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FULL PAPER

Claudia Ordóñez-Valencia · Alejandro Alarcón
Ronald Ferrera-Cerrato
Laura Verónica Hernández-Cuevas

In vitro antifungal effects of potassium bicarbonate on *Trichoderma* sp. and *Sclerotinia sclerotiorum*

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Abstract Bicarbonates are often utilized in the food industry to avoid fermentation and to improve pH, flavor, and texture. In the same manner, bicarbonates have been demonstrated to control postharvest phytopathogens; however, there are no reports describing the effects of these chemical compounds either on soil-borne pathogens such as *Sclerotinia sclerotiorum* or on antagonist fungi such as *Trichoderma* species. This study evaluated the antifungal effect of increasing concentrations (0, 2, 4, 6, 8, 10, 25, and 50 mM) of potassium bicarbonate ($KHCO_3$) on the growth of *Trichoderma* sp. strain R39 and *S. sclerotiorum* under in vitro systems. Applications of $KHCO_3$ greater than 8 mM significantly inhibited ($P < 0.001$) the growth of both fungi. Concentrations of $KHCO_3$ lower than 25 mM did not affect the antagonistic effect of *Trichoderma* on the growth of *S. sclerotiorum*; however, this fungal interaction was not observed when exposed to 50 mM $KHCO_3$ because of its strong inhibition of fungal growth. In addition, $KHCO_3$ concentrations higher than 8 mM caused significant ($P < 0.001$) reduction of the sclerotium formation of *S. sclerotiorum*. Sclerotium germination and de novo sclerotium formation were significantly ($P < 0.001$) inhibited as the concentrations of $KHCO_3$ increased. Results show the potential benefits of potassium bicarbonate for controlling both growth and development of *S. sclerotiorum*, although it also exerts negative effects on the *Trichoderma* strain that is a natural antagonist to *S. sclerotiorum*.

Key words Antagonism · Antifungal properties · Bicarbonates · Sclerotium germination

C. Ordóñez-Valencia · A. Alarcón (✉) · R. Ferrera-Cerrato
Área de Microbiología, Postgrado de Edafología, Colegio de Postgraduados, Campus Montecillo, Carretera México-Texcoco km 36.5, Montecillo, Estado de México, CP 56230, México
Tel. +52-595-952-0200, ext. 1280; Fax +52-595-952-0287
e-mail: aalarconcp@gmail.com

L.V. Hernández-Cuevas
Laboratorio de Micorrizas, Centro de Investigaciones en Ciencias Biológicas, Universidad Autónoma de Tlaxcala, Tlaxcala, México

Introduction

Bicarbonates are mainly utilized in the food industry to avoid undesirable fermentation processes, to regulate pH, and to improve texture and flavors (Aharoni et al. 1997; Smilanick et al. 1999; Bombelli and Wright 2006). Because bicarbonates possess wide-spectrum antimicrobial properties, their efficacy in controlling postharvest fungal phytopathogens has been successfully tested (Palmer et al. 1997; Bombelli and Wright 2006). In accordance with the United States Environmental Protection Agency (USEPA), bicarbonates are considered safe both for human health and for the environment (Palmer et al. 1997; Bombelli and Wright 2006); thus, their utilization may contribute to significant reduction of the use of other chemical products such as pesticides. Some sodium, potassium, or ammonium bicarbonates and carbonates have been demonstrated to inhibit the growth of several fungal pathogens during postharvest of fruits, vegetables, and ornamental species (Olivier et al. 1998; Palou et al. 2001; Karabulut et al. 2003; Arslan et al. 2006). For instance, a brief immersion of citrus fruits in sodium carbonates or bicarbonates resulted in reduced incidence of green mold (Smilanick et al. 1999). In the same manner, the foliage application of sodium or potassium bicarbonates diminished the severity of the disease caused by *Leveillula taurica* in pepper plants (Fallik et al. 1997). Thus, the application of bicarbonates represents an effective technique to control fungal pathogens for horticultural crops (Aharoni et al. 1997; Bombelli and Wright 2006).

The mechanisms by which bicarbonates act on fungal pathogens are based on alterations of membrane permeability and on the inhibition of oxidative phosphorylation reactions (Olivier et al. 1998). Nevertheless, the efficiency of these chemical compounds depends on their concentration (0.2%–3%) and on the susceptibility of the target microorganism; however, the mechanisms by which microorganisms are inhibited are not well understood (Olivier et al. 1998). In the case of fungi, potassium bicarbonate ($KHCO_3$) provokes the collapse of hyphal cell walls and ultimately cell death (USEPA 1998; Zavaleta-Mejia 1999).

As examples, *Helminthosporium solani* showed reduced formation of conidia when exposed to $KHCO_3$ (Olivier et al. 1998), whereas the growth of *Botrytis cinerea* was inhibited when exposed to several bicarbonates, including $KHCO_3$ under in vitro systems (Palmer et al. 1997; Bombelli and Wright 2006).

Sclerotinia sclerotiorum (Lib.) de Bary is an aggressive and destructive pathogen for many of the most important agricultural crops (Fernando et al. 2004; Hegedus and Rimmer 2005). This fungus (Ascomycota, Helotiales, Sclerotiniaceae) typically produce sclerotia, which are specialized structures that confer persistence and a viable source of inoculum for long periods under unfavorable conditions (Bolton et al. 2006; Wu and Subbarao 2006; Bae and Knudsen 2007). Once sclerotia germinate, the mycelium may infect the root system, producing rot root and plant wilting; on the other hand, sclerotia may also produce apothecia that eventually release ascospores which will potentially infect plant hosts (Mónaco et al. 1998; Bolton et al. 2006).

To control the negative effects of *S. sclerotiorum*, several agronomic practices have been implemented such as crop rotation, soil fumigation, and pesticide application (Bae and Knudsen 2007). However, biological control is an environmentally friendly alternative to reduce the negative effects of plant pathogens. For instance, fungal microorganisms such as *Trichoderma* species are antagonists that inhibit and parasitize either mycelium or sclerotia of different *Sclerotinia* species (Howell 2003; Ezziyyani et al. 2004; Fernando et al. 2004; Harman et al. 2004; Bolton et al. 2006; Abdullah et al. 2008). *Trichoderma* is a natural fungal genus that may be saprophytic or mycoparasitic, producing antifungal metabolites by which *Trichoderma* species may compete, inhibit, or cause lysis of several structures of plant fungal pathogens (Zago et al. 2001; Howell 2003; Benítez et al. 2004; Ezziyyani et al. 2004; Harman et al. 2004; Corrêa et al. 2007).

Although the benefits of *Trichoderma* species have been well demonstrated during biological control of *Sclerotinia* species, and the efficacy of bicarbonates to control pathogens during crop postharvest has been successfully tested, there is no available information about the response of *Trichoderma*, *Sclerotinia*, or their interactions when exposed to nontoxic chemical compounds such as bicarbonates. To propose the utilization of potassium bicarbonate as an alternative chemical agent for controlling *S. sclerotiorum*, the objectives of this research consisted of evaluating the effect of increased concentrations of potassium bicarbonate via in vitro systems on (1) the individual growth and the interaction of *Trichoderma–Sclerotinia* and (2) the germination and on de novo sclerotium formation by *S. sclerotiorum*.

Materials and methods

Phase I. Tolerance and growth of *Trichoderma* sp. and *Sclerotinia sclerotiorum* to increasing concentrations of potassium bicarbonate

Sclerotinia sclerotiorum and *Trichoderma* sp. R39 strains were obtained from the microbial collection belonging to the Area de Microbiologia, Colegio de Postgraduados. Both fungal strains were originally isolated from garlic crops established at El Bajio, Guanajuato (Mexico). For this study, *S. sclerotiorum* was selected based on their pathogenic effects on lettuce (data not presented), while the *Trichoderma* R39 isolate was selected from 11 effective strains with mycoparasitic activity on *S. sclerotiorum* (Ibarra-Medina 2008).

Petri dishes with potato dextrose agar (PDA; Merck, Darmstat, Germany) were prepared by adding different potassium bicarbonate concentrations on the following basis: 0, 2, 4, 6, 8, 10, 25, or 50 mM ($KHCO_3$; Fermont, Monterrey, Mexico). The pH of each $KHCO_3$ concentration was 6.5, 7.0, 7.0, 7.1, 7.3, 7.4, 7.9, and 8.0, respectively. In the center of each Petri dish, a disk of PDA with active growth mycelium (~9 mm diameter) of the respective fungal strain was placed. The fungal cultures were incubated at room temperature (about 22°C), and an approximate photoperiod of 12 h, for 6 days. The growth of each fungal colony was measured daily. At the end of the experiment the number of sclerotia from *S. sclerotiorum* cultures was also determined.

This experiment consisted of a completely randomized design that included eight treatments in triplicate for each fungal strain. Data were analyzed via analysis of variance (ANOVA) and the mean comparison test (Tukey, $\alpha = 0.05$) by using the SAS system for windows ver. 9.1 (SAS Institute, Cary, NC, USA).

In addition, *Trichoderma* sp. R39 and *S. sclerotiorum* were confronted in vitro by placing both fungi on opposite sides of Petri dishes, in triplicate, containing the several concentrations of $KHCO_3$ as previously described. Fungal cultures were incubated as already described; after 4 days, the antagonism of *Trichoderma* against *S. sclerotiorum* was visually examined.

Phase II. Increasing concentrations of potassium bicarbonate on germination and on de novo sclerotium formation by *Sclerotinia sclerotiorum*

Sclerotia were obtained from pure cultures of *S. sclerotiorum* that were grown on PDA. Sclerotia were collected, placed in sterile microtubes, and kept under refrigeration (4°C) until their utilization. Then, sclerotia were surface disinfested to avoid proliferation of bacterial contaminants. Disinfection consisted of exposing sclerotia to 70% ethanol for 40 s, washing with sterile distilled water, adding a solution of gentamicine (200 µg/ml), and keeping them at 4°C overnight. Afterward, sclerotia were placed on sterile absorbent paper towels to eliminate excess antibiotic solution. Once dried, ten sclerotia were placed on Petri dishes containing PDA enriched with the previously mentioned concentrations of $KHCO_3$. The Petri dishes were incubated at room temperature (about 22°C) for 11 days, and the number of germinated sclerotia was daily recorded for each treatment, as well as the number of newly formed sclerotia.

The experiment consisted of a completely randomized design that included eight treatments with four replicates each. Data were analyzed via analysis of variance (ANOVA) and the mean comparison test (Tukey, $\alpha = 0.05$) by using the SAS system for Windows ver. 9.1 (SAS Institute, Cary, NC, USA).

Results

Phase I. Tolerance and growth of *Trichoderma* sp. and *Sclerotinia sclerotiorum* to increasing concentrations of potassium bicarbonate

Potassium bicarbonate significantly inhibited ($P < 0.001$) the growth of both fungal strains. *Trichoderma* started growing after 24 h, but as the concentration of $KHCO_3$ increased its growth was significantly reduced ($P < 0.001$), especially at concentrations greater than 10 mM (Fig. 1A). After 48 h, the mycelium growth of *Trichoderma* at 8 mM bicarbonate was reduced 50% as compared to the control (0 mM); however, at 144 h the maximun fungal growth was reached in all concentrations, excepting that at 50 mM, in which the growth of *Trichoderma* was reduced 50% in comparison to the control.

In contrast, *S. sclerotiorum* started growing after 48 h, but its growth significantly decreased ($P < 0.001$) as the concentration of bicarbonate increased (Fig. 1B). At 72 h it was observed that the complete surface of the PDA was covered by *S. sclerotiorum* at concentrations from 0 to 6 mM, but at concentrations greater than 8 mM fungal growth was significantly inhibited. Nevertheless, at 144 h, *S. sclerotiorum* completely covered the agar surface for all concentrations lower than 25 mM; however, the fungal pathogen did not show growth when exposed to 50 mM $KHCO_3$.

In addition, sclerotium formation by *S. sclerotiorum* was significantly inhibited by bicarbonate application. After 144 h, sclerotia were completely formed at concentrations from 0 to 6 mM, but from 8 to 10 mM bicarbonate initiation of sclerotium formation was observed by the presence of white cotton-like conglomerated hyphae; in contrast, neither primordia nor complete sclerotium formation was detected at 25 and 50 mM (Fig. 2A). The number of sclerotia formed

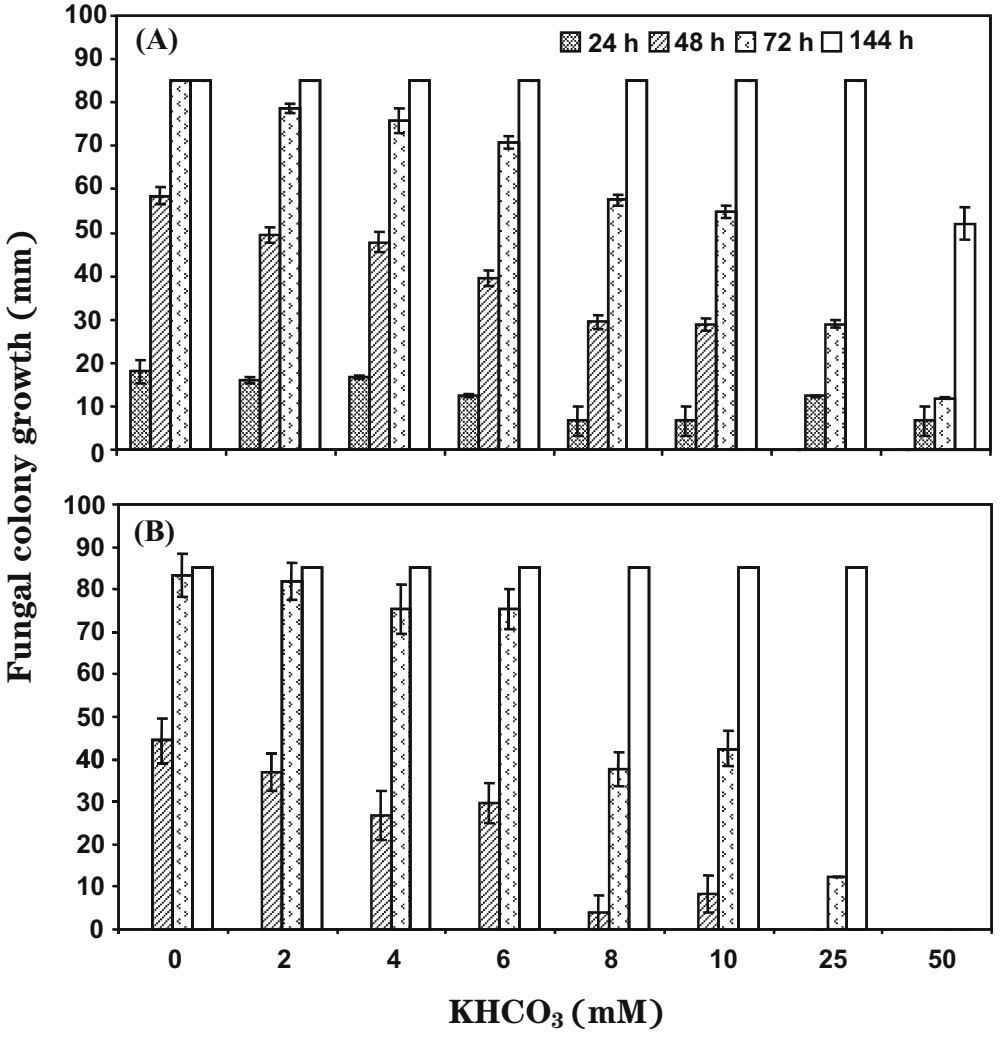
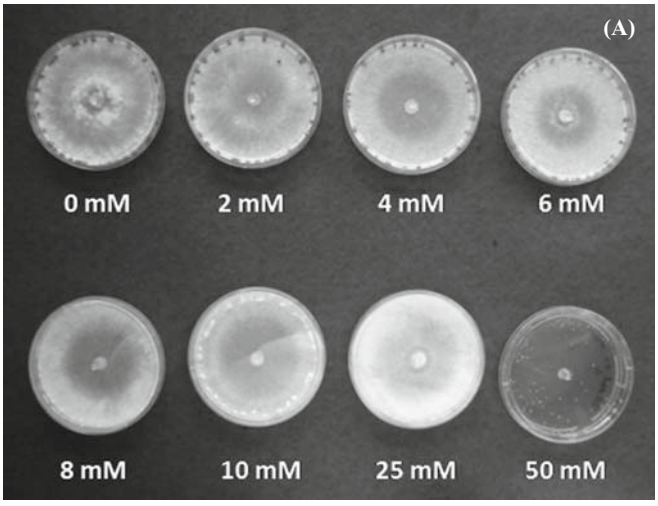


Fig. 1. Growth responses of *Trichoderma* sp. R39 (**A**) and *Sclerotinia sclerotiorum* (**B**) at 24, 48, 72, and 144 h after exposure on Petri dishes with increasing concentrations of potassium bicarbonate ($KHCO_3$). *Bars* ± standard error ($n = 3$)



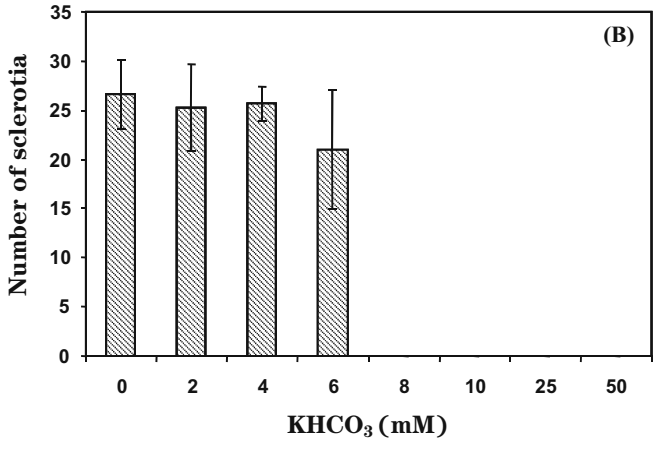


Fig. 2. Effect of potassium bicarbonate ($KHCO_3$) concentrations on sclerotium formation (**A**) and on the number of sclerotia (**B**) of *Sclerotinia sclerotiorum*, after 144 h. *Bars* ± standard error ($n = 3$)

at concentrations from 0 to 6 mM ranged between 20 and 30 (Fig. 2B); the number of sclerotia in the process of formation was 28 for both 8 and 10 mM.

In respect to the fungal confrontation test, it was observed that *Trichoderma* showed antagonistic effects to *S. sclerotiurum* when exposed to concentrations of bicarbonate from 0 to 25 mM (Fig. 3). Nevertheless, as bicarbonate concentration increased, the appearance of mycelia of both fungi was less dense and thinner, especially at 25 mM; here, despite these fungal morphological changes, the antagonistic effect of *Trichoderma* against *Sclerotinia* was maintained (Fig. 3). In contrast, exposure to 50 mM bicarbonate resulted in drastic growth inhibition for both fungi; consequently, fungal confrontation was not truly observed at this concentration (Fig. 3).

Phase II. Increasing concentrations of potassium bicarbonate on germination and on de novo sclerotium formation by *Sclerotinia sclerotiorum*

Application of $KHCO_3$ caused significant retardation ($P < 0.001$) of sclerotium germination. After 3 days, sclerotia germinated (100%) at control plates (0 mM), and for the rest of the treatments, sclerotium germination started at the fourth day. However, the greater the bicarbonate concentration, the lower the estimated percentage of sclerotium germination. At the seventh day, all sclerotia germinated from at concentrations from 0 to 25 mM, but only 82% germination was achieved at sclerotia exposed to 50 mM (Fig. 4).

During evaluation of sclerotium germination percentage, the production of de novo sclerotia, which was significantly reduced ($P < 0.01$) by $KHCO_3$ application, was also recorded. In the control treatment (0 mM), de novo sclerotia were formed after the 6th day; for the rest of the treatments, new sclerotia started forming at the 7th or 8th day (Fig. 5). At the 11th day, the sclerotia at 25 mM were in the process of being formed. Although sclerotium germination was observed at 50 mM $KHCO_3$, the growth of the mycelium was scarce, with no new sclerotia being produced at this concentration (Fig. 5).

Discussion

Because most of the literature about bicarbonate applications has focused on controlling postharvest disease causal agents (Fallik et al. 1997; Bombelli and Wright 2006), there is no information on bicarbonate effects, either on the growth and development of soil-borne pathogens such as *Sclerotinia sclerotiorum* or on that of beneficial fungi such as *Trichoderma* via in vitro systems.

$KHCO_3$ exerted significant inhibition on the growth of mycelium of *S. sclerotiorum* and *Trichoderma*, although either fungus behaved differentially when exposed to the various concentrations of bicarbonate. For instance, after 144 h, *Trichoderma* growth at 50 mM showed a 38.8% reduction when compared to its respective control (0 mM); *S. sclerotiorum* at low and intermediate bicarbonate concentrations had an average growth reduction of 63.9%, but at 50 mM this pathogen did not show any mycelium growth (see Fig. 1). Thus, these growth responses indicate that *S. sclerotiorum* was more sensitive and susceptible to high concentrations of bicarbonate than the *Trichoderma* strain.

Bicarbonate effects on growth inhibition of both fungi may be partially explained by changes in medium pH, which became more alkaline as bicarbonate concentration increased. Control PDA plates had a pH value of 6.2, and those plates in which PDA was enriched with 50 mM bicarbonate had a pH of 8.0. In this respect, soil fungi are more active under acidic pH values (Alexander 1977). Under such changes on pH induced by bicarbonates, both fungi are limited in growth; however, although it was not evaluated, fungi must have a physiological mechanism by which they may modify pH and consequently have better opportunities to develop under such adverse conditions. For instance, *S. sclerotiorum*, during mycelium development, produces oxalic acid and other organic acids, causing acidification of infected tissues that favors proliferation and

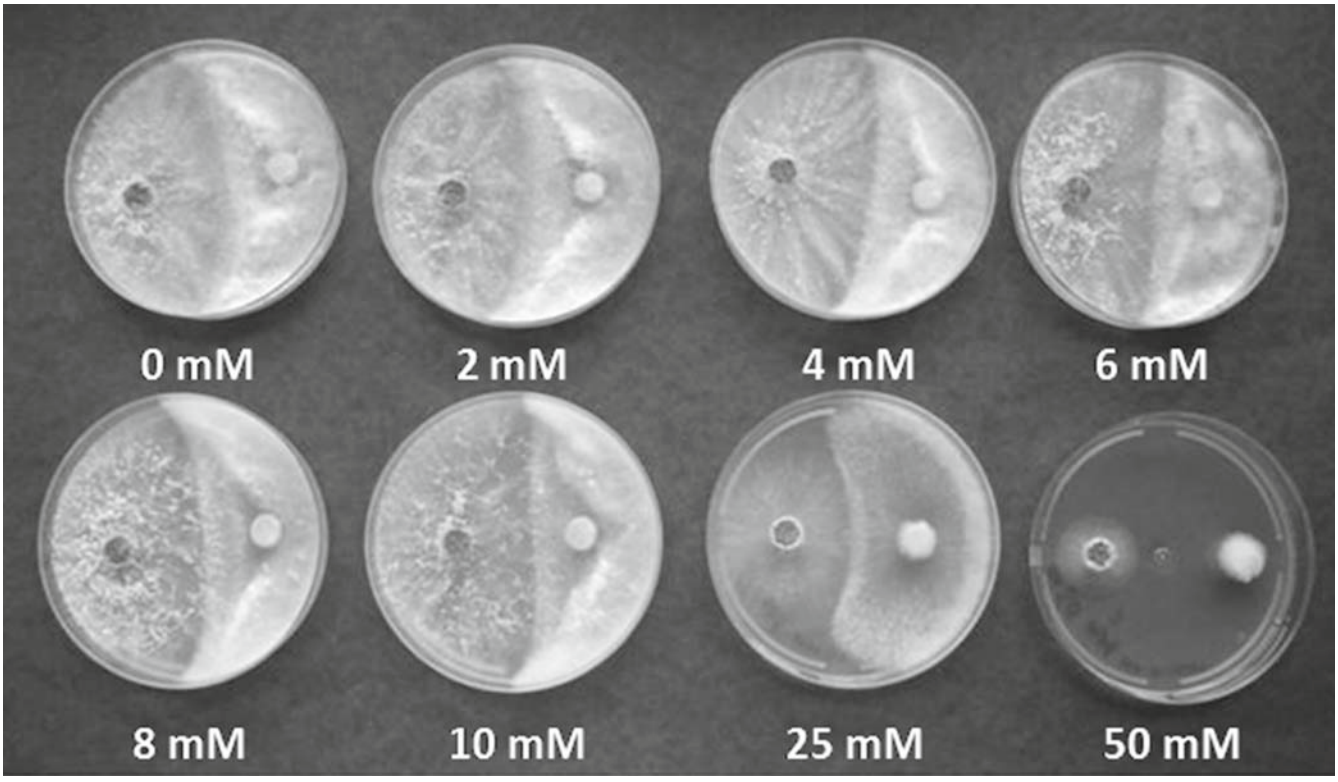


Fig. 3. Antagonism of *Trichoderma* sp. strain R39 (on the *left* of each plate) against *Sclerotinia sclerotiorum* (on the *right* of each plate) as an effect of increasing concentrations of potassium bicarbonate ($KHCO_3$), after 4 days of fungal confrontation

infectivity of this pathogen (Rollins and Dickman 2001; Durman et al. 2005). On the other hand, *Trichoderma* strains are also able to induce acidification in the surrounding media, by which means they are more active and reduce the virulence of pathogens (Benítez et al. 2004).

Increasing concentrations of $KHCO_3$ caused growth reduction in both fungal strains. Similar inhibition effects were observed for *Botrytis cinerea* when exposed to 20 mM of either $KHCO_3$, NH_4HCO_3, or $NaHCO_3$ using in vitro systems (Palmer et al. 1997). Besides changes to pH that affect fungal growth, bicarbonates may also affect membrane permeability and alter physiological processes such as oxidative phosphorylation (Olivier et al. 1998).

Application of $KHCO_3$ also caused modifications of growth patterns of both fungal strains. For instance, *S. sclerotiorum* at 25 mM showed a less dense and thinner mycelium, whereas at lower concentrations of bicarbonate the fungal colony was more compact with abundant mycelium. On the other hand, the colony of *Trichoderma* had reduced growth and mycelium production as the bicarbonate concentrations increased; although it was not considered for evaluation, sporulation of *Trichoderma* was also reduced at high concentrations of $KHCO_3$ (data not shown).

The aforementioned fungal alterations caused by bicarbonate application may explain in part the observed growth inhibition of both fungi; however, more research efforts are needed to understand the physiological, biochemical, and molecular responses of fungi exposed to critical concentrations of bicarbonates.

In the present work, antagonistic effects of *Trichoderma* against *Sclerotinia sclerotiorum* were maintained at concentrations no greater than 25 mM, but this fungal interaction was not detected at 50 mM. Under bicarbonate-free culture media, *Trichoderma* strains typically express their antagonistic and mycoparasitic capabilities against several plant pathogens, including *S. sclerotiorum* (Bae and Knudsen 2007; Reyes et al. 2007). However, under our experimental conditions the induction of alkalinity by bicarbonate application (for instance, 50 mM), represented an extreme adverse condition that not only affected the growth but also the effectiveness of *Trichoderma* to antagonize *Sclerotinia sclerotiorum*. In this respect, Benítez et al. (2004) have stated that *Trichoderma* species are more effective in inhibiting plant pathogens under acidic environments.

This research is one of the first reports describing the negative effects of $KHCO_3$ on sclerotium germination and on de novo sclerotium formation by *Sclerotinia sclerotiorum*. Either sclerotium germination or de novo sclerotium formation was significantly reduced by bicarbonate application. Sclerotium germination at 100% was achieved at 0 and 25 mM; in contrast, the germination of sclerotia at 50 mM was 82%, although mycelium growth was scarce. In addition, the exposure of increasing concentrations of bicarbonate resulted in limited de novo sclerotium formation, which was strongly inhibited when $KHCO_3$ was

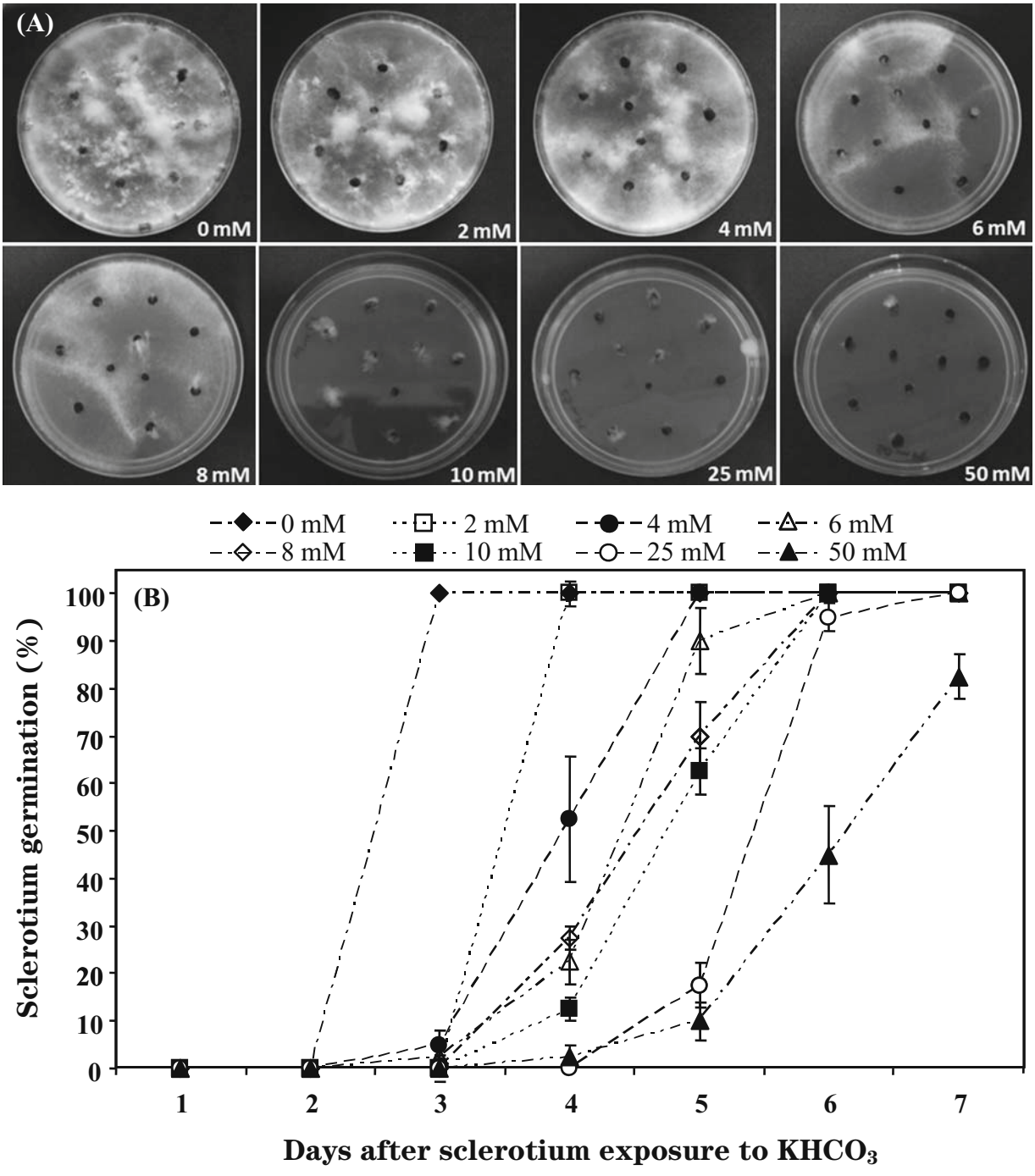


Fig. 4. Germination of sclerotia of *Sclerotinia sclerotiorum* (**A**) and its percentage (**B**) as an effect of increasing concentrations of potassium bicarbonate ($KHCO_3$) after 7 days. Means ± standard error ($n = 4$) are shown in **B**

applied at 50 mM. Few studies on germination and production of sclerotia with bicarbonate application have been reported. In this regard, germination of sclerotia from *Sclerotinia rolfsii* has been demonstrated to be inhibited when exposed to water agar enriched with 50 mM NH_4HCO_3 (Punja and Grogan 1982). There are no previous reports about the influence of bicarbonates on the production of new sclerotia derived from those germinated sclerotia; however, some researchers agree that pH affects several processes in *Sclerotinia sclerotiorum*, including the formation of sclerotia, especially at neutral or alkaline pH values (Rollins and Dickman 2001; Chen et al. 2004). Thus, our study gives more information about the influence of potassium bicarbonate on inducing alkalinity and on its consequent negative effects on either fungal growth and sclerotium formation and germination.

In spite of having detected sclerotium germination at 50 mM, the growth of the developed mycelium was aerial

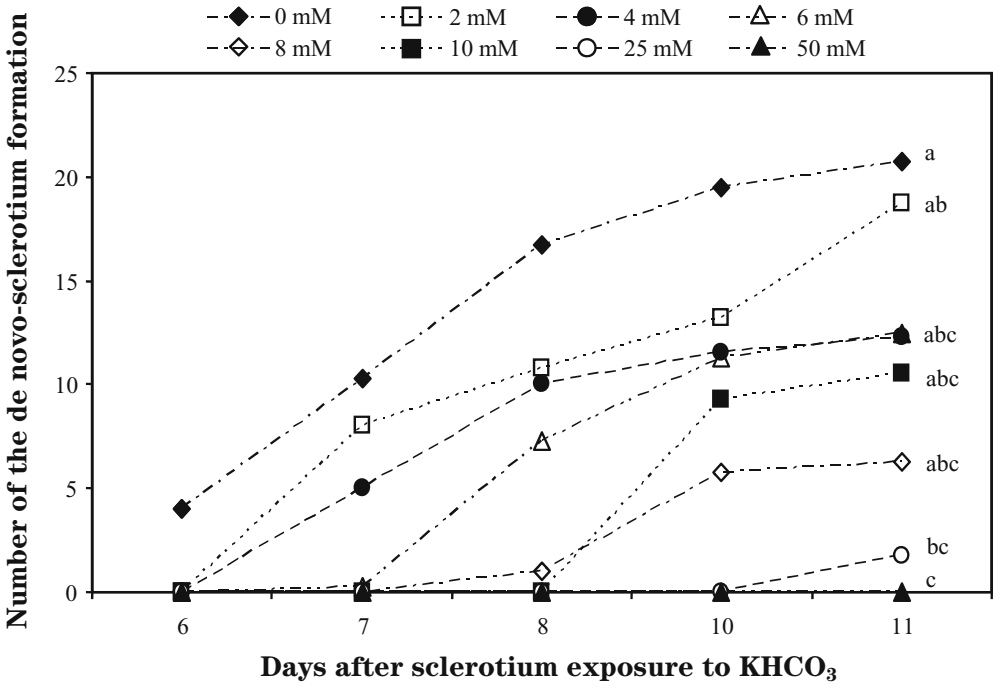


Fig. 5. Effects of increasing concentrations of potassium bicarbonate ($KHCO_3$) on de novo sclerotium formation by *Sclerotinia sclerotiorum* after the sixth day of sclerotium germination ($n = 4$). Means with the same letter for the treatments at day 11 are not statistically different (Tukey, $\alpha = 0.05$)

rather than diametrically on the agar surface. This mycelium growth behavior was also detected at 10 and 25 mM at the beginning of sclerotium germination, but eventually the mycelium completely covered the agar surface (data not shown). In the same manner as the previous experimental phase (Fig. 3), the mycelium of *Sclerotinia* had a cotton-like appearance that visually diminished in amount and density (thinner) as the bicarbonate concentrations increased (data not shown).

This research provides fundamental evidence about the potential use of potassium bicarbonate as a chemical control agent against *S. sclerotiorum* under in vitro systems. Nevertheless, it is necessary to evaluate the application of bicarbonate concentrations in combination not only with this pathogen but also with the target plants. In addition, further research is needed to evaluate the effectiveness of $KHCO_3$ for controlling the attack and severity of *S. sclerotiorum* on lettuce established in agricultural soils preinoculated with antagonist and/or beneficial fungi.

Conclusions

The growth of both fungal strains, but especially that of *Sclerotinia sclerotiorum*, was significantly inhibited as the concentration of $KHCO_3$ increased. Besides inducing morphological changes on both fungi, bicarbonate concentrations greater than 8 mM affect sclerotium formation by *S. sclerotiorum*. The antagonistic effect of *Trichoderma* against *S. sclerotiorum* was maintained at 25 mM bicarbonate, but this antagonism failed to be expressed at 50 mM. The process of sclerotium germination and de novo sclerotium formation was inhibited by increasing concentrations of $KHCO_3$. Although $KHCO_3$ was effective on controlling the growth and development of *S. sclerotiorum*, further research is needed for considering potassium bicarbonate as an alternative compound for disease management and for clarifying its effects on plants in which this fungal pathogen is an important threat for crop production.

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