Effect of potassium bicarbonate on fungal growth and sclerotia of *Sclerotium cepivorum* and its interaction with *Trichoderma*

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ORIGINAL

Efecto del bicarbonato de potasio en el crecimiento fúngico y esclerocios de *Sclerotium cepivorum* y su interacción con *Trichoderma*

Resumen. Se estudió el efecto del bicarbonato de potasio ($KHCO_3$) como agente antifúngico en el crecimiento de los hongos *Sclerotium cepivorum* y *Trichoderma* cepa R39. Se evaluó el crecimiento del micelio, el número y germinación de esclerocios formados por *S. cepivorum*, así como el antagonismo de *Trichoderma* sobre el patógeno, en el medio de cultivo papa dextrosa agar (PDA, Baker®) enriquecido con siete concentraciones de $KHCO_3$ (2, 4, 6, 8, 10, 25 and 50 mM). Con 50 mM de $KHCO_3$, el crecimiento de *S. cepivorum* fue inhibido en 34.5% y el de *Trichoderma* en 83.1%. Con la aplicación de 10, 25 y 50 mM de $KHCO_3$, la formación de esclerocios por *S. cepivorum* fue significativamente inhibida (51.5%, 77.0% y 100%, respectivamente), al igual que la germinación de los esclerocios (18%, 78% y 86%, respectivamente). Al confrontar ambos hongos en el medio de cultivo enriquecido con $KHCO_3$, no se afectó la capacidad antagónica de *Trichoderma* hacia el patógeno. Después de 144 h, *Trichoderma* mostró una colonización del 70% sobre el micelio de *S. cepivorum*, ante 50 mM. Los beneficios potenciales del $KHCO_3$ para inhibir tanto el crecimiento y desarrollo de *S. cepivorum* fueron evidentes al reducir el crecimiento fúngico, la formación y germinación de esclerocios.
Palabras clave: compuesto antifúngico, antagonismo, fitopatógeno, biocontrol.

Abstract. The effect of potassium bicarbonate ($KHCO_3$) as an antifungal agent was tested on the fungal growth of *Sclerotium cepivorum* and *Trichoderma* strain R39. The growth of the fungal colony, the number and germination of sclerotia formed by *S. cepivorum*, as well as the antagonism of *Trichoderma* on the pathogen were evaluated in potato dextrose agar (PDA, Baker®) culture media amended with seven concentrations of $KHCO_3$ (2, 4, 6, 8, 10, 25 and 50 mM). At 50 mM, the growth of *S. cepivorum* was inhibited 34.5%, whilst for *Trichoderma* inhibition was 83.1%. At the concentrations of 10, 25 and 50 mM of $KHCO_3$ sclerotia formation by *S. cepivorum* was significantly inhibited (51.5%, 77.0% and 100%, respectively), likewise the sclerotia germination (18%, 78% and 86%, respectively). When both fungi were confronted in a $KHCO_3$-enriched PDA, this chemical compound did not affect the antagonistic capability of *Trichoderma* towards the pathogen. After 144 h, *Trichoderma* showed an invasion of 70% over the colony of *S. cepivorum*, at 50 mM. The potential benefits of $KHCO_3$ to inhibit both growth and development of *S. cepivorum* were evident, since it reduced either fungal growth or both formation and germination of sclerotia.
Keywords: antifungal compound, antagonism, plant pathogen, biocontrol.

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Introduction

Mexico at the end of 2010 had 19,225 ha cultivated with onion (*Allium cepa* L.), and average yield of 15,933 ton ha^{-1} (SIAP, 2011). However, this crop is affected by white rot, caused by the fungus *Sclerotium cepivorum* Berk*,* which spreads rapidly and causes significant crop losses (Pinto *et al.,* 2000; Ponce-Herrera *et al*., 2008). This fungus forms sclerotia, which may remain in the soil for up to 20 years. The occurrence of the disease is related to the presence and abundance of sclerotia in soil, since these structures remain dormant in the absence of the crop, and its germination is stimulated by root exudates of *Allium* species, which contain volatile compounds such as allyl sulfide and *n*-propyl (Entwistle *et al*., 1982; Tariq and Magee, 1990).

In order to reduce the damage caused by this plant pathogen, several methods have been used, but the most common is the chemical control. However, the excessive use of fungicides has caused pollution problems like leaving residues in the harvested crop, and most important it has also induced pathogen resistance (Fushiwaki *et al*. 1990; Ma and Michailides, 2005; Klose *et al*., 2010; Van Dyk and Pletschke, 2011). Therefore, less environmentally harmful control alternatives have been sought. For example, biological control which is based on the use of antagonistic microorganisms and on the use of innocuous chemical compounds, such as bicarbonates (Quiroz-Sarmiento *et al*., 2008; Ordóñez-Valencia *et al*., 2009; Ibarra-Medina *et al*., 2010). The aim of these alternatives is to contribute on soil conservation, as well as to ensure quality food sources for humans and to maintain a healthy environment (Bombelli and Wright, 2006).

Laboratory and greenhouse studies have successfully proven the effects of microbial antagonists on plant pathogens (Arzate-Vega *et al.,* 2006). In this way, species of the genus *Trichoderma* are efficient and long-lasting agents for controlling pathogenic fungi such as *Phytophthora*, *Sclerotinia*, *Sclerotium*, *Rhizoctonia*, *Fusarium*, among others (Tsahouridou and Thanassoulopoulos, 2002; Vinale *et al*., 2008; Ibarra-Medina *et al*., 2010), by producing toxic metabolites, inhibiting or parasitizing mycelium of soil-borne fungi (Zago *et al.,* 2001).

On the other hand, bicarbonates are commonly used in food industry to avoid fermentation, control pH, and develop adequate textures or flavors (Karabulut *et al*., 2001; Bombelli and Wright, 2006). Their efficiency has also been proven in the control of many phytopathogenic fungi (Palmer *et al*., 1997; Gamagae *et al*., 2003). The bicarbonate ion has been identified as the probable cause of growth inhibition in some bacteria and fungi. In the case of fungi, hyphal walls collapse in the presence of bicarbonate, then causing either growth inhibition or fungal death (Palmer *et al*., 1997; Ordoñez-Valencia *et al*., 2009).

There are reports about the effectiveness of potassium bicarbonate ($KHCO_3$) on the reduction of foliar damage produced by fungi, as well as the benefit of *Trichoderma* species for controlling soil-borne fungi (Tsahouridou and Thanassoulopoulos, 2002; Vinale *et al*., 2008). However, the effect of $KHCO_3$ on the growth of *S. cepivorum* and on its interaction with *Trichoderma* is unknown. This study evaluated the effect of $KHCO_3$ on the growth and development of *S. cepivorum*, and on its interaction with *Trichoderma* strain R39.

Materials and methods

Phase I. Effect of potassium bicarbonate on the mycelial growth of *Trichoderma* and *Sclerotium cepivorum*

Cultures of *Trichoderma* sp. strain R39 and *Sclerotium cepivorum* were obtained from the Departamento de Microbiologia, Colegio de Postgraduados. *Sclerotium cepivorum* was isolated from a garlic crop field at Guanajuato, and the *Trichoderma* strain was selected due to its antagonistic and mycoparasitic ability against a wide range of species of pathogenic fungi of cultivated plants, including *S. cepivorum* (unpublished data).

Disks of medium and mycelium (0.9 cm of diameter) of either *Trichoderma* sp. R39 or *S. cepivorum* were placed at the center of Petri dishes containing Potato Dextrose Agar (PDA, Baker®) enriched with seven concentrations of $KHCO_3$ (2, 4, 6, 8, 10, 25, and 50 mM) whose pH was 7.0, 7.0, 7.1, 7.3, 7.4, 7.9 and 8.0, respectively. Five Petri dishes were used for each concentration of $KHCO_3$. PDA-dishes cultures without $KHCO_3$ were used as control (pH 6.5). Fungal cultures were incubated at room temperature (20°C) for four days, and the fungal growth of each fungus was measured each 24 h. Afterwards, the Petri dishes with *S. cepivorum* were kept at room temperature (20°C) for 7 days (168 h), to count new-formed sclerotia at each concentration of $KHCO_3$.

A completely randomized experimental design was used for the experiment which included eight treatments for each fungus with five replicates each (n=5). Data were analyzed using analysis of variance and the mean comparison test (Tukey, α=0.05) using SAS statistical program (SAS Institute Inc, 2002).

Phase II. Effect of potassium bicarbonate germination of sclerotia of *Sclerotium cepivorum*

Sclerotia of *S. cepivorum* were collected from Petri dishes without $KHCO_3$ from the previous experiment. Sclerotia were surface disinfected with 80% alcohol for 30 seconds, followed by several rinses with sterile distilled water. The sclerotia were transferred to a gentamicine solution (20 µg mL^{-1}) and incubated at 4°C for 24 h. Excess of antibiotic solution was removed by blotting dry sclerotia on sterile paper. Ten sclerotia were placed in each Petri dish containing PDA and $KHCO_3$ at the concentrations and pH described in the previous section. Plates were incubated at 20°C, and thus, germinated sclerotia were counted daily during seven days.

A completely randomized experimental design was used for the experiment that included eight treatments with five replicates each (n=5). Data were analyzed using analysis of variance and the mean comparison test (Tukey, α=0.05) using SAS statistical program (SAS Institute Inc, 2002).

Phase III. Effect of potassium bicarbonate on the antagonism *in vitro* of *Trichoderma* R39 on *Sclerotium cepivorum*

Petri dishes containing PDA and $KHCO_3$ at the concentrations and pH indicated in the previous section were inoculated with PDA disks with mycelium. *Trichoderma* R39 and *Sclerotium cepivorum* were placed in opposite sides of the Petri dishes. *Sclerotium cepivorum* was inoculated first, due to its slow growth, and *Trichoderma* R39 was placed 24 h later. Fungal cultures were incubated at 20 °C and the growth of both fungi was measured every 24 h for four days. The invasion percentage of *Trichoderma* over the fungal colony of the pathogen was estimated according to the following equation (Rollan *et al.,* 1999): C=[(DCAP/DSP)x100], where: C=Invasion percentage; DCAP=distance covered by the antagonist on the pathogen colony over the axis which separates both fungi, DSP=distance between sowing points (6.5 cm).

A completely randomized experimental design was used for the experiment that included eight treatments with five replicates each (n=5). The data obtained were analyzed using analysis of variance and the mean comparison test (Tukey, α=0.05) using SAS statistical program (SAS Institute Inc, 2002).

Results

Phase I. Effect of potassium bicarbonate on the mycelial growth of *Trichoderma* and *Sclerotium cepivorum*

Concentrations of $KHCO_3$ produced gradual increases on the pH in culture media (from 7.0 to 8.0) when compared to the control (pH=6.5). After 24 h, concentrations of 2 and 6 mM of $KHCO_3$, significantly ($P<0.001$) inhibited the growth of *Trichoderma* by 20.6% and 50.3%, respectively; the concentrations of 10 mM and 25 mM caused an inhibition of 58.1% and 84.2%, while in 50 mM the growth of the fungal culture was entirely inhibited (Figure 1A). After 96 h, concentrations of 2 and 6 mM inhibited fungal growth by 4.0% and 7.3% while concentrations of 10, 25 and 50 mM caused an inhibition of 15.7%, 17.5 and 67.9%, respectively in comparison to the control (Figure 1A).

After 24 h, concentrations greater than 2 mM significantly ($P<0.001$) reduced the fungal growth, in comparison to the control (Figure 1B). In contrast, after 96 h, the application of 2, 10 and 25 mM, caused fungal growth inhibition of 1.5%, 5.8% and 6.4%, respectively; however, at 50 mM the fungal growth reduction was 34.5% in comparison with the control (Figure 1B).

$KHCO_3$ caused significant ($P<0.001$) reductions on the number of sclerotia. After 168 h, fully formed sclerotia were observed in the concentrations from 0 to 10 mM; in contrast, at 25 mM the sclerotia formation just started, while sclerotia did not form at 50 mM (Figure 2). At the concentrations of 2 and 10 mM of $KHCO_3$, the number of sclerotia decreased 9.1% and 51.5% respectively, while at 25 and 50 mM, the inhibition on the number of sclerotia was 77.0% and 100% respectively, in comparison to the control (Figure 2).

Phase II. Effect of potassium bicarbonate germination of sclerotia of *Sclerotium cepivorum*

$KHCO_3$ significantly inhibited ($P<0.001$) the germination of sclerotia. After 24 h, sclerotia germination percentage was 26% and 20% in the control (0 mM) and in the concentration of 2 mM, respectively. After 48 h, sclerotia germination at control, 2 and 4 mM was 46%, 36% and 12% germination, respectively, whilst from concentrations from 6 to 50 mM, germination was not observed (Figure 3). After 168 h, 100% germination of sclerotia was observed for concentrations between 0 and 8 mM, while at 10, 25 and 50 mM, germination was 80%, 22% and 14%, respectively (Figures 3 and 4).

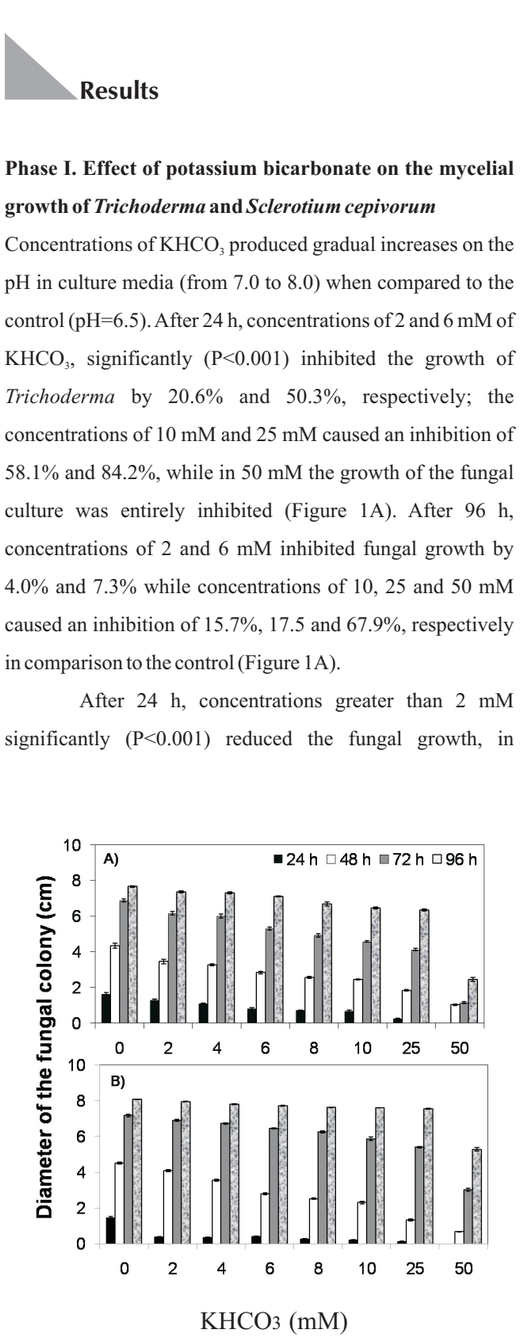

Figure 1. Effect of increasing concentrations of potassium bicarbonate ($KHCO_3$) on the individual growth of *Trichoderma* R39 (A) and *Sclerotium cepivorum* (B). Means±Standard error. n=5.

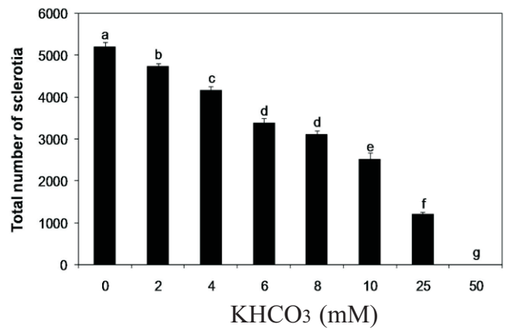

Figure 2. Effect of increasing concentrations of potassium bicarbonate ($KHCO_3$) on the formation of sclerotia by *Sclerotium cepivorum*, after 168 h. Means±Standard error. n=5.

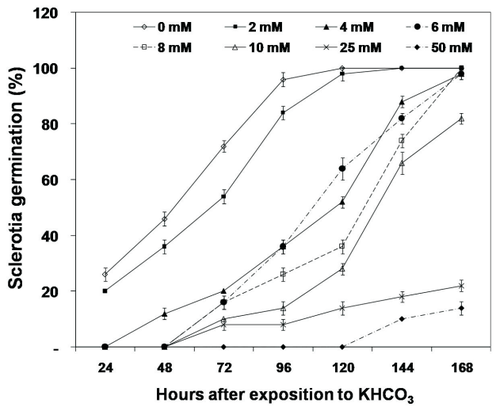

Figure 3. Effect of increasing concentrations of potassium bicarbonate ($KHCO_3$) on the sclerotia germination of *Sclerotium cepivorum* at 24, 48, 72, 96, 120, 144, and 148 h of exposure. Means ± Standard error. n=5.

Phase III. Effect of potassium bicarbonate on the antagonism *in vitro* of *Trichoderma* R39 on *Sclerotium cepivorum*

Concentrations of $KHCO_3$ up to 6 mM resulted in significant differences ($P<0.001$) in the antagonistic capability of *Trichoderma* towards the pathogen (Figure 5). After 96 h, invasion of *Trichoderma* on the colony of *S. cepivorum* at concentrations of 0 to 8 mM was approximately of 58%. At 10 and 25 mM the average percentage invasion of *Trichoderma* was 45%, while at 50 mM was 30% (Figure 5). After 120 h, the antagonism of *Trichoderma* on *S. cepivorum* persisted at concentrations from 0 mM (control) to 25 mM, in which the average invasion percentage was 78% (Figure 5). At 50 mM,

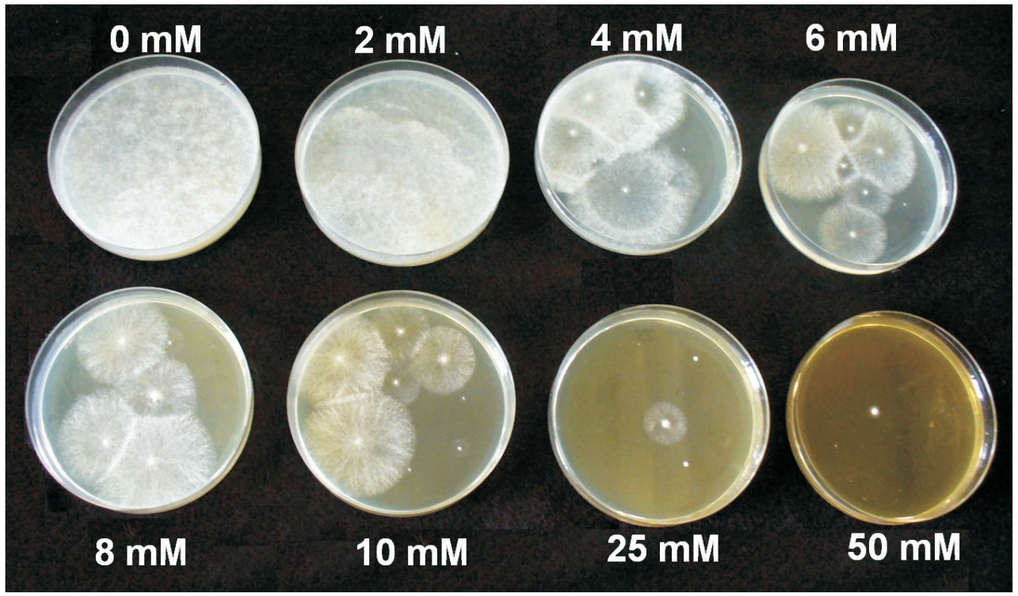

Figure 4. Effect of increasing concentrations of potassium bicarbonate ($KHCO_3$) on the sclerotia germination of *Sclerotium cepivorum*, after 168 h.

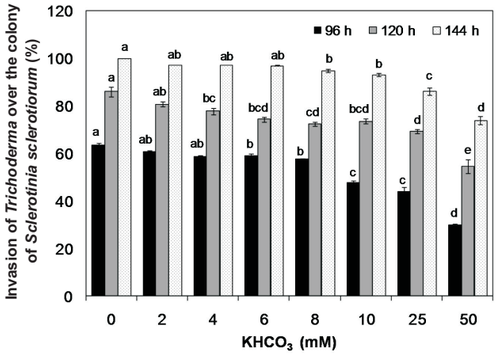

Figure 5. Effect of increasing concentrations of potassium bicarbonate ($KHCO_3$) on the invasion of *Trichoderma* (expressed in percentage) on the colony of *Sclerotium cepivorum* under *in vitro* conditions. Identical letters on bars at each sampling time are not significantly different (Tukey, =0.05). Means ± Standard error. n=5.

the invasion percentage was 53% (Figure 5) although the pathogen showed less growth than *Trichoderma* (Figure 6). After 144 h, *Trichoderma* grew over the colony of the pathogen by 96% at concentrations from 0 to 10 mM, while at 25 and 50 mM, the invasion of the antagonist on *S. cepivorum* was 80% and 70%, respectively (Figure 5).

Discussion

Results show that $KHCO_3$ exerted significant effects on the growth of *Trichoderma* R39 and *S. cepivorum*. For *Trichoderma* R39, the concentration of 50 mM inhibited in 67.9% the fungal growth. In contrast, the growth of *S. cepivorum* at 50 mM was inhibited in 34.5%. These results denote that *Trichoderma* was more sensitive to the presence

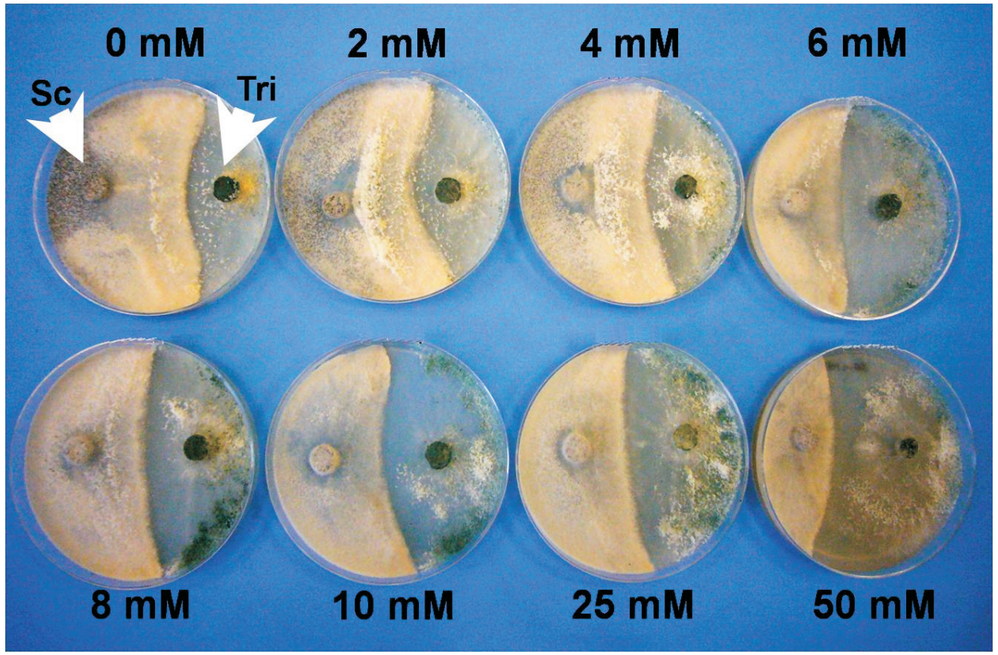

Figure 6. Effect of increasing concentrations of potassium bicarbonate ($KHCO_3$) on the antagonism of *Trichoderma* (Tri) on *Sclerotium cepivorum* (Sc), after 120 h.

of bicarbonate in comparison to *S. cepivorum*. This inhibitory effect can be explained partly due to the pH changes that bicarbonate concentrations induced in the culture media, thus affecting the acidic requirements for fungal growth (Steyaert *et al*., 2010). For instance, Harman and Taylor (1994) indicated that a pH 4.5 was more suitable for the growth of *Trichoderma* sp. *in vitro*, whilst at pH of 2.0 and/or 8.0 the fungal growth is slow. The antifungal effect of $KHCO_3$ observed on *S. cepivorum* is in accordance to findings that show the growth inhibition of *S. rolfsii* and *Botrytis cinerea* under *in vitro* conditions, when bicarbonates were applied at 20 or 40 mM (Palmer *et al*., 1997; Corral *et al*., 1998; Bombelli and Wright, 2006).

This study also shows that the number of sclerotia formed by *S. cepivorum* was significantly inhibited at $KHCO_3$-concentrations higher than 10 mM. Punja and Grogan (1982) and Palmer *et al*., (1997) found that the carbonates and bicarbonates applied in a concentration of 50 mM inhibited the formation and germination of sclerotia formed by *S. rolfsii* and *B. cinerea*. The decrease in the number of sclerotia observed in this study can be related to the pH increase due to $KHCO_3$. In this sense, several findings indicate that the pH affects both growth and sclerotia formation by fungal phytopathogens such as *B. cinerea*, *S. rolfsii*, *Sclerotinia sclerotiorum* and *Rhizoctonia solani* (De Pasquale and Montville, 1990; Ordóñez-Valencia *et al.*, 2009).

In addition, the present research shows the effects of $KHCO_3$ on the fungal confrontation between *Trichoderma* sp. and *S. cepivorum*. The antagonic effect of *Trichoderma* on *S. cepivorum* was maintained in all $KHCO_3$-concentrations, but the pathogen showed more growth inhibition at 50 mM. Although the antagonism of *Trichoderma* species on fungal pathogens is well documented (Vinale *et al*., 2008), the *in vitro* confrontation of this antagonist with *S. cepivorum* under culture media amended with $KHCO_3$ has not been previously reported.

The growth of *Trichoderma* was more sensitive to 50 mM of $KHCO_3$ than *S. cepivorum* in single cultures. In contrast, when both fungi were confronted, the growth of *S. cepivorum* was more limited. This effect can be in part explained due to the fungal antagonism in combination with the increased pH in the culture media by $KHCO_3$. In this regards, *Trichoderma* species preferably grow up in acid environments than in alkaline ones (Benitez *et al.,* 2004; Steyaert *et al*., 2010), and their antagonistic capabilities may be influenced by the pH in the culture media (Bell *et al*., 1982; Vinale *et al*., 2008). Likewise, the enrichment of culture media with $KHCO_3$ may result in modifications on growth patterns of the fungal colony. For example, *Trichoderma* at concentrations from 0 to 25 mM had greater sporulation, whereas *S. cepivorum*, exposed to 50 mM showed less abundance of mycelia, which has finer filaments (data not shown).

Our results agree with those from Tsahouridou and Thanassoulopoulos (2002) and De Souza *et al*. (2008) in the sense that *Trichoderma* species have antagonistic effects against pathogens such as *S. rolfsii* and *Moniliophthora perniciosa.* There are no earlier reports about the inhibitory effects of $KHCO_3$ on the antagonistic capability of *Trichoderma* sp. on *S. cepivorum*, therefore the importance of this study.

An inhibitory effect of $KHCO_3$ on the formation and germination of sclerotia at concentrations up to 10 mM was observed. An 80% inhibition in sclerotia germination of *S. rolfsii* in the presence of 40 mM of $KHCO_3$ was reported (Ricker and Punja, 1991). This effect shows the potential of bicarbonates to control not only the growth of *S. cepivorum* but also to inhibit the germination of sclerotia. In this regards, Wilson (1999) showed that carbonates and bicarbonates (sodium and potassium) are effective chemical compounds against *B. cinerea* which is the causal agent of postharvest diseases in grapes. Likewise, applying 50 mM of ammonium

bicarbonate (NH_4HCO_3) reduced the number of sclerotia en *S. rolfsii* under *in vitro* conditions (Punja and Grogan, 1982), while Palmer *et al.* (1997) indicated that *B. cinerea* did not produce sclerotia at concentrations greater than 50 mM of $KHCO_3$. The negative effects of bicarbonates on the formation and/or germination of spores have been demonstrated for fungal plant pathogens such as *Colletotrichum gloesporiodes*, *Penicillium* spp. (Korsten *et al*., 2000; Gamagae *et al*., 2003).

Conclusions

$KHCO_3$ had negative effects on the growth of both *S. cepivorum* and *Trichoderma* R39 *in vitro*. The formation of sclerotia by *S. cepivorum* was inhibited by 77% and 100% with 25 and 50 mM of $KHCO_3$. Sclerotia germination of *S. cepivorum* was significantly inhibited as the concentration of bicarbonate in the culture media increased. Potassium bicarbonate had significant effects during the confrontation of both fungi, nevertheless *Trichoderma* kept its antagonistic effect on *S. cepivorum*, even at the highest concentration (50 mM).

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