Control of major citrus postharvest diseases by sulfur-containing food additives

Victoria Martínez-Blay, Verònica Taberner, María B. Pérez-Gago, Lluís Palou*

Laboratori de Patologia, Centre de Tecnologia Postcollita (CTP), Institut Valencià d'Investigacions Agràries (IVIA), 46113 Montcada, València, Spain

* Corresponding author: L. Palou
E-mail: palou_llu@gva.es, Tel.: +34 963424117

Abstract

Sodium metabisulfite (SMBS), potassium metabisulfite (PMBS), aluminum sulfate (AlS) and aluminum potassium sulfate (AlPS), common sulfur-containing salts used as food additives, were evaluated for their antifungal activity against *Penicillium digitatum*, *Penicillium italicum* and *Geotrichum citri-aurantii*, the most economically important pathogens causing postharvest diseases of citrus fruits. In vitro radial mycelial growth was measured on potato dextrose agar (PDA) Petri dishes amended with five different concentrations of the salts (10, 20, 30, 50, 100 mM) after 7 d of incubation at 25 °C. SMBS and PMBS at all concentrations, and AIS and AIPS above 20 mM, completely inhibited the growth of these fungi. The curative antifungal activity of the four salts to control citrus green (GM) and blue (BM) molds and sour rot (SR) was evaluated on 'Valencia' oranges artificially inoculated in rind wounds with *P. digitatum*, *P. italicum* and *G. citri-aurantii*, respectively. In vivo primary screenings showed no significant antifungal activity of AlS and AlPS to control the three diseases at any dose tested, but SMBS and PMBS reduced the incidence and severity of GM, BM and SR at various concentrations. Effective salts and concentrations were selected for in vivo dip treatments in small-scale trials. Dips at room temperature (20 ºC) in SMBS and PMBS at 20 and 50 mM for 60 or 120 s significantly reduced the incidence and severity of GM and BM, with PMBS at 50 mM for 120 s the most effective treatment. Conversely, dips in SMBS and PMBS at 50 mM for 60 or 120 s did not reduce SR incidence and severity. SMBS and PMBS treatments are potentially new tools to be included in reduced-risk non-polluting strategies to control Penicillium diseases, but not SR, on citrus fruits.

Key words: antifungal activity, GRAS salts, oranges, *Citrus sinensis*, *Geotrichum citri-aurantii, Penicillium digitatum*, *Penicillium italicum*.

1. Introduction

Citrus are one of the most important crops in the world with a global production over 120 million tonnes. About 25 million tonnes are produced in the Mediterranean Region (primarily in Spain, Egypt, Turkey, Italy, Morocco and Greece), mainly destined for the fresh fruit market (FAO, 2017). Like many other fruits for fresh consumption, citrus are susceptible to different postharvest diseases and their relative importance is influenced by the climate of the production area (Smilanick et al., 2020). Green mold (GM), blue mold (BM) and sour rot (SR), caused by *Penicillium digitatum* (Pers.:Fr.) Sacc, *Penicillium italicum* Wehmer and *Geotrichum citri-aurantii* (Ferraris) Butler, respectively, are the most economically important postharvest diseases in citrus areas with low summer rainfall, such as Mediterranean countries, California and South Africa. They are wound pathogens and infect the fruit through injuries inflicted during harvest, transportation, handling, storage or retail sale, leading to significant economic losses (Hao et al., 2010; Palou, 2014; Smilanick et al., 2020).

Control of postharvest diseases of citrus fruits has relied for many years on repeated applications of synthetic chemical fungicides. However, the proliferation of resistant fungal biotypes and the increasing concern about chemical residues related to human health and environmental contamination are factors limiting this practice (Brent and Hollomon, 2007; Palou et al., 2016). Therefore, new consumer trends and legislative restrictions compel the adoption of alternative approaches. Alternative methods to control postharvest diseases include physical treatments, such as heat or irradiation, biological control with antagonistic microorganisms and the use of safe low-toxicity chemicals, such as natural compounds or food additives (Moscoso-Ramírez et al., 2013; Palou, 2014, 2018; Palou et al., 2002, 2016; Papoutsis et al., 2019; Platania et al., 2012; Talibi et al., 2014).

Food additives are widely used as preservatives for controlling food pH, taste or other qualities. Among them, many organic and inorganic salts have antimicrobial action and may offer a good alternative to the use of synthetic fungicides (D'Aquino and Palma, 2020; Palou et al., 2016). Some advantages of using salts as fungicides include their low environmental and mammalian toxicity, broad spectrum of activity, relatively low cost and high solubility in water (Deliopoulos et al., 2010; Mills et al., 2004). These salts are classified as generally recognized as safe (GRAS) and approved for use in foods by the United States Food and Drug Administration (US FDA) and by the European Food Safety Authority (EFSA) (Mills et al., 2004; Palou, 2018). Many studies have

demonstrated the potential of GRAS salts, such as carbonates, bicarbonates, sorbates, benzoates, parabens, etc. to control postharvest diseases of citrus fruits as both aqueous solutions and ingredients of edible coatings (Askarne et al., 2013; Guimarães et al., 2019; Montesinos-Herrero et al., 2016; Moscoso-Ramírez et al., 2013; Palou et al., 2002; Smilanick et al., 2008; Valencia-Chamorro et al., 2009). In previous studies, the antifungal activity of a variety of sulfur-containing salts was shown to include pathogens causing disease on important crops such as potato or carrot (Hervieux et al., 2002; Kolaei et al., 2012; Mecteau et al., 2008; Mills et al., 2004). The status as food additives of a group of sulfur-containing compounds included in these studies, the aluminum sulfate group (E-numbers E 520-523), was re-evaluated in 2018 by the EFSA and the Panel concluded that these compounds are of no safety concern in the current authorized uses and use levels, which now include only egg products and breath freshening micro-sweets (EFSA, 2018). Another important group, the sulfites group, including sulfur dioxide (SO_2) (E-numbers E 220-228), is also being re-evaluated by the EFSA since 2016, but they are currently approved for some uses on entire fresh fruits and vegetables (EFSA, 2016). In particular, postharvest technologies such as SO_2 fumigation and the application of in-package sodium metabisulfite pads are widely used worldwide by the table grape industry to control gray mold caused by the fungus *Botrytis cinerea* (Chaves Junior et al., 2019; Palou et al., 2010), and are also evaluated for the same purpose on other commodities such as blueberries (Pols et al., 2019) and figs (Cantín et al., 2011).

However, there is little information available about the potential use of sulfur-containing food additives to control major citrus postharvest diseases. The objectives of this study were: (1) to evaluate the in vitro antifungal activity of various sulfur-containing salts, at different concentrations, against *P. digitatum*, *P. italicum* and *G. citri-aurantii* and (2) to assess the in vivo curative activity of aqueous solutions of the most promising salts and concentrations to control GM, BM and SR. Initially, in vivo primary screenings were performed and, afterwards, the most effective compounds and rates were tested as dip treatments in small-scale trials.

2. Material and methods

2.1. Food additives

The names, acronyms, E-number, molecular formula, molecular weight, pH and solubility in water of the salts used in this work are given in Table 1 (Acros Organics,

2019; EC, 2012; Honeywell Fluka, 2019). Sodium metabisulfite (SMBS) and aluminum sulfate (AlS) were purchased from Honeywell Research Chemicals Fluka™ (Seelze, Germany), whereas potassium metabisulfite (PMBS), and aluminum potassium sulfate (AlPS) were acquired from Acros Organics BVBA (Geel, Belgium). The theoretical sulfur dioxide yield of the metabisulfite salts is 67.4% for SMBS and 57.6% for PMBS (EFSA, 2016).

2.2. Fungal pathogens

Isolates NAV-7 of *P. digitatum,* MAV-1 of *P. italicum* and NAV-1 of *G. citri-aurantii* were obtained from decayed mandarins or oranges from local packinghouses in the Valencia region (Spain). These fungal strains were isolated, identified and maintained in the culture collection of postharvest pathogens of the IVIA CTP. They were selected for their aggressiveness and uniform behavior on the most commercially important citrus cultivars. They were then also deposited in the Spanish Type Culture Collection (CECT, University of Valencia, Valencia, Spain) with the following respective accession numbers: CECT 21108, CECT 21109 and CECT 13166. Prior to the experiments, the fungal isolates were incubated on potato dextrose agar (PDA) (Scharlab S.L., Barcelona, Catalonia, Spain) Petri dishes at 25 °C for 7-14 d.

2.3. In vitro antifungal activity

The effect of the sulfur-containing food additives on the mycelial growth of *P. digitatum*, *P. italicum* and *G. citri-aurantii* was evaluated on 90-mm plastic Petri dishes with PDA medium amended, at 40-50 ºC, with sterile aqueous solutions of the respective salts. Stock solutions at 0.6 M of each food additive were prepared by dissolving the appropriate amount of the salt in sterilized bi-distilled water. These solutions were used to achieve final concentrations of 10, 20, 30, 50 and 100 mM of the salts in PDA media. PDA Petri dishes without salts served as controls. The center of each Petri dish was inoculated with a 5-mm diameter mycelial plug, produced with a sterilized cork-borer, from 7 to 14-d-old cultures of *P. digitatum*, *P. italicum* or *G. citri-aurantii*. The plates were incubated at 25 ºC in the dark, in a growth chamber. Radial mycelial growth was determined in each plate by calculating the mean of two perpendicular fungal colony diameters. These measurements were performed after 3, 5, 7 and 10 d of incubation. Results after 7 d are presented. Five replicates, each one corresponding with one 90-mm Petri dish, were used for each salt, concentration and

fungal pathogen. Results are expressed as the percentage of mycelial growth inhibition according to the formula: [(dc-dt)/dc] × 100, where dc = average diameter of the fungal colony on control plates and dt = average diameter of the fungal colony on Petri dishes amended with sulfur-containing salts.

2.4. In vivo curative activity

2.4.1. Fruit inoculation

In vivo experiments were conducted with 'Valencia' oranges (*Citrus sinensis* (L.) Osbeck). Oranges were collected from commercial orchards in the Valencia area (Spain) and used the same day or stored up to 1 week at 5 ºC and 90% relative humidity (RH) before use. No commercial postharvest treatments were applied before use in the experiments. Before fungal inoculation, fruit were selected, randomized, surface disinfected (4-min dips in diluted bleach (0.5% sodium hypochlorite)), rinsed with tap water and allowed to air dry at room temperature.

For inoculation, conidia of *P. digitatum, P. italicum* or *G. citri-aurantii*, from 7 to 14-d-old cultures, were taken from the PDA surface with a sterilized inoculation loop and transferred to a sterile aqueous solution of 0.05% Tween® 80 (Panreac-Química S.A., Barcelona, Catalonia, Spain). Conidial suspensions were filtered through two layers of cheesecloth. The density of the suspension was measured with a hemocytometer and dilutions with sterile water were done to obtain an exact inoculum density of 10^5 spores/mL (*P. digitatum* and *P. italicum*) or 10^7 arthrospores/mL (*G. citri-aurantii*). To prepare the final inoculum of *G. citri-aurantii*, fresh orange juice (10%), thiabendazole (50 mg/L; Textar® 60 T, Decco Ibérica PostCosecha, S.A.U., Paterna, Valencia, Spain) and cycloheximide (5 mg/L; Carl Roth GmbH + Co. KG, Karlsruhe, Germany) were added to the arthrospore suspension.

Each pathogen was wound inoculated in different sets of fruit. The tip of a stainless-steel rod, 1 mm wide and 2 mm in length, was immersed in the corresponding conidial suspension and inserted in the fruit rind afterwards. Each fruit was inoculated at one point in the equatorial zone. Inoculated fruits were kept in a temperature-controlled room for 24 h, until treatment. This temperature was 20 ºC for fruits inoculated with *P. digitatum* and *P. italicum* and 28 ºC for fruits inoculated with *G. citri-aurantii*. The RH inside these chambers was 90% for the three fungi.

2.4.2. In vivo primary screening tests

182Curative activity of SMBS, PMBS, AlS and AlPS to control GM, BM and SR was
183tested at three different concentrations on fruit previously inoculated with the
184pathogens. Sterile solutions of each salt at concentrations of 10, 50 and 100 mM were
185tested against GM and BM and concentrations of 10, 30 and 50 mM were tested against
186SR. These concentrations were selected according to previous results obtained in the in
187vitro tests.
188About 24 h after the inoculation of the pathogen, 30 µL of each salt at the corresponding
189concentration were placed, using a micropipette, in each inoculation rind wound.
190Control fruits were treated with 30 µL of sterile bi-distilled water. For each salt,
191concentration and pathogen, 4 replicates of 5 oranges each were used. Treated fruits
192were incubated at 20 ºC and 90% RH in the case of GM and BM and at 28 ºC and 90%
193RH in the case of SR. Disease incidence (percentage of infected fruit), severity (lesion
194diameter) and pathogen sporulation (percentage of fruit showing spores) were
195determined after 7 or 8 d of incubation. Severity and sporulation were assessed over the
196entire fruit sample, not only over infected and symptomatic fruits. Damage caused by
197the application of the droplet of each chemical solution was also visually assessed on
198the rind tissue surrounding each wound at the end of the incubation period.
199
2002.4.3. Dip treatments
201According to previous results from the in vivo primary screenings, small-scale dip trials
202were conducted with 'Valencia' oranges using the most convenient sulfur-containing
203salts and doses. Since the treatments lacked efficacy at 10 mM and were phytotoxic at
204100 mM, SMBS and PMBS were assayed against GM and BM at doses of 20 and 50
205mM and against SR at 50 mM. Curative activity of dip treatments at room temperature
206(20 ± 1 ºC) was determined on oranges wound inoculated with the pathogens about 24 h
207before treatment as described above. Stainless steel buckets containing 10 L of aqueous
208solution of each salt at each dose were used. Inoculated fruits were placed into 18 L
209multi-perforated wall stainless steel containers, exactly fitting in the above-mentioned
210buckets, and were completely immersed in the treatment solution for 60 or 120 s. After
211treatment, fruits were not rinsed and were allowed to air dry at room temperature.
212Control fruits were dipped for 60 s in bi-distilled water alone. Four replicates of 10
213fruits each were used per treatment. Treated fruits were arranged on plastic cavity
214sockets on plastic trays, wrapped with a large plastic bag to minimize contaminations
215and incubated at 20 ºC and 90% RH for the development of GM and BM and at 28 ºC

and 90% RH for the development of SR. Disease incidence and severity and pathogen sporulation were determined after 7 or 8 d of incubation. Severity and sporulation were assessed over the entire fruit sample, not only over infected and symptomatic fruits. Potential fruit phytotoxicity caused by salt dip treatments was visually assessed according to the following scale: 1 = none, 2 = slight (< 25% of the rind), 3= moderate (25-50% of the rind), and 4 = severe (> 50% of the rind).

2.5. Statistical analysis

Data from all experiments were subjected to analysis of variance (ANOVA) considering food additive and concentration as factors. In dip trials, immersion period was another independent variable. Disease incidence and pathogen sporulation were calculated as percentages and transformed to the arcsine of the square root of the proportion of infected or sporulated fruit to assure the homogeneity of variances. When appropriate, Fisher's Protected Least Significant Difference (LSD) test, at the 95% level of confidence (P = 0.05), was used for means separation. Non-transformed means are shown. All statistical analyses were performed with the software Statgraphics Centurion XVII (StatPoint Technologies Inc., Warrenton, VA, USA).

3. Results

3.1. In vitro antifungal activity

Table 2 represents the mycelial growth inhibition of *P. digitatum*, *P. italicum* and *G. citri-aurantii* on Petri dishes amended with different concentrations of sulfur-containing salts after 7 d of incubation at 25 ºC. According to the results, the four food additives tested greatly inhibited the growth of the three fungi. For *P. digitatum* and *P. italicum*, no significant differences were found among the tested salts and concentrations. Complete inhibition of mycelial growth was observed with SMBS, PMBS and AlPS at all concentrations. AlS provided almost 100% inhibition at 10 mM and inhibited completely the growth of both fungi at the other doses (Table 2). However, for *G. citri-aurantii*, complete inhibition of the fungal growth, at all concentrations, was only achieved with SMBS and PMBS. For AlS and AlPS, a reduction in mycelial development was observed as the concentration of the salts rose and complete inhibition was achieved at concentrations over 20 and 30 mM in the case of AlS and AlPS, respectively (Table 2).

Results showed that SMBS and PMBS were the most effective salts, with the growth of *P. digitatum*, *P. italicum* and *G. citri-aurantii* completely inhibited by all concentrations tested. However, AlS and AlPS were also effective against *Penicillium* pathogens and *G. citri-aurantii* at concentrations over 20 mM. Therefore, the four food additives were evaluated in subsequent in vivo tests.

3.2. In vivo curative activity

3.2.1. Effect of salt concentration

Fig. 1 shows that SMBS and PMBS significantly reduced the incidence, severity and sporulation of GM and BM on 'Valencia' oranges, with respect to the controls, at all concentrations tested. Moreover, the concentrations of 50 and 100 mM SMBS completely inhibited the development of GM. Likewise, 50 and 100 mM PMBS and 100 mM SMBS totally inhibited the development of BM. In contrast, AlS and AlPS, applied at the concentrations of 10 and 50 mM, did not significantly reduce the incidence, the severity or the sporulation of the molds. Both salts were effective at 100 mM against GM but no significant difference, with respect to the control, was observed against BM. The application of the four salts at 100 mM was phytotoxic on the fruit rind, causing apparent darkening and sinking at the point of inoculation (Fig. 1). SMBS and PMBS were effective against both GM and BM at the non-phytotoxic dose of 50 mM. In fact, after 7 d of incubation at 20 °C, 50 mM SMBS reduced GM and BM incidence by 100 and 70%, respectively, whereas 50 mM PMBS reduced these incidences by 70 and 100%, respectively. Similarly, pathogen sporulation was nil or below 5% after the application of SMBS or PMBS at 50 mM (Fig. 1). Thus, the selected concentrations of these food additives for the following preliminary screening against SR were 10, 30 and 50 mM instead of 10, 50 and 100 mM.

Fig. 2 shows that SMBS significantly reduced the incidence of SR on 'Valencia' oranges, with respect to the control, only at the concentration of 50 mM, while PMBS did at 30 and 50 mM and none of the concentrations completely inhibited the growth of the fungus. Likewise, SMBS reduced significantly the severity of SR at the concentrations of 30 and 50 mM and PMBS was effective at the three doses applied. Sporulation was not significantly reduced except for SMBS at 50 mM. On the other hand, AlS and AlPS were not effective to reduce SR incidence and severity and sporulation of *G. citri-aurantii* at any of the concentrations tested, being these values

even higher than those observed on control fruits. None of the treatments caused visible rind phytotoxicities in these SR screenings.

Only SMBS and PMBS were effective against SR, especially at the concentration of 50 mM. For example, after 8 d of incubation at 28 °C, 50 mM SMBS reduced the incidence of SR by 50% and 50 mM PMBS reduced it by 80% (Fig. 2). In accordance with these results, the subsequent dip treatments were performed only with SMBS and PMBS. Since these salts lacked efficacy at 10 mM and were phytotoxic at 100 mM, the concentrations of 20 and 50 mM were evaluated to control GM and BM. Further dips to control SR were performed only at the concentration of 50 mM.

3.2.2. Efficacy of selected dip treatments

The two salts tested (SMBS and PMBS) at both concentrations (20 and 50 mM) significantly reduced GM incidence and severity and the sporulation of *P. digitatum*, in comparison with controls, on 'Valencia' oranges dipped for 60 s (Fig. 3). The most effective treatments were 50 mM PMBS for incidence and sporulation reduction and 20 mM PMBS for severity reduction. Likewise, for fruits dipped for 120 s, all treatments tested reduced significantly the incidence, severity and sporulation, with 50 mM PMBS the most effective treatment against GM. Among all the treatments, 60 s dips in 50 mM SMBS was the least effective. On the other hand, no significant differences were observed between dip time (60 or 120 s) for each treatment.

Fig. 4 shows that for fruits dipped for 60 s, the two salts at both concentrations significantly reduced the incidence and severity of BM and the sporulation of *P. italicum* on 'Valencia' oranges in comparison with controls, with 50 mM PMBS and 50 mM SMBS the most effective treatments. Likewise, for fruits dipped for 120 s, all treatments, with the exception of 20 mM SMBS, reduced significantly the severity and the incidence of BM, while the pathogen sporulation was always below 20%. The most effective treatment in reducing BM incidence, severity and sporulation was 50 mM PMBS, followed by 50 mM SMBS. In addition, for each treatment, no significant differences were observed between dip times (60 or 120 s).

It can be observed in Fig. 5 that none of the tested treatments was effective against SR. SMBS and PMBS, at the dose of 50 mM, did not reduce significantly the disease incidence and sporulation neither for a dip time of 60 s nor for 120 s. Likewise, 120 s dips in 50 mM SMBS and 50 mM PMBS and 60 s dips in SMBS were ineffective to reduce SR severity. On the other hand, 60 s dips in 50 mM PMBS reduced significantly

disease severity, in comparison with the controls. However, despite the significant reduction, the value of SR severity was still high, with an average diameter of 39.1 ± 3.2 mm. Moreover, for each treatment, no significant differences were observed between dip times (60 or 120 s) (Fig. 5).

In all trials, no rind phytotoxicities were observed after dip treatments (injury scale value = 1 for all treatments; data not shown).

4. Discussion

The sulfur-containing salts used in this study (SMBS, PMBS, AlS and AlPS) are approved food additives for a variety of purposes and are considered as GRAS substances. SMBS and PMBS are used as preservatives. AlS and AlPS are permitted for multiple purposes; both are used as firming and pH-adjusting agents and AlS is also used as a starch modifier (Health Canada, 2017; Kolaei et al., 2012, 2013; US FDA, 2019). Herein, we evaluated the activity of these salts to inhibit the mycelial growth of *P. digitatum, P. italicum* and *G. citri-aurantii* and afterwards their ability to control citrus GM, BM and SR.

According to the in vitro results, the four salts were particularly effective to inhibit the mycelial growth of *P. digitatum* and *P. italicum*, and the development of both fungi was nil at all doses tested. Likewise, 100% of inhibition was achieved with SMBS and PMBS against *G. citri-aurantii*. AlS and AlPS were also very effective at concentrations of 30 mM or higher and also reduced reasonably the mycelial growth of *G. citri-aurantii* at low doses. Sulfur-containing salts have previously shown good activity in in vitro tests against different postharvest pathogens affecting important crops. Indeed, it has been reported that SMBS affected the in vitro spore germination of *B. cinerea* (Alaoui et al., 2017; Mills et al., 2004) and inhibited the mycelial growth of pathogenic fungi such as *Alternaria solani* (Kolaei et al., 2012), *Fusarium sambucinum* (Kolaei et al., 2012; Mecteau et al., 2002), *Fusarium solani* var. *coeruleum* (Mecteau et al., 2008), *Helminthosporium solani* (Hervieux et al., 2002), *Pythium sulcatum* and *Rhizopus stolonifer* (Kolaei et al., 2012). Likewise, PMBS inhibited the mycelial growth of *A. solani*, *B. cinerea*, *F. sambucinum* or *R. stolonifer*, at a minimum concentration of 10 mM (Kolaei et al., 2012). Regarding major citrus postharvest pathogens, previous in vitro studies have shown that SMBS at 20 mM completely inhibited the mycelial growth and sporulation of *P. italicum* (Askarne et al., 2011, 2013), while SMBS and PMBS at 2% (w/v) had the same effect on *P. digitatum* (Enginsu et al., 2018). Besides, Talibi et

al. (2011) showed that SMBS completely inhibited the arthrospore germination of *G. citri-aurantii* at the doses of 75 and 100 mM and significantly reduced it at 25 and 50 mM. Our positive results with SMBS and PMBS are in agreement with these former studies, highlighting the antifungal potential of metabisulfites. To our knowledge this is the first report on the effect of PMBS on the mycelial growth of *P. italicum* and *G. citri aurantii*. By contrast, the studies testing the antifungal activity of AlS and AlP are few. Mills et al. (2004) showed that AlPS was effective against *A. solani* and *B. cinerea*, but mycelial growth of these fungi was not completely inhibited at any of the concentrations studied (2, 20 and 200 mM). Kolaei et al. (2013) tested AlPS against *A. solani*, *B. cinerea*, *F. sambucinum* and *R. stolonifer*, but this salt was not effective at the concentration of 1 mM, having a minimum inhibitory concentration (MIC) of 10 mM. To our knowledge, this is the first report on the effect of AlS and AlPS on the mycelial growth of *P. digitatum*, *P. italicum* and *G. citri-aurantii*.

In our experimental conditions, in vivo primary screenings indicated that SMBS and PMBS were very effective to control GM and BM on oranges at all doses tested and, in general, the efficacy of the treatment increased as the application dose rose. After 7 d of incubation, BM incidence and severity were completely inhibited by both salts at 100 mM, while GM incidence and severity were significantly reduced. Nevertheless, the application of SMBS and PMBS at the concentration of 100 mM was phytotoxic on 'Valencia' oranges. In contrast, these salts applied at the concentration of 50 mM did not cause apparent damage to the rind of the treated fruits and were also very effective against both molds. Similarly, only SMBS and PMBS were effective in the in vivo primary screening against SR, especially at the highest concentration tested, 50 mM. Similar in vivo results were obtained in previous related research. Askarne et al. (2013) found that SMBS completely inhibited the development of BM on clementine mandarins at 100 and 200 mM, but these salt concentrations were phytotoxic to the fruit rind. However, SMBS at 50 mM significantly reduced the incidence and severity of BM without damaging the fruit. Talibi et al. (2011) obtained a significant reduction of SR incidence and severity on mandarins, compared with the control, with PMBS at the concentrations of 2 and 3% (w/v). In this study, however, the salt effect was preventive as the authors worked with fruit inoculated with the pathogen about 2 h after the treatment. Furthermore, Enginsu et al. (2018) indicated that SMBS and PMBS significantly reduced the severity of GM in both curative and protective applications. Herein, we first report on the curative effect of SMBS and PMBS against citrus BM and

384SR and our results confirm the potential of metabisulfite-containing salts to control
385major citrus postharvest diseases. Although less effective than metabisulfites, some
386sulfate-containing salts have provided significant inhibition of fungal growth and
387development of postharvest diseases such as dry rot and cavity spot in potato tubers and
388carrot roots, respectively (Kolaei et al., 2012, 2013). However, herein, AlS and AlPS
389did not provide significant reduction in disease incidence or severity in the in vivo
390preliminary screening tests. Thus, only SMBS and PMBS were considered for the
391subsequent in vivo dip treatments.
392The mechanisms by which fungi are tolerant or sensitive to these salts are not well
393understood. Salt-plant tissues interactions may involve different specific biochemical
394reactions. It is possible that the negative results obtained with AlS and AlPS may
395include, besides the limited direct fungicidal activity against the tested citrus pathogens,
396the lack of induction of biochemical defense mechanisms by which the treatments
397provide some degree of disease resistance to the fruit rind. In fact, it has been previously
398shown that some salts containing aluminum induced systemic disease resistance or host
399defenses, for example in potato tubers, that led to significant reduction of important
400fungal diseases (Jeandet et al., 2000; Kolaei et al., 2013; Mecteau et al., 2002).
401In the present work, in vivo dip treatments revealed that both salts, SMBS and PMBS,
402were effective against GM at the concentrations of 20 and 50 mM, reducing
403significantly both disease incidence and severity. Since both dip contact times tested (60
404and 120 s) were effective and gave similar significant disease reduction with respect to
405the control, the adoption of 60-s dips is recommended for future experiments. It is clear
406that, commercially, shorter dip times are preferred because they are simpler, faster
407(especially important during busy full season operations), cheaper (in terms of labor and
408energy costs) and can reduce the risks of phytotoxicity or other harm to fruit quality
409(Montesinos-Herrero et al., 2016). These positive results with aqueous solutions of
410SMBS and PMBS show the great potential of these food additives to control citrus GM
411and BM at the commercial level. Other researchers have previously reported the
412effectiveness of metabisulfite-containing salts when applied as aqueous solutions; for
413example, SMBS and PMBS gave very good results in controlling potato dry rot and
414carrot cavity spot diseases (Kolaei et al., 2012). Conversely, SMBS and PMBS dips
415were not able to reduce significantly SR incidence and severity at the concentration of
41650 mM, irrespective of the immersion time. Such negative results with SMBS against
417SR are in agreement with those reported by Talibi et al. (2011), who also obtained good

418results with SMBS in in vitro experiments, but not in in vivo tests with artificially
419inoculated mandarin fruits.
420In general, the effectiveness of 50 mM SMBS and 50 mM PMBS applied as aqueous
421dips against the three diseases was lower than that obtained in in vivo primary
422screenings, especially in the case of SR. This higher disease incidence on dip-treated
423oranges than on fruit treated with a salt solution drop may be possibly due to the
424increased contact time of the drop with the rind wound with respect to the dip contact
425time. However, the diseases were not more satisfactorily controlled by longer
426immersion times, suggesting that other factors may influence the treatment efficacy.
427Important other factors are likely to be pH, depletion of sulfite by oxidation to sulfate
428and the penetration capability of the active ingredient into rind wounds.
429In general, when sulfite salts are dissolved in water a percentage of them is released as
430sulfur dioxide (67.4% in the case of SMBS and 57.6% for PMBS; EFSA, 2016). Sulfur
431dioxide is a well-studied compound that has been reported as the responsible for the
432antimicrobial activity of the sulfite salts commonly used as food preservatives. Thus, it
433has been discussed that its accumulation in the cytoplasm inhibits microbial growth by
434interfering with intercellular processes and cell components (Davidson et al., 2003).
435Among them, for example, Avis et al. (2007, 2009) proposed that the mechanism of
436action of SMBS solutions against *F. sambucinum* and other fungal pathogens was the
437elevated levels of lipid peroxidation involving unsaturated fatty acids that lead to the
438loss of integrity of the fungal cell membranes. In aqueous solutions, sulfur dioxide is
439present as three different species, namely the undissociated free dibasic acid (H_2SO_3),
440which rapidly converts to "molecular" SO_2, bisulfite (HSO_3^-) and sulfite (SO_3^{2-}), and the
441equilibrium among them is dependent on the pH (Divol et al., 2012). In fact, Smilanick
442et al. (1990) found radical differences in sulfite toxicity over a relatively narrow range
443of pH on the germination of spores of *B. cinerea* and concluded that "molecular" SO_2
444was the prevalent contributor to the toxicity of sulfur dioxide solutions, especially as
445more acidic the medium was, while the ionized forms had little toxicity. Our results are
446in accordance with these studies. It would have been expected that SMBS solutions
447would be slightly more effective than equimolar PMBS solutions due to their higher
448release of sulfur dioxide when dissolved in water. However, in some experiments,
449PMBS was equal or even more effective than SMBS against citrus diseases. A possible
450explanation is that the pH of the SMBS solutions used in the experiments was slightly
451higher than that of the equimolar PMBS solutions and this may have reduced the

toxicity of SMBS. In this sense, it is worthy to note that, as it can be observed in Table 1, the pH of all salt aqueous solutions of sulfur-containing food additives was slightly lower as the salt concentration increased.

The particular chemistry involving the use of sulfur dioxide and sulfite salts makes them suitable for a variety of agricultural and environmental uses. Fumigations with sulfur dioxide gas or in-package use of SMBS pads that release the gas upon absorption of moisture are widely used commercially to control gray mold of table grapes because they are very effective to kill both mycelia and spores of *B. cinerea* (Smilanick and Henson, 1992; Smilanick et al., 1990). Since part of the sulfite (oxidation state of 4) is oxidized to the relatively benign form of sulfate (oxidation state of 6) when it reacts in an aqueous solution, sulfite salts are good candidates to be used, for example, for pH adjustment (acidification) of irrigation water or as reducing agents in the de-chlorination of waste water before it is released to the environment (Upton and Adams, 1982). The latter is a positive feature to be considered in fresh produce packinghouses, where it is very important to appropriately treat the discharge water to enhance its quality. Furthermore, sulfite-based postharvest technologies could also be useful in the packinghouse to kill or inactivate microorganisms of food safety concern potentially present on fresh horticultural products. Currently, this is especially important in the USA, under the Food Safety Modernization Act (FSMA) established by the US FDA. For every commercial packinghouse use, however, worker safety standards will certainly need to be seriously considered, as hazardous sulfur dioxide off-gassing may occur (Smilanick et al., 1995).

There are many studies showing the effectiveness of various organic and inorganic salts, classified as food additives or GRAS compounds, such as potassium silicate, sodium methylparaben, potassium sorbate and sodium benzoate to control decay caused by *P. digitatum*, *P. italicum* and *G. citri-aurantii* (Montesinos-Herrero et al., 2016; Moscoso-Ramírez et al., 2013; Moscoso-Ramírez and Palou, 2014; Palou et al., 2002; Smilanick et al., 2008). However, few provide some information about the effect of metabisulfite salts to inhibit these citrus pathogens (Askarne et al., 2011, 2013; Talibi et al., 2011). Although not prepared from metabisulfites, but from sodium bisulfite ($NaHSO_3$; E-222), heated sulfur dioxide solutions were as effective to control green mold on lemons as other treatments such as ethanol, sodium carbonate and the fungicide imazalil, and they were superior to hydroxide peroxide treatments (Smilanick et al., 1995). These workers observed that such treatments with sulfur dioxide solutions adjusted to pH 5

486satisfactorily eradicated disease caused by existing *P. digitatum* infections without
487producing dangerous off-gassing. A brief rinsing of treated fruit with tap water was
488sufficient to avoid rind injury on lemons and reduce sulfite residues below the threshold
489established for regulated fresh produce such as grapes, which is of 10 µg/g (US FDA,
4902019).

491In this work we have given new insights on the antifungal activity of four selected
492sulfur-containing salts against *P. digitatum, P. italicum* and *G. citri-aurantii* and the
493diseases that they cause. Overall, this study has revealed that SMBS, PMBS, AlS and
494AlPS have potent in vitro antifungal activity at relatively low concentrations (10 mM)
495against the three major citrus postharvest pathogens. However, only SMBS and PMBS
496were effective when applied to fruit tissues against GM and BM and none of the salts
497was effective against SR. In vitro tests are an important first step for selecting salts with
498antifungal potential. However, our results confirm that in vitro inhibition cannot predict
499the potential of a salt to control postharvest diseases and that the effect of pathogen-
500fruit-salt interactions needs to be taken into account. Apparently, the original toxicity of
501the salt solution to the pathogen in artificial growing medium may be altered by
502interactions in wounds with constituents of the fruit rind. Such interactions may alter the
503pH and/or other environmental conditions within the wound infection courts occupied
504by the fungus, thus affecting fungal development and the toxicity of the salts (Hervieux
505et al., 2002; Kolaei et al., 2013). Taking into account the previously mentioned large
506influence of pH on the mode of action of sulfite salts (Divol et al., 2012; Smilanick et
507al., 1990), it is clear that the toxicity of SMBS and PMBS will be higher if the pH
508within the infected rind wounds is lower than that of the surrounding tissue. It is known
509that among fungal pathogens attacking fresh fruits, some of them, such as
510*Colletotrichum* spp. or *Alternaria* spp., alkalinize the ambient in their infection courts as
511a key factor to assure pathogenicity; whereas others, such as *Penicillium* spp., including
512*P. digitatum* and *P. italicum*, acidify the ambient in infected fruit peel wounds as a
513procedure to enhance their virulence. This modulation mechanism ensures the
514expression of genes encoding the production of hydrolytic enzymes, especially
515polygalacturonases, which degrade the host cell walls and macerate the host tissue
516during the colonization phase of disease development (Prusky et al., 2004; Vylkova,
5172017). Therefore, such acidification could increase the activity of sulfite salts and
518consequently contribute to the significant reduction of the severity and sporulation of
519GM and BM observed on oranges treated with SMBS or PMBS at 50 mM. In contrast to

P. digitatum and *P. italicum*, to our knowledge, no information is available on the acidification capacity of *G. citri-aurantii* on citrus fruits, although the species *Geotrichum candidum* has been included in a list of fungal pathogens that promote an acidic environment (Prusky and Yakovi, 2003). As a hypothesis to explain our results, substantial differences in the degree of acidification may contribute to the different effectiveness of SMBS and PMBS to control GM and BM in comparison with SR. In any case, for now, the mode of action of SMBS and PMBS against major citrus postharvest diseases is not fully elucidated and further research is needed to understand the mechanisms by which diseases caused by *Penicillium* spp. are more effectively controlled by these sulfite salts than disease caused by *G. citri-aurantii*.

Information gathered from this study provides a basis for further research into the uses of sulfur-containing salts to control major citrus postharvest diseases. Our results indicate the possibility of applying SMBS and PMBS to inhibit or considerably reduce the incidence, severity and sporulation of Penicillium molds. These food additives are used in the food processing industry (i.e. wines, beverages) and are generally regarded as safe for human consumption, if applied at the appropriate concentrations (EFSA, 2016; US FDA, 2019). In the fresh fruit industry, they are currently allowed to be used as postharvest treatments of grapes, with a sulfite residue limit on treated fruit of 10 μg/g (Smilanick et al., 1995; US FDA, 2019). This research has focused on new potential applications of these salts as substitutes of polluting synthetic fungicides for citrus fruits and, according to the promising results, SMBS and/or PMBS, if properly handled and applied, could be a new tool to be included in integrated management programs for the control of GM and BM, although not SR. Hence, it would make sense for the citrus postharvest industry to pursue their registration as new postharvest treatments provided that they are cost-effective and suitable for citrus international markets with zero tolerance to chemical residues of conventional postharvest fungicides. However, further research is required to determine the optimal concentration of each salt for a given postharvest system and the sulfite residue levels on treated citrus fruit, to evaluate their effects on fruit quality of long-term cold-stored citrus fruits and also to assess their efficacy on a larger scale using naturally infected fruits.

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Declaration of interest

None.

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Table 1. Physicochemical properties of food additives used in this study.

Food additive	Acronym	Molecular formula	E-number[a]	Molecular weight (g/mol)	pH[b]	Solubility in water (g/ L at 20 ºC)
Sodium metabisulfite	SMBS	$Na_2 S_2 O_5$	E-223	190.11	3.50 (20 mM) 3.24 (50 mM)	Freely soluble, 667
Potassium metabisulfite	PMBS	$K_2 S_2 O_5$	E-224	222.33	3.25 (20 mM) 3.10 (50 mM)	Freely soluble, 450
Aluminum sulfate (hydrate)	AlS	$Al_2 (SO_4)_3 \cdot H_2O$	E-520	342.15 (anhydrous basis)	3.46 (10 mM) 3.20 (30 mM) 3.18 (50 mM) 3.05 (100 mM)	Freely soluble, 364
Aluminum potassium sulfate (dodecahydrate)	AlPS	$Al\ K\ (SO_4)_2 \cdot 12H_2O$	E-522	474.39 (dodecahydrate)	3.59 (10 mM) 3.28 (30 mM) 3.24 (50 mM) 3.10 (100 mM)	Freely soluble, 140

[a] E-number = codes for substances that are permitted to be used as food additives within the European Union (EFSA, 2016, 2018).

[b] pH of the solutions used in this study. Values for solutions used in preliminary screening tests are reported for AlS and AlPS, whereas values for solutions used in dip treatments are reported for SMBS and PMBS.

Sources: (Acros Organics, 2019; EC, 2012; Honeywell Fluka, 2019).

737**Table 2.** In vitro inhibition of radial mycelial growth of fungi causing citrus postharvest 738diseases on PDA Petri dishes amended with different concentrations of sulfur-containing food 739additives after 7 d of incubation at 25 ºC

Fungal pathogen	Food additive[b]	Inhibition (%) [a]				
		Concentration (mM)				
		10	20	30	50	100
Penicillium digitatum	SMBS	100 Aa[c]	100 Aa	100 Aa	100 Aa	100 Aa
	PMBS	100 Aa	100 Aa	100 Aa	100 Aa	100 Aa
	AlS	98.0 Aa	100 Aa	100 Aa	100 Aa	100 Aa
	AlPS	100 Aa	100 Aa	100 Aa	100 Aa	100 Aa
Penicillium italicum	SMBS	100 Aa	100 Aa	100 Aa	100 Aa	100 Aa
	PMBS	100 Aa	100 Aa	100 Aa	100 Aa	100 Aa
	AlS	98.2 Aa	100 Aa	100 Aa	100 Aa	100 Aa
	AlPS	100 Aa	100 Aa	100 Aa	100 Aa	100 Aa
Geotrichum citri-aurantii	SMBS	100 Aa	100 Aa	100 Aa	100 Aa	100 Aa
	PMBS	100 Aa	100 Aa	100 Aa	100 Aa	100 Aa
	AlS	82.1 Bb	100 Aa	100 Aa	100 Aa	100 Aa
	AlPS	59.9 Cc	83.6 Bb	100 Aa	100 Aa	100 Aa

740[a] Colony diameter reduction with respect to control treatments (non-amended PDA plates).

741[b] See Table 1 for acronym definitions.

742[c] Means in lines with different lowercase letters and means in columns with different capital 743letters are significantly different by Fisher's protected LSD test ($P < 0.05$) applied after an 744ANOVA.

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748**Fig. 1.** Effectiveness of sulfur-containing food additives (SMBS, sodium metabisulfite; PMBS,
749potassium metabisulfite,; AlS, aluminum sulfate; AlPS, aluminum potassium sulfate) at
750different concentrations to control citrus green (GM) and blue (BM) molds on in vivo primary
751screenings with 'Valencia' oranges artificially inoculated, 24 h before treatment, with
752*Penicillium digitatum* and *Penicillium italicum*, respectively, and incubated for 7 d at 20 ºC and
75390% RH. Control fruits (CON) were treated with water. For each disease and dependent
754variable, columns with different capital letters indicate significant differences among salts for
755each concentration and columns with different lowercase letters indicate significant differences
756among concentrations for each salt, according to Fisher's protected LSD test ($P < 0.05$) applied
757after an ANOVA. Thin vertical lines above columns designate standard error (SE). Incidence
758and sporulation values were arcsine-transformed. Non-transformed means are shown. *
759indicates treatments that caused visible rind phytotoxicity.
760
761**Fig. 2.** Effectiveness of sulfur-containing food additives (SMBS, sodium metabisulfite; PMBS,
762potassium metabisulfite,; AlS, aluminum sulfate; AlPS, aluminum potassium sulfate) at
763different concentrations to control sour rot (SR) on in vivo primary screenings with 'Valencia'
764oranges artificially inoculated, 24 h before treatment, with *Geotrichum citri-aurantii* and
765incubated for 8 d at 28 ºC and 90% RH. Control fruits (CON) were treated with water. For each
766dependent variable, columns with different capital letters indicate significant differences among
767salts for each concentration and columns with different lowercase letters indicate significant
768differences among concentrations for each salt, according to Fisher's protected LSD test ($P <$
7690.05) applied after an ANOVA. Thin vertical lines above columns designate standard error
770(SE). Incidence and sporulation values were arcsine-transformed. Non-transformed means are
771shown.
772
773**Fig. 3.** Curative activity of sodium metabisulfite (SMBS) and potassium metabisulfite (PMBS),
774at two different concentrations (20 and 50 mM), to control green mold (GM) on 'Valencia'
775oranges artificially inoculated, 24 h before treatment, with *Penicillium digitatum* and incubated

776for 7 d at 20 ºC and 90% RH. Control fruits were immersed in water for 60 s. For each dip time,
777columns with different capital letters indicate significant differences among salt treatments and,
778for each treatment, columns with different lowercase letters indicate significant differences
779between dip times, according to Fisher's protected LSD test ($P < 0.05$) applied after an
780ANOVA. Thin vertical lines above columns designate standard error (SE). Incidence and
781sporulation values were arcsine-transformed. Non-transformed means are shown.
782
783**Fig. 4.** Curative activity of sodium metabisulfite (SMBS) and potassium metabisulfite (PMBS),
784at two different concentrations (20 and 50 mM), to control blue mold (BM) on 'Valencia'
785oranges artificially inoculated, 24 h before treatment, with *Penicillium italicum* and incubated
786for 7 d at 20 ºC and 90% RH. Control fruits were immersed in water for 60 s. For each dip time,
787columns with different capital letters indicate significant differences among salt treatments and,
788for each treatment, columns with different lowercase letters indicate significant differences
789between dip times, according to Fisher's protected LSD test ($P < 0.05$) applied after an
790ANOVA. Thin vertical lines above columns designate standard error (SE). Incidence and
791sporulation values were arcsine-transformed. Non-transformed means are shown.
792
793**Fig. 5.** Curative activity of sodium metabisulfite (SMBS) and potassium metabisulfite (PMBS)
794at the concentration of 50 mM to control sour rot (SR) on 'Valencia' oranges artificially
795inoculated, 24 h before treatment, with *Geotrichum citri-auranti* and incubated for 8 d at 28 ºC
796and 90% RH. Control fruits were immersed in water for 60 s. For each dip time, columns with
797different capital letters indicate significant differences among salt treatments and, for each
798treatment, columns with different lowercase letters indicate significant differences between dip
799times, according to Fisher's protected LSD test ($P < 0.05$) applied after an ANOVA. Thin
800vertical lines above columns designate standard error (SE). Incidence and sporulation values
801were arcsine-transformed. Non-transformed means are shown.
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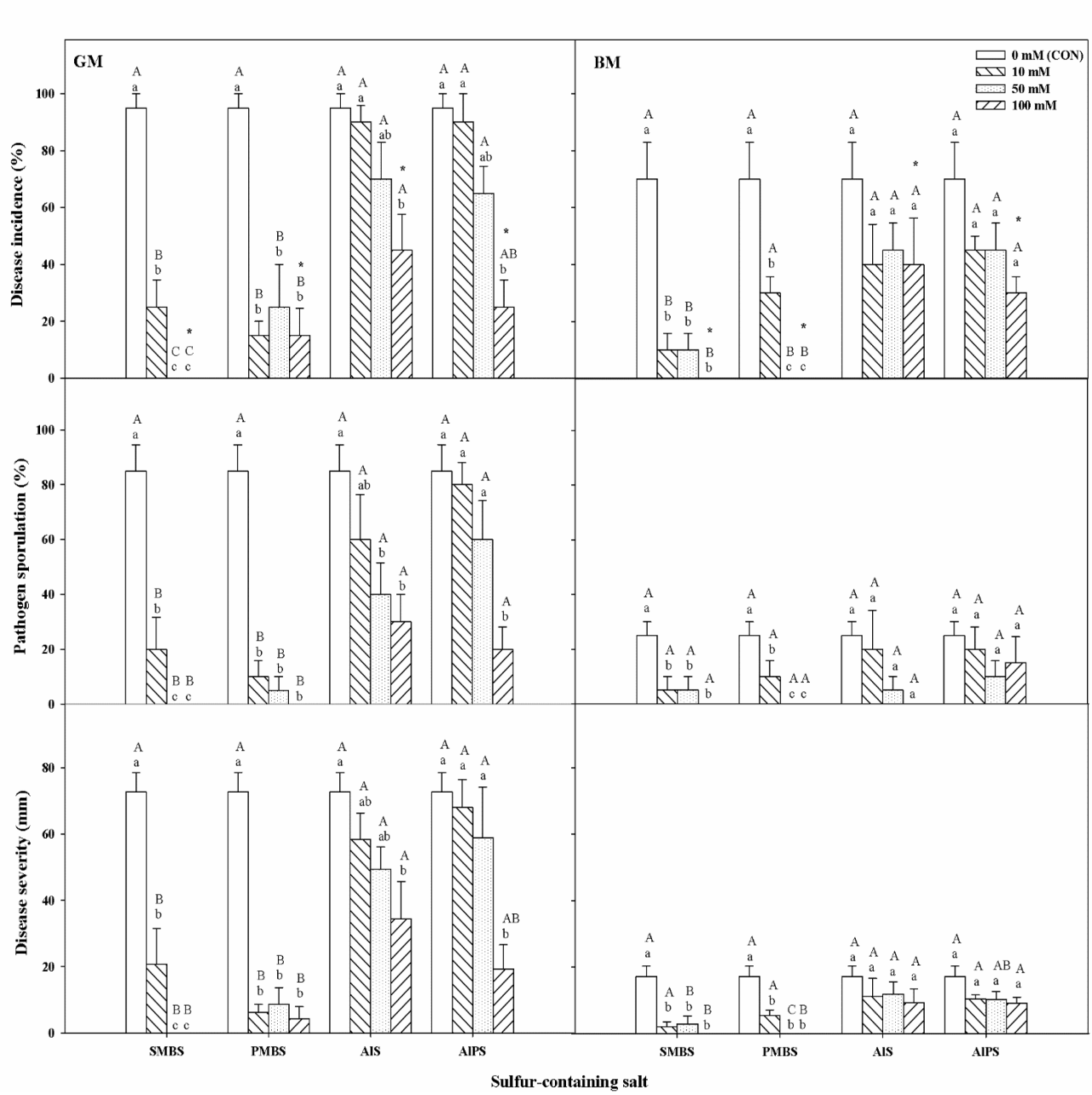

805**Fig. 1**

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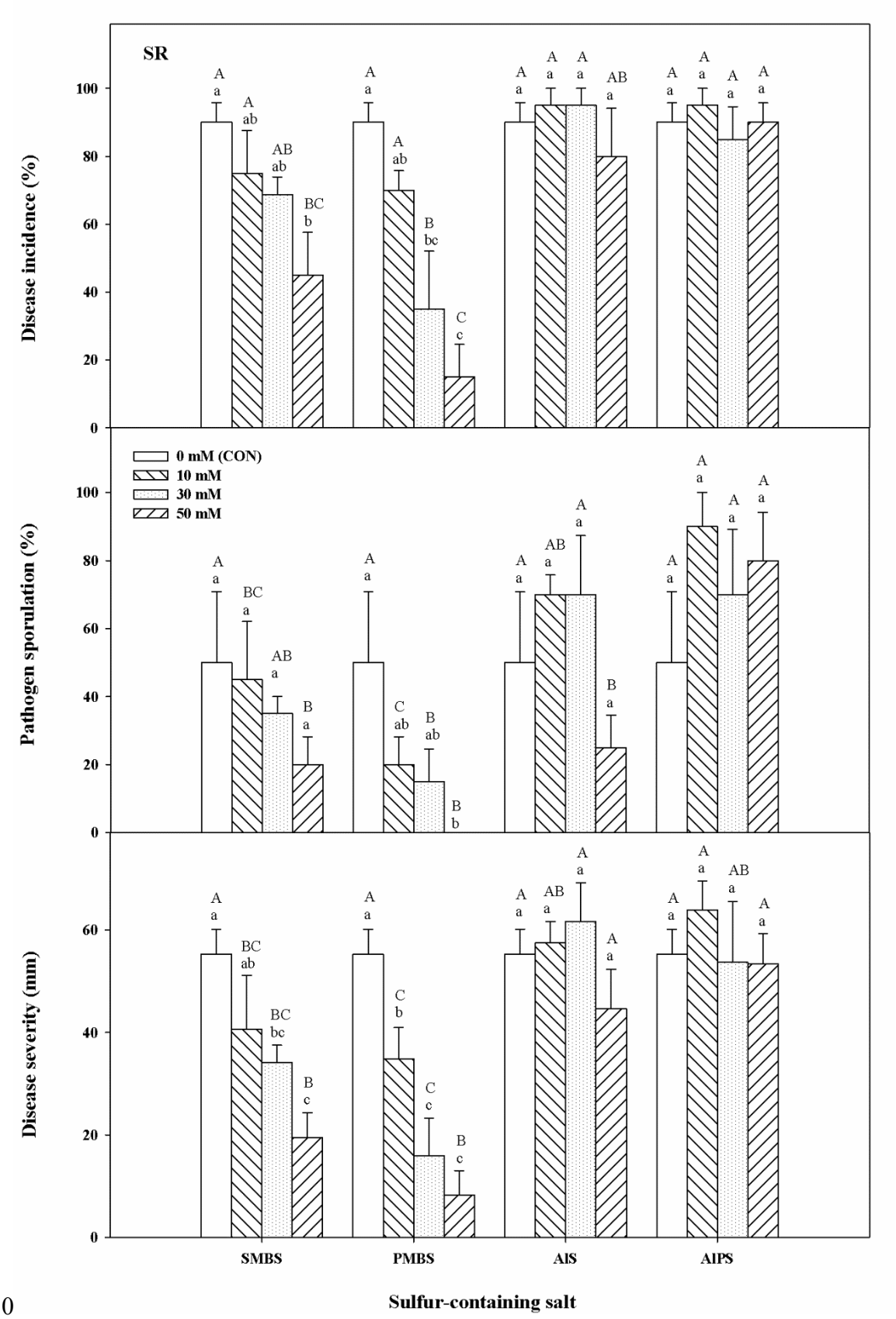
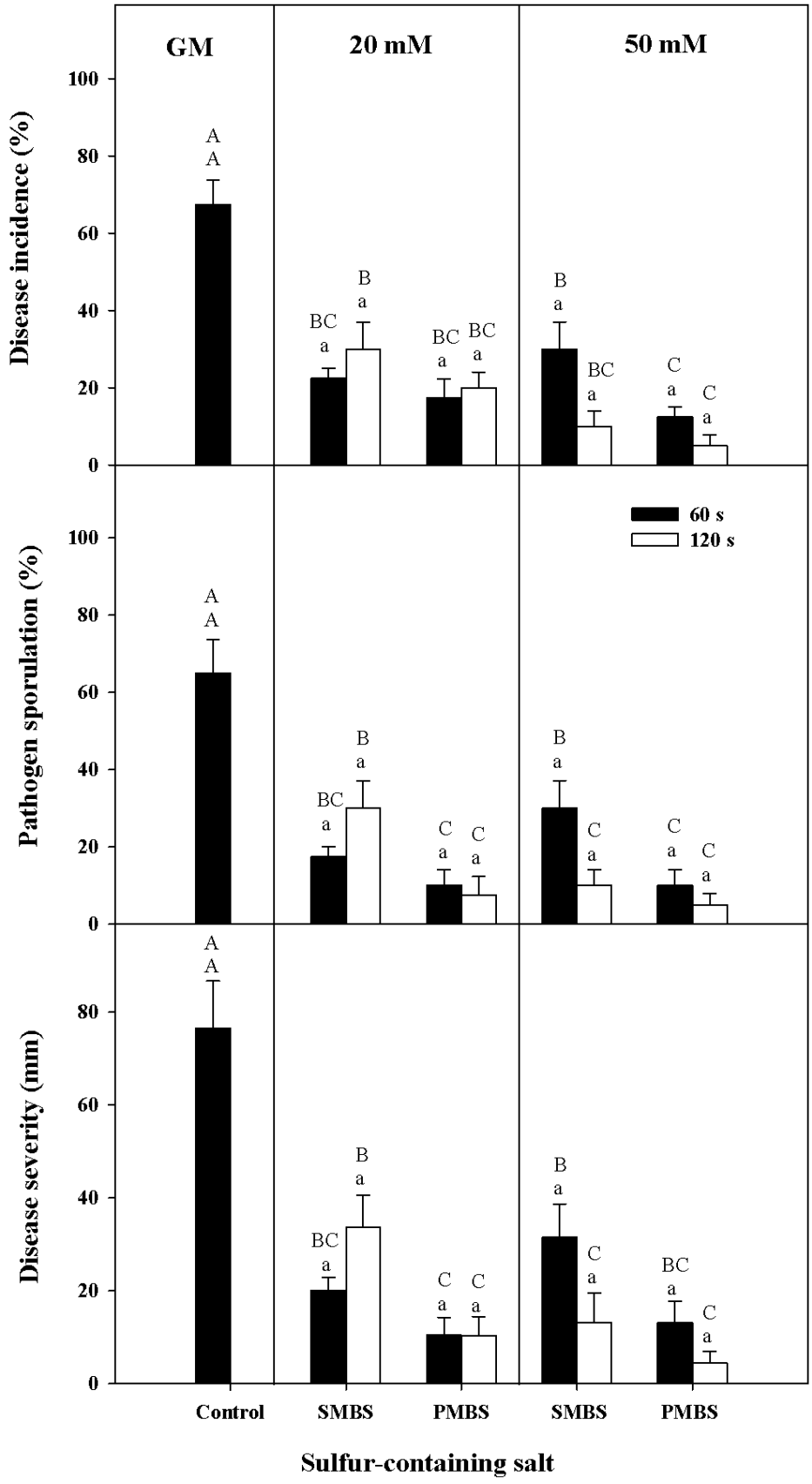
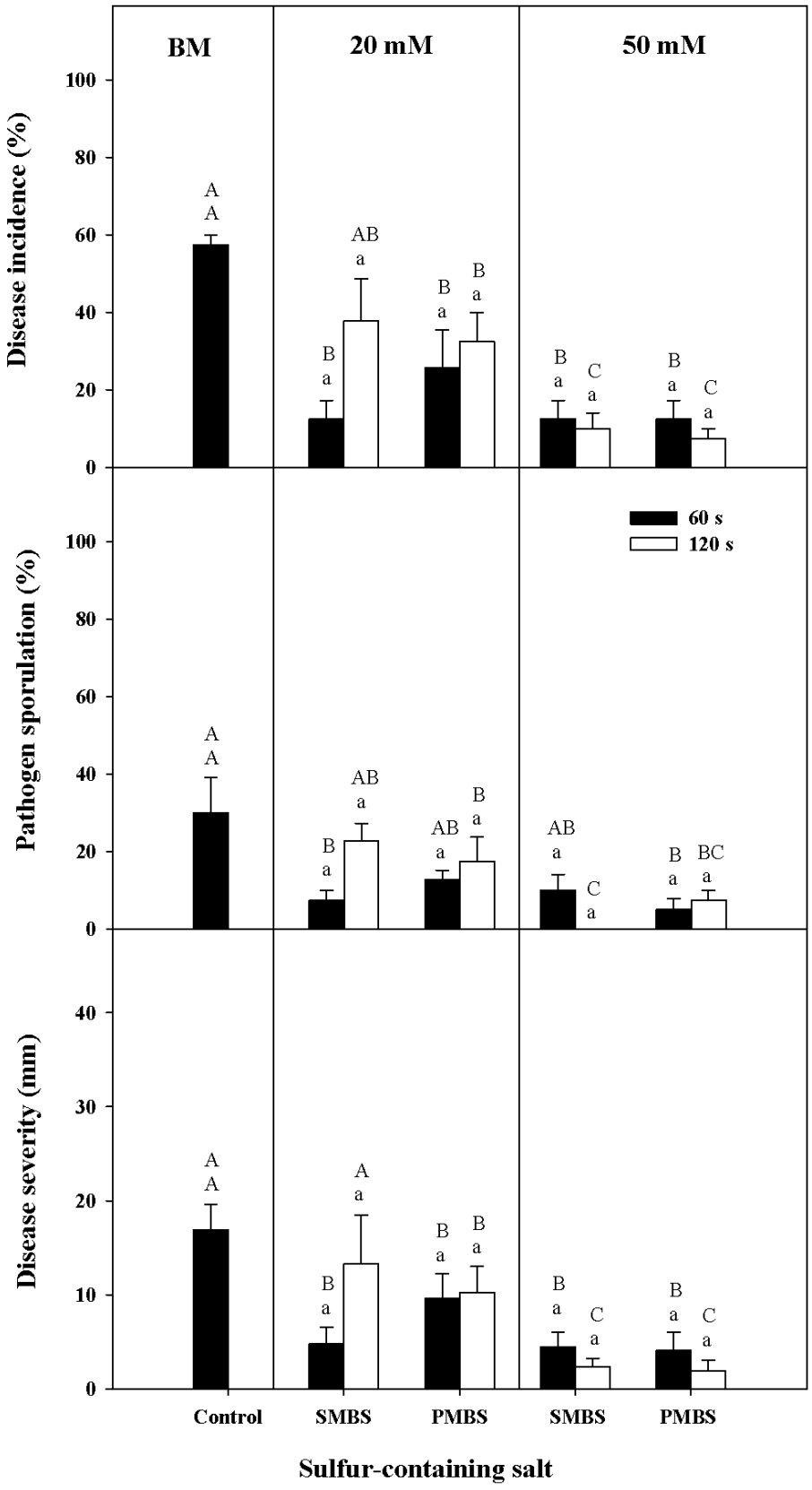

SR
Disease incidence (%)
Pathogen sporulation (%)
Disease severity (mm)
0 mM (CON)
10 mM
30 mM
50 mM
SMBS
PMBS
AIS
AIPS
Sulfur-containing salt

Fig. 2


GM
20 mM
50 mM
Disease incidence (%)
Pathogen sporulation (%)
Disease severity (mm)
60 s
120 s
Control
SMBS
PMBS
SMBS
PMBS
Sulfur-containing salt

813

814 **Fig. 3**



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816 **Fig. 4**

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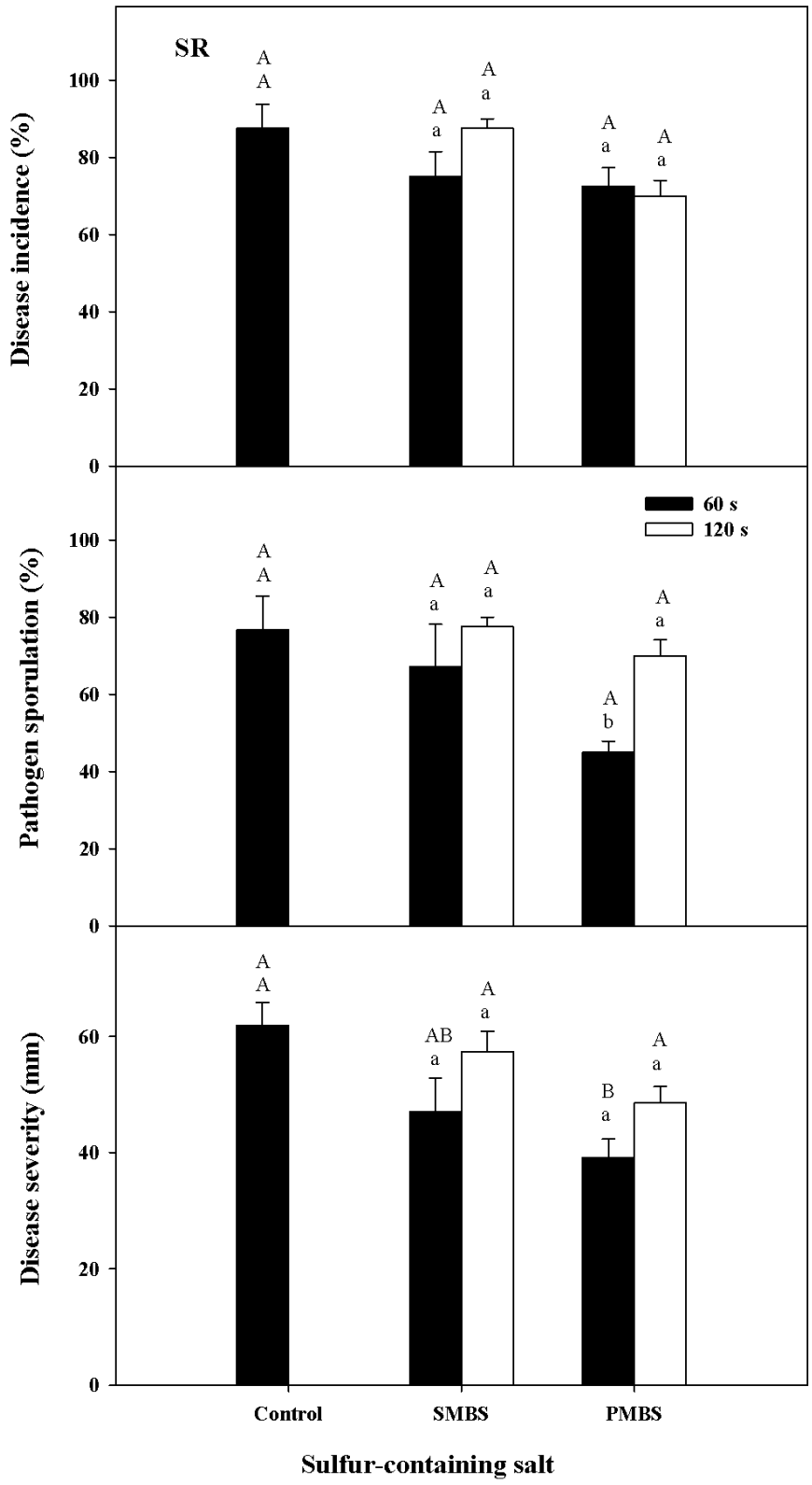
SR
Disease incidence (%)
Pathogen sporulation (%)
Disease severity (mm)
60 s
120 s
Control
SMBS
PMBS
Sulfur-containing salt

Fig. 5