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Scientific Article

$AgNO_3$ improved micropropagation and stimulate *in vitro* flowering of rose (*Rosa* x *hybrida*) cv. Sena

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Abstract

Rose is one of the most important cut flower in the world. Rose micropropagation was used for production of clonal and disease-free plantlets and to breeding purposes. However, many important rose cultivars showed physiological disorders as early-leaf senescence and very low multiplication rate under *in vitro* conditions. Our hypothesis is that these symptoms were associated with high sensibility of these cultivars to ethylene accumulation on *in vitro* environment. The rose cv. Sena was *in vitro* cultivated under different concentrations of $AgNO_3$ and two light sources, LED and fluorescent lamps, as a way to investigate *in vitro* similar symptoms to ethylene accumulation. $AgNO_3$ at 1.0-2.0 mg L^{-1} solved the main *in vitro* physiological disorders observed in this rose cultivar. Also, $AgNO_3$ stimulated induction of 50% of rose shoots to *in vitro* flowering at 2.0 mg L^{-1}. Higher concentrations also resulted in flowering induction, but with imperfect flower development.

Keywords: cut rose; micropropagation; physiological disorders; ethylene inhibitor, flower induction

Resumo

AgNO3 melhora a micropropagação e estimula o florescimento in vitro de rosa (*Rosa x hybrida*) cv. Sena

Rosa é uma das flores de corte mais importantes do mundo. A micropropagação de rosas tem sido utilizada para a produção de plântulas clonais e livres de doenças e para fins de melhoramento genético. No entanto, muitas cultivares importantes de rosas apresentaram distúrbios fisiológicos em condições *in vitro* como senescência foliar precoce e taxa de multiplicação muito baixa. Nossa hipótese é que esses sintomas estão associados à alta sensibilidade dessas cultivares ao acúmulo de etileno no ambiente *in vitro*. Para o experimento foi escolhida a cultivar de rosa chamada Sena sendo essa cultivada *in vitro* sob diferentes concentrações de $AgNO_3$, um inibidor da ação do etileno, e duas fontes de luz, LED e lâmpadas fluorescentes, como forma de investigar os sintomas *in vitro* comumente observados nesta cultivar. O $AgNO_3$ a 1,0-2,0 mg L^{-1} solucionou os principais distúrbios fisiológicos observados *in vitro* nesta cultivar rosa. Além disso, o $AgNO_3$ resultou na indução da floração *in vitro* em 50% das brotações cultivadas *in vitro*, utilizando a concentração de 2,0 mg L^{-1}. Concentrações mais altas também resultaram em indução de floração, mas com desenvolvimento imperfeito de flores, como exemplo a produção de botões florais sem pétalas.

Palavras-chave: rosa de corte**;** micropropagação; desordens fisiológicas; inibidor de etileno; indução de flores

Introduction

Roses (*Rosa* X *hybrida* L.) are the most economically and socially important cut flower industry around the world (Rezvanypour and Osfoori, 2011), and the main producing countries are Ecuador, Colombia, Kenya, Ethiopia and India, with focus on exportations (Vellekoop, 2018). Commercially, rose cultivars are propagated by cutting or grafting. However, there are reports in the literature about the use of micropropagation of different rose cultivars (Kanchanapoom et al., 2010; Jana and Sekhawat, 2011). Besides micropropagation aiming production of high quality and free-pest and disease plantlets, plant tissue culture has many other applications. As example, *in vitro* controlled environmental conditions are excellent for studies with *in vitro* flowering, especially due to the testing isolated factors as plant growth regulators or specific environmental factor, supporting in the study of the biological mechanism of flowering induction and

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led to development of new technologies, such as *in vitro* pollination and breeding (Silva et al., 2014).

Flower induction is considered a complex regulation mechanism controlled by both endogenous and environmental factors (Hirata et al., 2016; Kurokura et al., 2013), hampering studies with isolated factors under greenhouse or field conditions.

In vitro flowering induction has been reported for several species of plants (Panigrahi et al., 2018), including different cultivars of rose *Rosa x hybrida* (Kanchanapoom et al., 2009; Kanchanapoom et al., 2010; Vu et al., 2006; Zeng et al., 2013). Furthermore, *in vitro* flowering has a major impact on selective hybridization, especially in the case of pollen use in rare stocks, and may be the first step in the recombination of genetic material through *in vitro* fertilization (Murthy et al., 2012) and can be used in flowers breeding programs (Silva et al., 2014).

Khosh-Khui and Silva (2006) reported the potential of *in vitro* flowering technique for it successful application also for *Rosa x hybrida* cultivars, in Vietnam. Kanchanapoom et al. (2009) observed the *in vitro* flowering of rose cv. 'Red Masterpiece' in 5% of sprouts grown in MS medium containing 2.0 mg L^{-1} of BA.

Gaseous ethylene normally is associated in most species as inhibitory plant growth regulator for plants flower induction (Iqbal et al., 2017). In addition, ethylene, naturally biosynthesized and released by plants, accumulated in *in vitro* conditions may result in undesirable physiological effects in some species or more sensible-genotypes delaying or restraining the production of high-quality micropropagated plantlets (Cardoso, 2019). In roses cultivated *in vitro*, symptoms of ethylene accumulation, such as gradual yellowing of leaves were observed by Pratheesh and Kumar (2012).

Thus, chemical inhibitors of the ethylene action could be used in culture medium diminishing these symptoms (Pratheesh and Kumar, 2012). For instance, products that release or make it available Ag^+ ions to plants, such as $AgNO_3$, was reported as main action inhibitor of ethylene, preventing ethylene receptors from binding to it (Yang, 1987), improving the quality of micropropagated plantlets (Cardoso, 2019). Silver ion sources are considered anti-ethylene growth regulator and is reported promoting and accelerating flowering in cassava (*Manihot esculenta*), also showing other phenotypic responses (Hyde et al., 2020). Also, $AgNO_3$ induced *in vitro* flowering of different species (Panigrahi et al., 2018).

Also, the addition of $AgNO_3$ to the culture medium in specific concentrations may result in significant improvements in the regeneration of *in vitro* plantlets such as gloxinia (*Sinningia speciosa*) (Park et al., 2012) and *Anthurium andraeanum* (Cardoso, 2019).

The other actual and important factor affected *in vitro* plant regeneration and development are the replacement of conventional tubular fluorescent light by light emitting diodes (LED) (Ramírez-Mosqueda et al., 2017; Xu et al., 2020). Lighting affects plant development, in part by the changes of endogenous levels of plant hormones, such as cytokinins and ethylene (Zdarska et al., 2015)

Based on the several benefits reported by the use of Ag^+ sources for *in vitro* cultivation, the main objective of this study was to evaluate the effects of different concentrations of $AgNO_3$ and the use of LED lighting on the *in vitro* development of rose cv. Sena. This cultivar presented limited micropropagation due to the presence of physiological disorders similar to those described symptoms due to *in vitro* accumulation of ethylene.

Materials and Methods

Adult plants, propagated by cutting of rose cultivar 'Sena' and under cultivation in greenhouse conditions (35,000 lux and temperature of 15-28 °C) were used as mother plants. Young shoot tips with 1.0 cm length were excised, subjected to surface sterilization and used as explants. The asepsis consisted of immersion of 1-cm shoot tips in alcohol 70% for 30 sec, followed by sodium hypochlorite at 30% (2.0%-2.5% of active chlorine) for 20 min. Subsequently, they were submitted to three washings in autoclaved distilled water, before the shoot tips with approximately 0.3-0.5 mm being excised and inoculated in the culture medium.

The culture medium used for establishment and also for multiplication stage, was the Murashige & Skoog (1962) with reduction of macronutrients by half (MS½), with addition of 3% sucrose, myo-inositol 0.1 g L^{-1}, and 0.5 mg L^{-1} Benziladenine, with pH adjusted for 5.8 before addition of agar-agar at 6.5 g L^{-1}. The culture medium was autoclaved at 121°C and 1 kgf cm^{-2} for 20-min. This culture medium was previously tested in our laboratory conditions with success for establishment stage of rose micropropagation.

After four subcultures in the same culture medium, five in total and using nodal segments of 1.0-1.5 cm length with one leaf, important physiological disorders were observed in *in vitro* shoots, e.g. low multiplication rate and early-leaf senescence, which resulted in some required modifications of basic culture medium and the experiment with $AgNO_3$.

The MS culture medium was modified by the reduction of KNO_3 and NH_4NO_3 salts by ¼ and $CaCl_2$ increased two-fold from the original concentration of MS, maintaining 3% sucrose, myo-inositol 0.1 g L^{-1}, and 0.5 mg L^{-1} Benziladenine (BA). There were observed that reduction of nitrogen concentration and increased Calcium could present benefits to in vitro plant development of some woody species (Reed et al. 2013; Wada et al., 2015). To this new modified culture medium for rose in vitro cultivation, different concentrations of $AgNO_3$ added to culture medium were tested: 0, 1, 2, 3, 4 and 5 mg L^{-1} before pH adjustment. The pH was adjusted for 5.8 for all treatments and before addition of agar-agar at 6.5 g L^{-1}.

Four repetitions (vials) containing five nodal segments each, 0.8-1.0 cm in length, were used for the experiment. Shoots cultivated in culture medium with each $AgNO_3$ concentration were subjected to two sources of light, white fluorescent cold bulbs 40W and to white lighting-emiting diode (LED) bulbs 10W, as a way to test the use of LED light and to evaluate its viability and effects on

rose micropropagation. Shoots were cultivated for 60-days in culture medium containing different concentrations of $AgNO_3$. The shoot number and reduction of symptoms related to ethylene accumulation on in vitro conditions were analyzed. The percentage of shoots induced to flowering and with some abnormalities in flower development was realized between the 20 and 60-days of cultivation.

The experiment was conducted under completely randomized design, in a factorial of 6 concentrations of $AgNO_3$ and two types of light sources. The experiment was repeated twice. The data of percentage of flower induction were previously transformed by arcsen$\sqrt{(x+1)}$ and subjected to ANOVA and the means were compared by Tukey's test at 5% probability using Agroestat software for statistical analysis (Barbosa and Maldonado Junior, 2011). The data of percentage of abnormal flowers were subjected to regression analysis and correlated with concentrations of $AgNO_3$.

Results and Discussion

$AgNO_3$ avoids early-leaf senescence in *Rosa* sp. cv. Sena

The *in vitro* establishment resulted in successful introduction of rose explants, with low rate of contamination (<10%) and high rate of regenerated shoots (90%) that started the production of new leaves and elongation of stems after 30-d of *in vitro* inoculation.

The successive subcultures of shoots in the same culture medium increased the number of shoots until the second one. After the third subculture, symptoms of physiological disorders as early leaf senescence and drastic reduction in shoot proliferation propagation resulted in low quality shoot and poor shoot proliferation in 100% of in vitro cultivated shoots (Figure 1A). Symptoms of yellowing leaves were also observed after the first subculture of *Rosa indica* shoots (Prateesh and Kumar, 2012) under similar *in vitro* conditions used in actual study with cv. Sena.

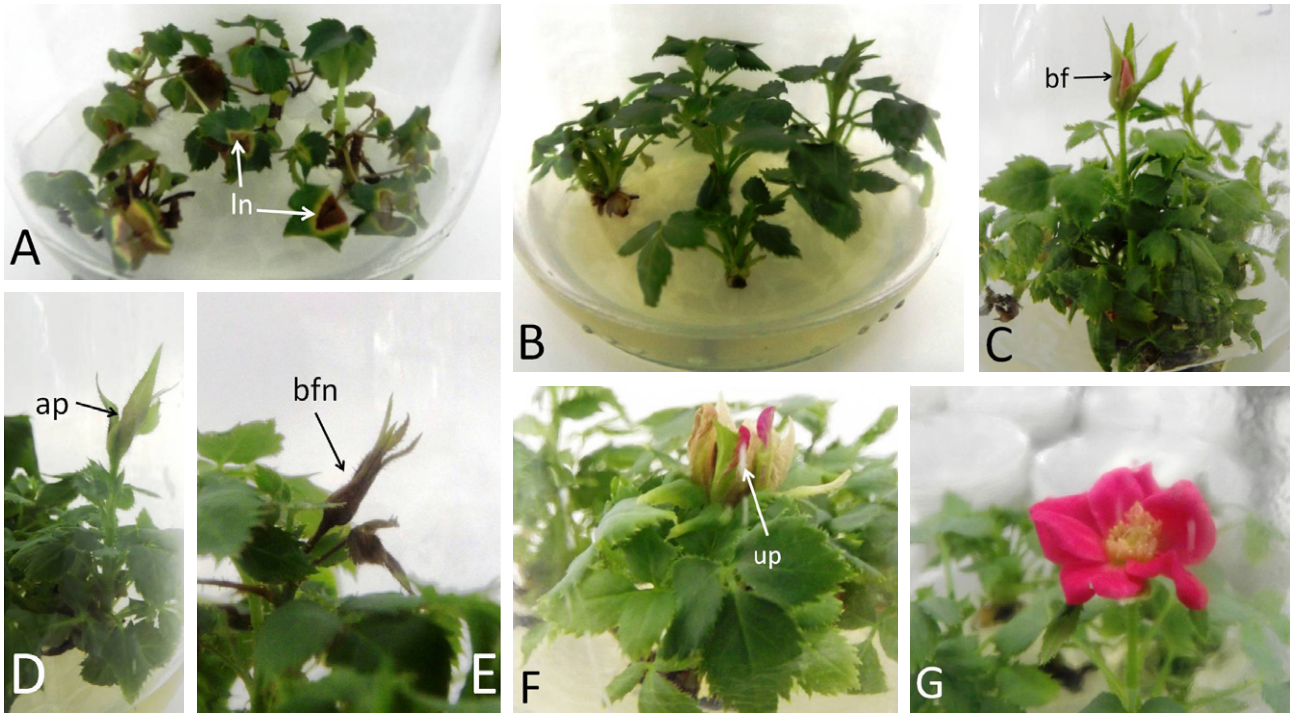

Figure 1. Effects of addition of $AgNO_3$ in culture medium for *in vitro* micropropagation of rose cv. Sena: A, early leaf senescence (ln) symptoms observed in rose cultivated in MS plus 0.5 mg L^{-1} BA; B, normal shoot development under modified MS medium plus BA 0.5 mg L^{-1} and $AgNO_3$ 1.0 mg L^{-1}; C, bud flower induction in shoots of rose cv. Sena in modified MS medium plus BA 0.5 mg L^{-1} and $AgNO_3$ 1.0 mg L^{-1}; D, E, F, main flower abnormalities observed in *in vitro* flower induction, absence of petals (ap) in bud flowers, bud flower necrosis (bfn) and undeveloped petals (up); G, normal rose flower anthesis on *in vitro* conditions;

These symptoms observed in rose micropropagation could be associated with the high biosynthesis and/or sensibility of the rose cv. Sena to accumulation of ethylene under *in vitro* conditions. Ethylene is commonly correlated with flowers senescence, mainly in ornamental plants (Olsen et al., 2015) and also can cause *in vitro* physiological disorders, as observed in Habanero peppers (Santana-Buzzy et al., 2006) and *Anthurium andraeanum* (Cardoso, 2019).

In rose micropropagation, the major peak of ethylene biosynthesis occurred after 4-8 d of culture in MS culture medium, but the authors did not observe any plant symptoms caused by ethylene accumulation in the cv. Madame Georges Delbard', since when the ACC precursor of ethylene was added (Gaspar et al., 1989). However, early leaf senescence associated with apical necrosis also was observed in another cultivar of rose, called 'Starina',

mainly when exposed to Indoleacetic acid (IAA) in rooting stage (Podwyszynska and Hempel 1988; Podwyszynska and Goszczynska 1998). In addition, leaf yellowing was observed after first subculture on *in vitro* shoots of *Rosa indica* grown in MS medium added 0.5 mg L^{-1} IAA and 1 mg L^{-1} BA (Pratheesh and Kumar, 2012). These results showed a genotype-dependent response of ethylene-sensibility in rose, with recurrent and similar symptoms observed with actual study in rose cv. Sena.

Similarly, in actual study, the use of $AgNO_3$ reduced drastically the symptoms observed on *in vitro* shoot culture of rose cv. Sena (Figure 1B). The shoots obtained in $AgNO_3$-added culture medium resulted in green coloration of stems and leaves (3-5/shoot), good shoot proliferation (2.5-2.8 shoots/explants) and absence of the *in vitro* previous symptoms reported for this cultivar, compared with culture medium without $AgNO_3$ (0.7-1.0 shoots/explants and no green leaves in shoots). These results showed that Ag^+ ions, added to the culture medium, was efficient to reduce the *in vitro* negative symptoms in shoots of rose cv. Sena, such as ealy leaf yellowing and senescence associated with reduced multiplication rates, showing that the main cause was due to ethylene accumulation in the *in vitro* conditions and the high ethylene-sensibility of this rose cultivar to this plant hormone, which resulted in poor in vitro development.

$AgNO_3$ influenced induction and development of *in vitro* flowers in rose cv. 'Sena'

Since at very low concentration of 1.0 mg L^{-1}, $AgNO_3$ added to the culture medium resulted in flowering induction of rose cv. Sena, (Table 1; Figure 1C-G).

Table 1. Effects of light-source and concentrations of $AgNO_3$ added in the culture medium on leaf senescence-abortion and on *in vitro* flowering induction of rosa cv. 'Sena'

		Flowering induction (%)	
[$AgNO_3$] (mg L^{-1})	**Aborted leaves at 20-d of *in vitro* cultivation**	**Fluorescent White Bulb (40W)**	**White LED light (10 W)**
0 (Control)	100	0 b	0 b
1	0	30 a	25 a
2	0	50 a	30 a
3	0	35 a	35 a
4	0	25 a	45 a
5	0	20 a	35 a
Mean		27.8 A	29.4 A
F lighting		0.37ns	
F $AgNO_3$		18.98**	
F interaction		1.79ns	
CV (%)		32.49	

[1]Means followed by the same letter don't differ statistically by the Tukey's mean test at 5% probability

Panigrari et al. (2018) also observed that addition of 0.1 µM of $AgNO_3$ also resulted in flowering induction of *Catharanthus roseus*, but the flowering induction in this species was only observed when $AgNO_3$ salt was combined with high concentrations of cytokinins (3 mg L^{-1} of BA and 3 mg L^{-1} Kinetin) in the culture medium. Also, in culture medium used for rose micropropagation BA was used at 0.5 mg L^{-1} as cytokinin source, which may have contributed for this interaction BA x Ag ions, this last acting as action repressor of ethylene.

The maximum percentage of flowering-induced shoots (50%) was observed at 2.0 mg L^{-1} concentration (Table 1). The use of major concentrations reduced or maintained the percentage of shoots with flowers, but increased the number of bud flowers and flowers with different types of abnormalities (Figure 1C-E; Figure 2).

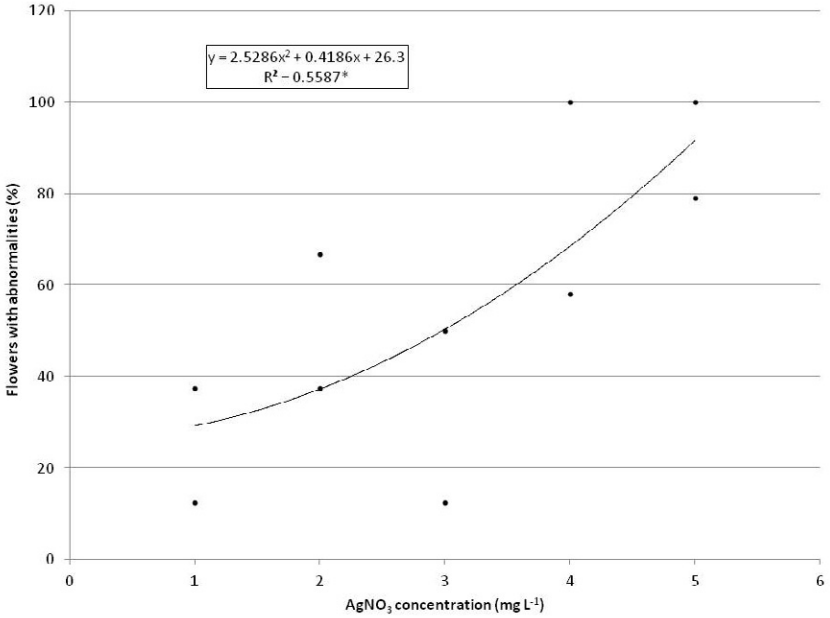

Figure 2. Regression analysis of correlation between $AgNO_3$ concentration added to the culture medium and the percentage of abnormal flowers of *Rosa x hybrida* developed *in vitro*. *significative at 5% probability.

These results showed two different response of $AgNO_3$ according to the stage of flowering, the induction phase and the flowers development. The stage of flower induction and flower development has different requirements, caused by differential gene expression (Hong and Jackson, 2015).

In this study addition of Ag^+ ions promoted the flowering induction in rose shoots, but high concentrations resulted in possible toxic effects to flower development. Silver ions acts causing block in ethylene receptors in plants, inhibiting ethylene effects (Santana-Buzzy et al., 2006; Faria et al., 2017). Thus, ethylene may be involved directly or indirectly in inhibiting of the flowering induction in roses. The response of floral transition to ethylene was species-dependent, but in *Arabidopsis*, ethylene delayed the flowering induction and is considered as inhibitor (Achard et al., 2007).

In roses, the response of flowering induction seems to be multi-factorial, but there were some reports with *in vitro* flower induction of roses (mean 20% of plantlets) with culture medium containing BA (Dobres et al., 1998), showing that cytokinins could be an important PGR for rose flower induction. Similarly, maximum *in vitro* flower bud induction in rose cv. 'Orange Parade' was reported by Wang et al. (2002) using the cytokinins TDZ at 0.5 mg L^{-1}(49.2%) or Zeatine at 0.5 mg L^{-1} (44.2%), combined with NAA at 0.1 mg L^{-1}. The use of major cytokinins concentration did not result in increases percentage of bud flower induction. These authors also reported that flowering induction in rose is highly genotype-dependent, with a range of *in vitro* bud flower induction from 17.3% to 50% depending on the genotype.

Interestingly, results of our actual study using modified MS medium, only the addition of cytokinin BA at 0.5 mg L^{-1} did not result in any shoots with flowers, while Wang et al. (2002) observed 12.5% bud flower of rose cv. Orange Parade induction using this same concentration of BA. Only addition of $AgNO_3$ resulted in rose cv. Sena *in vitro* flower induction and bud flower development (Table 1; Figure 1).

Cytokinins were reported as inhibitor of ethylene biosynthesis and flower senescence, while ethylene accelerates this physiological response in rose flowers (Wu et al., 2017), suggesting antagonistic effects of cytokinins and ethylene, at least for flower senescence. These results suggests that cytokinins and $AgNO_3$ could act together on flowering induction of rose cultivars, cytokinins acting reduced biosynthesis and Ag^+ ions reduced the sensibility of rose shoots to ethylene under *in vitro* conditions, which led to flowering induction of rose shoots. Other studies with cytokinins x ethylene interactions were very limited (Iqbal et al., 2017). Pratheesh and Kumar (2012) also reported the *in vitro* flowering of *Rosa indica*, but authors observed only one bud flower in a single shoot at 50 mg L^{-1} of $AgNO_3$, while in this paper 50% of shoots produced bud flowers at 2.0 mg L^{-1} of $AgNO_3$.

Another interesting phenomenon observed in *in vitro* flower-induced rose shoots is the formation of abnormal flowers, which increases frequency as $AgNO_3$ concentrations increase (Figure 1, Figure 2). Similar results were observed in *Anthurium andraeanum*, where concentrations above 2.0 mg L^{-1} of $AgNO_3$ resulted in non-desirable effects (Cardoso, 2019). These symptoms observed in rose and anthurium could be a result of plant phytotoxicity action by accumulation of high concentrations of Ag ions in plant tissues, similar to reported for tobacco (Cvjetko et al., 2018).

Ethylene also has important effects on flower development. Ethylene was associated with petal cell expansion (Liu et al., 2013) and recent studies have associated ethylene as a regulator of homeotic genes associated with flower development (Iqbal et al., 2017). These affirmatives

were coincident with the results of this study, as the main abnormalities observed was the non-developed petals in the bud flowers (>70% of the total abnormalities observed) (Figure 1D), while another abnormality consisted of bud flower necrosis (18.5%, Figure 1E) or defected petals (Figure 1F).

The prevalent color of *in vitro* petals was pink (Fig. 1G), while in greenhouse conditions red color of flowers characterize the cultivar. Also, the number of petals obtained *in vitro* is reduced compared to normal flowers under greenhouse with the same cultivar.

The type of light has low influence on *in vitro* shoot development and flowering induction in rose cv. 'Sena'. According to Terfa et al. (2013) rose was irradiance-dependent flowering, but the comparison between LED (80% Red; 20% Blue) and high-pressure sodium (low blue) lamps did not result in differences in flowering for *Rosa x hybrida* cv. Toril. In this study, the use of white LEDs (10W potency) reduced the percentage of abnormal flowers obtained *in vitro* and could be used for rose micropropagation, with similar results to white fluorescent tubular lamps (40W potency), reducing the costs with electrical energy for micropropagation.

The link between flowering in rose and ethylene was discussed, and two main hypotheses could be formulated with actual experiment, the hypothesis of inhibition of flowering induction by ethylene in rose and the importance of ethylene for flower petal development. *In vitro* flowering also has physiological interest, helping to understand the mechanism of rose flowering, but could also be used for breeding purposes, using early *in vitro* flowering for techniques such as *in vitro* pollination and fertilization (Zulkarnain et al., 2015).

Conclusions

The use of $AgNO_3$ in rose micropropagation increased the shoot proliferation and quality of shoots by avoiding physiological disorders observed in Ag-free culture medium, confirming the sensibility of rose cv. Sena to *in vitro* ethylene accumulation. In addition, $AgNO_3$ added to the culture medium also induced *in vitro* flowering of rose cv. 'Sena', which could be used for better comprehension of flowering physiology in rose species, and could be applied for emerging technologies, as *in vitro* breeding.

Author Contribution

JCC: contributes with the main idea, installation and conduction of the experiments, data analysis, writing and final edition of the paper, **AVCSM**, **BSO** and **MEBSO** contribute with data collection, software statistical analysis and paper writing.

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