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PLANT MATERIAL IN SOME STONE FRUIT SPECIES ROOTSTOCKS

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Abstract

This paper present results regarding the sterilization effectiveness of plant material applied to peach and plum rootstocks explants necessary for the initiation of the in vitro culture. For peach and plum rootstocks tissue culture initiation, two pre-sterilization agents (sanitary alcohol and ethanol) and two sterilization agents (sodium hypochlorite and calcium hypochlorite) where tested in 16 different variants. The Mirobolan dwarf explants were cultured in Murashige and Skoog, 1962 basal medium and the Adaptabil explants were cultured in Querin and Lepoivre, 1977 basal macronutrients and micronutrients and Linsmaier and Skoog, 1965 vitamins. The growth chamber for the in vitro culture had 22±2˚C temperature, with a photoperiod of 16h day light and 8h dark. For Mirobolan dwarf rootstock a good survival rate of explants was obtained both in sterilization variant 15 (56.67%) and in sterilization variant 16 (63.33%).With the Adaptabil rootstock, good sterilization results were also obtained in variant 16 (70%). A good percentage of survival with a lower contamination rate was obtained also in variant 1 (69.67%). Both for Mirobolan dwarf and for Adaptabil rootstocks, the sterilizing agents from variant 16 can be used to sterilize the plant material.

Key words*: rootstock, in vitro culture, sterilization, explants, sodium hypochlorite, calcium hypochlorite.*

INTRODUCTION

The interest in the use of rootstocks grew along with the development of fruit growing as a commercial activity, imposed by the need to produce a large number of trees in specialized nurseries, from the varieties demanded by the consumer market. Vegetative rootstocks propagated on a commercial scale appeared at the beginning of the 20th century, in England, for seed species. In Romania, the interest in rootstocks necessary for the propagation of fruit tree varieties appeared with the establishment of commercial nurseries, but after 1990, both the number of research units in the field of fruit growing and nurseries decreased (Ştefan et al., 2018). The traditional propagation methods allow the clonal multiplication of varieties of interest but at relatively low propagation rates, which explains why the introduction of a new genotype into agricultural practices may take a number of years. Tissue cultures allow the rapid production of a large number of plants, even where normally the species has low multiplication rate. At the same time, the space requirement for such multiplication is considerably smaller. Therefore, *in vitro* propagation techniques provide an efficient and effective method of creating quality-planting material that is true to type in less time and space (Thorpe, 2007). Propagation by seed can generate a large amount of genetic variation so efforts have been made to propagate plum and peach rootstocks *in vitro*. There are several rootstocks for peach, like Nemaguard, which imparts excellent scion vigour and productivity (Handoo et al., 2004), but is sensitive to fungal root rots, iron chlorosis and root waterlogging (Nyczepir et al., 1983; Zehr et al., 1976). The seedlings of Nemared rootstock have few lateral branches, but it is highly susceptible to bacterial crakers (Reighard et al., 2008). Other rootstocks are Guardian, Lovell and Halford, Bailey, Flordaguard, GF-677, which are sensitive to root waterlogging (Loreti et al., 2006). Adaptabil is a interspecific hybrid (Prunus besseyi x open pollination) obtained at

RIFG Maracineni - Pitesti that is recommended as rootstock for peach, nectarine and some plum and apricot variety (Duţu et al., 2002; Duţu et al., 2004; Mazilu et al., 2008). Also there are several rootstocks for plum, such as Brompton (from England), BNKR, Mirobolan C5, Saint Julien A and Pixy. Mirobolan Dwarf it was obtained at RIFG Maracineni - Pitesti and is characterized by low vigour (Duţu et al., 2002). Microbial contamination is one of the most serious problems in micropropagation (Leifert and Cassells, 2001). Successful tissue culture of all plant species depends on the removal of exogenous and endogenous contaminating microorganisms (Constantine, 1986; Buckley and Reed, 1994). The disinfectants usually used are sodium hypochlorite, calcium hypochlorite, ethanol, mercuric chloride, hydrogen peroxide and silver nitrate. Since these sterilization agents are toxic to the plant tissue, contaminants must be removed without killing the plant cells (Pranjić, 2013; Olew et al., 2014). Hypochlorite is known to be a very effective killer of bacteria. When diluted in water, the hypochlorite salts (NaOCl and $CaOCl_2$) lead to the formation of HOCl whose concentration is correlated with bactericidal activity (Nakagarwara et al., 1998). Determination of an effective explant sterilization procedure is essential to avoid contamination during in vitro culture, therefore, the objective of this work was to establish a protocol that is efficient and cost effective and has the highest grade of explants survival.

MATERIALS AND METHODS

Healthy and vigorous shoots of approximately 20-25 cm long, containing 10-15 axillary buds from the mother plantation of RIFG were collected for Mirobolan dwarf and Adaptabil rootstocks. The leaves were cut leaving 0.5 cm of the petiole and were washed under tap water. The shoots were cut into pieces of 1.5-2 cm each including an axillary bud, half of the explants was washed with tap water and the other half was washed with tap water and surfactant Tween 20 for 30 minutes to physically remove most of the microorganisms. Two pre-sterilization agents: sanitary alcohol and ethanol for 10 minutes and two sterilization agents: sodium hypochlorite - commercial bleach Ace with 5.25% active chlorine ingredient and calcium hypochlorite with 6% active chlorine were tested in 16 different variants. Two sterilization times were used: 10 and 20 minutes, and then the biological material was rinsed with distilled water, three times (Table 1).

Cultivation was done in test tubes containing 2 ml of Murashige and Skoog, 1962 (MS) basic medium for the Mirobolan dwarf rootstock, and for Adapatabil rootstock Querin and Lepoivre, 1977 (QL) basic medium with Linsmaier and Skoog, 1965 (LS) vitamins, without growth hormones, supplemented with 9 g agar/l and 20 g sucrose/l. In the experiment were used three repetitions on the variant with ten explants for each repetition. Results were taken after 28 days of in vitro culture and the following data were recorded: the number of survived and contaminated explants (fungus and bacteria). The results were presented as a percentage. Data were analyze using Microsoft Excel 2010 facility.

RESULTS AND DISCUSSIONS

The study showed that for Mirobolan dwarf rootstock, the most effective treatments with 63.33% survival rate was in the case of using Tween 20, pre-sterilizing with ethanol and sterilizing with 6% calcium hypochlorite for 20 minutes and with 56.67% survival rate in the same conditions for 10 minutes. These treatments exceeded those with Ace as source of sodium hypochlorite (Table 2).

These results are similar to the studies of Vujovic et al., 2012, which reference that bleach, as a source of sodium hypochlorite, proved ineffective in disinfecting explants derived from greenhouse-grown plants of Fereley Jaspi and Gisela 6.

The results obtained with Adaptabil rootstock showed that 6% calcium hypochlorite for 20 minutes with Tween 20 and ethanol as pre-sterilizing agent is the most effective treatment with 70% survival rate. For Adaptabil rootstock good results (66.67% survival rate) were also observed in the case of treatment with bleach as the source of sodium hypochlorite for 10 minutes and sanitary alcohol as pre-sterilizing agent without Tween 20 (Table 2).

Gertlowski and Petersen, 1993, reported the efficiency of sodium hypochlorite for superficial sterilization of explant. This method was efficient and did not injure the explants. Also, Al. Ghasheem et al., 2018, worked on peach explants and they found that sodium hypochlorite was the most effective treatment with 50% survival rate at 15% NaOCl for 5 min and 10% NaOCl for 10 min. This result was an important one because the costs in the case of *in vitro* cultures are high, and the use of sanitary alcohol, instead of ethanol, would contribute to reducing the costs of mass production of plant material in vitro.

Regarding the Mirobolan dwarf rootstock, the least effective sterilization protocols with a survival rate of 10% are found in the cases of washing without Tween 20, pre-sterilization with sanitary alcohol, sterilization with 6% calcium hypochlorite, for 10 minutes and in the case of washing without tween, pre-sterilization with ethanol and sterilization with Ace as a source of sodium hypochlorite, for 20 minutes.

Regarding the Adaptabil rootstock, the least effective sterilization protocols with a survival rate of 30% are found in the cases of washing with tween 20, pre-sterilization with sanitary alcohol, sterilization with 6% calcium hypochlorite, for 10 minutes and in the case of washing without tween, pre-sterilization with ethanol and sterilization with calcium hypochlorite, for 10 minutes (Table 2).

For the Mirobolan dwarf rootstock the most effective sterilization treatments with the lowest contamination rate of 10% were in the case of washing with Tween 20, pre-sterilization with ethanol, sterilization with 6% calcium hypochlorite for both 10 minutes and 20 minutes.

Another variant with a contamination rate of 10% is washing without Tween 20, pre-sterilisation with ethanol, sterilization with 6% calcium hypochlorite for 20 minutes. And in the case of washing without Tween 20, pre-sterilization with sanitary alcohol, sterilization with 6% calcium hypochlorite, a contamination rate of 10% was also obtained (Table 2).

For Adaptabil rootstock, the most effective disinfection protocol with a 10% contamination rate was in the case of washing without Tween 20, pre-sterilization with ethanol, sterilization with Ace for 10 minutes (Table 2).

For the Mirobolan dwarf rootstock the least effective sterilization protocols with the highest contamination rate of 60% were for variants 5, 8, and 12. For the Adaptabil rootstock, the least effective sterilization protocol with a 50% contamination rate was for variant 3 (Table 2).

In the case of the two studied rootstocks, Mirobolan dwarf and Adaptabil, in the sterilization variant that offered the highest percentage of survival and growth of the explants (V16), a low degree of contamination was also recorded.

The decontamination process of the explants is not dependent only on the sterilization agents or the method used, a fact proven by the results obtained in this experiment.

It turned out that the results were uneven, in this sense the human factor could contribute to the increase in the degree of contamination.

On total experiment regardless of the pre-sterilization agent or the sterilization agent used or the exposure time, the average survival rate in the case of the Adaptabil rootstock is higher than the average survival rate in the case of the Mirobolan dwarf rootstock (Figure 1).

Regarding the contamination, we notice that the average rate of contamination in the Adaptabil rootstock is lower than in the case of the average rate of contamination in the Mirobolan dwarf rootstock (Figure 1).

The behavior during pre-sterilization and sterilization showed that the Adaptabil rootstock has a higher survival and a lower and distinctly significant contamination ($r = 0.4883$), in other words the percentage of survival decreases with the increase of the percentage of contamination (Figure 2).

Table 1. Types of sterilizing agents used in different concentrations with varying time of sterilizing on peach and plum rootstocks

Variants	Tween 20	Pre-sterilization substance disinfectans	Concentration (%)	Exposure time (min)	Surface sterilizer	Concentration (%)	Exposure time (min)
V1	-	Sanitary alcohol	70	10	Sodium hypochlorite	5.25	10
V2	-	Sanitary alcohol	70	10	Sodium hypochlorite	5.25	20
V3	-	Sanitary alcohol	70	10	Calcium hypochlorite	6	10
V4	-	Sanitary alcohol	70	10	Calcium hypochlorite	6	20
V5	+	Sanitary alcohol	70	10	Sodium hypochlorite	5.25	10
V6	+	Sanitary alcohol	70	10	Sodium hypochlorite	5.25	20
V7	+	Sanitary alcohol	70	10	Calcium hypochlorite	6	10
V8	+	Sanitary alcohol	70	10	Calcium hypochlorite	6	20
V9	-	Ethanol	70	10	Sodium hypochlorite	5.25	10
V10	-	Ethanol	70	10	Sodium hypochlorite	5.25	20
V11	-	Ethanol	70	10	Calcium hypochlorite	6	10
V12	-	Ethanol	70	10	Calcium hypochlorite	6	20
V13	+	Ethanol	70	10	Sodium hypochlorite	5.25	10
V14	+	Ethanol	70	10	Sodium hypochlorite	5.25	20
V15	+	Ethanol	70	10	Calcium hypochlorite	6	10
V16	+	Ethanol	70	10	Calcium hypochlorite	6	20

Table 2. Effect of surface sterilizer, various concentrations and exposure time on survival and contamination of Mirobolan Dwarf explants and Adaptabil explants (%)

Variants	Surface sterilizer	Concentration (%)	Exposure time (min)	Mirobolan dwarf		Adaptabil	
				Survived (%)	Contamination (%)	Survived (%)	Contamination (%)
V1	Sodium hypochlorite	10	10	16.67	13.33	69.67	13.33
V2	Sodium hypochlorite	10	20	53.33	10	50	30
V3	Calcium hypochlorite	6	10	10	33.33	40	50
V4	Calcium hypochlorite	6	20	46.67	26.67	33.33	23.33
V5	Sodium hypochlorite	10	10	13.33	60	43.33	23.33
V6	Sodium hypochlorite	10	20	46.67	33.33	56.67	26.67
V7	Calcium hypochlorite	6	10	26.67	40	30	30
V8	Calcium hypochlorite	6	20	16.67	60	43.33	26.67
V9	Sodium hypochlorite	10	10	36.67	33.33	42.33	10
V10	Sodium hypochlorite	10	20	10	60	56.67	23.33
V11	Calcium hypochlorite	6	10	13.33	40	30	23.33
V12	Calcium hypochlorite	6	20	26.67	10	46.67	16.67
V13	Sodium hypochlorite	10	10	40	33.33	36.67	30
V14	Sodium hypochlorite	10	20	6.67	53.33	53.33	26.67
V15	Calcium hypochlorite	6	10	56.67	10	40	13.33
V16	Calcium hypochlorite	6	20	63.33	10	70	20

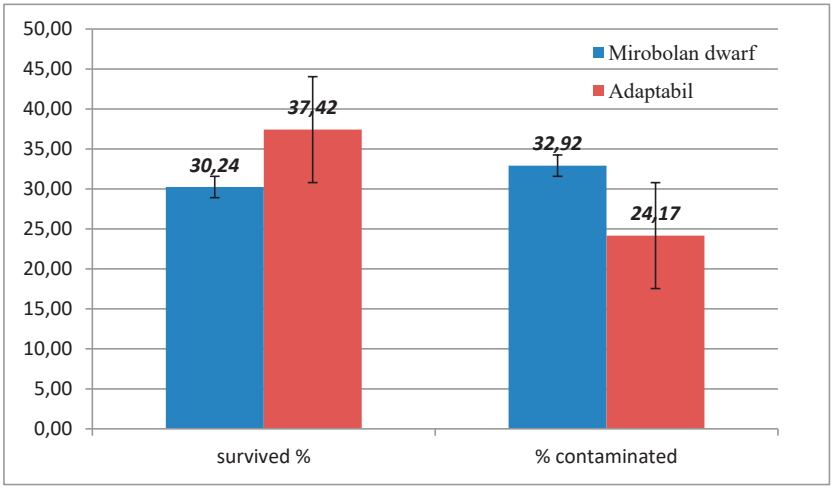

Figure 1. Average survival rate and average contaminated rate for Mirobolan dwarf and Adaptabil roostocks

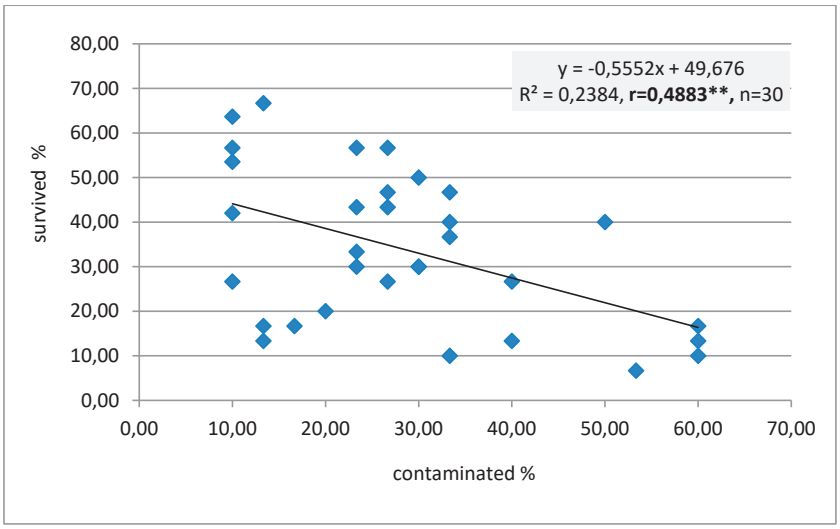

Figure 2. The correlation between the contamination and the survival of the explants in the case of the explants of Mirobolan dwarf and Adaptabil rootstocks

CONCLUSIONS

The establishment of aseptic culture is the first important step in in vitro propagation process. This study presents an effective protocol for surface sterilization of Mirobolan dwarf and Adaptabil rootstocks with promising results for large-scale propagation.

For the two rootstocks studied, Mirobolan dwarf and Adaptabil, the best sterilization results were obtained using the same sterilization protocol. The effective sterilization protocol consists of washing in the presence of Tween 20, presterilization with ethanol and sterilization with 6% calcium hypochlorite, for 20 minutes. The survival percentages were 63.33% in the case of Mirobolan dwarf rootstock and 70% in the case of Adaptabil rootstock.

For Adaptabil rootstock, the combination of sanitary alcohol, commercial bleach Ace as a source of sodium hypochlorite, without Tween, can also be used with good results. This is useful for the practice as it leads to cost savings.

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