academicJournals

Vol. 14(8), pp. 657-660, 25 February, 2015
DOI: 10.5897/AJB2014.14266
Article Number: 3ED5BBC50686
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Optimization of explants surface sterilization condition for field grown peach (*Prunus persica* L. Batsch. Cv. Garnem) intended for *in vitro* culture

Wegayehu Felek[1]*, Firew Mekibib[2] and Belayneh Admassu[3]

[1]Debre Berhan University, College of Agriculture and Natural Resource Science, Debre Berhan, Ethiopia.
[2]Haramaya University, Collecge of Agriculture and Environmental Science, Dire Dawa, Ethiopia.
[3]National Agricultural Biotechnology Laboratory, Holeta Agricultural Research Center, Holeta, Ethiopia.

Received 22 October, 2014; Accepted 16 February, 2015

The aim of this work was to sterilize nodal explants, so as to mitigate microbial contamination in peach micropropagation. The nodal explants were treated with three concentrations levels (0.15, 0.25 and 0.5% (w/v) active ingredient of chlorine) of locally produced bleach, sodium hypochlorite (NaOCl) for varying exposure times (10, 15 and 20 min). These treatments were carried out in randomized complete design with three replications. Data were recorded on the number of contaminated, dead and survived (clean) cultures after 10 days of culturing. The highest explant contamination (100%) and least explant survival (0%) were recorded when explants were treated with 0.15% active chlorinated local bleach for 10 min. The least culture contamination and minimum tissue death of 9.51 and 4.75%, respectively, and the highest culture survival (85.71%) were recorded when explants were disinfected with 0.25% active chlorinated local bleach for 15 min.

Key words: Explants, local bleach, micropropagation, *Prunus persica,* surface sterilization.

INTRODUCTION

Peach (*Prunus persica* (L). Batsch) belongs to the *Prunoideae,* a sub family of *Rosaceae,* with eight basic and 16 somatic chromosome numbers (2n = 16) (Hesse, 1975). *Prunus* include several species approximately 430 adapted primarily to the temperate regions of the northern hemisphere (http://en.wikipedia.org/wiki/Prunus). China is the native home for peach and was domesticated there 4000-5000 years ago (Aranzana et al., 2010). Peach is grown for its edible fruit consumed as fresh or processed. The total world production of peaches during the year 2012 was 18.1 million tons (Bruke and Change, 2013). The top producer of peaches is typically China, followed by the European Union (EU) and the United States (Daniel et al., 2007). Introduction of temperate fruits especially peach to Ethiopia and North Africa was in sixteenth and seventeenth centuries (Scorza and Sherman, 1996). Because of its early introduction, peach is relatively well established in many highland areas and is introduced much earlier than apple and plum. Recently introduced (2011), Garnem is one of the peach rootstock cultivar introduced from Spain to the country. The production

*Corresponding author. E-mail: dear.nayadi@gmail.com.

Table 1. The treatment combination for sterilization experiment.

Treatment code	NaOCl (Cl %)	Time (min)
ST1	0.15	10
ST2	0.15	15
ST3	0.15	20
ST4	0.25	10
ST5	0.25	15
ST6	0.25	20
ST7	0.5	10
ST8	0.5	15
ST9	0.5	20

potential of peach (McRed is 46 ton/ha) exceeds other temperate fruits production potential, like apple and plum (Endale and Kerssa, 2006).

Recently, breeding practices in *Prunus* have been advanced by the development and application of micropropagation (Martinez-Gomez et al., 2005). Micro-propagation offers the possibility of scale multiplication planting material. Explants surface sterilization is one of the critical steps in micropropagation of plants.

Microbial contamination is one of the most serious problems in micropropagation. Contamination with microorganisms is considered to be the simple most important reason for losses during *in vitro* culture of plants. Such microorganisms include viruses, bacteria, yeast, fungi, etc (Omamor et al., 2007). These microbes compete adversely with plant tissue cultures for nutrients. The presence of these microbes usually result in increased culture mortality but can also result in variable growth, tissue necrosis, reduced shoot proliferation and reduced rooting (Kane, 2003). Determination of effective explant surface sterilization procedure is essential to avoid the problem of contamination during *in vitro* culture. No single sterilization procedure would do for all the species. Even for the same species or the same variety, a single formula may not work at different time. This is due to the fact that load and type of microorganism on explants is dependent on seasons (George and Sherrington, 1984).

Disinfectants such as ethanol, NaOCl, and Tween 20 hamper the growth rate of fungi and bacteria on the growth media (Odutayo et al., 2007). Hypochlorite is known to be a very effective killer of bacteria; even micromolar concentrations are enough to reduce bacterial populations significantly. However, little is known about the exact mechanisms of its bacteriocidal activity. When diluted in water, the hypochlorite salts (NaOCl, $Ca(OCl)_2$) lead to the formation of HOCl whose concentration is correlated with bactericidal activity (Nakagarwara et al., 1998). Sodium hypochlorite is readily available and can be diluted to proper concentrations. A balance between concentration and time must be determined empirically for each type of explants because of phytotoxicity. Therefore, the aim of this study was to assess the effective peach explant-surface sterilization.

MATERIALS AND METHODS

The study was conducted at the Plant Tissue Culture Laboratory, National Agricultural Biotechnology Laboratory, Holeta Agricultural Research Center from October 2013 to January 2014.

Source and choice of plant materials

Young and healthy shoots (4-6 cm long), containing axillary buds (third, fourth and fifth nodes; from shoot apex), were excised and collected from three years old field grown Garnem fruit crop by cutting with sterile scissor and used as explant. Actively growing shoots (juvenile plants) were taken as they are more responsive to shoot regeneration and proliferation than shoot explants from adult forms as reported by (Naghmouchi et al., 2008).

Explant surface sterilization

Before explants were placed on a medium (inoculated), it must be sterilized to make them free of all microorganisms. The leaves were removed from the explants and soaked in tap water and brought to laboratory. The explants were then thoroughly washed with tap water 3-5 times followed by liquid soap for 30 min with agitation to physically remove most microorganisms. Then the explants were treated with 70% ethanol for 30 s under laminar air flow cabinet. After pretreatment with ethanol, the explants were rinsed with distilled water three times, to lower the toxic effect of ethanol. They were then treated with three concentration levels (0.15, 0.25 and 0.5% (w/v) active ingredient of chlorine) of locally produced bleach, sodium hypochlorite (NaOCl) with 5% active ingredient of chlorine for varying exposure times (10, 15 and 20 min) (Table 1). To increase the efficiency of NaOCl, a drop of Tween-20 per 50 ml solution was added as wetting agent. After decanting the sterilizing solutions under safe condition, the explants were washed three times each for 5 min with sterile distilled water to remove traces of NaOCl.

Both ends of the sterilized explants were trimmed under aseptic condition to provide a newly cut surface and to remove any cells damaged by sterilant. Then, the dorsal portion (1 cm long) nodal explants containing a single node were trimmed in a 'V- shape', to expose the xylem and increase the surface area of absorption, under aseptic conditions and cultured on test tubes, containing 10 ml of MS (Murashige and Skoog, 1962) medium fortified with 1 mgL^{-1} 6-benzylaminopurine (BAP), 3% sucrose, and 0.4% agar (agar agar, Type 1). The test tubes with cultured explants were properly sealed with parafilm and labeled. After wards, the cultures were transferred and randomly placed on the growth room shelf with a photoperiod of 16/8h light/dark using cool-white fluorescent lamps (photon flux density, 40 µmol m^{-2} s^{-1} irradiance) at 25 ± 2°C and relative humidity (RH) of 70 to 80%. For each sterilization treatments seven test tubes were line up randomly in completely randomized design (CRD) with three replications. The sterilization experiments data recorded include the number of contaminated, dead and survived (clean) cultures after 10 days of culturing. The data were converted into percentage.

Data collection and Statistical analysis

The sterilization experiments data recorded include the number of contaminated, dead and survived (clean)

Table 2. ANOVA for the effect of local bleach and exposure time on contamination, death and survival of explants.

Source of variation	DF	Mean square		
		Contamination (%)	Mortality (%)	Survival (%)
NaOCl	2	14582.01***	4450.61***	6265.76***
Time	2	1519.09***	1868.03***	211.47***
NaOCl * Time	4	283.62***	643.16***	1742.29***
Error	18	15.06	7.53	7.55
R^2 (%)		0.99	0.99	0.99
CV (%)		12.87	15.28	5.28

***Highly significant (P≤ 0.0001) at α=0.05 significant level; R^2 = coefficient of determination; CV = coefficient of variation; DF, degree of freedom.

Table 3. Effect of different concentrations of local bleach and length of exposure time on contamination, mortality and survival of explants.

NaOCl (local bleach) level (%)	Exposure time (min)	Contamination (%)	Mortality (%)	Clean survived explant (%)
0.15	10	100[a]	0	0
0.15	15	71.43[b]	0	28.57[f]
0.15	20	57.14[c]	0	42.86[e]
0.25	10	28.57[d]	0	71.43[c]
0.25	15	9.51[e]	4.75[e]	85.71[a]
0.25	20	0	28.57[c]	71.43[c]
0.5	10	4.75[ef]	14.29[d]	80.95[b]
0.5	15	0	42.86[b]	57.14[d]
0.5	20	0	71.43[a]	28.57[f]
CV (%)		12.87	15.28	5.3

Means in column with the same letter are not significantly different by DMR test at α=0.05 significant level.

cultures. The data were converted into percentage. The data were subjected to two-way analysis of variance (ANOVA) by SAS computer software (version 9.1). Significant difference between means were assessed by Duncan's multiple range test (DMRT) (*P = 0.05*) (Gomez and Gomez, 1984).

RESULTS AND DISCUSSION

Analysis of variance (ANOVA) (Table 2) revealed that concentration of local bleach and exposure time, and the interaction effect had highly significant difference (P≤ 0.0001) on the contamination of growth media, death and survival level of explants.

The data (Table 3) revealed that as the exposure time increased from 10 to 20 min for all levels of local bleach, the contamination decreased, and the same was true when concentration of local bleach increased from 0.15 to 0.5% (w/v) for all levels of exposure time.

The highest explant contamination (100%) and least explant survival (0%) were recorded when explants were treated with 0.15% active chlorinated local bleach for 10 min. This might be due to the insufficiency of the concentration of active chlorine in local bleach and short exposure time to take life of microorganisms from cultured explants. The least culture contamination and minimum tissue death of 9.51% and 4.75%, respectively, and the highest culture survival (85.71%) were recorded when explants were disinfected with 0.25% active chlorinated local bleach for 15 min. These findings agree with the result of Ahmad et al. (2003): the application of NaOCl at 0.25% (w/v) for 10 min gave minimum death (5%) and maximum survival (55%).

Surface sterilization with higher concentration (0.5% w/v) of local bleach at and beyond 15 min resulted in no contamination but high rate (71.43%) of explants mortality. This could be due to phytotoxic effect of 0.5% chlorinated local bleach at longer exposure time. Ervin and Wetzel (2002) had also noticed that high concentration of sterilant causing plant tissue death. Surface sterilization should not kill or break off the biological activity of explants, but the contaminants. Explants must be surface sterilized only by treatment with

disinfectant solution at suitable concentrations for a specified period (Oyebanji et al., 2009). Therefore, 0.25% (w/v) local bleach for 15 min exposure time was found to be the most effective one for explants taken from peach shoots.

Conflict of interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

We are indebted to Haramaya University and Holeta Agricultural Research Center, National Agricultural Biotechnology Laboratory for funding and facilitating the working environment, respectively.

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