THE EFFECTS OF THE CULTURE MEDIA COMPONENTS BY *IN VITRO* MULTIPLICATION OF 'Mr. S 2/5' ROOTSTOCK

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ABSTRACT: The rootstock for *Prunus* CV. Mr. S. 2/5 does not produce seeds so, their propagation is only possible through asexual methods. For this type of plant material, in vitro propagation can be a viable strategy for high multiplication rate in a short period of time. Thus, this study sought to identify the best saline media composition, pH, carbohydrate source and IBA concentration on a culture media prepared to establish the conditions for the best multiplication rate of in vitro of rootstock 'Mr. S. 2/5'. The study was divided in three experiments carried out with 1 cm long explant in a growth room at 25±2°C, 16 hours photoperiod and light flux density of 48 μmol $m^{-2}s^{-1}$s. The evaluations were made each 30 days after the installation of the experiments. MS media with pH 5.2 supplemented with 0.06 mg L^{-1} IBA produced the highest number of shoots per explant. The source and carbohydrate concentration did not influence the number and length of formed shoots.

KEYWORDS: *Prunus* spp, micropropagation, salts, pH, carbohydrate, IBA

RESUMO: O porta-enxerto para *Prunus* CV. Mr. S. 2/5 não produz sementes, portanto a sua propagação só é possível por via assexuada. Para esse tipo de material vegetal, a propagação in vitro pode ser uma estratégia viável para se obter alta taxa de multiplicação em curto espaço de tempo. Sendo assim, o presente trabalho buscou identificar a melhor composição salina do meio, pH, fonte de carboidrato e concentração de AIB no meio de cultura, que proporcionam as melhores taxas de multiplicação in vitro do porta-enxerto 'Mr. S. 2/5'. Este estudo foi dividido em três experimentos, sendo todos conduzidos a partir de explantes com 1 cm cultivados em sala de crescimento a 25±2°C, fotoperíodo de 16 horas e densidade de fluxo luminoso de 48 μmol $m^{-2}s^{-1}$. As avaliações foram realizadas sempre aos 30 dias após a instalação de cada experimento. Em meio MS com pH 5,2 suplementado com 0,06 mg L^{-1} de AIB obteve-se o maior número de brotações por explante. Entretanto, a fonte e a concentração de carboidrato não exerceram influência sobre o número e o comprimento das brotações formadas.

PALAVRAS CHAVE: Prunus spp., micropropagação, sais, pH, carboidrato, AIB

INTRODUCTION

The peach is a crop of great economic importance in Rio Grande do Sul (RS) state, which is responsible for about 60% of Brazilian production, dominated the cultivation of fruit for industry. However, the RS productivity (8.95 t ha^{-1}) is 60% lower than the productivity of the state of São Paulo (21.06 t ha^{-1}), being the third largest peach producer (IBGE, 2013). The low productivity in Rio Grande do Sul state, in part, is attributed to crop management problems and uncertainty in the use of rootstocks for different producing regions, a practice associated with a higher incidence of disease problems and the so called syndrome of early death peach (Mayer et al., 2009). Thus, identifying or selecting cultivars for specific purposes as rootstocks, which have the characteristics of interest such as resistance to pests and soil diseases, tolerance or resistance to root suffocation, good grafting compatibility, could contribute to increase crop productivity.

It is important to develop safe clonal multiplication protocols to different rootstocks of *Prunus* spp. for the propagation industry, because of the need of uniform seedlings and lack of fruit or seed observed in some rootstocks varieties like 'Mr. S. 2/5'

(*Cherry plum* (L.) x *Prunus spinosa* (L.)) (Fachinello and Loreti, 2000). According Loreti et al. (2008), this rootstock is an interspecific hybride that has resistance to *Agrobacterium tumefaciens* and root suffocation, can survive for relatively long periods of time under soil hypoxia conditions. It also has important agronomic characteristics like inducing low vigor in the scion, early production, strong and fast root system growth and good vegetative activity in the first years after the implementation of the orchard.

In vitro culture is a viable alternative to propagate some woody fruit species and, in some cases, it can be faster than traditional methods of asexual propagation such as cutting and grafting (Couto et al., 2004). Nevertheless, several authors observed that for some species of the genus *Prunus*, there are several difficulties in the multiplication phase, as the small number of shoots formed and the slow growth of the explants, that can frequently limits this type of propagation (Rodrigues et al. 2003; Radmann et al, 2009;. Rocha et al, 2009).

The MS is the most widely used media for plant tissue culture of many species, however, for the genus *Prunus* should be considered its dilutions or even other media formulations like SH (Schenk e Hildebrandt, 1972) QL (Quoirin and Leproive, 1977), WPM (Lloyd, McCown, 1980), according to Couto et al. (2004), Radmann et al. (2009) and Rocha et al. (2009).

Regarding growth regulators, cytokinins exert determining effect on multiplication, as it is very effective for adventitious shoot induction and to overcome apical dominance (Garden et al., 2010; Gajdosova et al., 2011). A 6-benzylaminopurine (BAP) is the most commonly used cytokinin in plant tissue culture as it can effectively induce adventitious buds, shoot multiplication and, besides, it is less expensive than other cytokinins (Barrueto and Cid, 2000). Some multiplication media are also enriched with auxins, such as indolbutíric acid (IBA), to balance hormone concentrations, promote elongation of shoots and decrease the inhibitory effect of cytokinin in their growth (Santos and Serejo et al., 2006).

A carbon source must be provided in the culture media, since the explants are not able to photosynthesize enough to support their own growth and development. Therefore, carbohydrates provide energy and contribute to balance the osmotic potential in the media (Pati et al., 2006). In tissue culture, sucrose is the most commonly used carbohydrate at concentrations that vary from 2 to 3%, which can affect the osmotic conditions of the media and the explants metabolism (Santos and Serejo et al., 2006). Other sugars such as sorbitol has also been used as carbon source, especially for species of the Rosaceae family (*Prunus Pyruse*, *Malus*), since it is their main translocation sugar (Ahmad et al., 2007).

The adjustment of the pH of the culture media is also a factor that affects growth and development of the explants, as this may compromise the solidification of the agar, inducing changes in membrane permeability, influencing the uptake of ions and molecules from the culture media (Ribeiro et al., 1997). The pH of the culture media for *in vitro* multiplication of Prunaceae is generally adjusted to 5.8 or 5.9 (Ruzic et al., 2008; Radmann et al, 2009; Radmann et al., 2011; Aghave e Yaadollahi, 2012). However, some rootstocks of *Prunus* as Mr.S 1/8 and Mr.S 1/14 (Campos et al., 2007) and Mr.S 2/5 (Rocha et al., 2009) grows in more acidic media (pH 5.2) with good development of the explants.

The objective of this work was to study the influence of the salt composition, pH, source and concentration of carbohydrate and the concentration of IBA, in the multiplication *in vitro* of 'Mr.S 2/5' *Prunus* rootstock.

MATERIAL AND METHODS

To establish *in vitro* Mr. S. 2/5 rootstock well developed branches of vigorous mature plants were collected and separated into nodal segments of 1.0 cm containing an axillary bud. After disinfestation with sodium hypochlorite 2% for ten minutes, the nodal segments were inoculated in MS medium supplemented with 7 g L^{-1} agar, pH adjusted to 5.8, and placed in a growth room with 25° C, 16 hours photoperiod and light flux density of 48 μmol $m^{-2}s^{-1}$ for 30 days. The shoots produced from these nodal segments were used in three multiplication experiments, as follows.

Experiment 1 - Effects of pH and composition of the culture media

In this study was used four mineral salts media (MS, WPM, SH and QL) supplemented with MS vitamins, BAP (0.5 mg L^{-1}), IBA (0.01 mg L^{-1}), myo-inositol (100 mg L^{-1}), sucrose (30 g L^{-1}), agar (7 g L^{-1}), and pH adjusted to 5.2 or 5.8. Apical segments with approximately 1.0 cm long were used as the initial explants, cultured at 25 ± 2°C, 16 hours photoperiod and light flux density of 48 µol $m^{-2}s^{-1}$, for 30 days. At the end of this period, the number and length of the shoots were evaluated. The experiment was carried out in a completely randomized design with a 2x4 factorial, two pH (5.2 and 5.8) and four media (WPM, SH, QL and MS), with four replicates and five explants per plot. The experimental data were subjected to analysis of variance and means compared by Tukey test at 5% probability using the program Winstat 2.0 (Machado and Conceição, 2005).

Experiment 2 - Effects of the sources and concentrations of carbohydrates

In this experiment was evaluated the effect of sucrose (Sac) and sorbitol (Sor) in the following concentrations and combinations: 30g L^{-1} Sac, 10g L^{-1} Sor + 20g L^{-1} Sac, 20 g L^{-1} Sor + 10g L^{-1} Sac and 30g L^{-1} Sor. Apical explants of 1.0 cm long were inoculated in MS media supplemented with 0.5 mg L^{-1} BAP and 0.01 mg L^{-1} IBA, pH 5.2, with all the constituents, conditions and variables used in the first experiment. The experiment was carried out in a completely randomized design with four treatments (sucrose and sorbitol combinations described above), four replicates and five explants per plot, and analyzed in the same way of the first experiment.

Experiment 3 - Effects of IBA concentrations

Apical shoots of 1.0 cm long were inoculated in MS media supplemented with 0.5 mg L^{-1} BAP, 100 mg L^{-1} of myo-inositol, 30 g L^{-1} sucrose, 7 g L^{-1} agar, different concentrations of IBA (0.0; 0.01; 0.03 and 0.06 mg L^{-1}), pH adjusted to 5.2, cultured in the same conditions used in previous experiment. Thirty days after installation of the experiment, the following variables were analyzed: number of shoots per explant and shoot length (cm) of the explants. It was used a completely randomized design with four treatments (0.0, 0.01, 0.03 and 0.06 mg L^{-1}) , four replicates and five explants per plot, and the data analyzed by polynomial regression using the program Winstat 2.0 (Machado and Conceição, 2005).

RESULTS AND DISCUSSION

Experiment 1 - Effect of salt composition and pH

The composition and pH of the media presented significant interaction in the number and length of shoots. The highest number of shoots (5.5 shoots per explant) was obtained on MS medium with pH 5.2 (Table 1). It was also found that the number of shoots per explants cultured in media with pH 5.2 was superior to those obtained at pH 5.8 in MS and SH media, but with no significant difference for the media WPM and QL. However, for the average length of shoots, differences were found in pH 5.8 with longer shoots in MS and HS compared to QL and WPM media (Table 1).

Table 1 .Number of shoots per explant and the length of these rootstock 'Mr. S 2 / 5' cultivated for 30 days on various culture media adjusted to pH 5.2 and 5.8

Culture media	Average number of shoots by explants		Average length of shoots (cm)	
	pH 5,2	pH 5,8	pH 5,2	pH 5,8
MS	5,5 Aa	4,5 Ab	0,4 Aa	0,4 Aa
SH	4,0 Ba	2,8 Bb	0,3 Aa	0,4 Aa
WPM	3,1 Ba	2,8 Ba	0,3 Aa	0,1 Bb
QL	3,0 Ba	2,8 Ba	0,3 Aa	0,1 Bb

* Means followed by capital letters in the column differ factor to the culture media, and medium followed by different lower case letters on the line, differ factor for pH, by Tukey test 5%.

In tissue culture, media is used to provide the essential nutrients for growth of the explants and control, largely, the pattern of *in vitro* development in each species (Cordeiro et al., 2014). The same basic biochemical and metabolic pathways that function in plants are kept in cells or *in vitro* cultured tissues, although some processes such as photosynthesis, can be inactivated by the growing conditions and the state of differentiation of cells (Storck and Zaffari, 2007). A variety of culture media has been used for different species, however, the salt formulation of the MS media is most frequently used, which is considered more concentrated when compared to other salt formulations such as QL, SH and WPM. Although some woody plants have shown better responses in less concentrated media salts (Villa et al., 2009; Radmann et al., 2009), several species and/or cultivars has shown better responses in media with higher concentrations of salts, as seen in this study and Chaves (2003) with Mr. S. 1/8 rootstock.

The higher efficiency of the MS media in the present study may be related to the fact that other media are less concentrated. The WPM media, for example, has 25% concentrations of nitrate ions and ammonia as compared to MS (Melo et al., 1999; Soares et al., 2009), nevertheless, its concentration of potassium and sulfate ions is higher. Nitrogen is the main inorganic nutrient in culture media and deppending on its chemical form that is provided strongly influences growth and morphogenesis *in vitro*. It is essential for formation of organic molecules, nucleotides of the nucleic acids (RNA and DNA), amino acids and proteins, being present as well in every chlorophyll molecule (Russowski, 2001).

Mercier et al. (1997) found that the source of nitrogen (NH_4^+ and NO_3^-) in the media culture direct influences the levels of endogenous IAA and cytokinins. Thus, depending on the species or cultivar, different substances present in the media containing nitrogen may induce different responses during the multiplication phase.

The pH of the media is another decisive factor that can influence the availability of nutrients and growth regulators, agar solidification and enzyme activity (Pasqual et al., 2002; Ilyas et al., 2013). A study of Pasqual et al. (2002), showed that when pH was adjusted to a range considered ideal it promoted a better use of the nutrients by the explant, influencing, for example, the uptake of nitrogen present in the culture media.

One of the assumptions in the scientific community is that the pH is directly linked to increased concentration of nitrogen in the form of ammonia in MS media compared to other culture media. This hypothesis agrees with the results obtained by Ribeiro et al. (1997) in experiments with Orange cv. Pera that showed best results with MS media at acidic pH, from values less than 5.2.

Thus, the results obtained with the cultivar 'Mr. S. 2/5 ', demonstrates that lower pH influenced directly on the variables tested. These experimental results can be correlated with the classical chemistry knowledge when an ionic liquid media dissociates chemically acid, electro active generating protons in the culture media that can be used by the plant in its metabolic pathway to produce chemical energy and subsequent conversion into biochemical energy through the ATP synthase. By using proton present in the media, the plant will metabolize existing ions to generate the electromotive force, allowing more easily ADP + Pi junction generating ATP (Nelson and Cox, 2014).

Experiment 2 - Effect of supply and carbohydrate concentration

No difference between treatments was found for both number and length of shoots, although the medium containing sucrose alone presented approximately 60% more shoots formed than the medium containing only sorbitol (Table 2).

Table 2. Number and length of shoots (cm), obtained from the rootstock Mr.S.2/5, cultured for 30 days in MS media with two carbohydrate sources

Treatments	Number of shoots	Shoot length (cm)
30 g L^{-1} de sucrose	3,46 A	0,65 A
10 g L^{-1} de sucrose + 20 g L^{-1} de sorbitol	2,62 A	0,57 A
20 g L^{-1} de sucrose + 10 g L^{-1} de sorbitol	2,43 A	0,55 A
30 g L^{-1} de sorbitol	2,06 A	0,42 A

Means followed by capital letters in the column differ factor for the concentration of sucrose and sorbitol at 5% Tukey test.

Borkowska and Szczerba (1991) obtained similar results for number and length of shoots in *Prunus cerasus* L. and found that explants of the cultivar North Star showed no difference between sucrose and sorbitol, but in cultivar Schattenmorelle the number of shoots was two fold in the sucrose media compared to sorbitol. On the other hand, in the multiplication rootstock in vitro of peach GF-677 '(*Prunus amygdalus* x *P. persica*), Ahmad et al. (2007) observed that sorbitol was the most effective source of carbon, compared to sucrose, providing more number and length of sprouts in the concentration of 30 g L^{-1}.

The carbohydrates provide energy and contribute to balance the osmotic potential of the culture media, affecting the proliferation and growth *in vitro* explant (PATI et al., 2006) and according to Faria et al. (2004), sucrose is the main source of carbohydrate used in a concentration of 2 to 3%. Some authors suggest that the superiority of sucrose is related to the transport of photoassimilates from source and drain in higher plants (Hackel et al., 2006).

Several enzymatic systems are involved in sucrose metabolism, where the enzymes invertase, sucrose synthase, and sucrose-6-phosphate synthase act in catalysis of reactions. The invertase catalyzes the irreversible hydrolysis of sucrose to fructose and glucose, sucrose synthase (SS) that catalyzes the reversible reaction of UDP-Glucose + Fructose Sucrose + UDP, while invertase produce glucose and fructose, SS produces fructose and UDPglucose. Since sucrose-6-phosphate synthase, a soluble cytoplasmic enzyme that catalyzes the reaction of UDP-Glucose + Fructose 6-Phosphate Sucrose 6-phosphate + UDP (Huber and Huber, 1996).

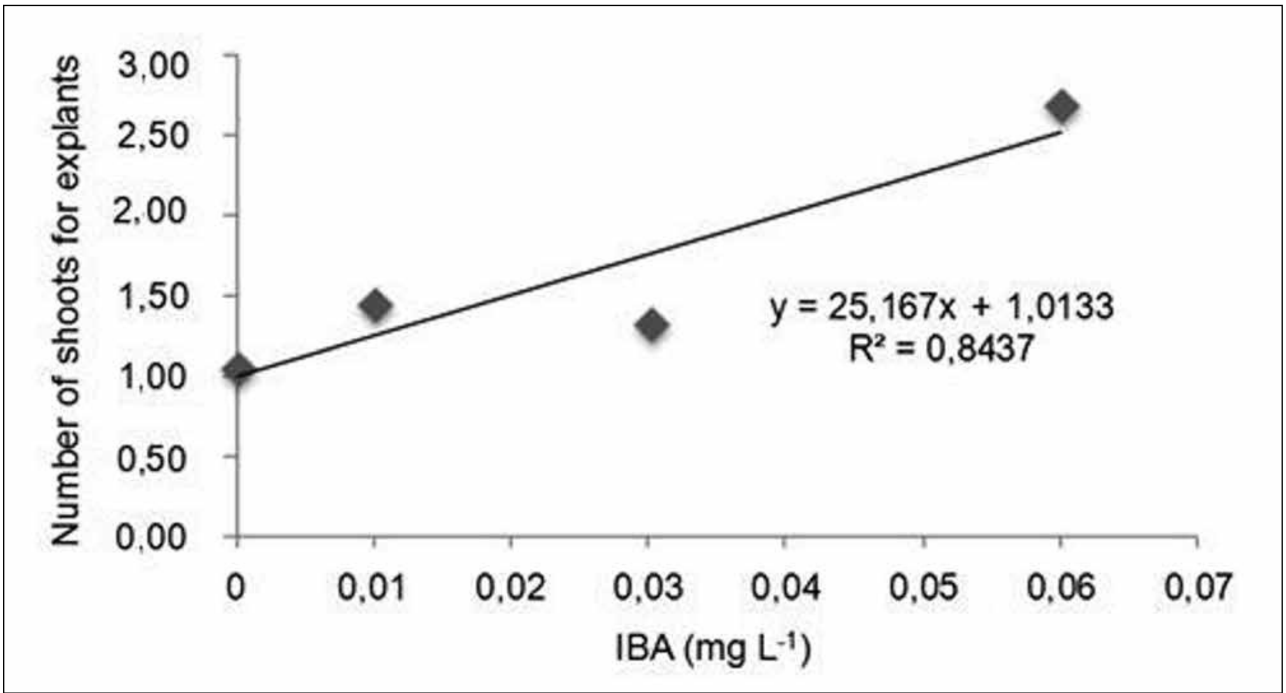
Sorbitol, a sugar alcohol or a polyol, is the main sugar translocation of Rosaceae family. Ruzic et al. (2008) reported that with the exception of *Malus spp*. and possibly *P. persica*, there is a certain correlation between the plant and the sorbitol content that could be metabolized *in vitro*.

According Wallaart (1980), the presence of sorbitol in woody plants of the family Rosaceae is variable (0.0 to 16.8%), being observed a range of 0.3 to 6.6% for the *Prunus* genus. Plants need specific enzymes such as the sorbitol dehydrogenase to metabolize this sugar (Marino et al., 2003). Thus, the use of sorbitol as a carbon source for *in vitro* propagation is directly related to the species, as well as sufficient availability of enzymes that hydrolyze that sugar alcohol.

Experiment 3 - Effect of concentration of IBA

The number of shoots per explant increased linearly with the concentration of IBA treatments with about 1,3 to 2,7 shoots/explant with 0.01and 0,06 mg L^{-1} of AIB, respectively (Figure 1). Regarding the other variables, length of shoots, fresh and dry mass, there was no significant difference between treatments obtaining a general average 0,81cm, 1,63g L^{-1} and 0.25 g L^{-1}, respectively (data not shown).

Figure 1. Average number of shoots obtained from the rootstock 'Mr. S. 2/5 'cultured for 30 days in MS media supplemented by different concentrations of IBA.



The main growth regulators used in the multiplication stage are cytokinins, however, some species are benefited by the use of auxins. The ratio between them is determinant for differentiation and growth of roots and shoots of the aerial plant tissues cultured (Santos and Serejo et al., 2006).

In a study conducted by Wagner Junior et al. (2003), with the 'Julior' rootstock (*Prunus* spp.), It was found that the IBA had no influence on the number and length of shoots. Silveira et al. (2002) found that the multiplication of *Prunus* rootstocks Mirabolano 29C', 'GxN22' and 'Marianna' responded positively to the presence of auxin, increasing the average number of shoots.

Therefore, the addition of IBA in the culture in the multiplication phase is important for some cultivars, as this growth regulator voids of the inhibitory effect of cytokinin promotes on growth of sprout, or the reaction of a fabric *in vitro* depends on the hormonal balance between these two classes of growth regulators (Santos and Serejo, 2006).

The results presented here show that the use of MS culture media with pH adjusted to 5.2 is more effective for in vitro multiplication of peach rootstock Mr. S. 2/5 than WPM, SH or QL media. Furthermore, the addition of 0,06 mg L^{-1} IBA together with 0,5 mg L^{-1} BAP determines an increase at the proliferation rate of shoots, independent of the source and concentration of sugar used in this work.

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