensively used for the control of fungal and bacterial diseases in several crop plants. In this context a study was undertaken to test the efficacy of copper sulphate to overcome the interference of systemic bacteria on the *in vitro* propagation of black pepper (Albright *et al*, 1974).

Copper compounds are extensively being used for controlling fungal and bacterial diseases in several crop plants. Toxic mechanisms of copper in microorganisms include interactions with proteins, enzymes, nucleic acids and metabolites at the cell wall, cell membrane and protoplasm and the sterilization method is easy and the material is easily available, less costly and less toxic to human compared to other disinfectant(Sato *et al*, 1986)

Several plant species, especially the perennial ones, have been observed to have mutually beneficial association with endophytic microorganisms, under *in vivo* conditions. However, when their explants are cultured *in vitro*, the microbes come in contact with the nutrient media and multiply at a much faster rate than that within the conducting vessels under normal *in vivo* conditions. Within a short period they multiply and become toxic to the explants. Copper sulphate in the present instance seemed to maintain the bacterial growth to a level comparable to that of the *in vivo* conditions, without allowing for excessive multiplication and consequent destruction of the explants. However, it did not totally eliminate the bacteria (Leifert, *et al*, 2001)

2. Materials and Methods

2.1. Description of the Experimental Area

The experiment was conducted during 2015/16 at the Tissue Culture Laboratory of Plant Biotechnology Division in Jimma Agricultural Research Center (JARC), South Western

Ethiopia. The center is located 361 km far from Addis Ababa in Southwest of Ethiopia at $7^0 40'$ 15''N latitude and $36^0 49'33''$E longitude and at an altitude of 1750 m.a.s.l. (EARO, 2000)

2.2. Sources of Experimental Material

Ripe seeds were collected from four years old healthy plants of piper nigrum L. cultivar Tato/380 from Teppi national spices center in October 2016 cropping season.

2.3. Experimental Procedure

3.3.1. Explants surface sterilization

Before seeds were inoculated on a medium, it must be sterilized to get read of all microorganisms. Seeds were washed with tap water to remove debris and the pulps were removed by using hand towel. The pulped seeds were rinsed in distilled water and soaked for two hours. Then the imbibed seeds were washed by DW with savalon plus 2-3 drops of Tween-20 for 25 minutes with agitation to physically remove most microorganisms and to remove some debris.

Then the seeds were treated with 70% ethanol for 10 minutes under laminar air flow cabinet. After pretreatment with ethanol, the explants were rinsed with autoclaved distilled water three times, to lower the toxic effect of ethanol. Also the seed were treated with 0.1% of mercuric chloride ($HgCl_2$) for 10minutes.

They were then treated with four concentration levels (50, 75, 100 and 125 mg/l) of copper Sulphate ($CuSO_4$) for varying exposure times (20, 25, and, 30). To increase the efficiency of $CuSO_4$, 2-3 drop of Tween-20 per 200 ml solution was added as wetting agent, after decanting the sterilizing solutions. The seeds were washed three times each for five minutes with autoclaved distilled water to remove traces of $CuSO_4$.

The sterilized seeds were then inoculated on to semi solid basal MS medi (PGR free media) fortified with 3% sucrose and 0.7% agar, for germination and induction of direct organogenesis. The jars with cultured seeds were properly sealed with parafilm and labeled. Thereafter, the cultures were transferred and randomly placed on the growth chamber with a photoperiod of 16/8h light/dark using cool-white fluorescent lamps (photon flux density, 40µmol m^{-2} s^{-1} irradiance) at 25 ± 2^0C and relative humidity (RH) of 70-80%. For each

treatments combination three jars each with three seeds per jar were placed in factorial arrangement randomly in completely randomized design (CRD).

2.4. Data recorded

- **Number of contaminated explants**: number of explants infected by microorganisms within 15 days of culture was counted from each replication and converted into percentage.
- **Number of clean explants**: number of explants which are free from microorganisms and

  survived was counted after 15 days of culture from each replication and converted into percentage.
- **Number of germinated explants:** number of explants germinated was counted from each replication after 60 days of culturing and converted into percentages.

2.5. Data Analysis

The Data were subjected to analysis of variance (ANOVA) using the SAS software Packages (version 9.2) and significant differences among mean values was compared using Least Significant Difference (LSD) at 5% of probability level.

3. Results and Discussion

3.1. Sterilization of the Explants

The results and discussion are presented in sterilization technique, of *invitro* propagated plantlets. Analysis of variance (ANOVA) revealed that concentration of copper sulphate, exposure time, and their interaction had highly significant difference ($P \leq 0.01$) on overcoming contamination of growth media and improving survival and germination level of *piper nigrum in vitro* seed culture (Appendix Table 1).

Among the different disinfectant treatments investigated, 50 mg/l $CuSo_4$ for 15 min were recorded the highest contamination (94.69 %), the minimum germination (1.40%) and the highest days to germination (85 days).the minimum days to germination(42.3 days) was obtained from 125mg/l $CuSo_4$ for 15 minutes.

The minimum contamination (4.37 %) and average days to germination (58 days) was obtained from 100mg $CuSo_4$ for 20 min duration, but, stastically the same in 125mg/l $CuSo_4$ for 15 in terms of contamination percentage (5.92%).

The obtained result revealed that 100 mg/l for 30 min, 125mg/l for 25 min, and 125mg/l for 20 min were as par stastically with germination of 53.82%, 47.20% and 42.55% respectively.
This result indicated that the concentration of cupper sulphate and exposure time used for disinfectant were interrelated factors to obtain microorganism free explants (Table 1).These results indicated that the chemical concentration and the duration at which the black pepper seeds exposed to the disinfectant time affected the seed germination percentage significantly. The data also revealed that the contamination percentage was dramatically decreased as the exposure time increased within the same level of CuSo4 solution. However, disinfection with higher concentration (100mg/l) of CuSo4 solution for 20 minute resulted in least contamination (4.37%) but no enough seed does germinated (14.4%)

This may be due to phototoxic ability of Cu^{++} at longer time exposure Comparing the interaction effect posed by the chemical and the duration of disinfection against the aim of sterilization, disinfection of black pepper seed with 125mg/l CuSo4 solution for 20 min was the most effective sterilization treatment, which gave highest clean explant percentage, lowest contamination, and moderately germination percentage. This sterilization method is easy and the material is easily available, less costly and less toxic to human compared to other disinfectant (e.g. $AgNO_3$). It doesn't also require special handling and waste removal.

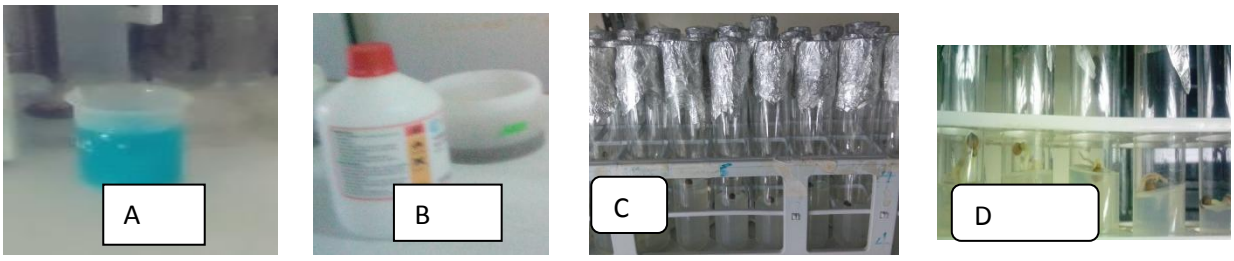

Fig.1.
Sterilization of seed with copper sulphate. A. Copper sulphate solution B. Black pepper seed ready for sterilization C. Decontaminated black pepper seed. D. A 45 days old seed

Table 1 Effect of $CuSO_4$ concentration and their exposure time on contamination, clean, and germination of explants *in vitro*

$CuSO_4$ mg/l	Exposure Time	Cont (%)	Ger (%)	DTGR
50	15	94.69 ± 3.51^{A}	1.40±1.22^{F}	85.0±5^{A}
50	20	80.19 ± 3.94^{B}	7.07±2.57^{E}	71.6±1.52^{B}
50	25	72.60 ± 5.01^{C}	10.41±3.63^{D}	66.0±2.64^{C}
75	15	63.11 ± 6.02^{D}	12.36±7.74^{C}	51.6±2.08^{D}
75	20	52.98 ± 3.52^{E}	19.29±6.39^{B}	54.6±3.05^{D}
75	25	42.99 ±5.61^{F}	20.85±7.79^{B}	47.3±0.57^{E}
100	15	18.20 ±3.70^{G}	42.55 ±3.60^{A}	52.6±4.16^{D}
100	20	4.37 ±1.22^{I}	47.20± 1.45^{A}	56.0±4.35^{D}
100	25	8.48 ±1.67^{HI}	53.82±12.68^{A}	60.6±5.68^{C}
125	15	6.92 ±1.85^{I}	20.99±8.60^{B}	42.3±2.51^{F}
125	20	7.57 ±1.22^{I}	14.40±7.50^{C}	58.0±2^{D}
125	25	8.85 ±1.93^{HI}	11.52±8.08^{D}	46.6±2.08^{E}
MEAN		38.79	20.82	57.72
CV (%)		9.56	18.39	5.74
LSD		3.61	6.61	2.79

Note: Means with the same letter (s) in the same column are not significantly different at P≤0.05 value using Fisher's LSD test. CV= Coefficient of Variation (%), LSD= Least Significant Difference, ± = Standard Deviation. Cont: contamination, Ger: Germination, DTGR=days to germination

4. Summary Conclusion and Recommendations

4.1. Summary and Conclusion

Establishments of contamination free plants from green house grown plants was very difficult due to endogenous bacterial contamination .The *in vitro* establishments of black pepper is also greatly hampered by the high incidence of bacterial and fungal contamination. It was found that *in vitro* germinated seedling would serve as a good source of explants to establish contamination free cultures. Since copper sulphate was a bactericidal anti fungal properties it can minimize the growth of systemic bacterial contamination in *invitro* regeneration of black pepper.

The concentration of copper sulphate, exposure time, and their interaction had highly significant difference on overcoming contamination of growth media and improving survival and germination level of *Piper nigrum in vitro* seed culture.

Among the different disinfectant treatments investigated, 50 mg/l $CuSO_4$ for 15 min duration recorded the highest contamination and, the lowest clean explants percentages. The lowest contamination and maximum clean explants percentages was recorded from 100mg $CuSO_4$ for 20 min duration. Therefore during *in vitro* regeneration of black pepper using 125mg/l of copper sulphate is better to minimize the occurrence of microorganism.

4.2. Recommendations

While this is the first attempt *in vitro* propagation of *piper nigrum* from seed as an explant, based on the results obtained thereof the following recommendations can be made: The use of well developed explants-surface sterilization (different concentration of copper sulphate with various exposure times) is effective and can be followed in the future when seed is used as explant to establish *in vitro* plantlet propagation.

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Appendices

AppendixTable 1. ANOVA for the Effect of Cupper Sulphate and Exposure Time on Contamination, Clean and Germination of Seeds

		Mean Square		
Sources	Df	Contamination (%)	Clean (%)	Germination (%)
Copper	3	11445.48**	14303**	2432.17**
Time	2	461.92**	475.85**	114.16ns
Copper*Time	6	102**	142.43**	98.68ns
Error	24	13.77	6.98	46.22
Cv(%)		9.56	5.60	18.39
Mean		38.79	47.10	20.99

Note: **=highly significant ($P \leq 0.01$) at $P \leq 0.05$ significant level; CV = coefficient of variation; DF= Degree of freedom; %=percentage, ns=non significant