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Biocontrol of postharvest *Alternaria* decay in table grapes from Argentina

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ABSTRACT

Postharvest diseases cause considerable losses of fruit during transportation and storage. Synthetic fungicides are primarily used to control them; however, the recent trend is to shif toward safer and more eco-friendly alternatives for the control of postharvest decays. In Argentina, Alternaria genus was cited as a postharvest pathogen in table grapes for the first time during 2010-2012 seasons. The aim of this study was to identify by morphological and molecular techniques *Alternaria* spp. strains isolated from table grapes cv. Red Globe in different phenological stages of vine and postharvest storage in Mendoza region. In addition, we intended to propose an alternative postharvest management to the use of sulphur dioxide (SO_2) generators (traditional method). We searched for a lower environmental impact substitute; using the yeast strain *Metschnikowia pulcherrima* RCM2 and chitosan coatings as possible control agents. The results showed that the pathogen was present in all the stages studied and it was determined that the 100% of the strains belonged to the species-group *Alternaria alternata*. On the other hand, chitosan coatings showed to be a good alternative method to SO_2 generators. This is the first study, to our knowledge, about *Alternaria* spp. incidence during the phenological cycle and postharvest storage of table grapes cv. Red Globe; and also the first study about the evaluation of alternative strategies to the use of SO_2 for the control of this disease during the prolonged cold storage.

Keywords: Postharvest, table grapes, *Alternaria* spp., biological control, chitosan

1. Introduction

Worldwide, fruits are vital components of the human diet, occupying the principal slice in the food wheel. Table grapes (*Vitis vinifera* L.) are one of the most economically important fruit crops in the world; in 2016, the total cultivated area was 7 516 000 ha and the production was 75 800 000 tons (OIV, 2017). This crop has been widely cultivated in Argentina, which is one of the main producers of the south hemisphere (FAOSTAT, 2016), with a planted area of 12 823 ha and an annual fruit yield of 9 300 tons. During 2016, 1 115 tons of table grapes were exported from Argentina, being the 7th most exported fresh fruit (Instituto Nacional de Vitivinicultura, 2016). The principal commercial destinies were Brazil, Russia and Germany (Ministerio de Agroindustria, 2017). San Juan and Mendoza provinces are the most important grape producers of the country. They concentrate 95% of the cultivated area of grapes for fresh consumption, with 12 823 implanted hectares (Instituto Nacional de Vitivinicultura, 2017).

Grapes are highly perishable non-climacteric fruits with reduced shelf-life due to a loss of firmness, berry drop, stem discoloration, desiccation and fungal rot (Meng *et al*., 2008). There are different physiological disorders, collectively called cold damage, which can develop due to the low temperatures during storage and affect the quality of the fruit. These disorders are, commercially, very important, since the problem is not acknowledged until the fruit reaches the consumers (Aubert *et al*., 2014). Extending the shelf-life of table grapes during postharvest becomes very important in order to increase the period of commercialization and to obtain better prices in the market; whose positive response is limited to products that meet certain quality requirements like good visual appeal, high palatability, adequate physical qualities and resistance to cold storage and transport (Llorente, 1991).

After harvest, fresh fruits are susceptible to be attacked by saprophytic or parasitic pathogens. This is due to their high content of water and nutrients and because, once detached from the plant, they lose most of the intrinsic resistance that protects them during their development. Their organic acid content is sufficient to produce pH values lower than 4.6, favoring that the predominant microbial forms in the fruit are of fungal origin (Viñas, 1990); mainly of the genera *Penicillium*, *Botrytis*, *Alternaria*, among others. Fungal decay of table grapes during transportation and storage severely affects the income of producers. Experts estimate that losses between 10 and 40% of the total grape production throughout the world are due to this issue (Acuña *et al.*, 2013).

One postharvest table grapes pathogen that has not been cited in Argentina until 2010-2012 is *Alternaria* spp. (Rodríguez Romera *et al.*, 2012). This genus includes saprophytic or parasitic plant pathogens, responsible for the deterioration of fruits and vegetables in the field and also at postharvest, during transport and storage, that cause considerable economic losses. Members of the genus Alternaria are a frequent cause of infections in which the fungus penetrates the tissue where it remains dormant until conditions for infection are favorable (Pavón Moreno *et al.*, 2012). Environmental requirements for infection depend on the Alternaria species and the host, but the interaction of a suitable temperature and a film of water produced by rain, dew condensation or overhead irrigation is always necessary. Optimum temperature for mycelial growth is around 18-25 ºC, but spores are able to germinate and infect in a range of 4 to 35 ºC. Under optimal temperature conditions, at least 5-8 hours of wetness are required for infection (EFSA on Contaminants in the Food Chain, 2011).

Previous studies carried out in the Instituto Nacional de Tecnología Agropecuaria (INTA) Estación Experimental Agropecuaria (EEA) Mendoza during 2010-2012 seasons, showed that the pathogen *Alternaria* spp. was detected in table grapes in a high

percentage and that it colonizes berries, pedicels and rachis during the entire bunch development period. After harvest, the pathogen can remain in bunches, producing lesions; and as Alternaria is able to grow at low temperatures, stress factors during the postharvest period could predispose the bunch decay produced by this pathogen. The rot caused by *Alternaria* spp. during postharvest storage is characterized by superficial, firm and dark brown lesions on berries, usually near the pedicels; and presence of gray mycelium in rachis, pedicels and berries (Rodríguez Romera *et al.*, 2012).

Chemical synthetic fungicides are the primary means to control postharvest diseases. The most used commercial treatment to reduce or avoid rachis browning and postharvest table grapes decay is the application of SO_2 generators. However, it can damage the fruit when access routes, such as skin wounds, detachment of the pedicel and not suberized lenticels, facilitate its entrance. In addition, SO_2 can cause spicy flavor and irritating smell, deteriorate berries by whitening and increase the rate of water loss; causing, therefore, premature browning of the stems (Swart and Holz, 1994).

According to World Health Organization reports, there are 20 000 unintentional deaths and 2 million poisonings each year, mainly caused by the misuse of synthetic fungicides in third world countries. Their use in food commodities storage has had several side effects on human health, such as carcinogenicity, teratogenicity, hormonal imbalance, among others. (Gilbert, 2012). Nowadays there is a global demand to reduce the use of pesticides and to protect the environment and consumers health. Many countries that import table grapes have set increasingly stringent limits on the concentration of SO_2 (Pássaro Carvalho *et al.*, 2012; Rivero and Quiroga, 2010).

Given these reviewed facts, it turns quite apparent that effective, safe and eco-friendly strategies must be developed to reduce postharvest losses as well as to compensate for the shortage of synthetic fungicides. Biological control has been proposed as an

alternative strategy to reduce the use of SO_2 at postharvest storage (Montealegre and Pérez, 2013). Among the microorganisms considered as potential biological control agents, yeasts are an interesting option. They have the ability to colonize plant surfaces or wounds for long periods under dry conditions (Dimakopoulou *et al.*, 2008), survive in a wide range of environmental conditions, grow rapidly on inexpensive substrates in fermenters and they are easy to produce in large quantities (Spadaro *et al*., 2010). In a previous study, we have demonstrated that an indigenous yeast strain of *Metschnikowia pulcherrima* (RCM2) isolated from grapes, was able to control the growth of *Aspergillus* section *Nigri* (Ponsone *et al*., 2012a) while showing the ability of growing at 0-1 ºC. Alternatively, edible films or covers represent an interesting option, without environmental costs or adverse effects on human health. They consist of thin layers that pre-form or form directly on the surface of plant products as protective wraps; they are biocompatible, biodegradable and non-toxic (Cruañes and Locaso, 2011). Chitosan is a polysaccharide used for this purpose; it is obtained from the exoskeleton of crustaceans (Ramos-García *et al*., 2010), wings of some insects, fungal cellulose, algae and others by partial deacetylation of chitin (Kucukgulmez *et al.*, 2011). It has biological activity as antimicrobial against a broad spectrum of bacteria, yeasts and filamentous fungi (Cruañes and Locaso, 2011).

The aim of this work was to detect and quantify the presence of the pathogen *Alternaria* in different stages of the vine cycle, in table grapes cv. Red Globe, from flowering to postharvest storage, in Mendoza region. We also aimed to propose a replacement for SO_2 for extending the shelf-life of table grapes, using chitosan and the yeast strain *M. pulcherrima* RCM2 as biocontrol agents.

2. Material and methods

2.1. Flowers and grapes sampling

The field sampling was performed during the 2014-2015 seasons from successive vine phenological stages: flowering, pepper-corn size (4 mm diam.), pea-size (7 mm diam.), veraison and harvest; from a table grapes vineyard cv. Red Globe implanted in 2010 and situated in Junín, Mendoza. A simple random sampling was used, with plots completely randomized.

The berries were superficially disinfected with 70° alcohol for 30 seconds and with 1% hypochlorite for a minute; they were drained and then rinsed with distilled sterile water three times. Unfortunately, the fragility of the flowers precluded surface disinfection.

Some of the collected bunches at harvest time were stored in cold storage at 0-0.5 ºC, with relative humidity (RH) of 90-95%. Then, samples were taken at 30, 60 and 90 days of storage.

2.2. *Alternaria* spp. isolation

Isolation was made by direct sowing method. Groups of 10 flowers, collected from the flowering stage, and groups of 10 pieces of berries from pepper-corn size, pea-size, veraison, harvest and post-harvest stages (30, 60 and 90 days) were plated onto Dichloran 18% Glycerol Agar (DG18) and Dichloran Rose Bengal Chloramphenicol Agar (DRBC) media. Twenty repetitions were made for each stage.

After an incubation period of 7 days at 25 ºC, the percentage of infection was determinate for each sampled stage. Then, *Alternaria* spp. isolates were sub cultivated in synthetic nutritive agar (SNA) for 7 days at 25 ºC. The isolates were stored at 4 ºC.

2.2.1. Morphological and molecular identification of *Alternaria* spp. isolates

2.2.1.1. Morphological identification

A representative number of 45 strains belonging to Alternaria genus were selected. Monosporic cultures were transferred to Potato Carrot Agar (PCA) and V8 culture media (V8). The cultures were incubated under alternative cycles of white/black light (8/16 hours) at 22 ºC in order to induce the formation of the characteristic sporulation patterns. Morphological identification was made according to the methodology based on macroscopic and microscopic characteristics proposed by Simmons (2007).

2.2.1.2. Molecular identification

2.2.1.2.1. Extraction, purification and quantification of genomic DNA

Genomic DNA was extracted according to the method described by Liu *et al*. (2000). Fresh mycelium was collected from monosporic *Alternaria* spp. cultures.

Quality and quantity of DNA were checked by comparison with DNA of the bacteriophage λ digested with *Hind*III (New England, Bio Labs, Inc.), used as control DNA on 1% agarose gel.

In order to confirm the morphological identification of *Alternaria* spp. strains, the methodology proposed by Pavón *et al.* (2010) was used. This methodology is based on a PCR technique which uses oligonucleotides generated from the *Alt a1* gene as primers. This technique allows a rapid Alternaria genus detection and its subsequent identification at species-group level.

In the first place, the genus specific primers set, Dir5cAlta1 (GAGAACAGCTTCATGGACTTCTCTTT) and Inv4Alta1 (CGCGGCAGTAGTTGGGAA) was used, and an annealing temperature of 58 °C, for the genus identification.

Then, following the same methodology, the identification at species-group level was made using each primers set for the 4 defined species-groups; *A. alternata*: AaltDAlta1 (CGCATCCTGCCCTGTCA) and AinfIAlta1 (GTTGGTAGCCTTGATGTTGAAGC), *Alternaria infectoria*: AinfDAlta1 (CGCATCCTGCCCAGTTG) and AinfIAlta1 (GTTGGTAGCCTTGATGTTGAAGC), *Alternaria radicina*: AraDAlta1 (CCCGCCAGGACAACGCT) and AsolIAlta1 (GTTGGTGGCCTTGATGTTGAAG), and *Alternaria porri*: AsolDAlta1 (CGCATCCTGCCCCGTCT) and AsolIAlta1 (GTTGGTGGCCTTGATGTTGAAG). Annealing temperatures of 65, 63, 65 and 63 °C were used, respectively.

The PCR reactions were carried out in an Eppendorf Gradient thermocycler using the following conditions: an initial denaturation step at 95 °C for 1 minute; 35 cycles of denaturation at 94 °C for 30 seconds, 30 seconds at annealing temperature and extension at 72 °C for 45 seconds; and a final extension at 72 °C for 5 minutes.

The amplification products were examined by electrophoresis on 1.7% agarose gel stained with ethidium bromide and visualized with a trans illuminator under UV light. The size of the obtained fragments was estimated by visual comparison with control DNA size molecular markers (Invitrogen) (50 bp ladder for species-groups and 100 bp ladder for gender identification, with reference bands that range between 50-1500 bp and 100-1500 bp, respectively).

2.2. Postharvest *in vivo* tests with controllers of natural origin

An *in vivo* test was carried out with asymptomatic grape bunches cv. Red Globe during the season 2016. The effectiveness of two inoculum levels of the yeast strain *M. pulcherrima* RCM2, previously obtained from red grapes (Ponsone *et al.*, 2012b), was evaluated as alternative postharvest treatment to SO_2 in table grapes cv. Red Globe. Also, we tested two concentrations of chitosan, obtained experimentally in the Instituto Nacional de Tecnología Industrial (INTI) of Mar del Plata, according to internal procedures from exoskeletons of prawns.

The experimental design included treatments with SO_2 generators (1g/kg) and potential biological controllers, chitosan and *M. pulcherrima* RCM2. The applied variables were two doses of chitosan (0.5 and 1%) and two levels of yeast inoculum (10^4 and 10^6 cells/mL); berries with and without wounds (3 wounds per berry made with hypodermic needle, 3 mm deep and 3 mm wide) and different moments of inoculation of an isolated postharvest fungus strain identified as *A. alternata*. These treatments were accompaniedby with their respective controls (berries without Alternaria inoculation) and an absolute control (berries with only sterile distilled water).

Each treatment was applied on 10 groups of 3 asymptomatic grape berries cv. Red Globe placed in plastic containers previously disinfected with 70º alcohol. Then, they were placed in cold storage rooms at 0-0.5 °C, with 90-95% RH, of the Postharvest Laboratory of the INTA EEA Mendoza. All treatments were carried out in triplicate.

The treatments were classified as Inoculation with "*Alternaria* before" the controller's application and Inoculation with "*Alternaria* after" the controller's application. In the first case, an inoculum of 10^4 conidia/mL of *A. alternata* was sprinkled on the berries and allowed to dry at room temperature for a period of 3 hours. Subsequently, the corresponding controller was applied; evaluating its activity as curative. In the second case, the corresponding biocontroller agent was applied first, and after 3 hours of

drying, an inoculum of 10^4 conidia/mL of *A. alternata* was sprinkled and left to dry again for 3 hours before being admitted to the cold room; evaluating the controller as preventive. SO_2 was only evaluated as curative because it is traditionally used that way. At the end of the experiment, the incidence of the disease was determined by visual evaluations, counting the number of table grapes berries cv. Red Globe with symptoms of rot caused by *Alternaria* spp., at 15, 30, 60 and 90 days in cold storage. It was calculated as follows: Incidence (%) = (number of decayed berries/number of total berries) x 100.

2.3. Statistical analysis

Data on the *Alternaria* spp. isolation and the postharvest *in vivo* test were analyzed by an analysis of variance (ANOVA), followed by Fisher LSD test. The threshold for statistical significance was set at $p<0.05$. Statistical analyses were done using the Software Infostat (Infostat version 2013).

3. Results and discussion

3.1. *Alternaria* spp. isolation

The results obtained in this study revealed the presence of *Alternaria* spp. in all the studied stages. The highest incidence was detected in flowering with 98%; after this stage, the percentage was highly reduced. Subsequently, a marked increase in the incidence during veraison and postharvest at 30, 60 and 90 days, was observed (Fig. 1). That fluctuation of the incidence percentage could be explained by the use of field fungicides. Normally, they are applied during flowering phase and then reinforced with 2 to 3 repetitions during veraison. Our hypothesis is that the incidence of *Alternaria* spp. diminished after flowering because of a fungicide application carried out between this stage and pepper-corn size; therefore the presence of the pathogen in the fruit was

reduced. In addition, the increase in the incidence observed in veraison could be related to the increase of sugars available in the berries.

Our results coincide with those reported by Swart and Holz (1991), who pointed out that a latent infection in the field, not detectable during harvest, can play an outstanding role in the rot caused by *Alternaria* spp. in postharvest. This behavior has also been recorded by other authors in the first published work on the subject (Hewitt, 1974).

Rotem (1994) observed that many of the hosts of the genus *Alternaria* are susceptible to their respective diseases at two stages of their development. The first peak of susceptibility occurs in the juvenile stage and the second one in the senescent stage. These results also agree with those that we present in this study. *Alternaria* spp. colonizes berries, pedicels and rachis throughout the period of bunch development, but stress factors during cold storage could predispose the bunchs to decay by this pathogen. Also, species-groups of small spores of *Alternaria* spp. (*A. alternata*, *Alternaria tenuissima* and *A. infectoria*, mainly) have been previously reported as contaminants of cereals, fruits and vegetables, causing decay before and after harvest (Andersen *et al.*, 2015; Armitage *et al.*, 2015; Logrieco *et al.*, 2009).

3.2. Morphological and molecular identification of *Alternaria* strains

According to their sporulation patterns, all of the 45 Alternaria strains were morphologically identified as *A. alternata*. In addition, the molecular identification, based on the *Alt a1* marker, showed for all of them, a specific amplification of 195 bp corresponding to the primers set Dir5cAlta1-Inv4Alta1, confirming, in the first instance, its belonging to the genus Alternaria (Fig. 2 A).

Subsequently, by using the primers set derived from the gene *Alt a1*, AaltDAlta1-AinfIAlta1, corresponding to the species-group *A. alternata*, it was observed that a

fragment of 118 bp, specific for it, was amplified for all the strains (Fig. 2 B). There was no amplification for the primer sets corresponding to *A. infectoria*, *A. radicina* or *A. porri* species-groups of Alternaria genus, neither for negative controls (Fig. 2 C).

To have quick molecular techniques, capable of differentiating Alternaria species-groups, is important for the early detection of phytopathogenic species in sensitive crops and for the establishment of preventive and corrective measures, in order to reduce economic losses in the primary sector and minimizing risks to consumer health (Pavón *et al*., 2010).

The need for a proper identification is also related to the ability of many of the species belonging to Alternaria genus, to produce numerous secondary metabolites, being some of these metabolites, toxic (Lee *et al*., 2015; Zain, 2011). They can also play an important role in the pathogenesis of plants. *A. alternata* is involved in the production of mycotoxins in foods, including alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT), tentoxin (TEN), tenuazonic acid (TeA), and altertoxins (ATX) (López *et al*., 2016; Troncoso-Rojas and Tiznado-Hernández, 2014).

The presence of mycotoxins in food is a topic of concern to human and animal health. Furthermore, the food deterioration and the occurrence of mycotoxins can lead to economic losses and food safety issues (Sivagnanam *et al*., 2017).

Alternaria alternata has been isolated from different plant crops and has been reported as a contaminant in fruits such as tomatoes (Yang *et al*., 2017), pears (Pan *et al*., 2017), apple (Estiarte *et al*., 2017), citrus (Gabriel *et al*., 2017), *Hylocereus undatus* (pitahaya fruits) and *Selenicereus megalanthus* (dragon fruit) (Castro *et al*., 2017; Vilaplana *et al*., 2017). As in the present work, *A. alternata* was the only *Alternaria* species-group identified in healthy wine grapes of the varieties of Malbec, Chardonnay, Merlot, Cabernet and Bonarda during 2001 vintage in Mendoza, Argentina (Magnoli *et al.*,

2003). In addition, Prendes (2015) determined on the basis of the morphological and molecular characters of Malbec grapes fungal isolates at harvest time of the DOC San Rafael during 2011 and 2012 vintages, that all of them belonged to the species-group *A. alternata.*

3.3. Postharvest trials with potential biocontrol agents

Since the treatments with chemical fungicides in postharvest are not friendly to the environment, nor to the health of the consumers, and in addition, are not allowed in every country arround the world; the search for suitable alternatives aiming to counteract fruit rot, is necessary (Yan *et al.*, 2014). In the last 25 years, due to the increase in regulations and the demands of consumers for healthier products, interest in biological control has increased as an alternative method to the application of chemical fungicides for the control of fungal growth in fruits (Liu *et al.*, 2013).

In the present work, the effect of chitosan and the yeast strain *M. pulcherrima* RCM2 as biological control agents of the rot caused by *A. alternata* during postharvest, was evaluated. The results obtained in our assay showed, at 15 and 30 days postharvest evaluations, the average of the incidence of *A. alternata* were lower than 5% in all the treatments. These results agree with Swart and Holz (1991); who observed that the symptoms of rot caused by *A. alternata* in table grapes were only evident at the end of prolonged cold storage.

When we compared the treatments with respect to the absolute control at 60 days evaluation, it was observed that there was a significant difference with respect to the incidence of the pathogen?, being lower for all of them than for the absolute control ($p<0.05$). This indicates that all the treatments were effective, at that instance, in reducing the incidence of *A. alternata* (Fig. 3 A). Analyzing *M. pulcherrima* RCM2 treatments, the incidence of *A. alternata* was lower when the highest level of inoculum

(10^6 cells/mL) was applied, compared to the lowest one (10^4 cells/mL) ($p<0.05$) (Fig. 3 A). This occurred despite that the incidence of *A. alternata* was higher in treatments with *M. pulcherrima* RCM2 than in those where SO_2 was applied for all assessment dates. Even more, at the end of the assay (90 days evaluation) there was no statistically significant difference between them, nor with the absolute control ($p<0.05$) (Fig. 3 B). The yeast strain was non-effective at the *in vivo* tested conditions, possibly due to the inoculum level used.

Since antagonistic yeasts have been previously used successfully in the control of various rotten and toxicogenic fungi in table and wine grapes (Lemos Jr. *et al.*, 2016; Nally *et al.*, 2012, 2013; Prendes *et al.*, 2015; Ponsone *et al.*, 2011); we think that, in our case, it is necessary to test a higher inoculum level. Also, Suzzi *et al.* (1995) observed that yeasts isolated from grape berries possessed biocontrol activity against plant pathogenic fungi, including *A. alternata*.

Many different yeast species of the genus *Metschnikowia*, including *M. pulcherrima* (Oro *et al*., 2014; Saravanakumar *et al*., 2009), *Metschnikowia fructicola* (Kurtzman and Droby, 2001; Spadaro and Droby, 2016), and *Metschnikowia andauensis* (Manso and Nunes, 2011), have been used as biocontrol agents in postharvest. Iron competition was reported as the main mode of action of *M. pulcherrima* (Saravanakumar *et al*., 2008) to inhibit *Botrytis cinerea, A. alternata* and *P. expansum* development in apples stored at 1 °C for 8 months under controlled atmosphere (2% O_2 and 3% CO_2).

It is interesting to mention the assay of Karabulut *et al.* (2003) where *M. fructicola* was applied on table grapes 24 hours before harvest time, to control the incidence of postharvest diseases generated by *B. cinerea*, *Alternaria* spp. and *Aspergillus niger*. After storage for 30 days at 1 °C, followed by 2 days at 20 °C, the incidence of the

diseases were reduced by approximately 60% and the population of *M. fructicola* persisted in the grapes during their storage.

Regarding the moments of inoculation of *Alternaria* respect to *M. pulcherrima* RCM2, at 60 and 90 days evaluations, there were no significant differences between the inoculation of the pathogen before and after applying the yeast. Likewise, no significant difference was observed between incidences registered at both moments of inoculation, with the absolute control ($p<0.05$).

It was observed in both evaluations dates, for all the treatments, higher incidence of *A. alternata* in berries with wounds, when compared to berries without wounds (Fig. 4). Previous studies on other substrates, such as pepper, indicated that *Alternaria* spp. causes rottenness in its fruits; and in addition to pathogenic species, which colonize plants through flowers, saprophytic species can also infect fruits if their skin is injured by insects, cooling, sunburn or calcium deficiency (Hochmuth and Hochmuth, 2009; Wall and Biles, 1993).

In the evaluations performed at 60 and 90 days, it was observed that the most effective treatments to control the incidence of *A. alternata* were SO_2, 0.5% chitosan and 1% chitosan ($p<0.05$) (Fig. 4).The results for the treatments with application of chitosan, including both concentrations, different moments of application of the pathogen and berries with and without wounds; showed that there was no significant difference with respect to the traditional treatment in none of the 4 postharvest evaluations carried out in terms of control of *A. alternata* in table grapes cv. Red Globe ($p<0.05$). Therefore, this biocontroller is a very promising alternative to replace SO_2 generators during postharvest.

In previous studies, El Ghaouth *et al.* (1992) showed that chitosan coverings were effective at reducing straw rot caused by *B. cinerea* and *Rhizopus stolonifera* in *in vivo*

assays. After 14 days of storage of the fruit, chitosan coverages at 15 mg/mL reduced the rot caused by both fungi in a 60%. More recently, Sánchez Domínguez *et al.* (2007) studied the *in vitro* effect of chitosan on the development and morphology of *A. alternata* in tomato, in which an increase in the degree of inhibition and a reduction of growth of this fungus in 50.6%, was obtained.

Those results coincide with a large number of studies that demonstrate the efficacy of chitosan treatments applied in the conservation of table grapes, such as those of Romanazzi *et al.* (2002 and 2012) and Freitas *et al.* (2015), in strawberries and cherries (Romanazzi, 2010; Romanazzi *et al.*, 2013), in tomatoes (Badawy and Rabea, 2009) and in citrus fruits (Chien *et al.,* 2007) In these fruits, the percentages of gray rot (*B. cinerea*) and other rottenness, such as green and blue ones, caused by *Penicillium digitatum* and *Penicillium Italicum*, respectively, decreased significantly with respect to the control.

Chitosan allows prolonging the shelf-life of fruits and vegetables by forming a semi-permeable layer that regulates the exchange of gases and reduces humidity losses through transpiration.

Our results indicated that there was no significant difference between the two chitosan doses applied in postharvest, in terms of controlling the incidence of *A. alternata* ($p<0.05$), in none of the 4 evaluation dates.

Researchers reported that the level of inhibition of fungi was also highly correlated with chitosan concentration, indicating that chitosan performance is related to the application of an appropriate amount (Ziani *et. al.*, 2009). Rodríguez Navas *et al.* (2015) in their studies done *in vitro* on table grapes cv. Red Globe with inoculation of *B. cinerea* B24, showed that the growth of the fungal mycelium decreased 70% in those treatments where 0.5% chitosan was applied; 77% with 1% chitosan and 85% with 2% chitosan.

Although the lower growth of *B. cinerea* B24 was recorded in the treatments with the highest concentration of chitosan (2%), there was no significant difference ($p=0.01$) between this treatment and the growth recorded using 1% chitosan. It is important to note that not all fungi have the same sensitivity to chitosan and this may be due to the composition of membrane phospholipids and particularly to the nature of their charges (Ramos García *et al.*, 2010).

In the present study, the behavior of chitosan with respect to the moments of inoculation of *A. alternata,* was also analyzed. In fact, it was observed that *Alternaria alternata* incidence was lower when the pathogen was inoculated before the chitosan application ($p<0.05$), at 60 and 90 days evaluations (Fig. 5).

This work represents an early step for further research, as it is the first study about *Alternaria* spp. incidence during the phenological cycle and postharvest storage of table grapes cv. Red Globe. It is also the first step of the evaluation of alternative strategies to the use of SO_2 for the control of this disease, during the postharvest storage. Further studies must be done in order to assess the quality of the treated fruit during the conservation period; considering that organoleptic properties are very important in this product, which is marketed for fresh consumption.

3. Conclusions

Alternaria pathogen was detected in all stages of the phenological cycle of table grapes cv. Red Globe analyzed, and in postharvest. Its highest incidence value was observed in the flowering stage, followed by veraison and postharvest at 90, 60 and 30 days of cold storage. All the strains analyzed were identified as *A. alternata*.

The presence of wounds in the berries favored the development of the rot caused by *A. alternata* in postharvest. This evidences that taking care of the healthiness status of the table grapes constitutes another way of preventing the incidence of rottenness.

Chitosan was found effective as biocontrol in *in vivo* postharvest essays against *A. alternata*, producing incidence rates similar to those obtained with SO_2 (traditional control) in prolonged cold storage. Equally beneficial results were obtained by applying the different doses of chitosan, thus the option of using the lowest doses would reduce the residue of chitosan in the grape that will be marketed, and which is an encouraging result. In addition, it was observed that chitosan treatments were more effective in reducing the incidence of *A. alternat*a when applied after the *Alternaria* inoculum. This fact suggests that chitosan would work better being used as a curative traetment in grapes that come with a certain level of inoculum established from the vineyard. Taking into consideration the results presented, the use of chitosan could be suggested as a substitute for traditional chemical control for reducing the incidence of *A.alternata* in grape postharvest.

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5. Founding sources

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Figure 1

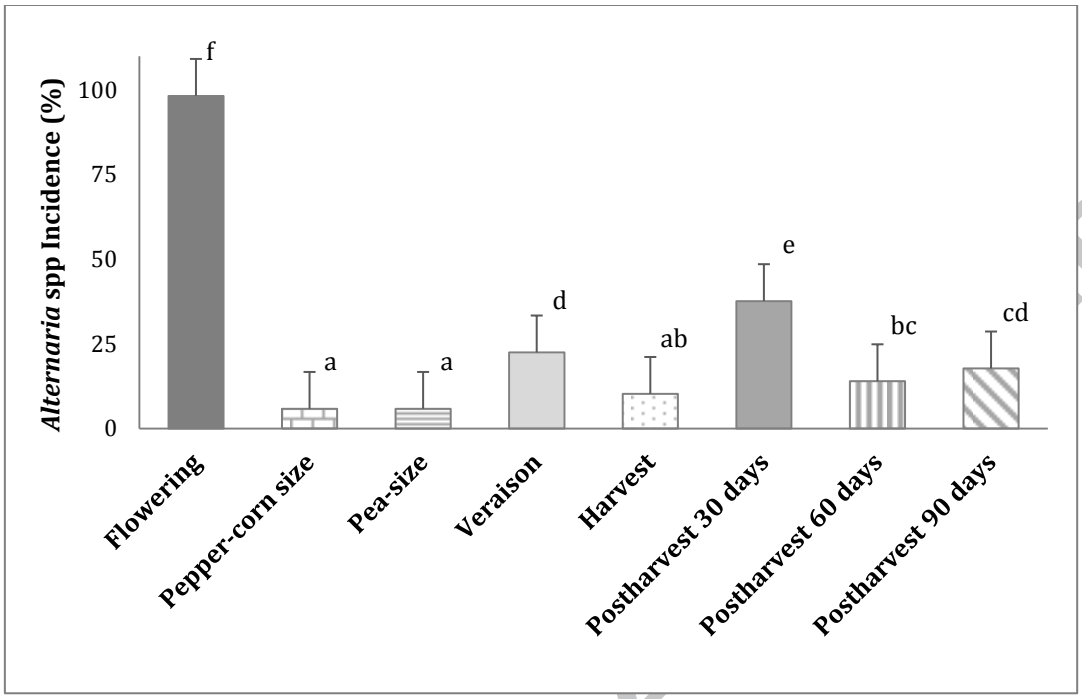

Figure 2

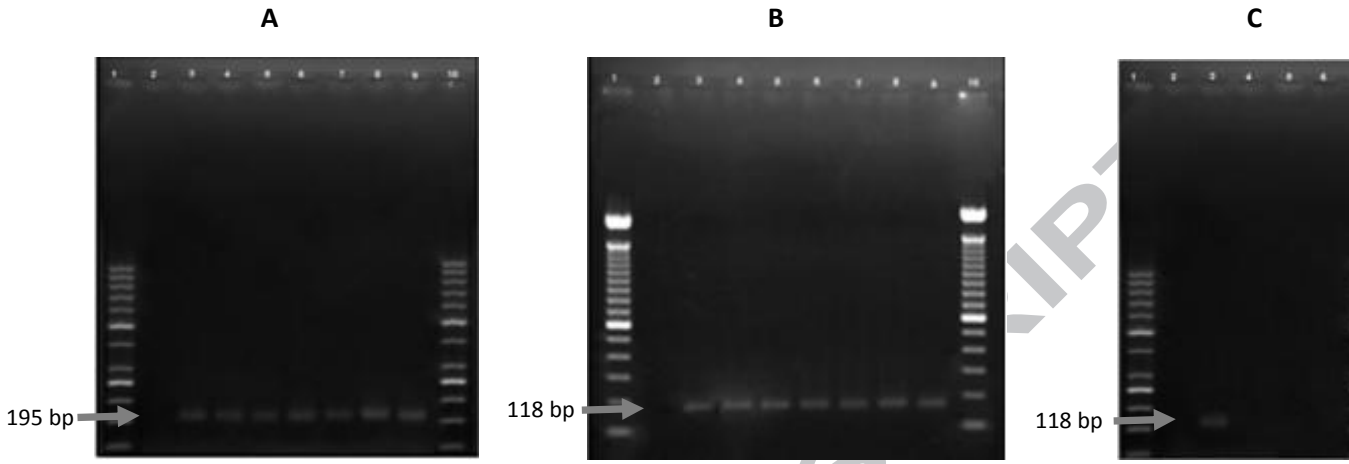

Figure 3

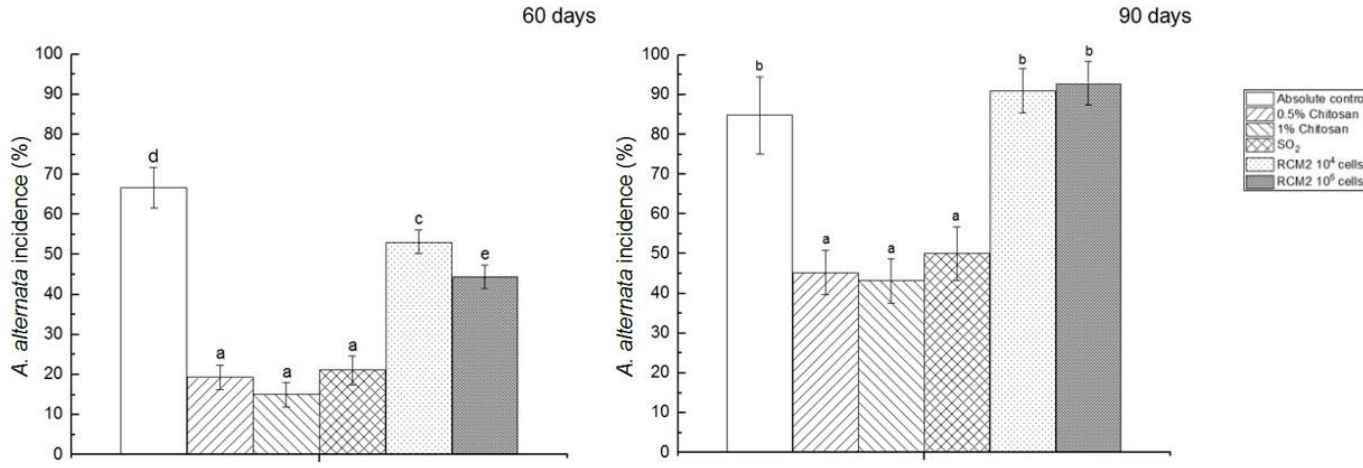

Figure 5

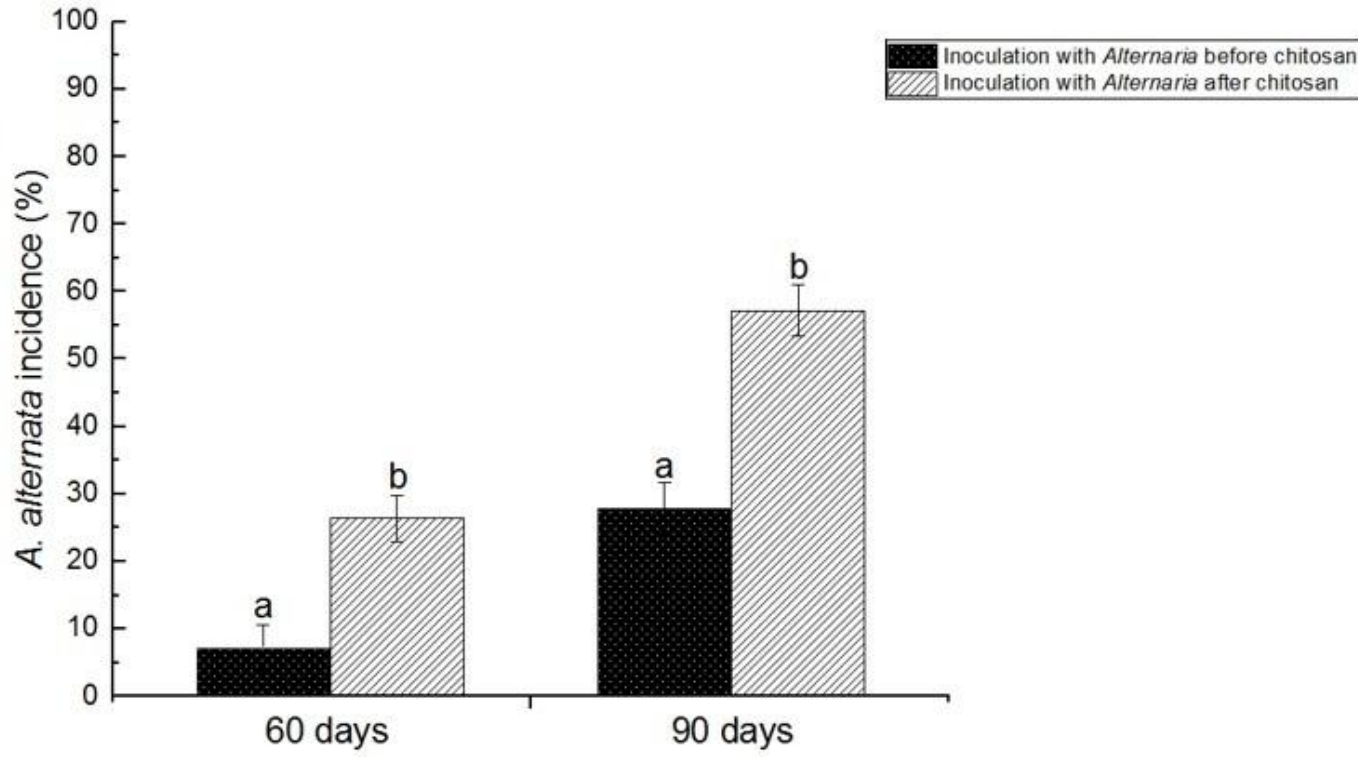

Table 1

60 days evaluation				90 days evaluation			
Treatments	Means	SE	LSD[a]	Treatments	Means	SE	LSD[a]
1% Chitosan	15	2.96	a	1% Chitosan	43.18	5.55	a
0.5% Chitosan	19.30	2.96	a	0.5% Chitosan	45.28	5.55	a
SO_2	21.11	3.62	a	SO_2	50	6.79	a
RCM2 10^6	44.45	2.96	b	Absolute control	84.81	9.61	b
RCM2 10^4	53.16	2.96	c	RCM2 10^4	91.07	5.55	b
Absolute control	66.67	5.12	d	RCM2 10^6	92.89	5.55	b

SE: Standard error

[a] *Values with the same superscript do not differ significantly (test of the minimum significant difference of Fisher LSD ($p<0.05$)).*

Figure and table legends

Fig. 1: Incidence of *Alternaria* spp. in the phenological stages and postharvest of table grapes cv. Red Globe, obtained from 2014/15 season.

Fig. 2: Agarose gels electrophoresis showing the PCR products.
(A) Lanes 1 and 10: 100 bp molecular weight marker with reference bands that range between 100-1500 bp (Invitrogen); lane 2: negative control; lanes 3-9: strains identified morphologically as *A. alternata*.
(B) Lanes 1 and 10: 50 bp molecular weight marker with reference bands that range between 50-1500 bp (Invitrogen); lane 2: negative control; lanes 3-9: strains identified morphologically as *A. alternata*.
(C) Lanes 1 and 7: 50 bp molecular weight marker with reference bands that range between 50-1500 bp (Invitrogen); lane 2: negative control; lane 3: primers set of the species-group *A. alternata*; lane 4: primers set of the species-group *A. infectoria*; lane 5: primers set of the species-group *A. porri*; lane 6: primers set of the species-group *A. radicina*; on a strain previously identified morphologically and molecularly as *A. alternata.*

Fig. 3: *A. alternata* incidence (%) at 60 and 90 days evaluation for the different treatments on table grapes cv. Red Globe inoculated with 10^4 cells/mL of *A. alternata*.

Table 1: *A. alternata* incidence (%) at 60 and 90 days evaluation for the different treatments on table grapes cv. Red Globe inoculated with 10^4 cells/mL of *A. alternata*.
SE: Standard error
[a] *Values with the same superscript do not differ significantly (test of the minimum significant difference of Fisher LSD p<0.05).*

Fig. 4: *A. alternata* incidence (%) at 60 and 90 days evaluations for treatments with berries with, and without artificial wounds on table grapes cv. Red Globe inoculated with 10^4 cells/mL of *A. alternata.*

Fig. 5: *A. alternata* incidence (%) at 60 and 90 days evaluations for treatments with inoculation of 10^4 cells/mL of *A. alternata* after and before the application of chitosan on table grapes cv. Red Globe.

Highlights

- The incidence of *Alternaria* spp was measured in different stages of the vine cycle in table grapes cv. Red Globe, from flowering to postharvest storage.
- Two alternative replacements for SO_2 were tested: a strain of *Metschnikowia pulcherrima* RCM2 and chitosan.
- The antagonistic activity against *Alternaria alternata* was tested under postharvest conditions.

Graphical Abstract