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Osmoregulators on *in vitro* encapsulation of potato 'BRS Clara' buds

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ABSTRACT

Synthetic or artificial seeds via encapsulated propagules could be used for *in vitro* regeneration and mass multiplication, for germplasm preservation and exchange of plant materials. An *in vitro* encapsulation of potato 'BRS Clara' buds efficient protocol was established. Lateral buds were immersed in a 4% sodium alginate matrix, dripped in $CaCl_2$ for 20 minutes in different osmoregulatory agents: sucrose (8.72, 15.00, 21.28 and 30.00 g L^{-1}) and mannitol (0.00, 11.63, 20.0, 28.37 and 40.0 g L^{-1}). They were then held for 30 days at approximately 4°C and subsequently immersed in KNO_3 for capsule decomplexing. Afterward, they were inoculated in MS medium with 3% sucrose and cultured in a growth room under irradiance of 43 µmol m^{-2} s^{-1}, 16 hours-photoperiod and 25±2 °C temperature. Sucrose and mannitol at concentrations of 15 g L^{-1} and 20 g L^{-1}, respectively, associated with the encapsulation matrix promoted 80% survival rate of 'BRS Clara' encapsulated buds.

Keywords: Germplasm preservation, Sodium alginate, *Solanum tuberosum*, Tissue culture

INTRODUCTION

In Brazil, about 118 thousand hectares are planted with potatoes, producing approximately 3.6 million tons annually, with an average yield of 30.1 t ha^{-1}, with Southeast and South regions being the largest producers (Instituto Brasileiro de Geografia e Estatística – IBGE, 2019). Commercially, the potato is propagated by seed tubers; however, the use of this type of material for repeated cycles can cause accumulation of diseases, mainly the viral ones, contributing to the crop degeneration (Fortes; Pereira, 2003). One alternative used for commercial seedling production is the *in vitro* cultivation of plant tissues (Assis, 1999), which allows the production and multiplication of pathogen-free plants (Assis, 1999; Lopes; Reifschneider, 1999; Pereira et al., 2001; Fortes; Pereira, 2003; Dutra et al., 2011; Morais et al., 2018).

In addition to the production of pathogen-free propagating material, tissue culture allows preserving the genetic diversity of species. *In vitro* encapsulation of lateral buds is an alternative for germplasm preservation as a conservation method (Ghanbarali et al., 2016; Doussoh et al., 2018). The use of storage at temperatures between 4° and 6.5 °C is suggested, aiming at minimum growth and optimization of the micropropagation stages (Fortes; Pereira, 2001; Lung'aho, et al., 2012). Prolonged *in vitro* maintenance of the explants was achieved by combining the effect of low temperatures with the use of osmoregulators, such as sucrose, mannitol, sorbitol and ancimidol (Nasiruddin; Islam, 2018; Mancilla-Álvarez et al., 2019). Non-metabolisable sugar-alcohols reduce the water availability to the growing cultures by imposing a water-deficit stress (Thompson et al., 1986). This stress may perhaps be responsible for slow-growth of potato microplants (Gopal et al., 2002).

Somatic embryos, apical and lateral buds, and meristematic tissues encapsulation in a hydrogel capsule have numerous advantages, among them,

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the propagules protection for the preservation in periods of slow growth (Guerra et al., 1999; Rai et al., 2009) and the resistance during manipulation and viability of cellular tissues preservation (Prewein; Wilhelm, 2003). In addition to, capsules can be stored, maintaining the genetic material integrity in a minimum storage and maintenance space (Rai et al., 2009).

Efficient encapsulation protocols of *Solanum* cultivars were established (Sarkar; Naik, 1997; Bouafia et al., 1996; Nyende et al., 2003; Ghanbarali et al., 2016) and thus, it is essential to develop a functional protocol applicable to genotypes adapted to the Brazilian conditions.

This work aimed to develop synthetic seeds in potato through alginate-mediated osmoregulators-encapsulation of *in vitro* 'BRS Clara' buds.

MATERIAL AND METHODS

Lateral buds excised from 'BRS Clara' potato shoots *in vitro* were mixed with the sodium alginate matrix (2.5%) containing sucrose (8.72, 15.00, 21.28 and 30.00 g L^{-1}) and mannitol (0.00, 11.63, 20.00, 28.37 and 40.00 g L^{-1}). These concentrations were obtained based on the experimental design proposed (Table 1). The experiment was conducted in a central rotational compound design (CRCD), as described by Rodrigues and Iemma (2005), with one central point, with five replicates of five test tubes containing each encapsulating unit. According to these authors, the use of CRCD is advantageous compared to the analysis of a variable at a time when it is desired to optimize a process. When applying CRCD, fewer trials are required to obtain quality information and maximize or minimize the desired response.

When using CRCD, once factorial points levels are defined for each variable, axial and central points levels are calculated. Or, as used in this experiment, when axial points levels are defined, then the central and factorial points are calculated. The coded and actual levels of the two independent variables are shown on Table 2.

Once the buds were mixed with the sodium alginate matrix containing combinations of sucrose and mannitol as shown in Tables 1 and 2, they were individually rescued and dripped with calcium chloride solution ($CaCl_2$ - 100 mM) for complexation during 20 minutes. Capsules were double washed in sterilized deionized water and immersed in potassium nitrate solution (KNO_3 - 100 mM) for decomplexing during 15 minutes and followed by a double wash. Afterward, the capsules were maintained at 6±1 °C in BOD for 30 days, when they were inoculated in MS culture medium (Murashige; Skoog, 1962), added with 3% of sucrose and kept in growth room under irradiance of 43 µmol m^{-2} s^{-1}, photoperiod of 16 hours and temperature of 25°± 2° C, for 15 days.

Table 1: Treatment codes of the proposed experimental design (CRCD) for optimizing sucrose and mannitol concentrations in 'BRS Clara' capsules. Combinations of +1 and -1 are factorial points, T6 (0,0) is the central point and combinations of 0 and +α or -α are axial points.

Treatment	Sucrose	Mannitol
	----------g L^{-1}------	
T1	-α	0
T2	-1	-1
T3	-1	+1
T4	0	-α
T5	0	+α
T6	0	0
T7	+1	-1
T8	+1	+1
T9	+ α	0

Table 2: Coded and actual levels of independent variables sucrose and mannitol.

	-α	-1	0	+1	+ α
Sucrose (g L^{-1})	0	8.72	15	21.28	30
Mannitol (g L^{-1})	0	11.63	20	28.37	40

Data were analyzed by statistical software SAS 9.2, using GLM procedures for continuous variables and GENMOD for discrete variables.

The highest root length and largest shoot, root number, shoot number and survival percentage were analyzed. To compare the treatments, the data of variables root length, shoot length and survival percentage were submitted to analysis of variance and means were compared by the t-test at the 5% level of error probability. For shoot and root number, the Poisson distribution was considered and generalized linear models were applied with the same rigor used for other variables.

RESULTS AND DISCUSSION

Response surface analysis was performed for variables that showed a significant difference between the factors applied to the encapsulated units. For survival rate, it is evident that the encapsulated 'BRS Clara' units respond better to the treatments combining intermediate concentrations of mannitol and sucrose, which is the center point of the design (Figure 1A). However, the encapsulated units have increased length (Figure 1B) and the number of roots (Figure 1C) when there are lower concentrations of both carbohydrates tested in the experiment. This increase in the number and length of roots in response to the lower availability of sugars may be the result of the search for the nutrient explant, due to greater availability of water and ions when the medium is less concentrated.

This result confirm that synthetic potato seed technology is feasible from the point of view of survival since most treatments provided percentages greater than 60%. Compared with other studies reporting survival rates between 15% and 70% (Sarkar; Naik, 1997; Nyende et al., 2002; Nyende et al., 2005; Kaczmarczyk; Rokka; Keller, 2011), these results can be considered satisfactory for the species.

The reduction in the carbon source of the encapsulated material results in the deceleration of the metabolism and consequently in its growth. Mannitol is a single-chain alcoholic sugar, and already in the first reaction to its conversion into fructose, it generates reducing energy (NADPH). After this oxidation process, mannitol is phosphorylated and its use in protein synthesis, energy metabolism or antioxidant metabolism is possible (Stoop; Williamson; Pharr, 1996; Patel; Williamson, 2016). These factors may be associated with the promotion of greater plant survival with the use of mannitol.

However, Nyende et al. (2005), studying the potato cultivars Désirée and Tomensa obtained the highest rates of synthetic seed re-growth, respectively, with 92.5 and 95.8%. Nyende et al. (2002) reported 100% germination in synthetic potato seeds in MS medium after six months of storage at 4 °C and 10 °C. These results demonstrate the feasibility of the encapsulation technique; however, it may vary according to the cultivar studied. Nyende et al. (2003) also using potato shoot which had been stored for 270 days obtained 100% germination of the capsules directly on substrate in a greenhouse.

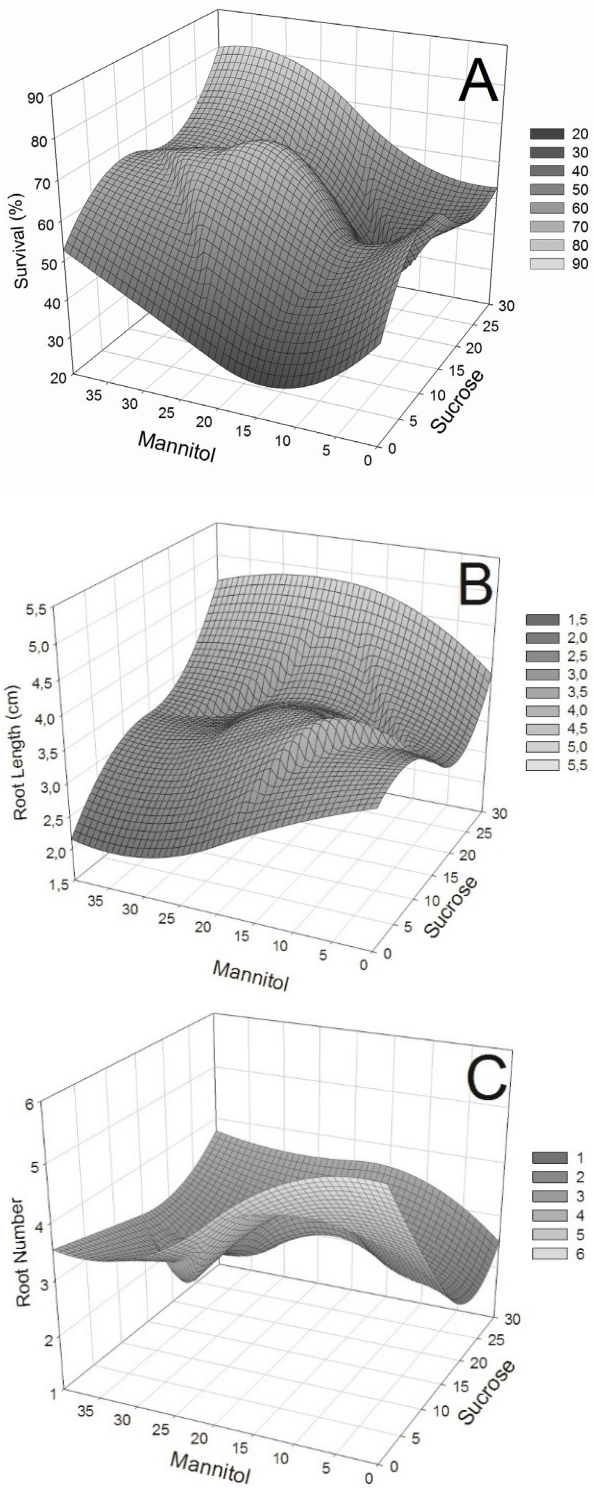

Figure 1: Survival rate (A), lenght (B) and number (C) of root response surfaces of *Solanum tuberosum* 'BRS Clara' encapsulated buds, cultivated under different sucrose and mannitol combinations.

In the absence of sucrose from the treatment containing only 20 g L^{-1} of mannitol, the lowest percentage of survival was observed, indicating the need of this carbon source. Similar results were reported by Silva et al. (2005), which obtained better growth rates of *Syngonanthus mucugensis* plants *in vitro* at 25 °C in a medium containing 15 g L^{-1} of sucrose.

Due to the metabolic mannitol pathway being efficient in the generation of reducing energy, in addition to having a low energy cost, this carbohydrate is commonly used in sink tissues in a wide variety of plants (Stoop; Williamson; Pharr, 1996). Therefore, sink tissues in plants generally have mannitol dehydrogenase (MTD); however, this enzyme is inhibited by excess of sucrose (Stoop; Williamson; Pharr, 1996; Patel; Williamson, 2016). In the present study, sucrose may have inhibited the enzymes involved in mannitol metabolism. Thus, the effects of mannitol were more evident when the concentration of sucrose was lower than that of mannitol itself.

However, the interaction between the osmotic agents had no effect on the development of the aerial system, since no significant differences were observed between the treatments in relation to the number and length of shoots formed. This effect can be attributed to the fact that plants subjected to water stress, with the addition of osmoregulatory agents, present a greater root system development to be able to absorb water at greater depths (Hadas, 1976).

This is due to the physical properties of the sugars used, mainly mannitol, which is used as an inducer of osmotic stress (Girotto et al., 2012; Llanes et al., 2016; Araújo et al., 2017). In this context, we have mannitol as an osmotic agent that reduces the free energy of water and limits its absorption. This physical property of mannitol may result in a reduction in growth rate due to its effect on water absorption and consequently nutrients, this would be an effect on the medium rather than the plant. These processes can be induced by ion excess as well as other carbohydrates, such as sucrose itself.

However, there are indications of the osmoprotective effect of mannitol on plants (Stoop; Williamson; Pharr, 1996; Patel; Williamson, 2016). In this context, mannitol contributes to tolerance to osmotic and/or dry stress. Plants tolerant to drought or salinity tend to accumulate mannitol in their tissues in order to avoid protein denaturation (Patel; Williamson, 2016). Thus, there is a possibility that the absorption and accumulation of mannitol may contribute to the greater survival of encapsulated materials. This is due to the synergism between its osmoprotective, antioxidant role and its physical properties, which reduce absorption of water and nutrients from the medium. In this sense, the encapsulation technique can be used for genetic material conservation in support of breeding programs, either by their direct effect on the plant or in the culture medium.

CONCLUSION

The combination of 15 g L^{-1} sucrose and 20 g L^{-1} mannitol provides a higher survival rate of encapsulated 'BRS Clara' potato shoot.

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