Treatment with nitric oxide preserves the quality of cold stored 'Cripps Pink' apples

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ABSTRACT: The objective of this work was to evaluate the effect of pre-storage fruit treatment with nitric oxide (NO) on the quality maintenance of cold stored 'Cripps Pink' apples. The following treatments were evaluated: 0 µL·L^{-1} of NO (control); 10 µL·L^{-1} of NO for 2 h; 10 µL·L^{-1} of NO for 4 h; 10 µL·L^{-1} of NO for 8 h; and 20 µL·L^{-1} of NO for 2 h. Fruit treated with NO, regardless of concentration and application time, had a greener skin background color than the control, especially those treated with 10 µL·L^{-1} for 8 h. After 7 days of shelf life (23 ± 3 °C/relative humidity, RH, of 65 ± 5%), fruit treated with 10 µL·L^{-1} for 8 h and 20 µL·L^{-1} for 2 h had lower respiration rate and a greener skin background color than the control. Fruit treated with NO, regardless of concentration and application time, had a lower incidence of flesh browning after storage. The use of NO (10 µL·L^{-1} applied for 8 h and 20 µL·L^{-1} applied for 2 h) pre-storage maintains the quality of 'Cripps Pink' apples, because it delays skin yellowing and reduces flesh browning. Nitric oxide has no effect on the maintenance of flesh firmness in 'Cripps Pink' apples.

Key words: *Malus domestica*; Pink Lady; physiological disorder; flesh browning; skin background color.

INTRODUCTION

'Cripps Pink' apple is one of three varieties that can be marketed as Pink Lady. Such fruits must meet a minimum standard of quality, which includes having more than 40% (in Asia > 40% and < 60%) of the surface covered with a strong pink to shiny red color, flesh firmness of at least 6.5 kg·cm^{-2} (± 64 N), soluble solids (SS) of at least 13 °Bx, and, on average, only 1% of fruits with internal defects (Pink Lady 2020).

'Cripps Pink' apples produced in South America are exported as Pink Lady to various countries on many continents, and can take, from harvest, up to 70 days to arrive at their destination. During this period, the apples are kept under refrigeration, during which fruit ripening and loss of quality, reduction of flesh firmness, skin yellowing and development of skin greasiness and flesh browning can occur. The apples are picked at the limit of the flesh firmness desirable, according to the quality standards, especially in orchards covered by an anti-hail net, where the development of the required red coloration is delayed. It is well established that flesh firmness at harvest is strongly correlated with storage potential, since fruits harvested with a lower flesh firmness tend to have less storage potential. So, it is essential to identify technologies complementary to refrigeration to allow a greater delay in ripening and to maintain quality for as long as possible.

Fruit ripening involves alterations initiated by ethylene. Thus, controlling ethylene during storage, transport and commercialization is essential (Keller et al. 2013). Some consolidated technologies are available for this purpose, for example using ethylene absorbers (Keller et al. 2013) and fruit treatment with 1-MCP (Williamson et al. 2018).

Ethylene absorption is mainly carried out using products based on potassium permanganate impregnated in materials, normally a mineral, of high surface area. The main disadvantages of this technology are related to the regenerative nature and the significant generation of by-product (residue), as well as the larger amount of product needed to effectively keep a low concentration of ethylene in the environment (Keller et al. 2013).

The 1-MCP treatment has shown excellent results in the maintenance of apple quality in cold storage (Amarante et al. 2010; Hackbarth et al. 2017; Williamson et al. 2018). However, this technology is expensive and has several disadvantages, such as its negative impact on the aroma of the fruit (Thewes et al. 2015) and is not effective on control postharvest fruit decay.

Nitric oxide (NO) is a reactive nitrogen species that is naturally produced in live cells and is involved in the regulation of many processes during plant development (Kolbert et al. 2019; Manjunatha et al. 2010; Palma et al. 2019). The exogenous application of NO, at low concentrations, delays the maturation process (Abdollahi et al. 2013; Mukherjee 2019). Its effect on maturation and senescence can be attributed, at least in parts, to the reduction of the synthesis of ethylene through the inhibition of the enzymes acid 1-aminocyclopropane-1-carboxylic (ACC) synthase and ACC oxidase (Manjunatha et al. 2012; Palma et al. 2019). Nitric oxide can also help maintain the postharvest quality of fruits through the control of oxidative stress via induction of enzymes, such as superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) and the suppression of lipoxygenase (LOX) (Manjunatha et al. 2010; 2012; Palma et al. 2019), being able to prevent the manifestation of physiological disturbances. However, this effect of NO is dependent of its concentration and the species of fruits (Manjunatha et al. 2010; Palma et al. 2019). Moreover, NO reduces the incidence of postharvest rot (Abdollahi et al. 2013; Palma et al. 2019). Nitric oxide gas treatment could be an option for maintenance the quality of 'Cripps Pink' (or Pink Lady) apples during storage and transport to distant markets.

The objective of this study was thus to evaluate the effect of treatment with NO (dose and application time) on the maintenance of the quality of cold stored 'Cripps Pink' apples.

METHODS

The experiment was carried out with 'Cripps Pink' apples harvested in 2018/2019, from a commercial orchard located in the municipality of Vacaria, RS (50°42" W; 28°33' S; 955 m of altitude), southern Brazil. The treatments evaluated were of 0 $\mu L \cdot L^{-1}$ of NO (control); 10 $\mu L \cdot L^{-1}$ of NO for 2 h; 10 $\mu L \cdot L^{-1}$ of NO for 4 h; 10 $\mu L \cdot L^{-1}$ of NO for 8 h; and 20 $\mu L \cdot L^{-1}$ of NO for 2 h. These NO concentrations were chosen because of results obtained in other studies with various types of fruits. The highest concentration was applied only during 2 h because, in theory, a higher concentration requires a shorter treatment time, and it was not possible to test the combination of all factors due to the limited amount of fruit and infrastructure available for this study.

For the application of NO, gas from a high-pressure cylinder was used (nitric oxide 1000 $\mu L \cdot L^{-1}$ + N^2 balance). Nitric oxide was added to the interior of air-tight chambers with a volume of 450 L, until it reached the concentrations required for each treatment (10 and 20 $\mu L \cdot L^{-1}$), with the fruits being exposed to the treatment, for the required time in an environment with 1 kPa of O_2 + ≤ 0.1 kPa of CO_2. The treatments were done in this environment (with low O_2) to prevent the reaction of NO with O_2 (forming NO_2), and thus guaranteeing its effectiveness (Liu 2016).

After treatment, the apples were kept for 70 days under cold storage (1.5 ± 0.2 °C and relative humidity, RH, of 94 ± 2%), simulating the period of transport for 'Cripps Pink' apple for exportation, followed by 7 days of shelf life (23 ± 3 °C and Relative Humidity of 65 ± 5%).

At harvest, the apples were evaluated for skin background color, percentage of red color, flesh firmness, titratable acidity (TA), SS and iodine-starch index. After storage, at the time of chamber opening, ethylene production and respiration rates, skin background color, greasiness and incidence of rot and cracks were evaluated. After 7 days of shelf life, in addition to the same attributes evaluated at the time of chamber opening, the variables flesh firmness, TA, SS and flesh browning incidence were evaluated.

To quantify ethylene production (ηmol $C_2H_4 \cdot kg^{-1} \cdot s^{-1}$) and respiration ($\eta$mol $CO \cdot kg^{-1} \cdot s^{-1}$) rates, the apples in each replicate were packed in 4.1 L containers, with an air-tight seal, according to Brackmann et al. (2017). After 30 min, a plastic syringe of 1.0 mL was used to collect three samples of air from the atmosphere of the free space from these containers. These samples were then injected into a gas chromatographer (Clarus 580, Perkin Elmer, USA), equipped with a Porapak N column of 3 m length (80–100 mesh) and a flame ionization detector. The temperatures of the column, the detector and the injector were 70, 250 and 130 °C, respectively. The flows of nitrogen, hydrogen and synthetic air were 70, 30 and 300 mL·min⁻¹, respectively. The respiration rate was quantified using an electronic gas analyzer (O_2 and CO_2) (Schelle, Germany), with the circulation of CO_2 in a closed circuit.

The iodine-starch index (scale of 1–5, where index 1 indicates the maximum starch content, and index 5 represents totally hydrolyzed starch) was determined by cutting the fruit through the equatorial region, and immersing the cut surface of the stem half in iodine solution (12 g metallic iodine + 24 g potassium iodine/1 L of distilled water) (Amarante et al. 2010).

The flesh firmness (N) was determined in two opposing regions, in the equatorial portion of the fruit, with a small portion of the skin previously removed, with the aid of a motorized penetrometer (Güss Manufacturing Ltd., South Africa), with an 11 mm diameter tip (Amarante et al. 2010).

The percentage of red coloring on the fruit was determined visually by examining the fruit surface, estimating the total area of the fruit that was covered by red color (Amarante et al. 2009). The background color of the skin was measured in terms of lightness (L), chroma (C) and angle hue (h°), in an area with the least red coloration, with the aid of a colorimeter (CR 400, Konica Minolta, Japan) (Brackmann et al. 2017).

The TA (% of malic acid) was determined in a sample of 5 mL of juice extracted from transverse slices taken from the equatorial region of the apples and shredded in an electric centrifuge. This sample was diluted in 45 mL of distilled water and titrated with an automatic titrator (Titro Line Easy, Schott Instruments, Germany), with a sodium hydroxide solution of 0.1 N until pH 8.1 (Amarante et al. 2010).

The SS content (%) was determined with a digital refractometer (PR 201α, Atago, Japan), using the juice extracted from the fruit as mentioned for TA (Amarante et al. 2010). The SS/TA ratio was then calculated.

The incidence (%) of rot, cracks and flesh browning was evaluated by counting the number of apples with these symptoms (Brackmann et al. 2017). To evaluate flesh browning, the apples were cut transversely in the equatorial region and analyzed for the presence of a diffuse brown color in the flesh.

The rate of skin greasiness was evaluated using methodology described by Yang et al. (2017), subjectively by rubbing fruit against the hand and scoring the degree of greasiness as none (0), slight (1), moderate (2), or severe (3).

The experimental design was entirely randomized. Each treatment was composed of five replicates, with each experimental unit composed of 30 fruits. The values in % were transformed by the formula arcsine $[(x+0.5)/100]^{1/2}$ prior to analysis. The data was submitted to variance analysis and the averages of the treatments were compared by LSD test ($p < 0.05$).

RESULTS AND DISCUSSION

At harvest the fruit presented the following attributes: flesh firmness of 82.9 N, TA of 0.48%, SS of 12.4%, iodine-starch index of 4.0, 55% of the surface of the fruit with a strong pink to red color, and the h°, C and L of background color of the skin were 103.7, 42.6 and 73.5, respectively.

At the time chamber opening, after 70 days of storage, the fruit treated with 10 µL·L⁻¹ of NO for 4 h presented the lowest ethylene production rate, but without differing significantly from the 10 µL·L⁻¹ of NO for 8 h treatment. However, after 7 days of shelf life, the ethylene production rate did not differ between treatments (Table 1). The effect of NO on ethylene biosynthesis is due to the stoichiometric reduction of ACC to 1-(malonylamino)cyclopropane-1-carboxylic acid (MACC) and to the formation of a stable ternary complex "ACC — ACC oxidase — NO", which hinders the oxidation of ACC to ethylene (Manjunatha et al. 2012). In 'Galaxy' apples there was no effect of NO on the ethylene production after storage in controlled atmosphere (CA) and plus 7 days of shelf life (Brackmann et al. 2017), as in most of the treatments evaluated in the current study.

Table 1. Ethylene production (ηmol $C_2H_4 \cdot kg^{-1} \cdot s^{-1}$) and respiration ($\eta$mol $CO \cdot kg^{-1} \cdot s^{-1}$) rates of 'Cripps Pink' apples stored during 70 days (1.5 ± 0.2 °C and RH of 92 ± 4%), at the time of chamber opening, and plus 7 days of shelf life (23 ± 3 °C and RH of 65 ± 5%), in function of the nitric oxide application (concentration/application time) at the beginning of the storage.

Nitric oxide (concentration/ application time)	Ethylene production rate	Respiration rate	Ethylene production rate	Respiration rate
	Time of chamber opening		7 days of shelf life	
0 µL·L⁻¹	1.54a	160.4a	3.30a	114.4a
10 µL·L⁻¹/2 h	1.89a	144.8ab	3.16a	124.7a
10 µL·L⁻¹/4 h	1.14b	132.4b	3.08a	118.1a
10 µL·L⁻¹/8 h	1.47ab	160.1a	3.43a	95.9b
20 µL·L⁻¹/2 h	1.56a	144.5ab	3.00a	97.3b
CV (%)	15.2	10.0	10.8	16.1

Note. *Average followed by different letters in the columns differ between themselves by the test LSD ($p < 0.05$). CV: coefficient of variation.

The respiration rate measured at the time of chamber opening was lower for fruit treated with 10 µL·L⁻¹ of NO for 4 h than fruit control and those fruit treated with 10 µL·L⁻¹ of NO for 8 h, but did not differ from that fruit treated with 10 and 20 µL·L⁻¹ of NO for 2 h. After plus 7 days of shelf life, respiration rate was lower in fruit from the 10 µL·L⁻¹ of NO for 8 h and 20 µL·L⁻¹ of NO for 2 h than other treatments (Table 1). Other studies observed that treatment with NO reduces the respiration rate of 'Galaxy' apples (Brackmann et al. 2017) and plums (Singh et al. 2009). Nitric oxide reduces cellular respiration through the reversible inhibition of cytochrome oxidase (Pandey et al. 2019).

The values of L and C for the background color of the skin did not differ between treatments. However, the h° value at chamber opening was higher in fruit treated with NO than fruit control, independent of the concentration and treatment time. After plus 7 days of shelf life, apples treated with 10 µL·L⁻¹ of NO for 8 h and 20 µL·L⁻¹ of NO for 2 h had a higher h° value than control and 10 µL·L⁻¹ of NO for 2 h treatments (Table 2). A higher h° value in the apple skin represents less yellow coloration, which indicates a lower degree of matureness, since the skin color is a trustworthy indicator of fruit maturation (Saure 1987). In 'Packhams Triumph' pears, a lower degree of yellowing of the skin was observed following the application of NO (Hendges et al. 2016). The lower h° value observed in the fruit control group after 70 days of storage and plus 7 days of shelf life visually indicated more mature fruit. The greener skin background color in NO-treated fruit can be attributed to a higher chlorophyll content. In broccoli, NO delayed the loss of chlorophyll a (Eum et al. 2009). Nitric oxide could be a protective molecule, preserving the chloroplast membrane against the toxicity of reactive oxygen species (Lazalt et al. 1997).

Table 2. Attributes of skin color of 'Cripps Pink' apples stored during 70 days (1.5 ± 0.2 °C and RH of 92 ± 4%), at the time of chamber opening, and plus 7 days of shelf life (23 ± 3 °C and RH of 65 ± 5%) in function of the nitric oxide application (concentration/application time) at the beginning of the storage.

Nitric oxide (concentration/ application time)	L	C	h°	L	C	h°
	Time of chamber opening			7 days of shelf life		
0 µL·L⁻¹	73.9a	39.9a	91.8c	71.7a	42.0a	84.3c
10 µL·L⁻¹/2 h	74.7a	41.1a	96.9ab	73.5a	42.9a	86.5bc
10 µL·L⁻¹/4 h	73.2a	40.1a	97.1ab	72.0a	42.5a	89.5ab
10 µL·L⁻¹/8 h	75.1a	41.5a	99.6a	72.8a	43.2a	90.4a
20 µL·L⁻¹/2 h	75.4a	40.9a	95.5b	73.1a	43.7a	90.0a
CV (%)	2.5	5.5	2.4	2.5	2.6	2.6

Note. *Averages followed by different letters in the columns differ between themselves by the LSD test ($p < 0.05$). CV: coefficient of variation.

There was no difference between treatments in flesh firmness, after 70 days of storage plus 7 days of shelf life (Table 3). In 'Galaxy' apples there was also no observed effect of the application of NO on the maintenance of flesh firmness after storage (Brackmann et al. 2017). However, in 'Feicheng' peaches (Sun et al. 2011; Zhu et al. 2006), 'Amber Jewel' plums (Singh et al. 2009) and 'Packhams Triumph' pears (Hendges et al. 2016), NO treatment led to higher flesh firmness after storage. This divergence of results between studies could be due to the different species of fruit, as well as to the experimental procedures adopted. The results show that NO is not effective at maintaining the flesh firmness of 'Cripps Pink' apples.

Table 3. Flesh firmness, titratable acidity (TA), soluble solids (SS), SS/TA ratio and flesh browning incidence in 'Cripps Pink' apple stored during 70 days (1.5 ± 0.2 °C and RH of 92 ± 4%) plus 7 days of shelf life (23 ± 3 °C and RH of 65 ± 5%), in function of the nitric oxide application (concentration/application time) at the beginning of the storage.

Nitric oxide (concentration/ application time)	Flesh firmness (n)	TA (%)	SS (%)	SS/TA ratio	Flesh browning (%)
0 µL·L^{-1}	66.3a	0.454a	15.0a	33.1b	62.1a
10 µL·L^{-1}/2 h	66.5a	0.432ab	14.6ab	33.9ab	10.1b
10 µL·L^{-1}/4 h	66.1a	0.445a	14.9ab	33.4ab	6.2b
10 µL·L^{-1}/8 h	66.3a	0.414b	14.5ab	35.0a	13.8b
20 µL·L^{-1}/2 h	64.4a	0.426ab	14.3b	33.6ab	11.6b
CV (%)	4.6	4.4	3.2	3.7	48.7

Note. *Averages followed by different letters in the columns differ between themselves by the LSD test ($p < 0.05$). CV: coefficient of variation.

TA was higher in fruit from the control group and in those treated with 10 µL·L^{-1} of NO for 4 h, but did not differ between fruit treated with 10 and 20 µL·L^{-1} of NO for 2 h (Table 3). In 'Amber Jewel' plums and longans (Duan et al. 2007) NO positively affected on maintenance of TA (Singh et al. 2009). However, in the present study there was no observed positive effect of NO on the maintenance of TA, as also observed in 'Galaxy' apples (Brackmann et al. 2017). Soluble solids were lower in the fruit treated with 20 µL·L^{-1} of NO for 2 h than fruit control (Table 3). However, all the treatments resulted in fruits with rates of SS above the minimum limit desired (13%) and, therefore, 'Cripps Pink' apples can be commercialized as 'Pink Lady' (Pink Lady 2020). The SS/TA ratio was higher, when compared to the control, in apples treated with 10 µL·L^{-1} of NO for 8 h (Table 3). This result can be explained mainly by the values of TA, which were lower apples treated with 10 µL·L^{-1} of NO for 8 h and higher in the control. In line with the quality standards required for 'Pink Lady' (Pink Lady 2020), SS is more relevant than TA, because 'Cripps Pink' apple has an elevated TA, which is maintained during storage (Table 3). Therefore, 'Cripps Pink' apples with an elevated SS/TA ratio can be considered more desirable.

All treatments with NO, independent of the concentration and the duration of exposure, reduced flesh browning after 70 days of cold storage plus 7 days of shelf life (Table 3). Flesh browning is due to oxidative stress that cause damage to cellular membranes and subsequent loss of cellular compartmentalization. Studies have shown that NO can reduce oxidative stress by inducing enzymes such as SOD, POD and CAT, and suppressing LOX (Manjunatha et al. 2010; 2012). Pristijono et al. (2006) reported the reduction of flesh browning with the application of NO in minimally processed 'Granny Smith', 'Royal Gala', 'Golden Delicious', 'Sundowner', 'Fuji' and 'Red Delicious' apples. The authors suggested that NO modulates the oxidative activity of the enzyme polyphenol oxidase and other oxidative enzymes, minimizing the browning reactions.

There were no differences between treatments in incidences of rot and fruit cracking or the skin greasiness (Table 4).

The results demonstrate that treatment with NO can help maintain the quality of 'Cripps Pink' apples after harvest, mainly because of the reduction in yellowing of the skin and flesh browning incidence, as well as the higher SS/TA ratio. The effects were most obvious with the lowest concentration applied for the longer application time (10 µL·L^{-1} of NO for 8 h) and with the highest dose evaluated (20 µL·L^{-1} of NO for 2 h) (Tables 2 and 3). Some studies have demonstrated the positive effect of NO with a combination of lower concentrations of NO and shorter application, than shown in the present study, for example in 'Amber Jewel' plums with 10 µL·L^{-1} for 2 h (Singh et al. 2009), 'Feicheng' peaches with 10 µL·L^{-1} for

1 h (Sun et al. 2011), and 'Pajero' strawberry with 10 µL·L^{-1} for 2 h (Soegiarto and Wills 2006). These differences can be attributed to the rate of absorption of NO, which varies according to the product (Manjunatha et al. 2010).

Table 4. Rot and fruit cracking incidences and skin greasiness in 'Cripps Pink' apple stored during 70 days (1.5 ± 0.2 °C and RH of 92 ± 4%), at the time of chamber opening (CO), and plus 7 days (7D) of shelf life (23 ± 3 °C and RH of 65 ± 5%), in function of the nitric oxide application (concentration/application time) at the beginning of the storage.

Nitric oxide (concentration/ application time)	Rot incidence (%)		Fruit cracking incidence (%)		Skin greasiness (1–3)	
	CO	7D	CO	7D	CO	7D
0 µL·L^{-1}	2.5a^s	2.5a	0.0a	0.0a	1.9a	2.0a
10 µL·L^{-1}/2 h	0.0a	2.5a	1.3a	1.3a	2.1a	2.2a
10 µL·L^{-1}/4 h	5.0a	5.0a	0.0a	0.0a	2.2a	2.3a
10 µL·L^{-1}/8 h	0.0a	0.0a	0.0a	0.0a	2.2a	2.2a
20 µL·L^{-1}/2 h	1.3a	1.3a	1.3a	1.3a	2.1a	2.3a
CV (%)	65.1	46.1	55.6	55.6	17.4	14.6

Note. *Averages followed by different letters in the columns differ between themselves by the LSD test (p < 0.05). CO: time of chamber opening. 7D: 7 days of shelf life. CV: coefficient of variation.

Nitric oxide was applied at low O$_2$ (1 kPa), as recommended by Liu (2016). However, according to Soegiarto and Wills (2004), short-term treatment with low concentrations of NO is able to extend the postharvest life, because NO at low concentration, such as those used in this work, has a relatively slow rate of degradation in air. For example, Soegiarto et al. (2003) found that the degradation of 30 µL·L^{-1} NO in air was much lower than expected with a retention of two-thirds of the original NO after 2 h of treatment. It is possible that 20 µL·L^{-1} NO for 2 h can be applied in air with positive results in relation to maintaining quality.

CONCLUSION

The use of NO (10 µL·L^{-1} applied for 8 h and 20 µL·L^{-1} applied for 2 h) pre-storage maintains the quality of 'Cripps Pink' apples, because it delays skin yellowing and reduces flesh browning.

Nitric oxide has no effect on the maintenance of flesh firmness in 'Cripps Pink' apples.

AUTHORS' CONTRIBUTION

Conceptualization: Steffens C. A.; **Methodology:** Steffens C. A., Miqueloto T., Fernandes R. C., Demari C. K. P., Anami J. M. and Lugaresi A.; **Investigation:** Steffens C. A., Miqueloto T., Fernandes R. C., Demari C. K. P., Anami J. M. and Lugaresi A.; **Writing – Original Draft:** Steffens CA., Miqueloto T., Fernandes R. C., Demari C. K. P., Anami J. M., Lugaresi A. and Amarante C. V. T.; **Writing – Review and Editing:** Steffens CA., Miqueloto T., Fernandes R. C., Demari C. K. P., Anami J. M., Lugaresi A. and Amarante C. V. T.; **Funding Acquisition:** Steffens C. A. and Amarante C. V. T.; **Supervision:** Steffens C. A.

DATA AVAILABILITY STATEMENT

Data will be available upon request.

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