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EFFECTS OF SUCROSE MEDIUM CONTENT AND STERILANT TREATMENT ON MICROBIAL CONTAMINATION OF SWEET POTATO CULTURES INITIATED *IN VITRO*

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ABSTRACT

The main objective of this study was finding solutions for reducing the level of microbial contamination occurred during in vitro cultivation of sweet potato introduced from the ex vitro environment. For this purpose, growth medium variants with different concentrations of sucrose (20 g/L, 30 g/L and 40 g/L) were tested as well as different periods of time during the biological material was in contact with the sterilizing agent: 70% ethanol for 3, 4 and 5 minutes followed by 1% sodium hypochlorite (NaClO) solution for 10, 13 and 16 minutes respectively. Culture medium variants with a sucrose content of 20 g/L and 30 g/L combined with an explants sterilant treatment in 70% ethanol for 4 minutes followed by 1% NaClO for 13 minutes were the most effective in reducing the percentage of microbial contamination.

INTRODUCTION

Having rich content in fiber, carbohydrates, proteins, vitamin A, B6 and C, iron and calcium, the sweet potato is considered a vegetable with high nutritional value (Ram Chandra Jena and Kailash Chandra Samal, 2011).

The frequent introduction and exchange of sweet potato cultivars and clonal propagation exposes the crop to viral infections (Xiansong, 2010 cited by Ogero, 2012). Cuttings sourced from old plants in propagation are often a channel for transmission of systemic infections from one generation to the other, leading to poor yields in successive seasons (Roca and Mroginski, 1991, cited by Hammond, 2014). The multiplication rate of cuttings is also very low compared to grain crops which are propagated by true seeds. *In vitro* propagation of sweet potato helps surmount these challenges by producing disease-free planting materials in large numbers (Hammond, 2014).

Plant tissue culture is uniquely suited for obtaining and maintaining mass propagation of specific pathogen-free plants. The provision of a steady supply of indexed planting materials through *in vitro* culture appears feasible economically and technologically (Mervat M.M. El Far, 2007).

Tissue culture allows the rapid clonal propagation of a large number of plantlets over a short period, as well as the maintenance of germplasm under controlled conditions in small spaces (Lizarraga et al., 1992). The micropropagation of sweet potato by *in vitro* culturing can be carried out continually and allows production of larger numbers of uniform, well-rooted plants using node explants (Doliński and Olek, 2013). Plant tissue culture carried out under aseptic conditions has important applications in plant biotechnology. However, microbes are common cause of contamination in tissue culture. Sterilization is one of the reliable means to control the pathogenic effect of microbes (De Almeida et al., 2007, cited by Hammond, 2014). The use of nodal explants for *in vitro* propagation

promotes direct regeneration of cultures, but may cause high levels of microbial contamination due to large size of the explants (Amissah et al., 2016).

In order to reduce the level of microbial contamination of sweet potato cultivated *in vitro*, this study intended to find an optimal amount of sucrose added to the growth medium and an optimal time for explants sterilization.

MATERIAL AND METHOD

The study was carried out at the National Institute of Research and Development for Potato and Sugar Beet (NIRDPSB) Brasov, Research Laboratory for Plant Tissue Culture. The biological material analyzed in the experiment was represented by five sweet potato varieties: Yulmi, KSC1, KSP1, Hayanmi și Juhwangmi. The sweet potato storage roots used for obtain vine cuttings were provided by Research and Development Center for Plant Growing on Sands (RDCPGS) Dabuleni, Dolj county, based on a joint research project (ADER 2.2.2.). Vine cuttings used to initiate *in vitro* culture were taken from mother plants obtained in protected area, approximately 80 days after tubers planting (Fig. 1).

Fig. 1 Sweet potato shoots 80 days after tubers planting

Healthy looking sweet potato vines were rinsed under running tap water for 3 minutes. After removing the leaves (keeping a petiole portion to protect the bud during sterilization), the shoots were cut into 2 cm segments containing one axillary bud. Explants were then transferred to clean containers, covered and sent to flow hood. All surfaces were wiped with sterile tissue paper soaked in 70% ethanol. Culture vessels, work tools, spirits, tissue paper and all other materials required for inoculation have been pre-sterilized and then placed in the hood.

For surface sterilization, sweet potato explants were subjected to different sterilant conditions. Thus, the uninodal segments were dipped in 70% ethanol for 3, 4 and 5 minutes respectively, after which, without rinsing, they were immersed in 1% sodium hypochlorite (NaClO) solution for 10, 13 and 16 minutes, respectively. Four drops of Tween-20 were added to the NaClO solution.

For initiation of *in vitro* sweet potato culture, a specific growth medium (Namanda et al., 2015) was used in which composition (Table 1), in addition to Murashige-Skoog (MS) salts and vitamins (Murashige and Skoog, 1962) other compounds were added: ascorbic acid, calcium nitrate, calcium pantothenate, arginine, putrescine-HCl, gibberellic acid, sucrose and phytoagar. In order to stimulate plant rooting, 1-naphthylacetic acid (NAA) was used.

For a more effective control of microbial contamination, a broad-spectrum product Plant Preservation Mixture (Plant Cell Technology) that inhibits the growth of pathogens in plant tissue cultures has been added to the medium.

The pH of the medium was adjusted to 5.7 and solidified with phytoagar. Test tubes containing the culture medium were sealed with aluminum foil and autoclaved at 121 °C for 20 minutes.

Table 1

Growth medium used for initiation of *in vitro* sweet potato culture

Components	Amount/1L of medium
MS salts including vitamins* (g)	4.4
Ascorbic acid (g)	0.2
Calcium nitrate (g)	0.1
Calcium panthotenate (g)	0.002
L-Arginine (g)	0.1
Putrescine-HCl (g)	0.02
Gibberellic acid (g)	0.01
Phyto Agar (g)	9
NAA (mg)	0.3
PPM (ml)	3
Sucrose (g)	**20; 30; 40**

*MS – Murashige-Skoog, 1962

The sweet potato explants were subjected to three sterilization treatments, further noted T_1, T_2 and T_3 (Table 2) and then repeatedly rinsed with sterile distilled water. After trimming, the explants were inoculated on the three medium variants (Table 2) containing different concentrations of sucrose (20 g/L, 30 g/L and 40 g/L respectively), using one explant per test tube. In the literature, culture medium supplemented with 30 g/L sucrose it is conventional for *in vitro* propagation of sweet potato. In this study, other amounts of sucrose were used to see if there was a connection between the sucrose content in the culture medium and the level of microbial contamination.

Table 2

Sterilant conditions and sucrose content in growth medium for *in vitro* initiation of sweet potato cultures

Sterilant conditions		Sucrose content (g/L)
T_1	70% ethanol for 3 minutes 1% NaClO for 10 minutes	20
		30
		40
T_2	70% ethanol for 4 minutes 1% NaClO for 13 minutes	20
		30
		40
T_3	70% ethanol for 5 minutes 1% NaClO for 16 minutes	20
		30
		40

After explants inoculation, the test tubes were closed with aluminum foil and sealed with Parafilm. The cultures were transferred to a growth chamber and exposed daily to 16 hours light. The growth chamber temperature was maintained at 24±2 ^{o}C. Observations and notes regarding the evolution of microbial contamination were performed every two days for 4 weeks.

The experiment was a two factor factorial arrangement of three sterilization treatments and three concentrations of sucrose in a completely randomized design with three replications. As a control, the variants with the lowest values of the two factors were chosen: for sterilization time, 3 minutes in ethanol 70% and 10 minutes in sodium hypochlorite 1%, and culture medium variant with 20 g/L sucrose. Statistical determination was made by analysis of variance (Săulescu N.A. and Săulescu N.N., 1967).

RESULTS AND DISCUSSIONS

Observations were made every two days for culture contamination and general growth of plantlets for 4 weeks. The first microbial contamination occurred 6 days after cultures initiation, on the medium with 40 g/L sucrose and T_1 and T_3 sterilization treatments.

Regarding the influence of sterilant treatment (Table 3) the lowest percentage of microbial contamination (4.69%) was obtained with T_2 variant which recorded a very significant negative difference (-8.70) compared to the control. The highest percentage of microbial contamination was recorded for T_3 variant (15.09%).

Table 3

The level of microbial contamination of sweet potato cultures using different sterilant treatments, 4 weeks after culture initiation

Sterilant treatments	Percentage of contamination (%)	%	Dif.	Semnif.
T_1 (control)	13.39	100	-	-
T_2	4.69	35.03	-8.70	ooo
T_3	15.09	112.70	1.7	*

DL 5% = 1.47; DL 1% = 2.44; DL 0.1% = 4.57

Table 4 presents the results of the culture medium sucrose concentrations influence on the percentage of microbial contamination. It can be seen that the lowest percentage of contamination was obtained on the medium variant with an addition of 20 g/L sucrose (4.32%), and the highest percentage of contamination was recorded on the medium variant with an addition of 40 g/L sucrose (18.57%).

Table 4

The level of microbial contamination of sweet potato cultures using different concentrations of sucrose in culture medium, 4 weeks after culture initiation

Sucrose content (g/L)	Percentage of contamination (%)	%	Dif.	Semnif.
20 (control)	4.32	100.00	-	-
30	10.28	238.15	5.96	***
40	18.57	430.12	14.25	***

DL 5% = 1.62; DL 1% = 2.36; DL 0.1% = 3.54

The combined influence of sterilant treatment and concentration of sucrose in the medium (Table 5) determined the lowest percentage of infection (0%) at the T_2 sterilization treatment and concentration of 20 g/L sucrose. On same sterilization treatment (T_2), but a concentration of 30 g/L sucrose a low percentage (4.55%) of microbial contamination was also obtained. The highest percentages of microbial contamination (Table 5) appeared in the medium variant with a concentration of 40 g/L sucrose and T_3 (28%) and T_1 (18,18%) sterilant treatments.

Table 5

The combined influence of sterilant treatment and concentration of sucrose in the culture medium on the level of microbial contamination, 4 weeks after culture initiation

Sucrose content (g/L)/ Sterilant treatments	**20**	**30**	**40**
T_1	7.69	14.29	18.18
Dif.	-	6.6	10.49
Semnif.	-	***	***
T_2	0	4.55	9.52
Dif.	-	4.55	9.52
Semnif.	-	***	***
T_3	5.26	12	28
Dif.	-	6.74	22.74
Semnif.	-	***	***

DL 5% = 0.76; DL 1% = 1.07; DL 0.1% = 1.52

In this study, beside the level of microbial contamination, the percentage of sweet potato plantlets regeneration was also determined. Explants that regenerated directly but got contaminated were still considered to have directly regenerated. Figure 2 shows healthy and contaminated sweet potato cultures 4 weeks after initiation.

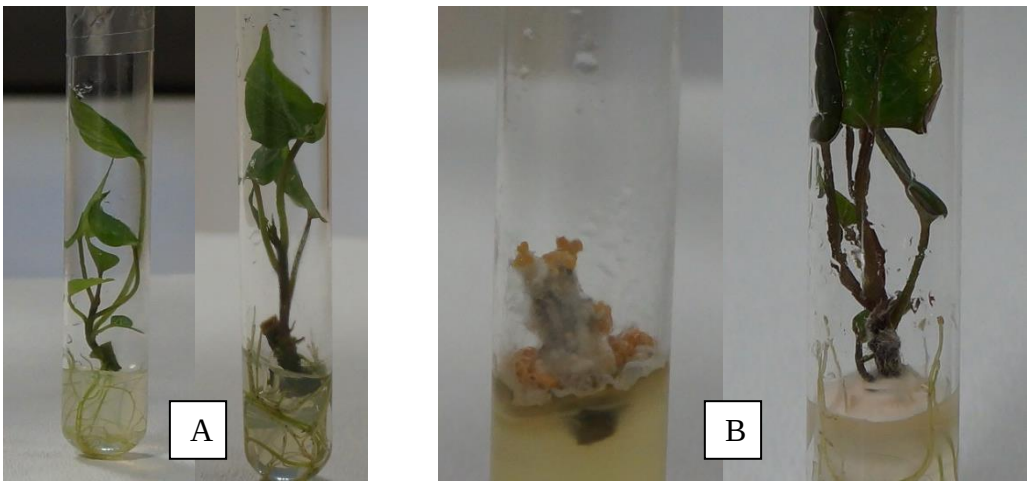

Fig. 2 Sweet potato plantlets 4 weeks after in vitro culturing: healthy cultures (A); contaminated cultures (B)

From the analysis of the combined influence of sterilant treatments and the sucrose concentration on the plant regeneration percentage (Table 6) it can be noted that by

applying T_2 treatment and by using in the nutrient medium of a quantity of 40 g/L sucrose a higher percentage of regeneration is obtained (95.24%), followed by T_3 sterilization treatment and using in the nutrient medium of a 20 g/L sucrose (94.74%).

Table 6

The combined influence of sterilant conditions and concentration of sucrose in the culture medium on plantlets regeneration percentage, 4 weeks after cultures initiation

Sucrose content (g/L)/ Sterilant treatments	**20**	**30**	**40**
T_1	80.77	80.95	72.73
Dif.	-	0.18	-8.04
Semnif.	-	ns	oo
T_2	83.33	81.82	95.24
Dif.	-	-1.51	11.91
Semnif.	-	ns	***
T_3	94.74	72	56
Dif.	-	-22.74	-38.74
Semnif.	-	ooo	ooo

DL 5% = 3.15; DL1% = 5.22; DL 0.1% = 9.76

The lowest percentage of regeneration was obtained in the T_3 sterilant treatment and the medium variant with a concentration of 40 g/L sucrose (56%).

CONCLUSIONS

By applying a suitable sterilization treatment and using a lower concentration of sucrose in the culture medium, it is possible to reduce the level of microbial contamination in the sweet potato initiated *in vitro* cultures.

In this study it was observed that as the concentration of sucrose in the culture medium was higher, the number of contaminated cultures increased. Also, the use of a higher amount of sucrose (40 g/L) combined with a longer exposure of the explants to the sterilizing agent (T_3 - 70% ethanol for 5 minutes followed by 1% NaClO for 16 minutes) led to the highest percentage of microbial contamination of the cultures and the lowest percentage of *in vitro* regeneration of sweet potato plantlets.

Based on the results obtained in this study, T_2 sterilization treatment (70% ethanol for 4 minutes followed by 1% NaClO for 13 minutes) combined with the addition of 20 g/L and 30 g/L sucrose respectively in the nutrient medium, is recommended to reduce the level of microbial contamination in sweet potato initiated *in vitro* cultures.

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