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ETHANOL AND NITRIC OXIDE IN QUALITY MAINTENANCE OF 'GALAXY' APPLES STORED UNDER CONTROLLED ATMOSPHERE[1]

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ABSTRACT - The aim of this work was to evaluate the effect of ethanol and two nitric oxide dose applications on the maintenance of the post-storage quality of 'Galaxy' apple during storage under controlled atmosphere (CA). Treatments evaluated were: [1] 1.2 kPa O_2 + 2.0 kPa CO_2; [2] CA + 20 μL L^{-1} of nitric oxide, [3] CA + 40 μL L^{-1} of nitric oxide; [4] CA + 1 mL of ethanol kg^{-1} fruit. Fruits received treatments before storage and were kept under CA during eight months and seven days of storage at 20 °C. Fruits had been kept on CA for eight months and seven days at 20° C. Fruits treated with ethanol showed higher ethylene production, low flesh firmness, high flesh breakdown, mealiness and acetaldehyde production. Fruits treated with 40 μL L^{-1} nitric oxide showed lower ethylene production, respiration rate and ACC oxidase ((1-aminocyclopropane-1-carboxylic acid) oxidase enzyme activity. Apples treated with 20 uL L^{-1} nitric oxide showed higher ethylene production, respiration rate, internal ethylene concentration CO_2 and ethanol concentration. Ethanol and nitric oxide application before storage have no benefits in maintaining fruit quality after storage under CA due to lower flesh firmness, higher mealiness incidence, flesh breakdown and decay incidence.
Index terms: Ethylene, respiration rate, fermentation compounds, *Malus domestica*.

ETANOL E ÓXIDO NÍTRICO NA MANUTENÇÃO DA QUALIDADE DE MAÇÃS 'GALAXY' ARMAZENADAS EM ATMOSFERA CONTROLADA

RESUMO - O objetivo do trabalho foi avaliar o efeito da aplicação de etanol e duas doses de óxido nítrico na manutenção da qualidade de maçãs 'Galaxy' durante o armazenamento em atmosfera controlada (AC). Tratamentos utilizados: [1] 1,2 kPa O_2 + 2,0 kPa CO_2; [2] AC + aplicação de 20 μL L^{-1} de óxido nítrico; [3] AC + aplicação de 40 μL L^{-1} de óxido nítrico; [4] AC + aplicação de 1 mL de etanol kg^{-1} de fruto. Os frutos foram mantidos em AC durante oito meses e sete dias a 20º C. Frutos tratados com etanol apresentaram maior produção de etileno, menor firmeza, elevada degenerescência, polpa farinácea e produção de acetaldeído. Já, os frutos tratados com 40 μL L^{-1} de óxido nítrico apresentaram menor produção de etileno, taxa respiratória e atividade da enzima ACC (1-aminociclopropano-1-carboxílico) oxidase. Maçãs tradadas com 20 μL L^{-1} de óxido nítrico apresentaram aumento na produção de etileno e respiração, etileno e CO_2 interno, e produzem mais etanol. A aplicação de etanol e óxido nítrico não apresenta benefícios na manutenção da qualidade dos frutos após o armazenamento em AC, devido à menor firmeza de polpa, maior ocorrência de polpa farinácea, degenerescência de polpa e incidência de podridões nos frutos.
Termos para indexação: Etileno, taxa respiratória, compostos da fermentação, *Malus domestica*.

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INTRODUCTION

The Brazilian production of apples reached more than 1.3 million tons in the 2014 harvest (IBGE, 2014). Most of this production is concentrated in the mountainous regions of Rio Grande do Sul and Santa Catarina. More than half of the production belongs to the 'Gala' cultivar and its mutants, such as 'Galaxy', which stands out for its excellent quality, attractive taste and intense red peel coloration, thus being more accepted by the consumer market (BRACKMANN et al., 2009), in addition to high succulence and good balance between acidity and soluble solids, (THEWES et al., 2015).

Given that the apple harvest is concentrated in a short period of time, from early February to late April, storage techniques are required to maintain fruit quality and increase the period of supply to the consumer market. A widely used technology is controlled atmosphere (CA). However, large losses still occur after storage under conventional CA due to loss of flesh firmness, decay occurrence and physiological disturbances, requiring the use of alternative products in conjunction with CA.

The application of post-harvest ethanol may be an advantageous technique for the conservation of fruits, vegetables and flowers (SUZUKI et al., 2004; ASODA et al., 2009). Due to the fact that ethanol is a natural compound, it can be used for the conservation of plant products in organic or agroecological systems (PESIS, 2005). The use of post-harvest ethanol may be feasible, since the treatment is simple and inexpensive, is easy to apply and has few negative effects when applied at the correct dose (ASODA et al., 2009). The application of ethanol has a suppressive effect on the transducing genes of ACC synthase enzyme BO-ACS1 and BO-ACS2, and ACC oxidase enzyme BO-ACO1 and BO-ACO2, both precursors of ethylene synthesis (PODD, VAN STADEN, 1998; ASODA et al, 2009), resulting in lower ethylene production and fruit quality conservation after storage, and according to Thewes et al. (2015), endogenous ethanol production decreases the activity of the ACC oxidase enzyme and reduces physiological disturbance in fruits maintained at 0.4 kPa O_2. The application of ethanol vapor in plum Reubennel cv. provided better maintenance of total soluble solids and reduced the microbial population (BENATO et al., 2015). Weber et al. (2015) verified that the application of 0.3 mL of ethanol kg^{-1} fruit inhibited the ACC oxidase enzyme activity and consequently ethylene production, and aided in the maintenance of flesh firmness and titratable acidity of 'Royal Gala' apples.

In addition to the application of ethanol, an alternative that needs further studies is the application of nitric oxide. Some studies have indicated that the application and nitric oxide maintains the quality of strawberries stored after harvest (WILLS et al., 2000, WILLS et al., 2007; ZHU; ZHOU, 2007). The exposure of fruits and vegetables to low concentrations of gaseous nitric oxide causes improvements in the maintenance of post-harvest life, delays ripening, and decreases ethylene synthesis (LESHEM et al., 1998), increases resistance to pests, diseases and reduces the activity of the ACC synthase enzyme in strawberries treated with sodium nitroprusside at concentration of 5.0 μmoL L^{-1} (ZHU; ZHOU, 2007). Yamasaki et al., (2001) suggest that nitric oxide acts on the suppression of ATP synthase in plant mitochondria, which can be attributed to the inhibitory effect on the cytochrome of the electron transport chain. In bananas, the exogenous application of nitric oxide can reduce damages caused by storage at low temperatures due to the increase of antioxidants in the defense system and secondary metabolites (WANG et al., 2013). Rudell and Mattheis (2006) reported that the application of sodium nitroprusside in 'Golden Delicious' apples inhibited the ACC synthase enzyme activity.

In this context, the aim of this study was to evaluate the effect of the application of ethanol and two doses of nitric oxide in the maintenance of the post-harvest quality of 'Galaxy' apples stored under controlled atmosphere.

MATERIAL AND METHODS

The experiment was conducted at the Laboratory of Post-Harvest Research, Department of Plant Science, Federal University of Santa Maria, Rio Grande do Sul (RS). The fruits used were harvested in a commercial orchard of Vacaria, RS. After harvest, apples were immediately transported to the where they were selected, discarding fruits that presented decay, injury or mechanical damage from transport. After homogenization of experimental samples, treatments were applied. A completely randomized design with four replicates of 30 fruits was used. Treatments evaluated were: [1] CA with 1.2 kPa O_2 + 2.0 kPa CO_2 (control); [2] CA + 20 μL L^{-1} nitric oxide; [3] CA + 40 μL L^{-1} nitric oxide; [4] CA + application of 1ml kg^{-1} ethanol. Ethanol was applied at the beginning of storage for three days. The ethyl alcohol concentration used was 1 mL of 92.5% kg^{-1} fruit monthly, where fruits were exposed to the compound within the experimental CA mini-chamber. Nitric oxide application was carried out

by injecting the gas into the storage chamber up to concentration of 20 μL L^{-1} for treatment 2 and 40 μL L^{-1} for treatment 3. Fruits were exposed to nitric oxide for the period of 24 hours and then, 1.2 kPa O_2 + 2.0 kPa CO_2 atmosphere was applied for CA storage.

Fruits were packed in CA mini-chamber, hermetically sealed, located inside a cold chamber at temperature of 1.5° C. CA was obtained through the dilution of oxygen, with the injection of nitrogen from an N_2 generator using the "Pressure Swing Adsorption" (PSA) principle. CO_2 injection from a high pressure cylinder was performed up to the pre-set level of 2 kPa. The mini-chamber atmosphere was monitored and corrected daily by automated equipment (Valis®, Lajeado, RS, Brazil), which corrected CO_2 and O_2 levels according to a pre-defined set point. Temperature monitoring was performed daily using high precision thermometer (± 0.1 ° C).

Laboratory analyses were performed after eight months of storage plus seven days of shelf life at 20°C. The following parameters were analyzed: a) ethylene production and respiratory rate: 10 fruits were packed in an airtight container with volume of 5 L, then sealed and kept at 20°C, after two hours, the respiratory activity was determined by the difference of CO_2 concentration (before and after 2 hours) inside the container. To determine ethylene production, 1 mL samples of the gas inside the container were collected with a syringe and then injected into a VARIAN® model CX 3400 chromatograph equipped with 1/8 "stainless steel column of 2.00 m in length, prepared with Porapak N80 / 100 and flame ionization detector (FID); results were expressed as μL C_2H_4 kg^{-1} h^{-1}; b) ACC oxidase enzyme activity: determined from peel samples (3g) collected from the equatorial region of fruits. These were immersed in a solution containing 0.1 mM ACC (1-carboxylic -1-aminocyclopropane acid) in a MES (2- (N-morpholino) ethanesulfonic acid) buffer solution at pH 6.0. After 30 minutes, samples were packed in 50 ml hermetically sealed syringes adding 1 ml CO_2 and after 30 minutes, the ethylene concentration contained in syringes was determined by means of chromatograph used to determine ethylene production, with data being expressed as nL C_2H_4 $g^{-1}h^{-1}$, according to Bufler (1986); c) mass loss: obtained by the difference between the initial weight of samples and their weight after storage, results expressed as percentage; d) flesh firmness: determined by drilling the fruit pulp with a penetrometer, equipped with 11 mm tip, on two opposite sides in the equatorial region of the fruit, where the peel was previously removed, and data were expressed in Newton (N); e) internal space: measured with a vacuum pump, which removed the air present inside the fruit, performing a suction pressure of 565 mm of mercury. Before and after the procedure, the sample was weighed and by subtraction between initial weight and final weight, the internal space percentage was determined; f) Total soluble solids (SS): value obtained by refractometry, with temperature correction, and data expressed as ° Brix; g) flesh breakdown: determined by the percentage of fruits that presented internal darkening, with data expressed as percentage. h) healthy fruits: obtained by counting fruits that did not present any decay, pulp crack, flesh breakdown and mealiness, expressed as percentage; i) internal ethylene and internal CO_2: determined according to methodology proposed by Brackmann et al. (2014), with values expressed as μL L^{-1} and mL 100 mL^{-1}, respectively; j) pulp crack: determined by percentage of fruits that had peel fissures, with results expressed as percentage; l) decay incidence: by counting fruits with internal and external signs of decay greater than 5 mm in diameter, with results expressed as percentage; m) mealiness: obtained by counting fruits with little succulence and dry pulp, with floury aspect, values expressed as percentage; n) Titratable acidity: determined by titration with 0.1 N NaOH of a solution containing 100 mL of juice diluted in 100 mL of distilled water, up to pH 8.1, with results expressed as meq 100 mL^{-1}; o) acetaldehyde, ethanol and ethyl acetate: according to the methodology of Saquet and Streif (2008), where samples were collected from the equatorial region of fruits and crushed in Philips Walita® centrifuge to extract the juice. Subsequently 10 ml of the juice were placed in 20 mL vial bottles. The juice sample was exposed to a 70 ° water bath for 30 minutes, so that the fermentation compounds volatilized into the flask headspace. Afterwards, a 100 μL sample of the headspace air was injected into Dani® gas chromatograph equipped with a 60m long capillary column (DN-WAX Dani®). The injector, oven and detector temperatures were 140, 60 and 250° C, respectively. Values were expressed in μL L^{-1}.

Data were submitted to analysis of variance (ANOVA). Percentage data were transformed by the arcsin $\sqrt{x}$ / 100 formula before being submitted to ANOVA. Data that presented significant difference in ANOVA were submitted to principal component analysis (PCA), but before that, data were scaled to obtain the same weights for all variables (mean = 0 and variance = 1), and for the accomplishment of PCA, The Unscrambler® X software (version 9.7, CAMO A / S, Trondheim, Norway) software was used. For comparison of means, the Scott-knott test

($p < 0.05$) was used.

RESULTS AND DISCUSSION

The application of ethanol increased fruit ethylene production at two and four days of shelf life (Figure 1). On the other days, the ethylene production rate did not differ among treatments. The increase in ethylene production may have been caused by the high dose applied, since according to Pesis (2005) and Plotto et al. (2006), the response of fruits to ethanol depends on several factors among them, the application dose. The ethanol application decreased the respiratory rate of fruits at two shelf life days when compared to control fruits (Figure 1). In respiration, fruits exposed to ethanol did not differ from treatment fruits with the application of 20 μL L^{-1} nitric oxide. The lowest respiration rate at two days at 20°C occurred in fruits receiving 40 μL L^{-1} nitric oxide (Figure 1). Fruits with nitric oxide applications showed a significant difference in respiration and ethylene production when compared to control fruits and with ethanol application at two days of shelf life. Higher ethylene production was observed at four days in fruits exposed to 20 μL L^{-1} nitric oxide compared to those receiving 40 μL L^{-1} nitric oxide (Figures 1), indicating that the nitric oxide dose of 20 μL L^{-1} was less efficient in reducing ethylene production.

Fruits submitted to 40 μL L^{-1} nitric oxide and ethanol presented lower ACC oxidase enzyme activity (Table 1). According to Zhu and Zhou (2007), the application of nitric oxide in strawberries has no effect on the conversion of ACC into ethylene, acting only on the inhibition of the ACC synthase enzyme. The ACC synthase enzyme acts on the formation of ACC, which is transformed into ethylene by ACC oxidase (LIEBERMAN, 1979; YANG; HOFFMAN, 1984). However, in ethanol treatment, lower enzyme activity did not result in lower ethylene production during shelf life. This fact may have occurred due to the depletion of the enzyme activity during the first days of shelf life at 20° C in which high ethylene production was observed, and at the moment when the ACC oxidase enzyme activity was evaluated, it was low, since the evaluation of the ACC oxidase enzyme activity was performed at 7 days at 20° C and the highest ethylene production occurred at 2 days.

Fruits submitted to ethanol application had lower flesh firmness (Table 2), probably because the dose applied was very high, which may have caused damage to the fruit tissue, resulting in higher ethylene production and its effects on the reduction of flesh firmness. Fruits did not tolerate the dose of 1mL kg^{-1} fruit at the beginning of storage, which may have stimulated cell wall degradation enzymes due to increased ethylene production (HARB et al., 2012). This damage may have led to a significant increase in flesh breakdown (Table 2) and mealiness (Table 1). According to Singh et al. (2009), the application of 20 μL L^{-1} gaseous nitric oxide in plums decreases the loss of flesh firmness up to six weeks of shelf life, which was not observed in the present work, where flesh firmness of fruits treated with 20 and 40 μL L^{-1} nitric oxide did not differ from control fruits.

The internal ethylene concentration (IEC) was higher in fruits submitted to the application of 20 μL L^{-1} nitric oxide and ethanol (Table 1). Fruits submitted to 20 μL L^{-1} nitric oxide also had higher internal CO_2 concentration (ICO_2) (Table 2). The application of 40 μL L_{-1} nitric oxide resulted in fruits with higher decay incidence after seven days of storage. At the exit of the chamber, no difference for this parameter was observed.

The production of fermentation compounds during the storage period is extremely important, since studies have indicated that the exogenous application of ethanol decreases the expression of ACC synthase and oxidase genes in broccoli treated with ethanol due to the suppression of transcription at molecular level (ASODA et al., 2009). The production of these compounds occurs naturally under normoxic oxygen conditions, in proportion of one part of acetaldehyde to 100 parts of ethanol (PODD; VAN STADEN, 1998; ZABALZA et al., 2009); however, when stored under hypoxic conditions, there is an increase in the synthesis of both. Compounds from fermentation have great influence on several metabolic routes, ethylene synthesis, aroma production, respiratory metabolism, and expression of some enzymes and proteins, thus acting on the post-harvest quality of fruits, vegetables and flowers (PODD; VAN STADEN, 1998, PESIS, 2005, ASODA et al., 2009, MORI et al., 2009, BOTONDI et al., 2012; LIU et al., 2012). However, when present in the fruit in large amounts, they may increase the occurrence of physiological disorders. The ethanol concentration of fruits was higher with application of 20 μL L^{-1} nitric oxide, comparing to fruits of the other treatments. The application of ethanol in fruits caused an increase in acetaldehyde concentration due to ethanol being converted into acetaldehyde through the alcohol dehydrogenase enzyme (ADH). No decrease in ethylene production was observed due to the action of acetaldehyde. Control fruits did not produce ethyl acetate, whereas fruits exposed to 40 μL L^{-1} nitric oxide had low ethyl acetate production, indicating a decrease in the

fermentative route (Figure 3). Fruits exposed to 20 µL L^{-1} nitric oxide and ethanol produced more ethyl acetate than the other treatments, probably because these compounds stimulate the activity of the alcohol acetyltransferase enzyme (AAT). According to Liu et al. (2012), ethyl acetate is produced through the action of the alcohol acetyltransferase enzyme (AAT), which converts ethanol into ethyl acetate and other esters responsible for fruit aroma.

To better visualize the effect of treatments, principal component analysis (PCA) was performed, where treatment fruits had different responses when compared to each other (Figure 2). The ACC oxidase enzyme activity and internal ethylene concentration showed correlation with fruits of the control treatment. The production of fermentation compounds, ethanol and ethyl acetate, had correlation with treatment of 20 µL L^{-1} nitric oxide applied to fruits, which can be explained by the lower correlation with the production of acetaldehyde, which is precursor of ethanol and ethyl acetate. During respiration, ethylene production at two and four days of shelf life, acetaldehyde production, flesh breakdown and mass loss, showed a correlation with fruits that received ethanol application, and the higher the mass loss of fruits of this treatment allowed a greater diffusion of gases from the pulp of fruits, explaining the lower ethylene and CO_2 concentration (BRACKMANN et al., 2014). The decay incidence in fruits at seven days of shelf life at 20ºC had correlation with the 40 µL L^{-1} treatment of nitric oxide, presenting unfavorable marketing conditions.

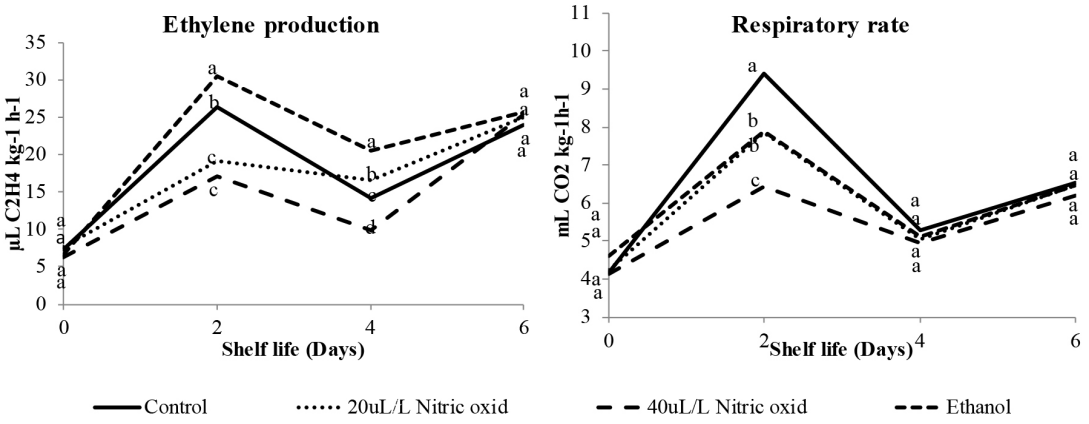

FIGURE 1- Ethylene production (a) and respiratory rate (b) of 'Galaxy' apples stored under controlled atmosphere plus seven days of shelf life at 20° C. Santa Maria, Brazil, 2015.

* Treatments with averages not followed by the same vertical letter differ by the Scott-knott test at 5% error probability.
ns = non-significant difference.

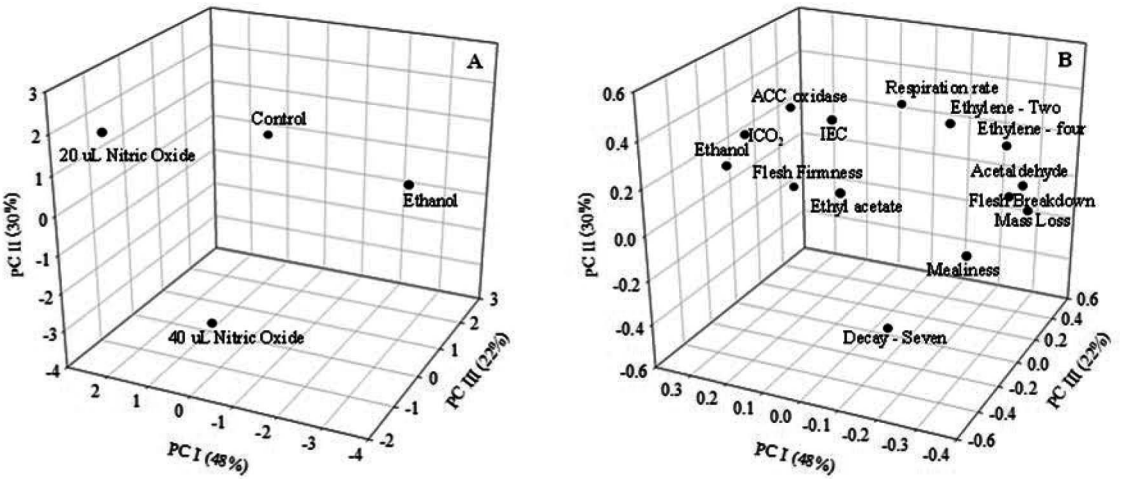

FIGURE 2- Principal Component Analysis (PCA) showing the three main components (A - Treatments and B - variables analyzed) of 'Galaxy' apples submitted to the application of nitric oxide and ethanol, and after controlled atmosphere storage for eight months. Santa Maria, Brazil, 2015.

TABLE 1-ACC oxidase enzyme activity, mass loss, internal space, soluble solids (SS), flesh firmness, farinaceous pulp, healthy fruits and internal ethylene concentration (IEC) of 'Galaxy' apples stored under controlled atmosphere. Santa Maria, Brazil, 2015.

Treatments	ACC oxidase ($\mu L\ C_2H_4\ g^{-1}h^{-1}$)	Mass loss (%)	Internal space ($g\ 100g^{-1}$)	SS °Brix
Initial analysis	0.27*	0.00	-	11.4
Control	17.4 a	1.62 b	15.7 a	12.2 a
20µL L^{-1} Nitric Oxide	18.0 a	1.13 b	17.6 a	12.2 a
40µL L^{-1} Nitric Oxide	12.9 b	1.49 b	17.3 a	12.1 a
Ethanol	13.5 b	2.18 a	16.4 a	12.4 a
VC (%)	19.10	26.36	9.77	1.82
Treatments	Flesh firmness (N)	Mealiness (%)	Healthy fruits (%)	IEC ($\mu L\ L^{-1}$)
Initial analysis	78.74	0.00	100	-
Control	70.9 a	13.5 c	88.5 a	49.1 b
20µL L^{-1} Nitric Oxide	68.7 a	17.7 b	81.2 a	90.0 a
40µL L^{-1} Nitric Oxide	68.9a	20.3 b	77.5 a	35.9 b
Ethanol	65.5 b	26.1 a	75.6 a	69.6 a
VC (%)	2.93	12.2	9.5	22.3

* Treatments with averages not followed by the same vertical letter differ by the Scott-Knott test at 5% error probability.

TABLE 2- Incidence of pulp cracking, decay incidence at the chamber opening and at seven days of shelf life at 20° C. flesh breakdown, internal CO_2 and titratable acidity values, of 'Galaxy' apples stored under controlled atmosphere, Santa Maria, Brazil, 2015.

Treatments	Pulp cracking (%)		Decay incidence (%)	
	Chamber opening	Seven days of shelf life	Chamber opening	Seven days of shelf life
Initial analysis	0.00	0.00	0.00	0.00
Control	0.00 a*	0.00 a	1.00 a	1.04 b
20µL L^{-1} Nitric Oxide	0.00 a	0.00 a	1.00 a	1.04 b
40µL L^{-1} Nitric Oxide	0.00 a	0.00 a	1.00 a	5.36 a
Ethanol	0.83 a	0.86 a	0.83 a	2.62 b
VC (%)	40.12	40.95	65.88	41.25

Treatments	Flesh breakdown (%)	Internal CO_2 (mL CO_2 L^{-1})	Titratable acidity (mEq 100 mL^{-1})
Initial analysis	0.00	-	5.22
Control	0.00 b	34.24 b	4.08 a
20µL L^{-1} Nitric Oxide	0.00 b	46.17 a	3.84 a
40µL L^{-1} Nitric Oxide	0.00 b	28.72 b	3.89 a
Ethanol	1.72 a	27.25 b	3.95 a
VC (%)	42.93	21.69	4.02

* Treatments with averages not followed by the same vertical letter differ by the Scott-knott test at 5% error probability.

TABLE 3- Fermentation compounds (acetaldehyde, ethanol and ethyl acetate) of 'Galaxy' apples, stored under controlled atmosphere after 8 months of storage and seven days shelf life at 20° C, Santa Maria, Brazil, 2015.

Treatments	Fermentation compounds (µL L^{-1})		
	Acetaldehyde	Ethanol	Ethyl acetate
Control	2.15 b*	48.7 b	0.00 b
20µL L^{-1} Nitric Oxide	1.60 b	67.2 a	0.87 a
40µL L^{-1} Nitric Oxide	1.74 b	49.5 b	0.35 b
Ethanol	3.33 a	43.1 b	0.71 a

* Treatments with averages not followed by the same vertical letter differ by the Scott-Knott test at 5% error probability.

CONCLUSION

The application of nitric oxide at doses of 20 and 40 µL L^{-1} and ethanol at 1 mL kg^{-1} did not bring benefits in maintaining the quality of 'Galaxy' apples stored under CA 1.2 kPa O_2 + 2.0 kPa CO_2, due to the lower flesh firmness, higher occurrence of mealiness, flesh breakdown and higher decay incidence. Further studies should be carried out evaluating other doses of these products, since the effect on quality is dependent on the dose applied.

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