Blueberry (*Vaccinium ashei* Reade) cv. Brightwell *in vitro* establishment with silver thiosulfate

Estabelecimento *in vitro* de mirtileiro (*Vaccinium ashei* Reade) cv. Brightwell cultivado com tiossulfato de prata

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ABSTRACT

During *in vitro* culture the ethylene accumulation inside the culture recipient can be detrimental to culture. An alternative to avoid the formation of ethylene that has brought good results in seedling development is the addition of silver thiosulfate (STS), an inhibitor of ethylene perception. Based on this knowledge, the present study aimed to evaluate the influence STS on the *in vitro* establishment of blueberry (*Vaccinium ashei* Reade) cv. Brightwell. Nodal segments were used, in WPM culture medium, supplemented with 5 mg L^{-1} 2iP and STS at concentrations of 0, 15, 30, 45 e 60 µM. After 60 days it was assessed: the percentage of surviving explants and that developed leaf primordia, number of buds with shoots, length of longer budding and number of leaves. The obtained data were submitted to analysis of variance and, when significant, compared by Duncan test ($p \leq 0.05$) and shown by polynomial regression. The effect of STS concentration used was significant at 5% probability for the parameters number of buds with shoots and leaves and favored by the use of 45µM STS.

Index terms: Ethylene; STS; micropropagation.

RESUMO

Durante o cultivo *in vitro* o acúmulo de etileno no interior do frasco pode ser prejudicial à cultura. Uma alternativa para evitar a formação de etileno e que tem trazido bons resultados no desenvolvimento das plântulas é a adição de tiossulfato de prata (STS), um inibidor da percepção deste hormônio. Baseando-se neste conhecimento, o presente trabalho teve por objetivo avaliar a influência STS no estabelecimento *in vitro* de mirtileiro (*Vaccinium ashei* Reade) cv. Brightwell. Foram utilizados segmentos nodais, em meio de cultura WPM, suplementado com 50 ml de 2iP [100 mg L^{-1}] e 0, 15, 30, 45 e 60µM de STS. Após 60 dias foram avaliadas as seguintes características: porcentagem de explantes sobreviventes e que desenvolveram primórdios foliares, número de gemas com brotações, comprimento da maior brotação e número de folhas. O efeito da concentração de STS utilizada foi significativo ao nível de 5% de probabilidade para os parâmetros número de gemas com brotações e de folhas e favorecidos pelo uso de 45µM de STS.

Termos para indexação: Etileno; STS; micropropagação.

INTRODUCTION

Blueberry is a small temperate berry fruit with its consumption in North America is widespread and is a pioneer species in Brazil, which has been popularized (Nascimento; Schuch; Peil, 2011; Spinardi; Ayub, 2013). Its propagation is done by cuttings or micropropagation because there is variability by seed (Pasqualini; Santos; Ayub, 2016).

Many *in vitro* studies of *Vaccinium spp* have been reported and involve the culture of meristem and the usage of segments containing both lateral and apical buds as explants (Litwińczuk; Wadas-Boroń, 2009; Ross; Castillo, 2009; Debnath, 2011a, b). However one of the problems observed during the *in vitro* establishment of the blueberry is that some explants show active growth, emitting elongated shoots with several buds and juvenile characteristics, while others, paralyze their growth after the emission of first shoot, without elongation, with large and mature leaves (Erig; Schuch, 2006; Jiang et al., 2009).

Plants cultivated *in vitro* synthetize ethylene (Steinitz et al., 2010), which regulates many aspects of plants cycle of life (Lin; Zhong; Grierson, 2009) since seeds germination up to the organs senescence (Bleecker; Kende, 2000). In tissue culture, the ethylene gas produced by the plant volatilizes and remains trapped in the cultivation flasks, around the explants, which may be unfavorable to the development of the culture (Steinitz et al., 2010). Silver ion is capable of specifically blocking

Received in August 19, 2016 and approved in August 16, 2017
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the action of exogenously applied ethylene in classical responses such as abscission, senescence and growth retardation (Beyer, 1976).

Silver thiosulfate (STS) is used to inhibit the ethylene action on the *in vitro* tissue culture (Marutani-Hert et al., 2011). The ethylene receptor, ETR1, contains an ethylene-binding site per homodimer and the bond is mediated by a cofactor Cu present at the ethylene-binding site (Kumar et al., 2009). The mechanism of inhibition is due to the substitution of the cofactor Cu by the Ag + ion, in a conformation such that continually represses the ethylene responses (Binder, 2008).

Silver ions are known to improve somatic embryogenesis, promote the formation of multiple shoots, act in vitro rooting, modify of sex expression, showing which its effect it's closely related to plant morphogenesis (Kumar et al., 2009).

Thus, the aim of this study was to evaluate the STS influence on the *in vitro* establishment of blueberry (*Vaccinium ashei Reade*) cv. Brightwell from nodal segments.

MATERIAL AND METHODS

Semi-woody branches of blueberry cv. Brightwell, collected in the experimental orchard located in Guarapuava, PR (25°23'36"S, 51°27'19" O and 1.120m asl) were cut into segments of 20 cm, placed in jars with water and taken to the heated air-conditioned room (temperature of 25 ^{o}C ± 2 ^{o}C), with 16 hours photoperiod and photon flux density of the light period of 27 µmol m^{-2} s^{-1}. The segments remained in this condition until shoot emission (Silva et al., 2008) of approximately 2 cm, which were detached from the branches. After removing the leaves, the disinfestation of the shoots was preceded in a laminar flow hood. Initially, the material was dipped in alcohol 70% for 30 seconds, followed by immersion in sodium hypochlorite (2,5% active chlorine) and addition of 2 drops of Tween 20 for 10 minutes (Silva et al., 2006). After the disinfection, the branches were washed three times with sterile water. Then the explants were prepared keeping two buds and approximately 1 cm long.

The culture medium utilized for the inoculation of the *in vitro* material was the WPM (Lloyd; McCown, 1980) supplemented with 5 mg L^{-1} 2iP, 30 g L^{-1} sucrose and 100 mg L^{-1} myo-inositol (Schuch et al., 2008). The pH was adjusted to 5.0 before the inclusion of 6 g L^{-1} agar. The culture medium was previously autoclaved at 121 ^{o}C and 1,5 atm for 20 minutes. Silver thiosulfate was at 0.02 M was prepared at the time of use, then sterilized by Millipore® membrane filters with a pore diameter of 0.22 µ and added to the base medium at a temperature of 50-60 ^{o}C.

The experimental design was completely randomized, with five concentrations of STS (0, 15, 30, 45 and 60µM), four replicates and experimental plot with 5 test tubes with an explant in which. After the explants inoculation, the tubes were sealed with plastic cover and maintained in a room with air conditioning (constant temperature of 25 ^{o}C ± 2 ^{o}C), in the dark for seven days (Silva et al., 2006); after that were analyzed: variable percentages of oxidized explants, fungal and bacterial contaminations.

Next the tubes were transferred to the light with a 16 hour photoperiod and photon flux density of the light period of 27 µmol m^{-2} s^{-1}. After 60 days of the experiment installation, the STS influence on the establishment of the *in vitro* blueberry cv. Brightwell was assessed trough the percentage of survivor explants (green) and explants that had developed leaf primordia, number of bud sprouting, length of the biggest sprouting, and number of leaves.

The data of the number of bud sprouting, length of the biggest sprouting, and number of leaves were transformed to √y+1. All of the data were submitted to the Barlett test, followed by analysis of variance and, when significant, compared by Duncan test ($p \leq 0,05$) and polynomial regression by the statistical program SAS (Statistical Analysis System) (SAS, 2008).

RESULTS AND DISCUSSION

After 7 days in the dark, the fungal contamination was 7% for the treatment with 60µM STS. As for the other treatments both bacterial and fungal contaminations was zero and explants oxidation didn´t occured. The phenolic oxidation can be observed in *in vitro* tissue culture during cell disruption, after the excision of explant (Das; Pal, 2005). The explants packaging in the dark during the first week of *in vitro* establishment helps to prevent this phenomenon (Dutra; Wendling; Brondani, 2009).

Data from percentage of survivor explants (green) and explants that developed leaf primordia, after 60 days of *in vitro* establishment, in culture medium containing

different concentrations of STS, are shown in Table 1. In the final evaluation of the experiment there was no significant difference between the treatments in relation with the percentage of survivor explants (green). Among the survivor explants that developed leaf primordia, the best results were observed in treatments that had STS in the composition of the culture medium. For all treatments, the mean percentage of survivor explants was higher than the mean percentage of explants that developed leaf primordia, which shows that not all of the explants that survived developed primordia.

Table 1 – Percentage of survivor explants and of survivor explants that developed leaf primordia, after 60 days of *in vitro* cultive, of blueberry *Vaccinium ashei* Reade cv. Brightwell, in culture medium containing different concentrations of STS.

STS (µM)	Mean Percentages of Survivor Explants	Mean Percentage of Explants with Leaf Primordia
0	86.67 ns	53.33 B
15	73.33	60.00 AB
30	86.67	80.00 AB
45	93.33	86.67 A
60	93.33	86.67 A
Quadratic regression*		ns
CV (%)	18.40	24.39

* Significant to the 5% level of probability. Ns = not significant. Means followed by the same letter do not statistically differ among each other by the Duncan test to the 5% level of probability.

Table 2 shows variance analysis results of the data transformed buds with shootings number, number of leaves, length of the biggest shooting, and Qui-square (χ^2) value from the Barlett test, which showed homogeneity of the treatments variances. To the variable number of buds with shooting and number of leafs, the treatments showed statistical difference. As for the length of the biggest shooting (cm), the treatments showed no statistical difference.

Table 3 shows the effect of the concentrations of STS on the average of the number of buds with shooting, of leaves, and length with the biggest shooting, which showed significant difference. However, the variable length of the biggest shooting did not showed significant difference among the treatments. For the results of the number of buds with shooting there was significance for the quadratic regression (Figure 1).

Table 2 – Variance analysis of number of buds with shooting, number of leaves, length of the biggest shooting in *in vitro* cultivation, of blueberry *Vaccinium ashei* Reade cv. Brightwell, in culture medium containing STS different concentrations.

Number of buds with shooting				
Sources of Variation	G.L.	Mean Square	F	A
Treatment	4	0.27539		
Error	95	0.05524	4.99*	0.0011
Variation Coefficient (%)			16.46	
Qui-square (χ^2)			3.79670*	
Number of leafs				
Sources of Variation	G.L.	Mean Square	F	A
Treatment	4	3.65979		
Error	95	0.60750	6.02*	0.0002
Variation Coefficient (%)			31.46	
Qui-square (χ^2)			1.19549*	
Length of the biggest shooting				
Sources of Variation	G.L.	Mean Square	F	A
Treatment	4	0.02644		
Error	95	0.01118	2.36 ns	0.0584
Variation Coefficient (%)			9.11	
Qui-square (χ^2)			1.21137*	

NS = not significant.
* Significant to the 5% level of probability.

With the use of STS there was a greater number of leafs and buds with shootings in concentrations higher than 30 µM (Table 3), indicating that there was a superior quantity of ethylene in the *in vitro* atmosphere, and that it was harming its development. The maximum number of buds with shooting (1.5561) was obtained with 45 µM STS and the first order derivate of the quadratic function at the inflexion point of the curve corresponding to 42 µM (Figure 1). A significant increase of buds and leaf area was also observed in *Solanum nigrum* L., when the action and the biosynthesis of ethylene were inhibited (Sridhar; Preethi; Naidu, 2011). In critical plants the STS altered the shoot growth at a concentration of 200 mM (Marutani-Hert et al., 2011).

Table 3 – Average of the number of buds with shooting, number of leaves, length of the bigger shooting *in vitro* cultive, of blueberry *Vaccinium ashei* Reade cv. Brightwell, in culture medium containing different concentrations of STS.

STS (µM)	Number of buds with shooting	Number of leaves	Length of the bigger shooting
0	1.2845 C	1.95 B	1.1299 B
15	1.3333 BC	2.12 B	1.1199 B
30	1.5221 A	2.65 A	1.1618 AB
45	1.5561 A	2.99 A	1.2082 A
60	1.4437 AB	2.66 A	1.1813 AB
Quadratic regression*	$y= 1,25151228+0,01269657x+0,00015146x^2$ $R^2=0,83$	ns	ns

Transformed data √(x+1). * Significant to the 5% level of probability. Means followed by the same letter do not statistically differ among each other by the Duncan test to the 5% level of probability.

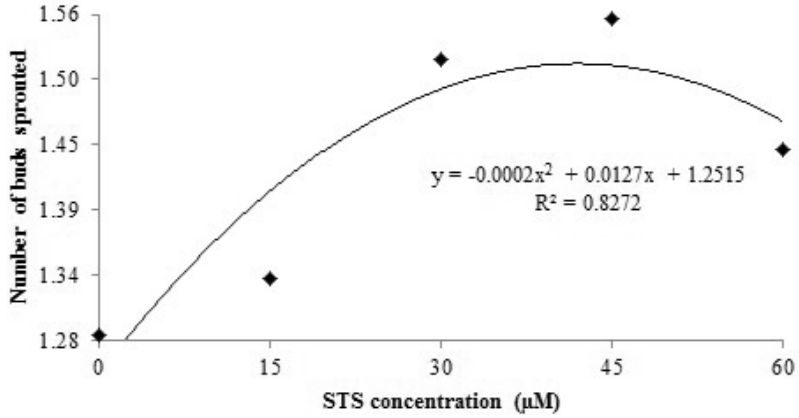

Figure 1 – Relationship between STS concentration (µM), and number of buds with shooting, in blueberry explants of *Vaccinium ashei* Reade cv. Brightwell, after 60 days of *in vitro* cultivation.

On the other hand, it is possible to observe that at the concentration of 60 µM there was a decrease on the average of the variables listed in Table 3, this way, the higher STS concentration can decrease the development of the shoot, due to a greater reduction in the quantity of ethylene, suggesting the need of small quantities of this phytohormone (Sgmma; Thomas; Muelo, 2015).

Ag+ ions present in STS block ethylene production, inhibiting premature aging of the plants (Sridhar; Preethi, Naidu, 2011). The inhibition of ethylene biosynthesis by Ag + ions favors the biosynthesis of polyamines, since both have a common precursor, S-adenosylmethionine (SAM) (Kumar et al., 2009). Evidence proves that ethylene favors the phenomenon of senescence and wich polyamines (PAs) are effective anti-senescence agents and are found to retard chlorophyll loss, membrane deterioration and increased RNase and protease activities, which helps to slow down the senescence process (Pandey et al., 2000). In this study, after 60 days of the experiment installation, the silver thiosulfate inhibited ethylene synthesis, maintaining green leaves, young tissue characteristics as explants without STS treatment had visual signs of senescence, with the presence of internerval anthocyanins, yellowing and loss of leaves (Figure 2). The senescence is the final developmental phase of a leaf which starts with nutrient salvage and ends with cell death (Jing et al., 2005).

Explants treated with STS showed curled leaves and necrotic points (Figure 3). Apricot leaves presented slight leaf necrosis after addition of STS, being related to the slight toxicity of the compound (Burgos; Albuquerque, 2003). It is suggested the use of STS in the first 30 days of the *in vitro* establishment, to encourage the emergence of shoots and leaves, followed by STS-free medium in order to avoid toxicity however, further tests are needed.

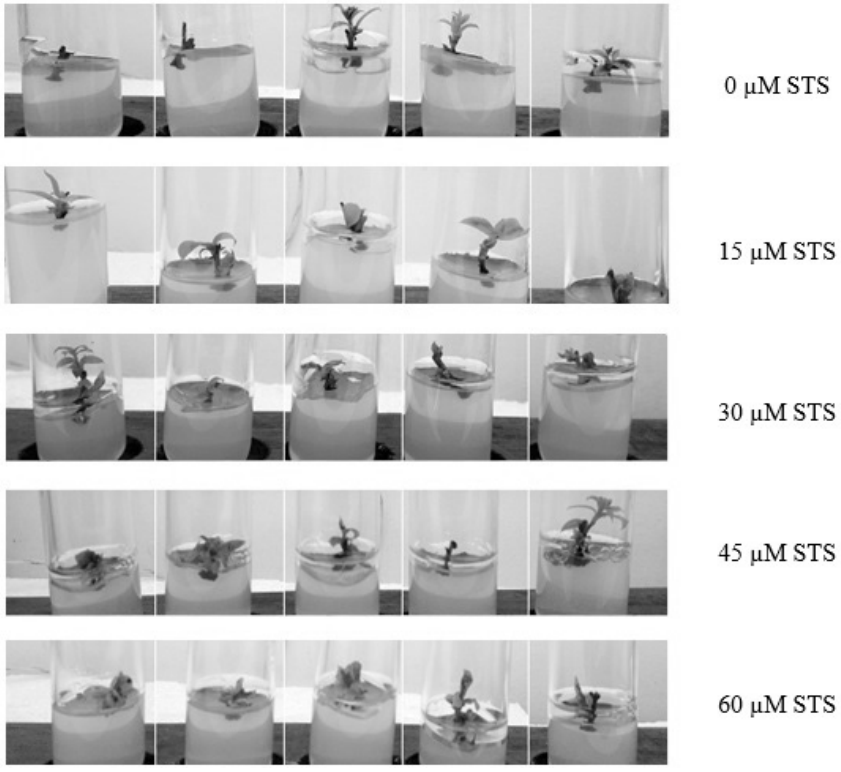

Figure 2 – Silver thiosulfate effect on *in vitro* blueberry (*Vaccinium ashei Reade*) cv. Brightwell; establishment after 60 days of cultivation.

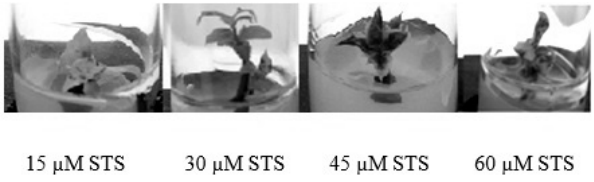

Figure 3 – Blueberry *Vaccinium ashei Reade* cv. Brightwell leaf necrosis of, treated with silver thiosulfate, after 60 days of *in vitro* cultivation.

CONCLUSION

The use of STS at 45 µM enables vegetative buds and leaves development in *Vaccinium ashei Reade* cv. Brightwell, and reduces leaf senescence.

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