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ORIGINAL ARTICLE

UV-C radiation for control of gray mold disease in postharvest cut roses

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Abstract

The shipment of cut flowers from Colombia and Ecuador to the United States, the biggest importer of this product in the world, has doubled in the last 20 years. One of the main constraints in cut roses production is the gray mold disease caused by the fungus *Botrytis cinerea*, which can destroy the flowers, in the crop, during storage and/or shipping. Since the resistance of the fungus to conventional fungicides has been increasing, as well as the health effects in rose growers, alternative approaches for controlling the disease are needed. The effect of UV-C light on the gray mold-development in cut roses was studied. Irradiation with 2,160; 1,080 and 540 J · m^{-2} UV-C, every 24 h for 5 days in a humid chamber, did not harm the roses. Instead, as seen by image analysis, a highly significant reduction of the area of the lesions by the disease and of the fungus germination was obtained at 1,080 J · m^{-2}. The addition of a 4-h dark period to the irradiation did not improve the effect of UV-C on the disease. The results of this work potentiate the use of UV-C light in the agro-industry as a low-cost and non-invasive alternative method to control diseases. They also reflect the application of optical approaches as image analysis in the evaluation of important agricultural features.

Keywords: gray mold, pest control, postharvest, roses, UV-C radiation

Introduction

Over the last 20 years, cut flower exports to the United States (the biggest consumer of this type of flower in the world) have more than doubled in size from about 4 billion USD in 1995 to more than 8 billion USD by 2015 (Loyola *et al.* 2019). Cut flower producer countries are primarily located near the equator, while the main consumers are in Europe and North America, thereby involving trade over long distances. This, in addition to the highly perishable nature of cut flowers, leads the biggest consumer country to import 81% of this kind of flower from nearby countries, such as South American countries (van Rijswick 2016).

The cut flower industry is a major agricultural activity in Colombia, occupying 7,335 ha and it is the second largest flower exporting country (after the Netherlands). This industry generates an income of more than 1 billion USD annually, creating jobs for people in the productive regions (Asocolflores 2017). Cut roses are one of the most popular flower crops in the world (Chu *et al.* 2015a). In Colombia, this is the most cultivated flower with 2,580 ha, representing 35% of total cut flowers (Asocolflores 2017). The production of this type of flower is constantly threatened by fungal diseases. Gray mold caused by *Botrytis cinerea* is the most destructive disease affecting its yield, not only in the crops, but also during storage and shipping (Elad *et al.* 1993; Costa *et al.* 2013; Chu *et al.* 2015a; Hao *et al.* 2017). This fungus affects different plant tissues,

such as flowers, stems, and leaves, but the greatest damage occurs on the flower petals (Elad 1988). The germination of conidia takes place 10–12 h after inoculation, with long germ tubes at 10–20 h (Cole *et al.* 1996). In rose flowers, after incubation for 24 h, lesions were visible at 100% relative humidity, causing severe blemishes after 48 h (Williamson *et al.* 1995); subsequently, these early lesions combine to form higher necrotized areas that may affect entire petals, sepals and receptacles. The infection may also lead to petal abscission and death of the flower (Gleason and Helland 2003).

Several studies have confirmed the resistance to fungicides by the fungal population (Timudo-Torrevilla *et al.* 2005; Bardas *et al.* 2008; Konstantinou *et al.* 2015; Álvarez-Medina *et al.* 2017; Rupp *et al.* 2017), which has led to increased doses and frequency of application of fungicides (Card *et al.* 2009; Álvarez-Medina *et al.* 2017; Rivera-Casignia *et al.* 2017), causing a threat to public health and high risks of environmental contamination (Kretschmer and Hahn 2008; Valera *et al.* 2018). The above has forced the industry to search for and/or develop alternative methods for disease management, such as biological methods (Chen *et al.* 2018; Jiang *et al.* 2018), application of defense activators (Ortega Martínez *et al.* 2017) and physical methods (Chu *et al.* 2015b; Janisiewicz *et al.* 2016; Usall *et al.* 2016).

Ultraviolet C (UV-C) light (200–280 nm) is used as a physical method to avoid microbial infections and it is mainly known to generate damage and mutations in the microorganisms' DNA (Sinha and Häder 2002). This light is used on several surfaces, as well as in air and water, due to its germicidal effects (Levetin *et al.* 2001; Yin *et al.* 2013). UV light is a viable alternative method to control postharvest diseases of fruits and vegetables, by acting directly on pathogens or indirectly through the induction of defenses both in plants as well as in harvested products (Ben-Yehoshua *et al.* 1992; Stevens *et al.* 1996, 1998; de Capdeville *et al.* 2002; Romanazzi *et al.* 2012; Gutiérrez *et al.* 2016; Mohamed *et al.* 2017; Valencia *et al.* 2017). This light has shown promising performance to control decomposition and to extend shelf life of postharvested plant products (Nigro *et al.* 1998; Vicente *et al.* 2005; Nigroa and Ippolito 2016; Jin *et al.* 2017; Karasahin Yildirim and Pekmezci 2017; Ortiz Araque *et al.* 2018). The UV-C effects on *B. cinerea* were previously studied in fressia (Darras *et al.* 2010), strawberry (Marquenie *et al.* 2002, 2003; Pan *et al.* 2004; Janisiewicz *et al.* 2016; Jin *et al.* 2017), lettuce (Ouhibi *et al.* 2015), peppers (Mercier *et al.* 2001), tomatoes (Charles *et al.* 2008a, b, c, d, 2009), grapes (Nigro *et al.* 1998), and carrots (Mercier *et al.* 1993, 2000). It has also been suggested that a dark period after UV-C irradiation enhances the effect of reducing the disease (Janisiewicz *et al.* 2016). Despite the benefits that have been found on the management of gray mold by UV-C irradiation, information on its effects particularly in cut rose crops has not yet been reported under production conditions.

The present work studied the UV-C light effects on fungal infection by *B. cinerea* in cut roses used for exportation. The experiments were carried out using a UV-C lamp, a camera to acquire RGB images of the samples, a custom-made program developed to measure lesion area, image analysis and bright field microscopy. We found that our alternative approach was easy to implement and it was an effective way to control *B. cinerea* in cut roses using the shelf optical elements techniques.

Materials and Methods

Floral material

Rose flowers (variety MovieStar), 10 weeks old and less than one week after cutting, were provided by a local company (Inversiones Coquette S.A.). Prior to the experiment, the flowers were washed with tap water and submerged for 5 min in distilled water. Petals with mechanical damage were removed.

Pathogen inoculation

Conidia of *B. cinerea* were collected weekly from postharvest roses of cash crops with typical symptoms and signs of gray mold disease. The samples were washed with tap water and left in a humid chamber, maintaining the temperature at 20 ± 2°C, with a relative humidity of 94% and 12 h of light per day, to induce the sporulation of the fungus. Subsequently, a solution of 1×10^4 conidia · ml^{-1} was prepared. The inoculation of the petals was carried out by placing an aliquot of 20 µl of conidia solution on the adaxial side of one petal per flower, to favor the development of the disease (Latorre *et al.* 2002). In the same way, placing an aliquot of 20 µl of sterile distilled water was used as a control treatment. Variations of the incubation times were made from 24 to 27 h after inoculation to see if there was any influence on the development of the disease when UV-C light was used.

UV-C treatment on flowers

The UV-C light was produced by a UV lamp (OSRAM Puritec Germicidal lamp HNS 8W G5®). The lamp has a characteristic spectral emission over the UV-C region with a specific irradiance peak of 254 nm, which is generally found in low pressure germicidal lamps. The bulb is cylindrical and the homogeneity depends on the distance from the central axis of the lamp. For the experiments, the UV-C lamp was housed in a wooden support and wrapped in aluminium to facilitate

handling and protection of the operator during irradiation. Irradiations were carried out on the flower head, directing the UV-C light on the adaxial side of the petal. The irradiance of the lamp was measured with a UV-C light meter (Sentry Optronic Corp®) at the center of the illumination field. We first examined doses that did not cause any visual phototoxicity on the petal. Then, we proceeded to perform UV-C treatments on the infected petals. The UV-C doses on petals infected with *B. cinerea* in an early stage of infection development reached at 24 and 27 h from inoculation, were 540 J · m^{-2}; 1,080 J · m^{-2} and 2,160 J · m^{-2}. These treatments were performed every 24 h (with or without a 4 h dark period) for 4 days with a final evaluation of disease development on the 6th day (time in which maximum development of disease is reached under conventional environmental conditions (Dik and Wubben 2007).

Evaluation of the UV-C effect on the disease

Symptoms of the disease are initially associated with small spots on the petals. Later as the infection progresses, small chlorotic spots develop and they are visible on the surface of the petal until it is totally discolored and profuse sporulation of fungus is expressed. The growth of the lesions caused by the disease was evaluated quantitatively by means of RGB images of the petals and digitally measuring the affected area using a custom-made program for image analysis.

Evaluation of the UV-C effects on conidia

The conidia were obtained from roses with 8 days of infection by *B. cinerea* as described above. The conidia were transferred to the surface of 3% agar in Petri dishes by gently touching it with the infected roses. Then, they were exposed to a UV-C dose of 1,080 J · m^{-2}. The control treatment was conidia not being exposed to UV-C light. The Petri dishes were then sealed with parafilm® and incubated at 22°C in darkness for 48 h. Finally, the treated conidia were examined with a light field microscope Olympus CX23 (200X). Conidia with clearly visible germ tubes, more than two times the size of conidia, were considered as germinated.

Image capture and analysis

The effectiveness of the UV-C light treatments was tracked in time by taking images of the petals before each UV-C irradiation. These RGB images were taken using a digital camera (CANON-T5i) and setting a background with a visible metric scale for reference. To obtain reliable quantitative data we measured the areas of the lesions using a custom-made image analysis program. The software was developed using the Matlab® platform and it allowed us to estimate the lesion area over the RGB images in an automatic way and more accurately than with the classical approach using a calliper. The program interface was designed to visually inspect the images and extract the area values in metric units using the reference calibration scale set in the capture process. To classify the points on the images as pertaining to healthy or infected zones, the program initially converts the original RGB values in the images in L*a*b* color values, by using the calculations proposed by Kang (2006). Points from lesion areas are used as color values of reference; they are selected by interactively clicking on them using the program interface. We defined a color difference image of a single channel for segmentation by calculating the CIEDE2000 color differences (Sharma *et al.* 2005) of every point in the image with respect to the mean of the reference color values previously selected. This color difference image is segmented as a binary image using the Fast Marching Method (Song *et al.* 2013). The pixels determined as pertaining to the area lesion are counted and converted to metric units of area.

Statistical analysis

A random design was used. Means were compared using Tukey's multiple range tests. The data analysis was performed in R program® (version 3.0.1, 2013). Experiments were repeated two times. Standard error of the mean is reported as s.e.m.

Results

Effect of UV-C application after different times of pathogen incubation without a dark period

First, healthy petals were exposed every 24 h for 6 days to five different UV-C treatments: 540; 1,080; 2,160; 3,240 and 6,480 J · m^{-2}. The UV-C doses of 3,240 and 6,480 J · m^{-2} generated a photo toxicity reaction on the petals, exhibiting necrotic lesions and alteration of the foliar tissue (data not shown). Doses of 2,160; 1,080 and 540 J · m^{-2} did not induce any visible damage on the petals; therefore, these exposure doses were chosen to study the effect of UV-C on the disease development.

Figure 1 shows the advancement of the lesions for the first and sixth days after the application of UV-C light over samples with different pathogen incubation times. The different incubation times were: 24 h (Fig. 1A), 25 h (Fig. 1B), 26 h (Fig. 1C), and 27 h (Fig. 1D). In these experiments, a dose of 1,080 J · m^{-2} of UV-C light was applied, for 4 consecutive days every 24 h. The greatest reduction of the disease was observed for the

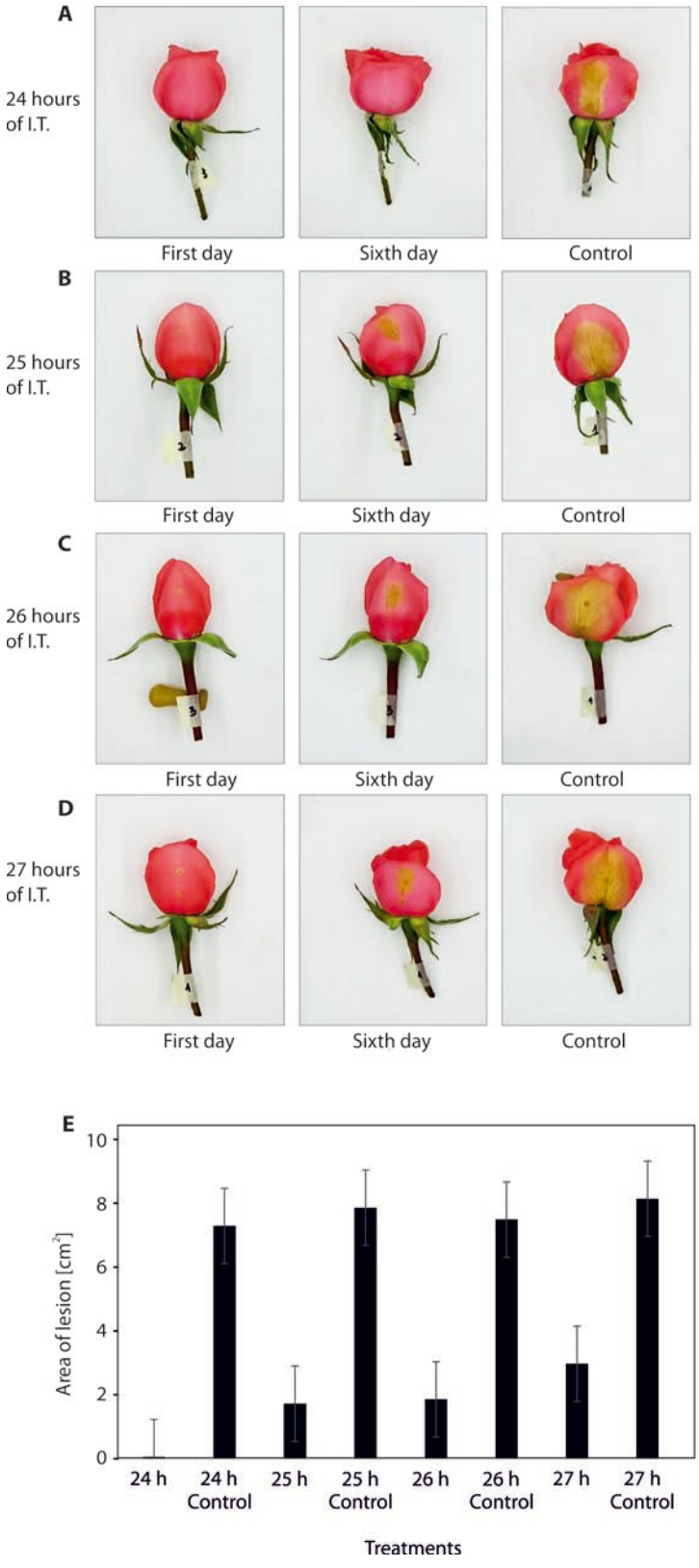

Fig. 1. Effect of application of a UV-C light with a dose of 1,080 J · m^{-2} over samples of rose petals with different pathogen incubation times (I.T.). Rose images on the first and last days of treatment for the pathogen I.T. of: A – 24 h, B – 25 h, C – 26 h, and D – 27 h; E – results of the advance of the area of the lesion for samples with different I.T. The UV-C irradiation treatments were applied every 24 h for 4 days (without a dark period after exposure of the samples). Controls were roses inoculated with the pathogen but not irradiated with UV-C. The reported area of the lesion corresponds to the last day of evaluation. These areas were measured by an image analysis program developed in Matlab® ($p < 0.05$), n = 15, mean ± s.e.m.

incubation time of 24 h (Fig. 1A) compared to the control. Figure 1E shows quantitatively, through measuring the area of the lesion on the 6th day, the results of disease control for the different incubation times and comparisons with the control.

Effect of UV-C application at different doses on gray mold development without a dark period

Irradiation with the selected doses with a 24 h of fungus incubation in petals without a dark period after the treatments generated a highly significant reduction of the disease with respect to the control as shown in Figure 2A. For a fungus incubation period of 27 h, the reduction of the lesion was smaller than for the period of 24 h, probably because the fungus at 27 h, had a higher colonization within petal tissues, which implies a higher mass of fungus; however, the treatments at this time, presented a significant reduction of lesion area compared to the control (Fig. 2B). As shown in Figure 2C, the area of the lesion was highly significantly reduced for all treatments with respect to the control for the two incubation times. It can also be observed from Figure 2C, that there were no significant differences between treatments applied over roses with the same incubation time. However, the treatments applied after 24 h of inoculation were able to almost completely stop the progress of the lesion.

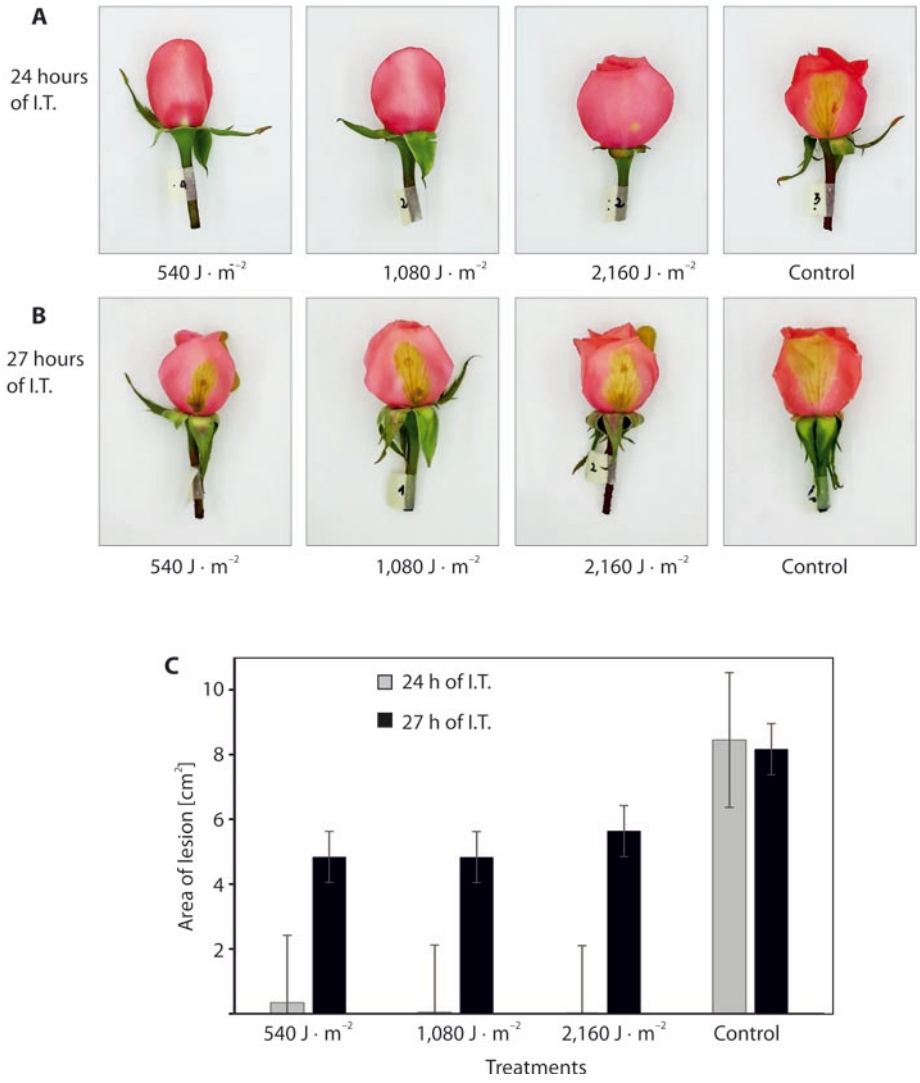

Fig. 2. Effect of application of UV-C light without a dark period over infected rose petals. Rose images on the last day for 2,160; 1,080 and 540 J · m^{-2}. UV-C treatments without a dark period and with a pathogen incubation time (I.T.) of A – 24 h and B – 27 h; C – results of the advance of the area of the lesion for samples with pathogen I.T. of 24 h and 27 h. The treatments were performed every 24 h for 4 days in early lesions, i.e., samples 24 h and 27 h after the infection with *Botrytis cinerea*. Controls were roses inoculated with the pathogen but not irradiated with UV-C. The reported area of the lesion corresponds to the last day of evaluation. These areas were measured by an image analysis program developed in Matlab® ($p < 0.05$), n = 15, mean ± s.e.m.

Effect of UV-C treatments followed by a dark period

For the same selected UV-C irradiation doses (540; 1,080; and 2,160 J · m^{-2}), but in this case followed by a dark period, experiments showed a significant reduction of the disease at 24 h and 27 h of fungus incubation (Fig. 3A and B, respectively). As in the case without a dark period, Figure 3C shows that for samples with 24 h or 27 h of pathogen incubation the results for the three applied doses were not significantly different. However, for samples treated with the same doses, but with different times of pathogen incubation there were significantly different results. When applying the treatments over samples for 24 h, the progress of the lesion was almost completely stopped. However, the results were different when applied to samples with 27 h of pathogen incubation. The dark period did not increase the potential of UV-C. As was stated above, a higher growth (colonization) of fungus within petals probably requires a higher dose of irradiation to control the gray mold disease.

Effect of the UV-C radiation on fungal germination and pathogenic capacity

The viability of fungal conidia after irradiation with a 1,080 J · m^{-2} UV-C dose was highly reduced compared to the control (Fig. 4). The percentage of germination of the non-irradiated conidia was 89.2%, while for the irradiated it was 2.8% (Fig. 4C). Despite the fact that some conidia still germinated (2.8%), they were not able to cause infection in healthy petals (data not shown).

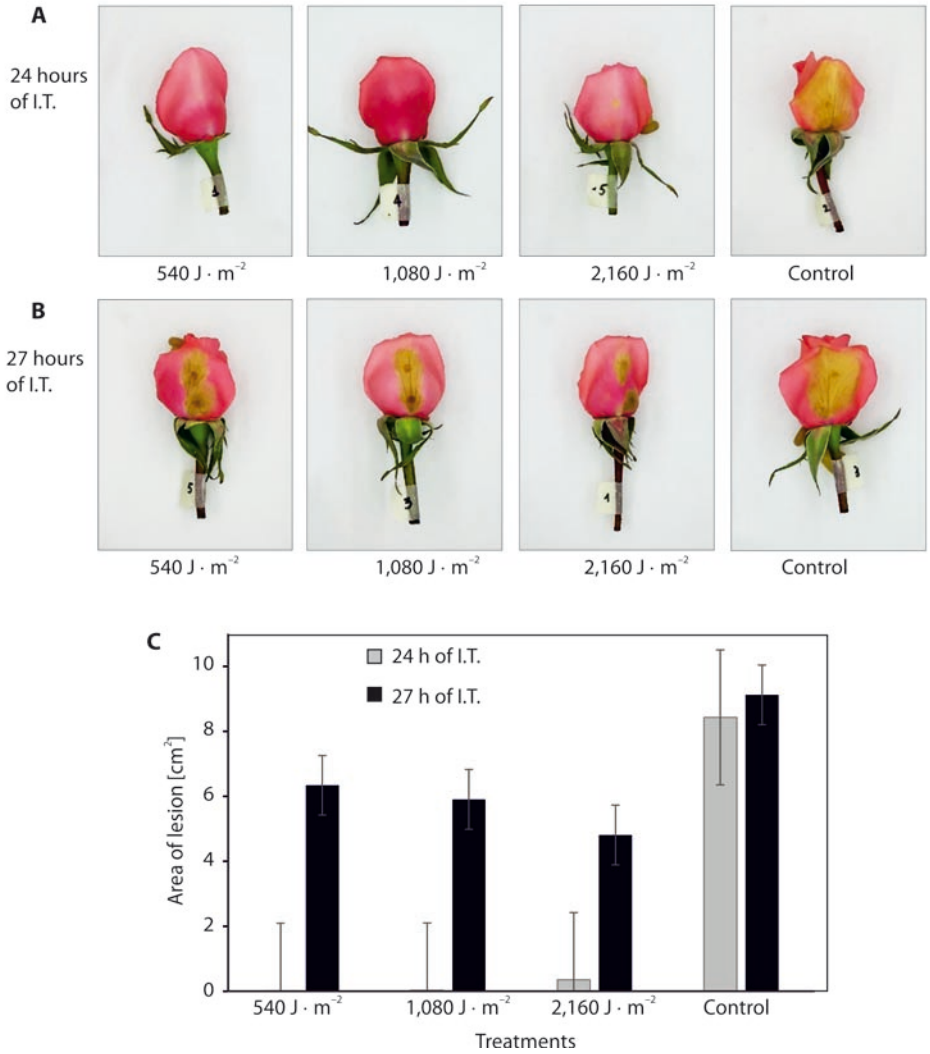

Fig. 3. Effect of UV-C application, followed by a dark period of 4 h, over infected rose petals. Rose images on the last day for treatments of 2,160; 1,080 and 540 J · m^{-2} followed by a dark period and with pathogen incubation times (I.T.) of A – 24 h and B – 27 h; C – results of the advance of the area of the lesion for samples with a pathogen I.T. of 24 and 27 h. The treatments were performed every 24 h for 4 days in early lesions, i.e., samples 24 and 27 h after the infection with *Botrytis cinerea*. Controls were roses inoculated with the pathogen but not irradiated with UV-C. The reported area of the lesion corresponds to the last day of evaluation. These areas were measured by an image analysis program developed in Matlab® ($p < 0.05$), n = 15, mean ± s.e.m.

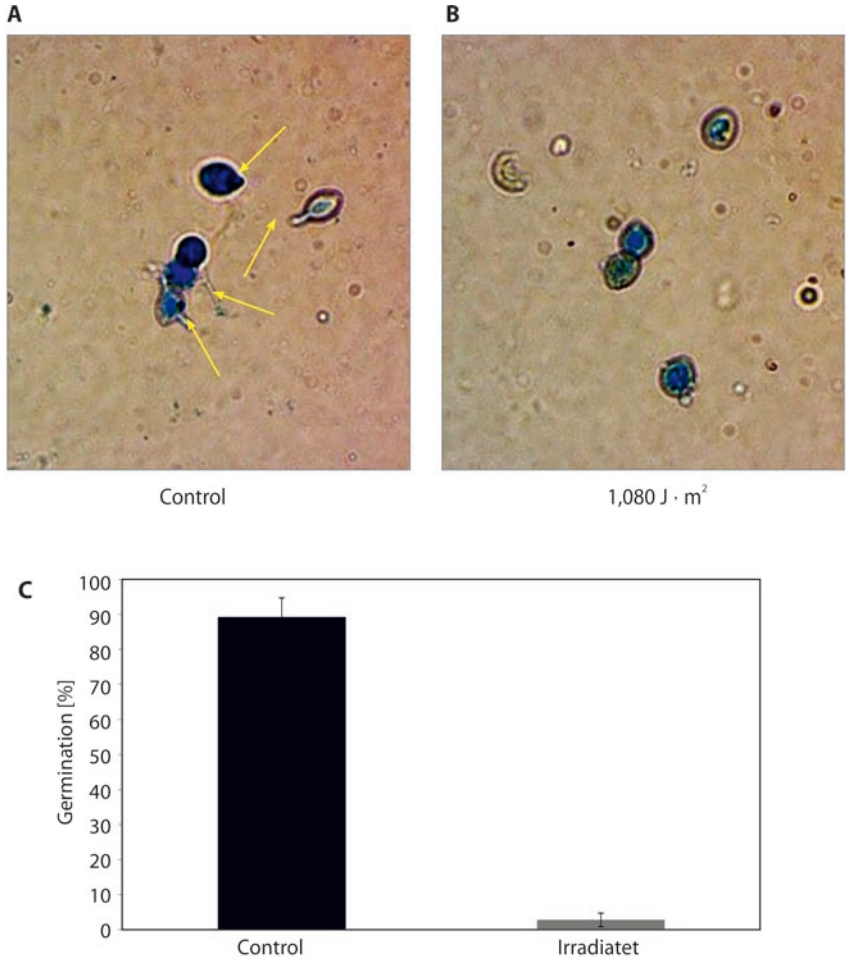

Fig. 4. Biological effect of exposure to UV-C light on the percentage of germinated conidia in agar with 48 h of incubation. Conidia were evaluated for visible germ tubes with an optical microscope (200X). A – Image example of conidia without UV-C treatment (a total of 244 conidia was counted); B – image example of conidia after a UV-C dose of 1,080 J · m^{-2} (a total of 260 conidia was counted); C – percentage of conidia germination. The arrows point to the germ tubes. Scale bar = 10 µm

Discussion

The present study highlights the effective application of UV-C light to control gray mold disease in postharvest cut roses supported by the image processing tool as a method to quantitatively evaluate the development of the disease. Using image acquisition for determining the lesion areas has proven to be a reliable method in other cases, but to our knowledge it has not been considered in combination with UV-C when evaluating radiation effects over the rose petals or the fungus *B. cinerea*. One of the advantages of digital determination of these areas is that there is no need for any sort of reference diagram development, i.e., in our case no comparison against a reference pattern was needed. Overall, the results showed that UV-C doses of 540; 1,080 and 2,160 J · m^{-2}, every day for 5 days, considerably reduced the lesion area, in comparison with the non-exposed roses, without causing visible damage on the petal. As several studies have shown, DNA is one of the key targets for UV-induced damage in a variety of organisms (Mercier *et al.* 2001; Lado and Yousef 2002; Sinha and Häder 2002).

The results demonstrate that a UV-C dose of 1,080 J · m^{-2} significantly reduced gray mold in the petal, after 24 h of inoculation with the fungus. Other studies showed similar results concerning reduction of gray mold disease in other species with UV-C treatments (Janisiewicz *et al.* 2016; Valencia *et al.* 2017). However, we found that after 27 h of inoculation, the treatment was not as effective as 24 h after inoculation. UV-C light prevents the formation of germ tubes (Zhu *et al.* 2019), which suggests that it affects the processes that take place during the first stages of germination and infection. This fact could explain the strong reduction of the infection observed. In the germination of *B. cinerea* it is known that the spores have sequential steps. The first stage is the establishment of relatively weak adhesive forces that bind the spores to a support; then, a hydration process is carried out before the formation of the germ tube and finally, after germination,

the spores adhere strongly through the secretion of an extracellular matrix (Doss *et al.* 1993). Therefore, at 27 h of fungus incubation, this event may have been previously generated and the total control of the disease possibly requires a stronger UV-C dose. Consequently the doses for this incubation time were not strong enough to completely control the disease. The optimal dose of UV-C (1,080 J · m^{-2}) used in this investigation was not sufficient to effectively control the disease at 27 h of pathogen incubation, unlike in other studies where the incidence of gray mold on strawberry was significantly reduced with doses of 2,000 J · m^{-2} (Jin *et al.* 2017) and 4,100 J · m^{-2} (Pan *et al.* 2004), and in harvested tomatoes where 1,300 J · m^{-2} also markedly reduced *B. cinerea* (Liu *et al.* 1993).

It has been reported that establishing a dark period after UV-C treatment could improve the action of UV-C due to deterioration in the photoreactivation of DNA repair (Janisiewicz *et al.* 2016; Zhu *et al.* 2019). However, in our study it was not necessary to appeal to this dark period at 24 h of pathogen incubation; and such a period did not improve the effectiveness of this light when the fungus had a longer pathogen incubation time (i.e. 27 h). The above implies that the use of UV-C for controlling gray mold in cut flowers must be applied during early steps of fungus development inside the flower, i.e., preventively from preharvest of roses. However, it is necessary to further study if higher doses of UV-C could stop the progress of fungus at times longer than 27 h of pathogen incubation.

The treatments with UV-C have demonstrated a high performance in their application for commercial conditions; several studies ratify and promote the use of UV-C in postharvest fruit treatments (Charles *et al.* 2008a, b, c, d). Also, other researchers obtained similar results in the application of UV-C in postharvest strawberries by helping the control of *B. cinerea* (Janisiewicz *et al.* 2016). These fruits and plants reach the market with greater acceptability due to their morphological and health conditions. According to Quintero *et al.* (2013), the use of UV-C in agro-industry means the reduction of postharvest losses, increased bioactive use, antioxidant activities and delayed enzymatic browning which helps to better preserve the processed products.

To be able to penetrate the surface of the host, a pathogenic fungi uses an infection structure (appressorium) that forms a penetration pin that breaks the cuticle (van Kan 2006). When the conidia of the fungi are irradiated with UV light, they lose the capacity to develop these structures, as also evidenced by Janisiewicz *et al.* (2016), Valencia *et al.* (2017) and Zhu *et al.* (2019). Additionally, the formation of dimers in DNA, caused by UV-C, impairs the action of DNA polymerase during elongation of germ tubes. Also, inhibition of cell wall biosynthesis has been attributed to biocide activity by UV-C (Suthaparan *et al.* 2016). Thus, the mentioned studies agree with our results that UV-C irradiation morphologically affects the development of the germ tube of fungus and therefore its capacity for infection. Other associate-UV-C effects on gray mold disease, such as: cell membrane detachment from cell wall, collapse and vacuolization of cytoplasm, and organelles disruption (Romero Bernal *et al.* 2019) can help to explain the high reduction capacity of UV-C on *B. cinerea* development.

An alternative action of UV-C in gray mold management is its preventive effect of stimulating the immunity in plants, e.g., accumulation of defensive secondary metabolites (Kuniga *et al.* 2015), increase of polyphenol oxidase (PPO) activity (Darras *et al.* 2012) and preformation of callose in plant cells as a barrier to penetration of fungus (López Cruz *et al.* 2017). These facts show the broad spectrum of possibilities of applying UV-C, in particular, the combination of a preventive effect with the post-infection effect of UV-C found in this study which could increase the reduction of fungal diseases on crops.

To bring the applied science to the producers, facilitate its use and grant an economic bonus to the experiment, simulations of export conditions are needed to determine if any negative aspects were generated in the course of the trip and life in vases of the flowers. According to our results, we suggest the manufacturing and implementation of a prototype that simulates the realization of this with a UV-C dose of 1,080 J · m^{-2} using low cost and commercial UV-C lamps, verifying and evaluating on a real scale the results obtained, hoping that the simplicity of the technique encourages economical interest and promotes the development and application of this long-term technology to different postharvest processes.

Finally, this study has demonstrated the effective control of the gray mold disease caused by fungus *B. cinerea* by UV-C irradiation in cut roses for exportation under postharvest conditions, being the first to demonstrate such an effect in this crop. This is especially true in developing countries. Physiological evidence has been found that helps in the understanding of its preventive and controlling characteristics. We showed with our approach that taking advantage of the current development and ubiquity of technology in illumination and imaging other structures of intervention for the issues of agriculture can be developed, becoming themselves possible substitutes for the more traditional procedures. In general, our methodology showed that photonic methods in agriculture can be reliable, environmentally friendly, safe, helpful, and, even as implemented here, of low cost due to the use of commercial elements.

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