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IN VITRO EFFECT OF VARIOUS STERILIZATION TECHNIQUES ON PEACH (*Prunus persica* (L.) Batsch) EXPLANTS

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Abstract

One of the major challenges in Romania in fruit growing sector is the production of certified planting material, considering the specificities of the local climate conditions and the consumers' preferences. Due to the damages produced by hard winters and late spring frosts and the lack of resistant and suitable peach varieties, in the last decades, this species was almost eliminated from the producers choices in establishing new orchards. One of the major research projects of the Faculty of Horticulture within the University of Agronomic Sciences and Veterinary Medicine of Bucharest is to identify and multiply the best peach varieties (Prunus persica L. Batsch) adapted to the Romanian harsh conditions. This paper presents different sterilization techniques applied to peach explants necessary for the initiation of the in vitro culture. The research was conducted at the Micropropagation Laboratory within the Faculty of Horticulture. For peach tissue culture initiation, four sterilization agents where tested in 18 different variants: Sodium hypochlorite (NaOCl) in three concentrations: 5%, 10% and 15%, for 5 and 10 min; Hydrogen peroxide (H_2O_2), in two concentrations: 5% and 10%, for 10 and 20 min; Captan (50%) fungicide, in four concentrations: 1%, 2%, 3% and 4%, for 5 min and Boric acid ($B(OH)_3$), in two concentrations: 1% and 2%, for 5 and 10 min. The explants (shoots-tip and nodes) were cultured in MS (Murashige and Skoog, 1962) basal medium supplemented with 30g sucrose, as carbon source and 7g agar. The growth chamber for the in vitro cultures had 22±2°C temperature and 70 to 80% relative humidity, with a photoperiod of 16 h day light and 8 h dark. The percentage of contamination, survival rate and shoot growth were studied during the initiation phase. Among the different sterilization variants, sodium hypochlorite was the most effective treatment with 50% survival rate at V5 (15% for 5 min) and 60% at V4 (10% for 10 min). After sterilization, shoots continued to grow vigorously and the multiplication phase initiated.

Key words*: shoot-tip, node, sodium hypochlorite, hydrogen peroxide, boric acid.*

INTRODUCTION

Peach (*Prunus persica* L. Batsch) is one of the most important stone fruits which are grown extensively in different parts of the world. Peaches are native to China and their planting dates refer to at least 4000 years (Wang and Zhuang, 2001). Peach trees are infected by numerous different pests and diseases. Micropropagation which encompasses cell culture, tissue culture, organ and embryo culture, has been a vital technique for considerable multiplication of plants, elimination of plant diseases through tissue culture technique, plant conservation and improvement production through gene transfer (Sarasan et al., 2011).

Tissue culture techniques are used for commercial and research purposes extensively to grow many different plants (Hussain et al, 2012). Aseptic conditions are usually practiced in micropropagation, plant tissues on their surfaces inherently have various bacteria and fungi, moulds etc. It is necessary that the explants be free from any surface contaminants prior to tissue culture since contaminants can grow in the culture medium, rendering the culture non activity (Hiremath, 2006). There is so much pathogen (microbial contaminants) which has been a major threat to tissue cultures due to their rapid proliferation characteristics, microbes can be come from explants, laboratory instruments, conditions in the laboratory and contaminants may be introduced with the staff during manipulations in the laboratory (Leifert and Cassells, 2001; Enjalric et al., 1998).

Contamination is a real problem that opposes the progress and development of tissue culture technology (Webster et al., 2003). These microbes compete adversely with explants for nutrients, and their presence often results in

variable growth or increased culture mortality or can also result, reduced shoot proliferation, tissue necrosis and reduced rooting (Oyebanji et al., 2009). A successful *in vitro* culture protocol, starts with effective explants sterilization, the sterilization chosen for an experiment depend on the type of explants, Sterilize material and plant genotype (Dodds and Roberts, 1985; Rezadost et al., 2013). There are several various sterilization factors are used to sterilize tissues, these disinfect materials are also toxic to explants tissues, and therefore select the correct concentration and the times of exposure to explants, must be elected to reduce the injury of plants (CPRI, 1992).

So there is a state of balance between sterilizing explants and killing the explants themselves (Qin et al., 2012; Olew et al., 2014). Many researchers have used these sterilizing agents successfully; also there are studies on the effect of fungicides and antibiotics on these kinds of contaminants (George, 1993; Rashid et al., 2008; Maqbool et al., 2010; Bakhsh et al., 2012). Several different mechanism are used to eliminate fungal and bacterial contamination, including the use of inactivation by heat and light, fungicides and antibiotics, the time of sterilization is dependent on the type of tissue (Haldeman et al., 1987; Kneifel and Leonhardt, 1992; Leifert et al., 1992).

Explants are commonly surface-sterilized using ethanol, sodium hypochlorite, mercuric chloride, hydrogen peroxide, fungicides and antibiotics. Therefore, the present study was conducted to compare different sterilizing protocols for peach micropropagation and to find out the best, efficient and cost effective sterilization procedure that may result in least or no contamination in peach tissue culture. In our study we compared several modifications of four surface sterilization methods based on the use of, sodium hypochlorite, hydrogen peroxide, captan (50%) and boric acid with using explants of peach accessions with different degrees of contamination.

However, in this study we did not used the mercuric chloride, this material is very dangerous because of more difficult to dispose and high toxicity (Li et al., 2005; Jean-Philippe et al., 2012). Sodium hypochlorite (bleach) is the most common sterilization agent used for seed and explants sterilization in many plants. Hypochlorite was used to surface sterilize wheat seeds (Sauer and Burroughs, 1986).

Also sodium hypochlorite has been reported to be very effective factors different types of bacterial strains (Nakagawara et al., 1998). Hydrogen peroxide solution as sterilizing agent has been reported for plants (Dumroese et al., 1988; Ogawa and Masaki, 2001; Rosner et al., 2003). It is recommended to use in the initial sterilization as ethanol (Stănică et al., 2002). Hydrogen peroxide solution as sterilizing factor and improved germination on many plants like wax, cotton, barley, pines, safflower and currant (Dumroese et al., 1988; Rosner et al., 2003; Cram and Fraedrich, 2009; Çavusoglu and Kabar, 2010; Lizarraga-Paulin et al., 2013). A report confirmed by Dolatabadian and Modarressanavy (2008) hydrogen peroxide is more effective than other sterilization factors for plant tissue. However, there are several studies indicates that hydrogen peroxide was ineffective for surface sterilization of explants and seeds (Miche and Balandreau, 2001). Captan 50% WP, agricultural fungicide for the control of certain fungus diseases of fruit, vegetables and ornamental crops are used in surface sterilization *in vitro*.

There are several studies like reported by Sohnle et al. (1998). Also the effectiveness of Bavistin was confirmed by Garla et al. (2011). Reported by Altan, et al. (2010), it failed to inhibit the microorganism and the activity of these sterilizing factors. Shields et al. (1984) analyzed the effects of fungicides against *in vitro* on tobacco cultures. They recommend two fungicides, carbendazim and fenbendazole. Boric acid in agriculture is used as an insecticide, herbicide and fungicide in food crops and orchards (EPA. U.S., 1993). Used in the United States as a fungicide on citrus (Olkowski et al. 1993). The results by Jan H. et al. (2002) indicate that use of boric acid (3% solution) as pre-plant seed treatment on potato was gave the lowest percent incidence of powdery scab diseases 4.3%.

MATERIALS AND METHODS

Explants surface sterilization

Shoot tips and nodes (0.5-1 cm in size) of peach (*Prunus persica* L. Batsch cv 'Florin')

were collected from the orchard of trees planted in the field of experiments to the Faculty of Horticulture, University of Agronomic Sciences and Veterinary Medicine of Bucharest to used as explants for *in vitro* culture establishment.

Explants were placed under running tap water with detergent for 30 min to remove any foreign contaminants.

After washing, explants were dissected and surface sterilized in a laminar air flow hood with rinsed with ethanol 70% for 2-3 min then were washed with distilled water three times for 2-3 min after that. For peach tissue culture initiation, four sterilization agents where tested in 18 different variants: Sodium hypochlorite (NaOCl) in three concentrations: 5%, 10% and 15%, for 5 and 10 min (Figures 3, 4); Hydrogen peroxide (H_2O_2), in two concentrations: 5% and 10%, for 10 and 20 min (Figure 1); Captan (50%) WP fungicide, in four concentrations: 1%, 2%, 3% and 4%, for 5 min (Figure 2) and Boric acid ($B(OH)_3$) in two concentrations: 1% and 2%, for 5 and 10 min. (Table 1).

Culture media and culture conditions

The explants (shoots-tips and nodes) were cultured in MS (Murashige and Skoog, 1962) basal medium supplemented with 30 g sucrose, as carbon source and 7 g agar. pH was adjusted between 5.7 and 5.8 by using either 1 N HCl or 1 N NaOH before the agar was added.

Media was then heated on a hot plate with continuous stirring using a magnetic stirrer until agar is dissolved and media put in the culture tubes. The culture tubes were covered with lids and put in trays and autoclaved. Autoclave was adjusted at a temperature of 121°C for 15 min. The growth chamber for the *in vitro* cultures had 22±2°C temperature and 80-85% relative humidity, with a photoperiod of 16 h day light and 8h dark. (Stănică et al., 2002)

Data collection

In the experiment ten replicates (one explants in one tube culture) were used for each treatment and the experiment was repeated twice.

Results were taken after 2, 4 weeks of planting and the following data were recorded; % of contamination (fungus+ bacteria and sterilizer); % of explants survived; mean length of shoots (cm); mean number of leaves per explant. Experiments were conducted as factorial experiments based on Completely Randomized Design (CRD).

RESULTS AND DISCUSSIONS

Effect of sterilization factors

The study showed there is an effect of substances used in sterilization, sodium hypochlorite the most effective treatment with 50% survival rate in 15% for 5 min and 60% in 10% for 10 min and has outperformed the rest of the other sterilizers which their results were not satisfactory as the results were hydrogen peroxide (H_2O_2) with 25% survival rate in 10% for 20 min; Captan 50% with 25% survival rate in 4% for 5 min; Boric acid ($B(OH)_3$) with 20% survival rate in 2% for 10 min (Table 2).

These results are similar to the studies Satish et al. (2012) on sugarcane; Siddique et al. (2018) on *Skimmia laureola*, when they used different substances in sterilization, where the results differed according to the sterilizers.

Studied by many researchers, the solution of sodium hypochlorite for superficial sterilization of explant was efficient and didn't injury the explants at appropriate focus (Gertlowski K. and Petersen M., 1993).

These results are similar with those of Hippolyte (2000), which reference that the high focus of sodium hypochlorite can be effective in sterilizing the superficial explants cultivated *in vitro*, but it is accompanied by the death of explants.

Many researchers have found these sterilizing agents successfully (Rashid et al., 2008; Maqbool et al., 2010; Bakhsh et al., 2012).

Effect of concentration and exposure time

The study showed that there was a correlation between the dipping period and the concentration of the substance used in the sterilization on the extent of their effect on the percentage of explants survived, explants contaminated by fungi, bacteria and explants dead due to the increased concentration of the material used (Tables 2, 3 and 4).

Increasing the exposure duration and sterile concentration had reduced the contamination

rate but highest number of loss explants resulted.

The influence of sterilizing chemical ruin the shape and functions of microbe's enzymes (George et al., 2008).

But the increasing exposure duration and concentration of sterilizes above certain optimum limit cause loss of explants because of the oxidant chemical ingredient ruin the plant tissue as well (Danso et al., 2011).

These findings are similar of the negative effects of Sodium hypochloride at high concentration were observed (Colgecen et al., 2011).

And a higher concentration of hydrogen peroxide 5% was reported to negatively affect in sunflower (Dolatabadian and Modarressanavy, 2008).

Figure 1. Fungal contamination on peach explants after 14 day from sterilization V9 (H_2O_2 10% for 10 min)

Figure 2. Fungal contamination on peach explants after 14 day from sterilization V11 (captan 1% for 5 min)

Effect of explants

The study showed there are differences in the extent of the response of the explants used in rate of plant growth (shoots length and leaves number), contaminated rate and survival rate (Tables 3 and 4). Also, the shoots had been registered the lowest rate of infection and the most effect to increase the concentration of sterile material compared to the contract also explained that the shoots and nodes gave the best rate of shoots length and leaves number formed when using concentrations less (Figures 3 and 4).

These results are similar with (Rezadost et al, 2013) who confirmed that the surface sterilize used for an experiment typically depend on the explants and plant species.

Figure 3. Peach explants (shoots-tip) after 28 day from sterilization V10 (NaOCl 10% for 10 min). There are leaves damaged because of the sterilizer

Figure 4. Peach explants (nodes) after 28 day from sterilization V10 (NaOCl 10% for 10 min)

Table 1. Types of sterilizing agents used in a different concentration with varying time of sterilizings on peach explants

Variants	Pre-sterilization Substance disinfectants	Concentration %	Exposure time (min)	Surface sterilizer	Concentration %	Exposure time (min)
V1	Ethanol	70%	2-3	Sodium hypochlorite	5	5
V2	Ethanol	70%	2-3	Sodium hypochlorite	5	10
V3	Ethanol	70%	2-3	Sodium hypochlorite	10	5
V4	Ethanol	70%	2-3	Sodium hypochlorite	10	10
V5	Ethanol	70%	2-3	Sodium hypochlorite	15	5
V6	Ethanol	70%	2-3	Sodium hypochlorite	15	10
V7	Ethanol	70%	2-3	Hydrogen peroxide	5	10
V8	Ethanol	70%	2-3	Hydrogen peroxide	5	20
V9	Ethanol	70%	2-3	Hydrogen peroxide	10	10
V10	Ethanol	70%	2-3	Hydrogen peroxide	10	20
V11	Ethanol	70%	2-3	Captan 50%	1	5
V12	Ethanol	70%	2-3	Captan 50%	2	5
V13	Ethanol	70%	2-3	Captan 50%	3	5
V14	Ethanol	70%	2-3	Captan 50%	4	5
V15	Ethanol	70%	2-3	Boric acid	1	5
V16	Ethanol	70%	2-3	Boric acid	1	10
V17	Ethanol	70%	2-3	Boric acid	2	5
V18	Ethanol	70%	2-3	Boric acid	2	10

Table 2. Effect of surface sterilizer, various concentrations and time exposure on % of contamination and % of survived on explants after 14, 28 days from culture

Variants	Surface sterilizer	Concentration %	Exposure time (min)	Shoot-tips			Nodes		
				Contamination %		Survived %	Contamination %		Survived %
				after 14 days	after 28 days		after 14 days	after 28 days	
V1	Sodium hypochlorite	5	5	70	100	00	80	100	00
V2	Sodium hypochlorite	5	10	50	90	10	70	100	00
V3	Sodium hypochlorite	10	5	45	70	30	50	70	30
V4	Sodium hypochlorite	10	10	20	40	60	35	40	60
V5	Sodium hypochlorite	15	5	25	50	50	40	50	50
V6	Sodium hypochlorite	15	10	50	70	30	70	80	20
V7	Hydrogen peroxide	5	10	70	85	15	70	85	15
V8	Hydrogen peroxide	5	20	65	85	15	65	85	15
V9	Hydrogen peroxide	10	10	65	80	20	65	80	20
V10	Hydrogen peroxide	10	20	50	75	25	60	80	20
V11	Captan 50%	1	5	35	90	10	50	100	00
V12	Captan 50%	2	5	35	90	10	70	100	00
V13	Captan 50%	3	5	30	80	20	40	85	15
V14	Captan 50%	4	5	30	75	25	45	85	15
V15	Boric acid	1	5	50	100	00	70	100	00
V16	Boric acid	1	10	50	85	15	60	100	00
V17	Boric acid	2	5	40	80	20	70	90	10
V18	Boric acid	2	10	45	80	20	80	85	15

Table 3. Effect of surface sterilizer, various concentrations and time exposure on % of contamination (fungus + bacteria and sterilizers) on explants after 28 days from culture

Variants	Surface sterilizer	Concentration %	Exposure time (min)	Shoot-tips			Nodes		
				Contamination %			Contamination %		
				after 28 days	fungus+ bacteria	sterilizer	after 28 days	fungus+ bacteria	sterilizer
V1	Sodium hypochlorite	5	5	100	100	00	100	100	00
V2	Sodium hypochlorite	5	10	90	90	00	100	100	00
V3	Sodium hypochlorite	10	5	70	59.5	10.5	70	63	7
V4	Sodium hypochlorite	10	10	40	30	10	40	36	4
V5	Sodium hypochlorite	15	5	50	15	35	50	42.50	7.50
V6	Sodium hypochlorite	15	10	70	30	40	80	24	56
V7	Hydrogen peroxide	5	10	85	85	00	85	85	00
V8	Hydrogen peroxide	5	20	85	85	00	85	85	00
V9	Hydrogen peroxide	10	10	80	64	16	80	72	8
V10	Hydrogen peroxide	10	20	75	52.5	22.5	80	68	12
V11	Captan 50%	1	5	90	90	00	100	100	00
V12	Captan 50%	2	5	90	90	00	100	100	00
V13	Captan 50%	3	5	80	56.25	23.75	85	76.50	8.50
V14	Captan 50%	4	5	75	56.25	18.75	85	67.25	12.75
V15	Boric acid	1	5	100	100	00	100	100	00
V16	Boric acid	1	10	85	85	00	100	100	00
V17	Boric acid	2	5	80	60	20	90	65.50	4.50
V18	Boric acid	2	10	80	60	20	85	67.25	12.75

Table 4. Effect of surface sterilizer, various concentrations and time exposure on mean shoots length (cm) and mean (no) leaves shoot on explants after 28 days from culture

Variants	Surface sterilizer	Concentration %	Exposure time (min)	Shoot-tips			Nodes		
				Survived after 28%	Mean shoot length (cm)	Mean shoot Leaves (on)	Survived after 28%	Mean shoots length (cm)	Mean shoot Leaves (on)
V1	Sodium hypochlorite	5	5	00	0.00	0.00	00	0.00	0.00
V2	Sodium hypochlorite	5	10	10	4.64	5.70	00	3.12	7.88
V3	Sodium hypochlorite	10	5	30	3.32	4.22	30	3.12	7.10
V4	Sodium hypochlorite	10	10	60	3.17	4.05	60	2.78	5.87
V5	Sodium hypochlorite	15	5	50	3.25	3.50	50	2.55	4.65
V6	Sodium hypochlorite	15	10	30	2.64	3.12	20	2.06	4.50
V7	Hydrogen peroxide	5	10	15	4.34	4.44	10	3.45	7.33
V8	Hydrogen peroxide	5	20	15	3.89	4.21	10	3.12	7.02
V9	Hydrogen peroxide	10	10	20	3.11	4.00	20	3.00	5.16
V10	Hydrogen peroxide	10	20	25	2.64	3.66	20	2.77	5.00
V11	Captan 50%	1	5	10	4.50	5.99	00	0.00	0.00
V12	Captan 50%	2	5	10	4.22	5.78	00	3.13	7.23
V13	Captan 50%	3	5	20	3.80	4.43	15	2.98	7.11
V14	Captan 50%	4	5	25	3.62	3.68	15	2.77	6.78
V15	Boric acid	1	5	00	0.00	0.00	00	0.00	0.00
V16	Boric acid	1	10	15	4.17	4.16	00	0.00	0.00
V17	Boric acid	2	5	20	3.87	4.20	10	3.01	6.89
V18	Boric acid	2	10	20	3.45	3.80	15	2.48	6.22

CONCLUSIONS

Among the different sterilization protocols tested for the successful establishment of *in vitro* culture of peach tissue culture. Our results showed that during the sterilization were different depend on the sterilization factors, exposure time and explants type was used for micro- propagation.

It is recommended for this study to be used among the different sterilization variants, sodium hypochlorite was the most effective treatment with 50% survival rate at V5 (15% for 5 min) and 60% survival rate at V4 (10% for 10 min). Recommended also hydrogen peroxide (H_2O_2) 10% for 20 min; Captan 50% 4% for 5 min; Boric acid ($B(OH)_3$) 2% for 10 min recommended to use in the initial sterilization.

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