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# Control of Contamination and Explants Phenolics in Ginger Accession (Zingiber officinale Rosc.) in Vitro Cultures

Berihu Mengs
Ethiopia institute of agricultural research, Jimma agricultural research center, P.O.Box 192, Jimma, Ethiopia

**Abstract**
Ginger which is an important tropical and sub-tropical herbaceous perennial plant, with the rhizome valued for its culinary and medicinal properties. *In vitro* growth performance of *Zingiber officinale* cultures is limited by contamination and phenolics. This study was aimed to minimize the effect of phenolic components and eliminate contamination for *in vitro* regeneration of *Zingiber officinale*. Our results showed that, the use of 3% (w/v) copper sulphate ($CuSo_4$), 0.1% (w/v) polyvinylpyrrolidone (PVP), 0.1% (w/v) ascorbic acid and 0.1% (w/v) citric acid for 6 hrs significantly reduced the sprout bud of explants browning. This pre-treatment was followed by 70% ethanol for 1 min, 1.5% (V/V) sodium hypochlorite and Tween 20 for 15 min for eliminating contaminants. Nevertheless, using whole pre-treatments browning (phenolics) were highly significant difference mean than aseptic and survival percentage. However, pre-treatments one at all periods were unable to effectively reduce contamination in bud cultures, but using 3% (w/v) copper sulphate ($CuSo_4$), PVP, ascorbic acid and citric acid as pre-treatment one for 6 hrs was the best for reducing contamination (33.3% clean) and browning (1.47), with a high survival rate (93.3%) on sprout bud explants. Streptomycin at 100 mg/ l was most effective against contaminating bacteria, along with the formation of the maximum number of ginger shoots, and had the least phytotoxicity. However, reduction in shoot growth and decreased rates of shoot multiplication and survivals were recorded in cultures grown on medium after the addition of 200 mg/ l of either antibiotic.
**Keywords:** *Zingiber officinale, in vitro* cultures, phenolic components, sterilization technique, copper sulphate, streptomycin.

## Introduction

Ginger (*Zingiber officinale* Rosc.), a member of the family Zingeberaceae, is an important tropical and sub-tropical herbaceous perennial plant, with the rhizome valued for its culinary and medicinal properties. Ginger production for the extraction of oleoresins and essential oils, as well as the direct use of rhizomes for culinary purposes is increasing worldwide (FAO, 2008). In 2007, it was the second widely cultivated spice 12.8 ton/ ha next to chilies in Ethiopia (Girma *et al.*, 2008).

Although, there are more than 45 ginger cultivars in the country (MoARD, 2008), their production and productivity has been lost due to *Pseudomonas solanacearum* (bacterial wilt), *Fusarium oxysporum* (yellow leaf), *Pythium aphanidermatum* (soft rot), *Phyllosticta zingiberi* (leaf spot), the major bottlenecks (Kavyashree *et al.,* 2009).

Plant micro propagation is an *in vitro* technique of growing “aseptic” plant cells, tissue or organs separate from the mother plant in an artificially prepared nutrient medium. It is particularly important for mass propagation especially when seed propagation and conventional vegetative techniques are difficult or unsuccessful. Although aseptic conditions are usually employed, *in vitro* contamination of tissue cultures by microorganisms is often the most serious problem in plant tissue culture (Omamor *et al*., 2007). Contamination is not always seen at the culture establishment stage; some internal (endophytic) contaminants become visible at later subcultures and difficult to eliminate (Reed *et al*., 1998). Chemicals like Copper sulphate have long been recognized to have fungicidal, algicidal, molluscicidal, bactericidal and herbicidal properties (Albright, L. J. and Wilson, E. M. 1974). Toxic mechanisms of copper in microorganisms include interactions with proteins, enzymes, nucleic acids and metabolites at the cell wall, cell membrane and protoplasm (McDonald, D. B. *et al*., 1986).

Apart from contamination, phenol of excised plant tissues and nutrient media occurs frequently and remains a major basis for recalcitrance *in vitro*. The severity of browning has varied according to species, tissue or organ, developmental phase of plant, age of tissue or organ, nutrient medium and other tissue culture variables (Huang *et al*., 2002). The browning phenomenon is usually imputed to oxidized phenolics compounds by polyphenol oxidase (PPO) and peroxidises (POD). However, phenol will be treating by applying antioxidants like activated charcoal, polyvinylpyrrolidone (PVP), ascorbic acid, citric acid and L-cystine in tissue culture media (Krishna *et al*., 2008). Phenol oxidation enzymes could be influenced by environmental factors such as light and high temperature raise browning rate by increasing the enzyme activity (Dobránszki and Teixeira, 2010).

A study was carried out to control contamination and phenol of cultures during the mass propagation of *Zingiber officinale* using sprout bud of explants fortified with various concentrations of plant growth regulators.

**Materials and Methods**
**Source of plant materials and explants**
National released elite cultivars of ginger were collected from Teppi National Spice Agricultural Research Centre (TNSARC). The seed rhizomes is heat treated by $50^{o}C$ for 30 min in water bath and sowing in sterilised sand to maintain humidity in poly bags under lat house with 70% shade. Sprout bud 1.5 cm length samples for establishment of explants were obtained after four weeks of planting.

**Initiation media contains**
The sprout bud was cultured in Murashige and Skoog (MS) medium fortified with 30g/1sucrose, 7 g/1 agar and 1mg/1 $N^6$-benzyladenine (BA), 0.05mg/l gibberellic acid $GA_3$ with 0.1mg/1 naphthalene acetic acid (NAA). The pH of medium had been adjusted to 5.7 prior to autoclaving at 121°C for 20 min. Therefore, Cultures were incubated in the dark for the first two weeks of establishment at 25 ± 2°C followed by 16/8 h photoperiod conditions and the light intensity of 40 µmol $m^{-2}s^{-1}$ provided by cool white fluorescent lamps.

**Pre-treatment for eliminating contamination and phenol**
The sprout buds were washed under running tap water and then given two types of pre-treatments. Pre-treatment 1 was contained 3% (w/v) copper sulphate ($CuSo_4$), 0.1% (w/v) PVP, 0.1% (w/v) ascorbic acid and 0.1% (w/v) citric acid. Pre-treatment 2, was the same as pre-treatment 1, but with addition of 0.1% (w/v) streptomycin. In both pre-treatments, explants were immersed for initial period 3, 6, 9 or 12 h and all treatments had been agitated on gyratory shaker (150 rpm). The explants were washed with sterile distilled water, followed by 70% ethanol for 1 min and 1.5% (V/V) sodium hypochlorite and three drop of Tween 20 for 15 min. Finally, the explants had been washed with sterile distilled water for three times and cultured on basal media. This experiment consisted of four treatments arranged in factorial based on completely randomized design with each treatment containing ten flasks. Each experiment was conducted in three replications. Contamination, phenol and survival data had been collected after four weeks of culture.

**Eliminating contamination**
Samples were exposed to one of four treatments (I, II, III and IV) and cultured (Table 1). This experiment was consisted of four treatments conducted in completely randomized design. Each treatment in this experiment consisted of 10 test tubes, where each test tube contained one explant and each experiment was repeated three times. Contamination data were collected after six weeks. Explants with no visible contaminant growth after four weeks on culture medium were placed into individual flasks with the same constituents for an additional two weeks to determine any undetected contaminants before sub culturing.

**Data analysis**
Data were analyzed using the analysis of variance procedure in the SAS statistical software (version 9.2) and the Duncan's new multiple range test (DNMRT) was used for comparison among treatment means. For all the data analysis, probability level of less than 5% ($P < 0.05$) was considered for statistical significance.

**Results**
**Pre-treatment for eliminating contamination and phenol**
Analysis of variance revealed that factor pre-treatment had a significant effect on all recorded parameters (Table 2). Result also indicated that factor period of time was highly significant on score of browning and percentage of survival for sprout bud explants at 6 hour. However, it was not significant for aseptic explants (Table 3). The interaction of pre-treatment x period of time was significant on score of percentage of aseptic explants and percentage of survival sprout buds. However, it was not significant difference score for browning (Table 4). Mean comparison among two pre-treatments showed that pretreatment 1 with 3% (w/v) copper sulphate ($CuSo_4$), PVP, ascorbic acid and citric acid was more effective than another pre-treatment 2 for sprout bud explants. Nevertheless, using whole pre-treatments browning (phenolics) were highly significant difference mean than aseptic and survival percentage (Table 3). It was also observed that pretreatment for six hrs was the best period for both aseptic, browning and survival explants (Table 4). All periods of pre-treatments were not effective for elimination contamination. However, contaminants were reduced to 33.3% of aseptic culture using 3% (w/v) copper sulphate ($CuSo_4$), PVP, ascorbic acid and citric acid as pretreatment for 6 hrs. Nevertheless, browning was reduced effectively and explants survival rate was increased to 93.3% using this pre-treatment (Table 4). The sprout bud explants that survived were greenish in color, while those that did not survive turned to browning in color. Results also indicated that pre-treatments at all periods were unable to effectively reduce contamination in bud cultures, but using 3% (w/v) copper sulphate ($CuSo_4$), PVP, ascorbic acid and citric acid as pre-treatment 1 for 6 hrs was the best for reducing contamination (33.3% clean) and browning (1.47), with a high survival rate (93.3%) (Table 4). The surviving sprout bud explants were observed to be yellowish in color, whereas non-

surviving explants turned to brown and black in color. Based on these results pre-treatments with or without streptomycin for 6 hrs were used in the subsequent experiment with contamination, phenolics and survival bud explants respectively.

**Eliminating contamination**

Treatment I resulted in 54.2% aseptic and 78.7% survival on sprout bud cultures, respectively, whereas treatment II resulted in 83.3% aseptic and 49.2% survival bud explants, respectively, where 49.2% of aseptic bud explants were necrotic. Utilizing streptomycin at both concentrations 100 and 200 mg/l was not as effective as using copper sulphate for microbial elimination in both explants. However, necrosis in sprout bud explants was also observed in higher rates when streptomycin was used (Table 5).

Table 1: Treatments were used to sterilize sprout bud explants

| Treatments | Protocol |
|---|---|
| I | Cutting explants to 1 cm and surface sterilizing with 1.5% (V/V) sodium hypochlorite for 10 min and rinsing three times with sterile distilled water. |
| II | Cutting explants to 1 cm, dipping into 1.5% (V/V) sodium hypochlorite for 10 min, rinsing three times with sterile distilled water, and culturing onto medium containing 100mg/l copper sulphate ($CuSo_4$). |
| III | Cutting explants to 1 cm, dipping into 1.5% (V/V) sodium hypochlorite for 10 min, rinsing three times with sterile distilled water, and culturing onto medium containing 100mg/l streptomycin. |
| IV | Cutting explants to 1 cm, dipping into 1.5% (V/V) sodium hypochlorite for 10 min, rinsing with sterile distilled water, and culturing onto medium containing 200 mg/l streptomycin. |

**Table 2.** Effect of pre-treatments on percentage of aseptic explants, browning scores and percentage of survival explants were examined after 30 days of culture.

| | Mean for sprout buds | | |
|---|---|---|---|
| **Pre-treatment** | **Aseptic explants (%)** | **Browning score** | **Survival (%)** |
| P1 | $24.9^{a}$ | $1.12^{a}$ | $93.3^{a}$ |
| P2 | $11.5^{b}$ | $1.10^{b}$ | $85.7^{b}$ |
| F-test | * | ** | * |

*, Significant at $p < 0.05$; **, significant at $p < 0.01$. Means within columns followed by the same letters are not significantly different at $p < 0.05$(DNMRT).

**Table 3.** Effect of different period of time on percentage of aseptic explants, browning scores and percentage of survival explants were examined after 30 days of culture.

| | Mean for sprout buds | | |
|---|---|---|---|
| **Period (h)** | **Aseptic explants (%)** | **Browning score** | **Survival (%)** |
| 3 | $16.55^{a}$ | $0.80^{b}$ | $80.7^{b}$ |
| 6 | $20.00^{a}$ | $1.28^{a}$ | $93.3^{a}$ |
| 9 | $19.99^{a}$ | $1.23^{a}$ | $66.9^{bc}$ |
| 12 | $13.33^{a}$ | $0.80^{b}$ | $53.6^{c}$ |
| F-test | ns | ** | ** |

ns, Non-significant; **, significant at $p < 0.01$. Means within columns followed by the same letters are not significantly different at $p < 0.05$(DNMRT).

**Table 4.** Effect of pre-treatments in different period of time on percentage of aseptic explants, browning scores and percentage survival of explants were examined after 30 days of culture.

| Treatment | Mean for sprout buds | | |
|---|---|---|---|
| | Aseptic explants (%) (± S.E.) | Browning score (± S.E.) | Survival (%) (± S.E.) |
| P1T1 | $13.3 \pm 6.67^{bcd}$ | $0.93 \pm 0.067^{bcd}$ | $93.3 \pm 6.67^{a}$ |
| P1T2 | $33.3 \pm 6.67^{a}$ | $1.47 \pm 1.13^{a}$ | $93.3 \pm 6.67^{a}$ |
| P1T3 | $26.7 \pm 6.67^{ab}$ | $1.20 \pm 0.20^{abc}$ | $66.7 \pm 6.67^{ab}$ |
| P1T4 | $26.7 \pm 6.67^{ab}$ | $0.87 \pm 0.24^{bcd}$ | $66.7 \pm 13.33^{ab}$ |
| P2T1 | $20.0 \pm 0.00^{abc}$ | $0.67 \pm 0.18^{d}$ | $66.7 \pm 17.64^{ab}$ |
| P2T2 | $13.3 \pm 6.67^{bcd}$ | $1.13 \pm 0.67^{abcd}$ | $86.7 \pm 6.67^{a}$ |
| P2T3 | $6.7 \pm 6.67^{cd}$ | $1.27 \pm 0.18^{ab}$ | $66.7 \pm 6.67^{ab}$ |
| P2T4 | $0.0 \pm 0.00^{d}$ | $0.73 \pm 0.67^{cd}$ | $40.0 \pm 0.00^{b}$ |
| F-test | ** | ns | * |

ns, Non-significant; *, significant at $p < 0.05$; **, significant at $p < 0.01$. Means within columns followed by the same letters are not significantly different at $p<0.05$ (DNMRT). P1, pre-treatment with copper sulphate ($CuSo_4$), PVP, ascorbic acid and citric acid; P2, pre- treatment with copper sulphate ($CuSo_4$),streptomycin, PVP, ascorbic acid and citric acid; T1, T2, T3 and T4, pre-treatment in 3, 6, 9 and 12 h respectively.

**Table 5.** Effect of sterilization method on percentage of aseptic explants and percentage of survival were examined.

| Treatment | Mean for sprout buds | |
|---|---|---|
| | Aseptic explants (%) (± S.E.) | Survival (%) (± S.E.) |
| Control | $54.7 \pm 4.17^{c}$ | $76.7 \pm 1.67^{a}$ |
| $CuSo_4$ (100 $mg^{-1}$) | $83.3 \pm 4.17^{a}$ | $49.2 \pm 7.94^{b}$ |
| Streptomycin (100 $mg^{-1}$) | $75.0 \pm 0.00^{ab}$ | $46.7 \pm 3.33^{b}$ |
| Streptomycin (200 $mg^{-1}$) | $62.5 \pm 7.22^{bc}$ | $22.2 \pm 5.55^{c}$ |
| F-test | ** | * |

*, Significant at $p < 0.05$; **, significant at $p < 0.01$. Means within columns followed by the same letters are not significantly different at $p < 0.05$ (DNMRT).

**Discussion**

The use of field grown plants as a direct source of explants for the establishment of aseptic *in vitro* cultures is generally considered a major setback, especially with sprout bud close to the ground (Webster *et al*., 2003). The higher percentage of contamination in sprout bud cultures was due to nearer position of sprout bud to the ground anchored. In this study, contamination was successfully reduced in sprout bud cultures using the modified sterilization protocol. However, browning became more easily necrotic and rejuvenation of explants from aseptic bud was more difficult when compared to survival bud cultures. It appears therefore that aseptic culture at 6 hrs was more suitable for *Zingiber officinale* micro propagation. Microbial contaminants could be affected by rainfall by enhancing humidity. The sprout bud phylotaxy of *Zingiber officinale* overlap one another which causes rain water to collect between sprout bud stalks, making it a suitable place for pathogen growth. This mechanism of pathogen contamination was minimized as the collected plants were cultured in lat house under rain shelter for at least two months to restrict microbial development. A postulate for successful tissue culture is the establishment of a sterilization technique. A common method of sterilizing explants is by using alcohol and sodium hypo chloride. However, optimizing the technique or even opting for another technique is often executed for contaminant free explants when explants are prone to high contamination. The presence of both gram positive and negative bacteria emphasizes the need for the use of copper sulphate and streptomycin which have been known to restrict both gram positive and negative bacteria, while the presence of fungi requires the use of copper sulphate, a fungicide. A serious limitation in *Zingiber officinale* micro-propagation is oxidation of polyphenols exuded from cut surfaces of explants and their release into the culture media causing browning and necrosis of explants. Browning has been described as enzymatic oxidation of phenolic substances by PPO (Chitbanchong *et al*., 2009). PPO is mainly in the vacuoles, while the enzyme is localized in plastids or chloroplasts. They do not come in contact with each other, but during excision cells are injured, and the browning reaction is initiated (Murata *et al*., 1997). Besides PPO, phenylalanine ammonia lyase (PAL) and peroxidase (POD) are also responsible for browning arising from wound as a catalyser of polyphenol biosynthesis (Krishna *et al*., 2008). Sprout bud which are considered as etiolated tissue, since they are raised from rhizomes of ginger, should show less browning than rhizomes. Wounded surface in Sprout buds emit higher exudates than rhizomes stem, as the cut surface of explants exude polyphenols that are easily oxidized

 

and cause explants necrosis and medium darkening (Abdelwahd *et al*., 2008). The results showed that pre-treatment with copper sulphate, ascorbic acid, citric acid and PVP for 6 hrs reduced 33.3% of aseptic bud microbial contamination in sprout bud. Although these were not efficient methods for contaminants elimination, it was observed that microbial contaminants were reduced in these treatments. The browning decreased significantly in both pre-treatments explants at 6 hrs. Pretreatment of explants for 3 hrs did not effectively reduce browning and contamination. Augmenting the pretreatment for more than 6 hrs did minimize browning, but when explants were stirred on an orbital shaker for more than 6 hrs the occurrence of necrosis was manifested. It could be summarized that pre-treatment with antioxidants for six hours minimized browning effectively, even with the high polyphenols exudation from wounded explants surfaces. In spite of the synergistic effect of PPO and POD in browning ( Krishna *et al*., 2008), PPO, POD and PAL play a vital function in plant defense against bacteria, fungi and other pathogens ( Houssien *et al*., 2010). Another interesting observation made in this experiment was significantly reduced contaminants level on survival percentage 1 and 2 for 6 hrs in sprout bud culture respectively. It can be assumed that since these pre-treatments were able to reduce phenol leaching efficiently in sprout bud and it thereby, helped maintenance of sufficiently high phenolics level in explants system to restrain the growth of pathogens (Krishna *et al*., 2008). Of the four protocols using three anti-microbial agents, 100mg/l copper sulphate ($CuSo_4$) proved to be more effective in reducing contamination in cultures in both explants. However, some contaminations were still observed after sub culturing on new medium after 30 days. This is attributed to latent pathogen effect (Rajmohan *et al*., 2010) using 100mg/l copper sulphate ($CuSo_4$) at a concentration for 10 minute resulted in minimal phytotoxicity to sprout bud explants and presented acceptable control of contamination. However, copper sulphate was phytotoxic to sprout bud explants the concentration increased. High losses have been reported in *in vitro* cultures due to bacterial contaminants that multiply and affect explants growth (Leifert *et al*., 1992). Streptomycin at 100 mg/ l was most effective against contaminating bacteria, along with the formation of the maximum number of ginger shoots, and had the least phytotoxicity. Streptomycin has more active against both Gram-positive and Gram-negative bacteria than 'first-' and 'second-generation' cephalosporins (Mbah *et al*., 2012). Reduction in shoot growth and decreased rates of shoot multiplication and survivals were recorded in cultures grown on medium after the addition of 200 mg/ l of either antibiotic. The role of antibiotics to eliminate bacterial contamination in micro propagated cultures of various crops was evaluated by (Fang *et al*., 2012). Our results agree with the observations of Leifert *et al*. (1991), who stated that high concentrations of antibiotics have been found to be phytotoxic to *in vitro* explants, and resulted in the death of almost all treated cultures.

**Conclusion**

Contamination by microbes has been a persistent problem for *in vitro* propagation of *Zingiber officinale.* Another serious constraint of micro-propagation of this plant is the presence of secondary metabolites which are oxidized after wounding and cause subsequent browning and necrosis of explants. It was easier to eliminate contamination and browning from sprout bud, but sprout buds were also easily damaged by antibiotics and at the concentration increased in sterilization procedures. Pre-treating copper sulphate with copper sulphate, PVP, ascorbic acid and citric acid (0.1%) for 6 hrs followed by dipping in ethanol (70%), surface sterilizing with, 1.5% (V/V) sodium hypochlorite for disinfecting with 100mg/l copper sulphate for 10 min, cutting explants into 1 cm, and transferring into for 1.5% (V/V) sodium hypochlorite 10 min was the best technique for reducing browning and contamination in sprout bud explants. However, browning became more easily necrotic and rejuvenation of explants from aseptic bud was more difficult when compared to survival bud cultures. This protocol could also be an efficient procedure to eliminate contamination and browning in monocot plants with higher potential for microbial contamination due to sprout apices being close to the ground.

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