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Chitosan and postharvest decay of fresh fruit: Meta-analysis of disease control and antimicrobial and eliciting activities

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Chitosan and Postharvest Decay of Fresh Fruit: Meta-Analysis of Disease Control and Antimicrobial and Eliciting Activities

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Short version of title: **Chitosan and Postharvest Decay of Fruit**

Abstract

Consumers are increasingly aware of the importance of regular consumption of fresh fruit in their diet. Since fresh fruit are highly sensitive to postharvest decay, several investigations focused on the study natural compounds alternative to synthetic fungicides, to extend their shelf life. A long list of studies reported the effectiveness of the natural biopolymer chitosan in control of postharvest diseases of fresh fruit. However, these findings remain controversial, with many mixed claims in the literature. In this work, we used random-effects meta-analysis to investigate the effects of 1% chitosan on (i) postharvest decay incidence; (ii) mycelium growth of fungal pathogens *Botrytis cinerea*, *Penicillium* spp., *Colletotrichum* spp. and *Alternaria* spp.; and (iii) phenylalanine ammonia-lyase, chitinase and β-1,3-glucanase activities. Chitosan significantly reduced postharvest disease incidence (mean difference [MD], –30.22; P <0.00001) and *in-vitro* mycelium growth (MD, –54.32; P <0.00001). For host defence responses, there were significantly increased activities of β-1,3-glucanase (MD, 115.06; P = 0.003) and chitinase (MD, 75.95; P <0.0002). This systematic review contributes to confirm the multiple mechanisms of mechanisms of action of chitosan, which has unique properties in the natural compound panorama. Chitosan thus represents a model plant protection biopolymer for sustainable control of postharvest decay of fresh fruit.

Keywords: defence related enzymes; fungal pathogens; natural antifungal compounds; plant protection; sustainable control of plant pathogens

1 INTRODUCTION

Postharvest fungal diseases can limit the storage period and shelf life, and thus market life, of fruit and vegetables, which results in serious economic losses worldwide (Oerke & Dehne, 2004; Romanazzi, Smilanick, Feliziani, & Droby, 2016; Palou & Smilanick, 2020). The global average loss due to the food postharvest reported by Food and Agriculture Organization, was estimated in North America, Europe and Oceania about 29%, compared to an average of about 38% in industrialized Asia, Africa, Latin America and South East Asia (Parfitt, Barthel, & Macnaughton, 2010; Food and Agriculture Organization of the United Nations, 2011; Sawicka, 2019).

The main fungal diseases (and their associated fungal pathogen) include: gray mold (*Botrytis cinerea* Pers.); *Rhizopus* rot (*Rhizopus stolonifer* Ehrenb.); anthracnose (*Colletotrichum* spp.); green mold (*Penicillium digitatum* Pers.); blue mold (*Penicillium italicum* Wehmer on citrus fruit, *P. expansum* Link on other fruit); and *Alternaria* rot (*Alternaria* spp.). The control of the causal fungal pathogens is therefore critical to extend the shelf-life of these fresh products (Prusky, 2011; Arah, Amaglo, Kumah, & Ofori, 2015). Despite the efficacy of synthetic fungicides in the control of postharvest decay, public concerns about chemical and toxic residues in food (Belden, McMurry, Smith, & Reilley, 2010; Mebdoua, 2018; Gonçalves et al., 2019; Liu, Yamdeu, Gong, & Orfila, 2020) and the increase in drug-resistant strains of many pathogens (Zuccolo et al., 2019) indicate the need for development of new strategies. Over the last few decades, there has been an increasing interest in the study of postharvest control methods that make use of natural resources (Palou, Smilanick & Droby, 2008; Talibi, Boubaker, Boudyach, & Ait Ben Aoumar, 2014; Souza, Yuk, Khoo, & Zhou, 2015; Guimarães, Abrunhosa, Pastrana, & Cerqueira, 2018; Ebrahimzadeh & Abrinbana, 2019; Liu et al., 2019; Liu, et al., 2020). Such alternative compounds can act as resistance inducers and/or activators of plant defence mechanisms, or they can have strong antimicrobial activities against the main postharvest fungal pathogens (Romanazzi, Feliziani, Baños, & Sivakumar, 2017; Ribes, Fuentes, Talens, & Barat, 2018). However, only a few such natural fungicides have been approved for use as control agents for postharvest diseases, due to the strict regulatory policies for food safety. Among these, chitosan is a natural biocompatible polysaccharide emerged as a promising eco-friendly alternative to synthetic fungicides (Muzzarelli,1983; Romanazzi, Feliziani, & Sivakumar, 2018; Betchem, Johnson, & Wang, 2019). To give some background, chitosan is a common name for the polysaccharide N-aceyl-D-glucosamine (Zargar, Asghari, & Dashti, 2015). The chitosan compound is obtained by deacetilation of chitin through exposure to NaOH solutions or to the enzyme chitinase. It is a functional cationic biopolymer that is widely studied and used across the world. Chitosan have many applications included food industry (Gutiérrez, 2017; da Silva, de Souza, & Dantas Lacerda, 2019; Morin-Crini, Lichtfouse, Torri, & Crini, 2019; Kabanov, & Novinyuk, 2020), cosmetology (Aranaz et al., 2018; Kaczmarek, Struszczyk-Swita, Li, Szczęsna-Antczak, & Daroch, 2019) and human medicine (Tungland & Meyer, 2002; Leung, Liu, Koon, & Fung, 2006; Kofuji et al., 2010; Zhao et al., 2018).

Concerning the agriculture applications, the chitosan was the first compound in the list of basic substances approved in the European Union for plant protection purposes (Reg. EU 66 2014/563), for both organic agriculture and integrated pest management. For several years now, chitosan has been of interest in many studies that have shown that it can be used to prolong storage of an array of fruit and vegetables worldwide, where it has been shown to have three major activities: including biofilm formation on treated surfaces (El Ghaouth, Arul, Ponnampalam, & Boulet, 1991; Valencia-Chamorro, Palou, & Del Río, 2011; Romanazzi et al., 2018); as an antimicrobial (Goy, De Britto, & Assis, 2009; Kong, Chen, Xing, & Park, 2010; Feliziani, Landi, & Romanazzi, 2015; Cheung, Ng, Wong, & Chan, 2015; Wang, Li, & Zhang, 2017; Pétriacq, López, & Luna, 2018; Duan et al., 2019); and as an elicitor of host defence mechanisms (Landi, Feliziani, & Romanazzi, 2014; Coqueiro et al., 2015; Landi et al., 2017; Colman et al., 2019; Xoca-Orozco et al., 2019; Obianom, Romanazzi, & Sivakumar, 2019). For these reasons, chitosan can be used as a biodegradable fungicide (Rebelo, Vila, & Fangueiro, R., 2016; Liang et al., 2017).

However, the heterogeneity of chitosan activities and its effectiveness across a wide range of experimental conditions have led to different interpretations of its primary use/ mechanism/ actions. As a result, different recommendations for chitosan treatments have been provided (Ramos-García et al., 2012; Bill, Sivakumar, Korsten, & Thompson, 2014; Xing et al., 2016; Flores et al., 2018; Betchem et al., 2019; de Souza, Lundgren, de Oliveira, Berger, & Magnani, 2019). Furthermore, based on reports of the evaluation of chitosan across similar and different fungal strains, its value for disease reduction can vary (Herrera-Romero, Ruales, & Caviedes, 2017; Hua et al., 2019; Zahid, Maqbool, Ali, Siddiqui, & Bhatti, 2019). Also, despite the many studies in the literature that have investigated a wide range of chitosan treatments and their influences, no single study has made all of the appropriate comparisons for a full evaluation. Thus, given the mixed claims in the literature, there is the need to define the overall effectiveness of chitosan, to highlight useful aspects for its future investigation.

Meta-analyses can be applied as a tool for analysis of large amounts of data across many primary studies, in which the main purpose is to integrate and interpret the findings, to provide conclusions that the individual studies alone cannot show clearly. This statistical procedure provides an integration of the data across several to many independent studies (Maestri, Pavlicevic, Montorsi, & Marmiroli, 2019). The combination of the resulting outcomes can also increase the statistical power, and make it possible to detect relatively small effects (Rosenberg, Garrett, Su, & Bowden, 2004; Nelson, Gent, & Grove., 2015; Schwingshackl, Hoffmann, Iqbal, Schwedhelm, & Boeing, 2018; Chen, Chen, Chen, & Huang, 2019; González-Domínguez et al., 2019).

The aim of the present study was to carry out a meta-analysis to quantitatively review the data across the available studies on the effectiveness of 1% chitosan, the most common concentration that has been tested in the control of postharvest decay (Romanazzi et al., 2018). Hence, the objectives were to determine the effectiveness of 1% chitosan on: (i) reduction of postharvest diseases of fresh fruit; (ii) *in-vitro* mycelium growth of the causal agents of postharvest decay; and (iii) phenylalanine ammonia-lyase (PAL), β-1,3-glucanase and chitinase activities associated with host defence mechanisms against these causal agents at 24 h post-treatment (hpt).

2. METHODS

2.1 Search strategy and study selection

A systematic literature search from 2007 to 2019 was performed using the databases of Scopus and Web of Science and the following terms: ‘chitosan’ and ‘fruit’. Studies that used chitosan mixed with other compounds were not considered. The selection of studies was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines (Moher, Liberati, Tetzlaff, Altman, & PRISMA Group, 2009).

Article selection for the meta-analysis used the following inclusion criteria: 1% chitosan; disease incidence; *in-vitro* mycelium growth according to specific postharvest fungi; and activity of the enzymes involved in plant defence mechanisms. The eligibility of the articles was assessed, with the exclusion of the studies with different chitosan concentrations, with no information on disease incidence, mycelium growth or defence enzymes, and with no known fungal species.

In more detail, three categories were included for the studies related to: (i) disease incidence published from 2010 to 2019, caused by gray mold, *Rhizopus* rot, anthracnose, green/blue mold and/or *Alternaria* rot, considered as subgroups; (ii) *in-vitro* mycelium growth published from 2007 to 2019 for the decay causing fungal pathogens *B. cinerea*, *Penicillium* spp., *Colletotrichum* spp. and *Alternaria* spp., considered as subgroups; (iii) enzyme activities associated with host defence mechanisms analysed at 24 hpt published from 2009 to 2018, for PAL, chitinase and β-1,3-glucanase, considered as subgroups. All of the studies included at least two treatments, as an untreated control and the 1% chitosan treatment. The fruit varieties, the 1% chitosan application and the detection timing varied across these studies. In some studies, the treatment application times and rates were reported. In such cases, only the treatments applied at the same time as the standard treatment were considered in the meta-analysis. The risk of bias and test for asymmetry for the funnel plots were used to evaluate the publication bias. Cochran's I^2 indices, Tau^2 and χ^2 tests were used to estimate the statistical heterogeneity of the studies (Tufanaru, Munn, Stephenson, & Aromataris, 2015). If the heterogeneity was significant (I^2 >75%; and/or $P < 0.05$), a random effects model was applied to all of the subgroups included in the postharvest decay disease incidence, the decay causing fungi mycelium growth, and the defence enzyme activity categories.

2.2 Data extraction

Data were recorded from the same days of chitosan treatments in each study. All of the studies that were related to the effects of chitosan towards disease incidence were calculated as percentage effects. The studies on the effects on mycelium growth resulted on three different measurement units (percentage, mm, cm), and again these were converted to percentages. To unify the different measurement units used across the studies of the defence enzyme activities, the values were converted into percentage of the mean (% mean) with respect to the normal control ([treatment mean/ normal control mean] × 100) (Viswanatha, Shylaj, & Moolemath, 2017). If the standard deviations (SDs) or standard errors (SEs) were not reported, the data were transformed according to the P values (Weir et al., 2018). Data were extracted from the Figures presented in the papers using Plot Digitiser software (Kadic, Vucic, Dosenovic, Sapunar, & Puljak, 2016). The change scores with the corresponding standard deviations were used, as based on the guidelines of the Cochrane handbook (https://www.cochranelibrary.com/cdsr/doi/10.1002/14651858.CD012276/epdf/full).

2.3 Data analysis

All of these meta-analyses were conducted using the Review Manager (RevMan) software, version 5.3. (Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2014; http://tech.cochrane.org/revman). The data type was selected as continuous. The statistical method was considered as inverse variance. Weighted means, effect sizes, 95% confidence intervals (CIs), which included 0, were calculated. In all of these analyses, P-value <0.05 was considered statistically significant. Differences among the groups were defined when the 95% CIs overlapped a vertical line. If the 95% CIs did not overlap, it can be suggested that the differences were significant (Yang, Scott, Mao, Tang, & Farmer, 2014; Dardiotis et al., 2018). The studies are presented as Forrest plots in the order of the statistical power.

3 RESULTS OF THE REVIEW

3.1 Chitosan-microbe interactions

The antimicrobial activity of chitosan is a complex process that depends significantly from intrinsic properties and environmental factors (Yilmaz Atay, 2019) as well as the type of bacteria, fungi or virus involved (Chirkov, 2002; Kong, et al., 2010; Hosseinnejad, & Jafari, 2016). The precise mechanism of chitosan antimicrobial activity is still not completely understood. Several studies have suggested that the antimicrobial action is mainly due to the polycationic structure of the chitosan. Several studies have suggested that the antimicrobial action is mainly due to the polycationic structure of the chitosan. This activity is carried out in a pH range among 5.6 and 6 (Romanazzi, Gabler, Margosan, Mackey, & Smilanick, 2009) that is below the pKa of chitosan. The chitosan, positively charged, reacts with negatively charged microbial cell membranes (Rabea, Badawy, Stevens, Smagghe, & Steurbaut, 2003; Goy et al., 2009; Kong et al., 2010). This bond alters the permeability of the membrane which is followed by an inhibition of DNA replication and subsequently cell death (Nagy et al., 2011; Divya, Vijayan, George, & Jisha, 2017). A chelating action was also observed. The chitosan molecule binds to the metallic elements present in the trace causing the inhibit of toxins production and microbial growth (Cuero, Osuji, & Washington, 1991; Chung, Wang, Chen, & Li, 2003). The effect of chitosan on fungal pathogens was to inhibits the radial growth, spore germination, and the elongation of the germ tube as well as the production of virulence factors (Palma-Guerrero, Jansson, Salinas, & Lopez-Llorca, 2008; Badawy, & Rabea, 2011).

3.2 Chitosan-plant interactions

The chitosan acts as a powerful elicitor able to inducing a defense response against pathogens in plant tissues by activating both, a local (Zuppini et al., 2003; Iriti, & Varoni, 2015) and systemic plant defense (Benhamou, Lafontaine, & Nicole, 1994; Xing, Zhu, Peng, & Qin, 2015) with the involvement several molecules related to defense mechanisms as pathogenesis-related (PR) proteins (Lopez-Moya et al., 2017; Corsi, Forni, Riccioni, & Linthorst, 2017), Reactive Oxygen Species (ROS) (Singh et al., 2019) and secondary metabolites with active roles in defense as lignin, callose, phytoalexins, PAL, peroxidases and chitinase (Ma, Yang, Yan, Kennedy, & Meng, 2013; Landi et al., 2014; Malerba, & Cerana, 2016). However, the chitosan elicitation activity depends on the reactivity of the host tissues (Romanazzi et al., 2016) as well as from the acetylation and degree polymerization of chitosan (Cord-Landwehr, Melcher, Kolkenbrock, & Moerschbacher, 2016; Li, Xing, Liu, & Li, 2016). Until now the chitosan binding receptors are undefined (Iriti & Faoro 2009; Hidangmayum, Dwivedi, Katiyar, & Hemantaranjanm, 2019). Some researches proposed that chitosan could also interact with chromatin and directly affect gene expression (Hadwiger & Polashock, 2013; Katiyar, Hemantaranjan, Bharti, & Nishant Bhanu, 2014). However, chitosan molecular signals are transduced by messengers such as ROS or phytohormones able to induce physiological and defense response by host (Yin, Li, Zhao, Du, & Ma, 2006; Hidangmayum et al., 2019).

An effect often observed on plants tissue after chitosan treatment was the inhibition of light-induced stomatal opening (Lee et al., 1999; Iriti et al., 2009). On this regard, the transcriptome analysis performed on sweet orange (Coqueiro et al., 2015) and strawberry (Landi et al., 2017) after chitosan treatments underline early impact of compound on the light photosynthetic process affecting imbalance/balance of ROS/redox signaling (Landi et al., 2017). These entire signaling molecules contribute to the adaptive mechanism in chitosan treated plants in response to stress.

3.3 Description of included studies

A flow chart of the screening of the studies identified for the effectiveness of 1% chitosan is shown in Figure 1, with a total of 56 articles finally available for the meta-analysis according to the search criteria. These covered 117 studies, of which 49 were related to disease incidence (total cases, 8,543 [for each of control and chitosan treatment]) (Figure 2), 41 to *in-vitro* mycelium growth (total cases, 1,072) (Figure 3), and 27 to changes in defence-mechanism-related enzymes (total cases, 1,332) (Figure 4). Some of the relevant details of the articles that were included in this meta-analysis are given in Table 1. All of the selected articles were included in the assessment for risk of bias. Also, blinding of outcome assessment in these studies (i.e., performance bias) was not necessary, so it was not included in the analysis for risk of bias. The domains considered for risk of bias were chosen based on each study that reported data and scientific information. All of the studies provided specific indication that the basic characteristics of the control and treatment groups were balanced and were treated under similar environmental conditions. None of these studies included misleading samples. As a result, the selection, detection, attrition and reporting were free of bias, and the publications were defined as at low risk of bias. The funnel plots constructed from the data for disease incidence, mycelium growth and defence enzyme activities did not reveal any significant asymmetry (Figure 5).

3.4 Effects of 1% chitosan on disease incidence

Based on this meta-analysis, the overall data demonstrated the significant effectiveness of 1% chitosan over the control treatment for reduction of disease incidence (studies, 49; total cases, 8,5473) (mean difference [MD], –30.22; 95% confidence intervals [CI], –36.48 to –23.96; I^2, 90.0%; P <0.00001) (Figure 2). The subgroup analysis here (Figure 2) showed that 1% chitosan was significantly effective for reduction of disease incidence against: gray mold (studies, 12; total cases, 1,473), (Shao, Tu, Tu, & Tu, 2012; Feliziani, Santini, Landi, & Romanazzi, 2013; Gao, Zhu, & Zhang, 2013; Romanazzi, Feliziani, Santini, & Landi, 2013; Feliziani et al., 2015; Kanetis, Exarchou, Charalambous, & Goulas, 2017; Zheng, et al., 2017; Gramisci, Lutez, Lopes, & Sangorrína, 2018; Hajji, Younes, Affes, Boufi, & Nasri, 2018) (MD, –23.97; 95% CI, –32.25 to –15.68; I^2, 77.0%; P <0.00001), as highly effective in 9 of these studies, (Shao et al., 2012; Gao et al., 2013; Romanazzi et al., 2013; Feliziani et al., 2015; Kanetis et al., 2017; Zheng, et al., 2017; Gramisci et al., 2018; Hajji et al., 2018); blue/green molds caused by *Penicillium* spp. (studies, 16; total cases, 1,968) (Xing, Xu, Che, Li, & Li, 2011; Shao et al., 2012; Cháfer, Sánchez-González, González-Martínez & Chiralt, 2012; Feliziani et al., 2013;

Romanazzi et al., 2013; Wang, Wu, Qin, & Meng, 2014; Lu et al., 2014; Shao et al., 2015; El Guilli, Hamza, Clément, Ibriz, & Ait Barka, 2016; Zheng, et al., 2017; Gramisci et al., 2018; Kharchoufi, et al., 2018; Liu, Sun, Xiu, Huang, & Zhou, 2018; Shi, Wang, Lu, & Deng, 2018) (MD, –30.85; 95% CI, –41.91 to –19.79; I^2, 90.0%; $P < 0.00001$), as highly effective in 9 of these studies (Xing et al., 2011; Romanazzi et al., 2013; Lu, et al., 2014; Shao et al., 2015; El Guilli et al., 2016; Zheng, et al., 2017; Liu et al., 2018; Shi et al., 2018); *Rhizopus* rot (studies, 5; total cases, 1,740) (Cia, Benato, Pascholati, & Garcia, 2010; Ramos-García et al., 2012; Romanazzi et al., 2013; Xing et al., 2015) (MD, –28.80; 95% CI, –46.13 to –11.47; I^2, 87.0%; $P = 0.001$), as effective in 3 of these studies (Cia et al., 2010; Ramos-García et al., 2012; Romanazzi et al., 2013); and anthracnose (11 studies; total cases, 2,134) (Maqbool, Ali, Ramachandran, Smith, & Alderson, 2010; Zahid, Ali, Manickam, Siddiqui, & Maqbool, 2012; Bill et al., 2014; Edirisinghe, Ali, Maqbool, & Alderson, 2014; Ali, Noh, & Mustafa, 2015; Gutiérrez-Martínez, Bautista-Banos, Berúmen-Varela, Ramos-Guerrero, & Hernández-Ibanez, 2017; Obianom et al., 2019) (MD, –46.64; 95% CI, –61.54 to –31.73; I^2, 92.0%; $P < 0.00001$), as effective in all of these studies. For *Alternaria* rot, 1% chitosan was not significantly effective (studies, 5; total cases, 1,228) (Meng, Yang, Kennedy, & Tian, 2010; Yan et al., 2011; López-Mora, Gutiérrez-Martínez, Bautista-Baños, Jiménez-García, & Zavaleta-Mancera, 2013; Feliziani et al., 2015; Guo, Xing, Yu, Zhao, & Zhu, 2017) (MD, –8.50; 95% CI, –15.75 to –1.25; I^2, 27.0%; $P = 0.24$), although in 1 of these studies (Guo et al., 2017) its effect reached significance.

3.5 Effects of 1% chitosan on in-vitro mycelium growth

The overall data here showed the significant effectiveness of 1% chitosan over the control treatment against *in-vitro* mycelium growth of these fungal pathogens that are involved in postharvest diseases (studies, 41; total cases, 1,072) (MD, –54.32; 95% CI, –64.35 to –44.28;

I^2, 95.0%; P <0.00001) (Figure 3). The subgroup analysis here (Figure 3) showed that 1% chitosan was significantly effective against *in-vitro* mycelium growth for: *B. cinerea* (studies, 5; total cases, 37) (Kanetis et al., 2017; Xu et al., 2007; Feliziani et al., 2013; Munhuweyi et al., 2017; Flores et al., 2018). (MD, -49.38; 95% CI, –72.98 to –25.79; I^2, 94.0%; P <0.0001), as medium high effects for all of these studies; *Penicillium* spp. (studies, 9; total cases, 65) (Xing et al., 2011; Abdel-Kader, El-Mougy & Lashin, 2011; Nisia, Noreña, & Brandelli, 2012; Wang et al., 2014; Waewthongrak, Pisuchpen, & Leelasuphakul, 2015; Shao et al., 2015; Munhuweyi et al., 2017; Madanipour, et al., 2019) (MD, –73.00; 95% CI, –89.71 to –56.30; I^2, 92.0%; P <0.00001), as the highest effects seen, and for all of these studies; *Colletotrichum* spp. (studies, 24; total cases, 955) (Jitareerat, Paumchai, Kanlayanarat, & Sangchote, 2007; Rahman, Mahmud, Kadir, Abdul Rahman, & Begum, 2008; Munoz, Moret, & Garces, 2009; Maqbool et al., 2010; Zahid et al., 2012; Mohamed, Clementine, Didier, Gérard, & Noëlle, 2013; Ali et al., 2014; Bill et al., 2014; Edirisinghe et al., 2014; Ali et al., 2015; Varela, Coronado Partida, Ochoa Jiménez, López, & Martínez, 2015; Gutiérrez-Martínez et al., 2017; de Oliveira, Berger, de Araújo, Camara, & de Souza, 2017; Ramos-Guerrero, González-Estrada, Hanako-Rosas, & Bautista-Banõs, 2018; Xoca-Orozco, Aguilera-Aguirre, López-García, Gutiérrez-Martínez, & Chacón-López, 2018) (MD, –48.18; 95% CI, –62.83 to –33.53; I^2, 96.0%; P <0.00001), as the lowest effects seen based on the point estimate, with the highest effects for 16 of these studies (Jitareerat, et al., 2007; Rahman, et al., 2008; Maqbool et al., 2010; Zahid et al., 2012; Bill et al., 2014; Ali et al., 2014; Varela et al., 2015; de Oliveira et al., 2017; Ramos-Guerrero et al., 2018; Xoca-Orozco et al., 2018); and *Alternaria* spp. (3 studies; total cases, 15) (Yan et al., 2011; Feliziani et al., 2013; López-Mora et al., 2013) (MD, –55.20; 95% CI, –80.50 to –29.90; I^2, 90.0%; P <0.0001), as significant for all of these studies.

3.6 Effects of 1% chitosan on enzyme activities associated with host defence

The overall data for the effects of 1% chitosan on the activities of the enzymes associated with host plant defence at 24 hpt showed significantly increased activity over the control treatment (studies, 27; total cases, 1,332) (MD, 74.58; 95% CI, 41.15 to 108.01; I^2, 99.0%; $P < 0.0001$) (Figure 4). For the details of the subgroup analysis here (Figure 4), in the treated fruit, 1% chitosan did not induce any significant difference compared to the control at 24 hpt for the PAL activity (studies, 9; total cases 575) (Zahid et al., 2012; Landi et al., 2014; Bill et al., 2014; Shao et al., 2015; Waewthongrak et al., 2015; Song et al., 2016; Jongsri, Rojsitthisak, Wangsomboondee, & Seraypheapa, 2017;.Shen & Yang, 2017; Silva et al., 2018) (MD, 37.06; 95% CI, –17.28 to 91.40; I^2, 99.0%; $P = 0.18$). However, 5 of these studies (Landi et al., 2014; Bill et al., 2014; Shao et al., 2015; Waewthongrak et al., 2015; Shen & Yang, 2017) showed significant increases in PAL activity. Furthermore, significant increases were seen overall for chitinase activity (10 studies; total cases, 491) (Hewajuliage, Sultanbawa, Wijeratnam, & Wijesundara, 2009; Feliziani et al., 2013; Bill et al., 2014; Landi et al., 2014; Ali et al., 2014; Shao et al., 2015; Jongsri, et al., 2017;. Shen, & Yang, 2017) (MD, 75.95; 95% CI, 36.18 to 115.73; I^2, 99.0%; $P = 0.0002$), as 8 of these with significance increases (Hewajuliage, et al., 2009; Feliziani et al., 2013; Landi et al., 2014; Bill et al., 2014; Ali et al., 2014; Jongsri, et al., 2017; Shen, & Yang, 2017), and overall for β-1,3-glucanase activity (8 studies; total cases 266) (Hewajuliage, et al., 2009; Wang & Gao, 2013; Landi et al., 2014; Bill et al., 2014; Ali et al., 2014; Shao et al., 2015; Jongsri, et al., 2017;. Shen, & Yang, 2017) (MD, 115.06; 95% CI, 38.24 to 191.88; I^2, 100.0%; $P = 0.003$), as 5 of these with significance increases (Hewajuliage, et al., 2009; Wang & Gao, 2013; Landi et al., 2014; Bill et al., 2014; Ali et al., 2014).

4 DISCUSSION

This study brings together and summarises the results from the literature of the effects of 1% chitosan on postharvest diseases and pathogens, according to disease incidence, *in-vitro* mycelium growth, and induction of host defence responses through monitoring of the most commonly analysed enzymes linked to defence mechanisms. This meta-analysis emphasises the primary role of 1% chitosan against the main diseases and pathogens associated with postharvest decay (Romanazzi et al., 2018; Betchem et al., 2019). These pooled estimates highlighted that 1% chitosan is effective against the main postharvest diseases caused by several fungal pathogens that infect different plant species. Although some of these data show high heterogeneity, they also show low risk of bias and high validity for each study, with no substantial baseline differences seen between the control and treatment groups. Indeed, the funnel plots as a method to assess the potential role of publication bias (Harbord, Egger, & Sterne, 2006) indicate that no bias was detected across the studies included. Therefore, these values of I^2 >90% indicate real differences in these studies.

Our study underlines the transversal effectiveness of chitosan in postharvest disease management. Here, the subgroup analysis of *in-vitro* mycelium growth emphasises that the most powerful growth reduction was for *Penicillium* spp., followed by *Alternaria* spp. and *B. cinerea*, while lower effectiveness was seen against *Colletotrichum* spp..

These data also show that chitosan has differential effects across these fungal species, potentially through the control of fungal development and lytic enzyme activation by chitosan (El Gueddari, Rauchhaus, Moerschbacher & Deising, 2002; Geoghegan & Gurr, 2016; Geoghegan, Steinberg, & Gurr, 2017; Ramos-Guerrero et al., 2018; Ramos-Guerrero, González-Estrada, Romanazzi, Landi, & Gutiérrez-Martínez, 2020). There are direct links between the cell wall and cell membranes, as the synthesis of key cell-wall components (e.g., glucans, chitin) occurs at the plasma membrane, with the associated synthase enzyme complexes (Maddi, & Free, 2010). The chitin is localized in the membrane proximal portion of the cell wall and is incorporated into the wall matrix by being cross-linked to the glucans (Patel & Free, 2019). Previous studies have investigated the role of plasma membrane in the sensitivity of fungi to chitosan showing that the plasma membrane of chitosan-sensitive fungi is more fluid and richer in polyunsaturated free fatty acids than in chitosan-resistant fungi (Palma-Guerrero et al., 2009 and 2010). The authors evidenced that chitosan binds to negatively charged phospholipids. This alter plasma membrane fluidity to inducing the membrane permeabilization, which was greatest in membranes containing elevated content polyunsaturated lipids.

While this meta-analysis highlights the different reactions between the fungal species and chitosan effectiveness, it also underlines the key role of plant species in this complex relation that significantly affects the outcome of chitosan-pathogen interaction.

For this reason, the fungal pathogens can react differently to chitosan in terms of disease incidence and in *in-vitro* tests. Indeed, the meta-analysis summarized studies related to disease incidence, show significantly reducing postharvest disease incidence, although the results linked to singular disease show the highest effectiveness of chitosan against anthracnose, while it is less effective against blue/green mold, Rhizopus rot, gray mold, and particularly Alternaria rot. Therefore, it is not excluded that the involvement of mainly different fruits species on anthracnose incidence, as banana, papaya, dragon, bell pepper, soursop and avocado, not tested for the other diseases, the chitosan, could be elicited a different defence response.

This study also confirms that disease incidence is the result of a combination of the chitosan effects on film-forming, plant defence eliciting, and its antimicrobial properties (Romanazzi et al., 2018). In this context, chitosan can be considered to be a modulator of plant defences (Lopez-Moya, Suarez-Fernandez, & Lopez-Lorca, 2019). Chitosan application to plants fits into the delicate relationship between the host and pathogenic fungi and involves the primary cell-wall defence mechanisms. A link between pathogenicity and the enzymes that synthesise the fungal cell wall has been demonstrated in numerous studies (Arana et al., 2009; Levdansky et al., 2010; Lenardon, Munro, & Gow, 2010; Oliveira-Garcia, & Deising, 2013;

Geoghegan et al., 2017; Patel & Free, 2019), and depolymerisation of the cell walls of plant pathogenic fungi following the infection, evading plant immune recognition, has been reported (Geoghegan et al., 2017). It has been reported that the strategy of some fungal pathogens to evade plant immunity is to convert chitin into chitosan (Lopez-Moya, et al., 2019). Thus, both chitosan and chitin will have key roles in the control of plant immunity.

According to the concepts of systemic acquired resistance (Pieters et al., 1998; Durrant & Dong, 2004) and induced systemic resistance (Heil & Bostock, 2002; Timmermann, González, & Ruz, 2020), chitosan can induce resistance in the plants to control postharvest fungal pathogens of their fruit and as vegetables (Nandeeshkumar et al., 2008; Jia, Meng, Zeng, Wang, & Yin, 2016; Jia, Zeng, Wang, Zhang, & Yin, 2018). On this basis, the meta-analysis data related to the eliciting of the host defence enzymes by chitosan through activation of induced resistance can help us to understand this aspect (Mandal, Kar, Mukherjee, & Acharya, 2013; Walters, Ratsep, & Havis, 2013).

Although a meta-analysis of publicly available data, related to transcriptome investigations of plants defense priming, evidenced a common set of conserved transcriptional changes on plants upon stress conditions, (Baccelli, Benny, Caruso, & Martinelli, 2020), the detailed role of the chitosan in the induction of defence mechanisms has been shown for sweet oranges (Coqueiro et al., 2015) and strawberries (Landi et al., 2017). The most common approaches related to the study of enzyme activities (Wang & Gao, 2013; Ali et al., 2014; Pasquariello et al., 2015; Shao et al., 2015; Adiletta, Zampella, Coletta, & Petriccione, 2019) and the expression of individual genes (Ma et. al., 2013; Landi et al., 2014; Petriccione et al., 2017; Fooladi vanda, Shabani, & Razavizadeh, 2019; Chun & Chandrasekaran, 2019) have been investigated, both of which are associated with reactive oxygen species, specific PR proteins, cell-wall enzymes and secondary metabolites. Usually, these individual studies have shown wide variability associated with host fruit species, application methods and times of treatment.

In the present study, we analysed the most studied of the plant defence enzymes, PAL, which is associated with the phenylpropanoid pathway (Dixon, Lapthorn, & Edwards, 2002; Yadav et al., 2020), and chitinase and β-1,3-glucanase, which are linked to cell-wall hydrolysis (Gupta et al., 2015; Pusztahelyi, 2018), at the main analysis time point of 24 hpt. These data do not show any significant effects of chitosan on PAL activity at 24 hpt, while high increases in the activities of chitinase and β-1,3-glucanase were detected, independent of the host species. These findings are in agreement with the plant immunity mechanisms that indicate that chitinase and β-1,3-glucanase release the glucan oligomers from the chitin of the fungal cell walls to trigger the plant immune responses (Jones & Dang, 2006; Fesel & Zuccaro, 2016; Lopez-Moya et al., 2019;), although the induction of these defence mechanisms can vary greatly according to the time of treatment. The present study suggests that the analysis of the chitinase and β-1,3-glucanase activities at 24 hpt represents a marker for verification of induction of the plant defences by chitosan, while activation of PAL has generally been reported to occur at later times (Landi et al., 2014; Bill et al., 2014).

5 CONCLUSIONS

The present work established the first comprehensive investigation of chitosan effectiveness on postharvest pathogens using meta-analysis approach. This study provides knowledge based on three robust findings, as the effects of 1% chitosan on disease incidence, mycelium growth of decay-causing fungi, and the activities of two important defence enzymes in particular, chitinase and β-1,3-glucanase. This investigation shown the chitosan have antifungal properties against different phytopathogens highlight the versatile properties of this natural biopolymer.

It was demonstrated there are enough data about the effectiveness of chitosan in the control of postharvest diseases, also inducing resistance on fruit to postharvest pathogens.

The outcomes of this study aim to contribute to a better understanding concerning the role of chitosan in the control of postharvest decay of fresh fruit, that will be relevant for the conceptualization and measurement of future studies. Collectively, these data confirm the multiple mechanisms of action of chitosan, which has unique properties in the panorama of activities of natural compounds that define it as a model plant-protection agent for sustainable control of postharvest decay of fruit and vegetables.

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Author Contributions

R.R. performed the literature research, analysed the data, and contributed to write the manuscript; L.L designed the analysis, analysed the data, and wrote the manuscript; G.R. designed the analysis, supervised and complemented the writing, and coordinated the study.

Conflicts of Interest: The authors declare that they have no competing interests.

TABLE 1. Main characteristics of datasets that have included 1% chitosan effects on postharvest fungal pathogens.

First author	**Year**	**Fungal pathogen**	**Chitosan effects measures**			**Defence enzyme**
			Disease incidence (fruit)	***In-vitro* mycelium growth**	**Plant defence mechanism (fruit)**	
Xu	2007	*B. cinerea*		Yes		-
Jitareerat	2007	*Colletotrichum* spp.		Yes		-
Rahman	2008	*Colletotrichum* spp.		Yes		-
Hewajulige	2009	-			Papaya	Chitinase, β-1,3-glucanase
Munoz	2009	*Colletotrichum* spp.		Yes		-
Meng	2010	*Alternaria* spp.	Pear			-
Maqbool	2010	*Colletotrichum* spp	Banana			-
Cia	2010	*Rhizopus* spp.		Yes		-
Yan	2011	*Alternaria* spp.	Jujube	Yes		-
Abdel-Kader	2011	*Penicillium* spp.		Yes		-
Xing	2011	*Penicillium* spp	Jujube			-
Nisia	2012	*Penicillium* spp.		Yes		-
Ramos-Garcia	2012	*Rhizopus* spp.	Tomato			-
Shao	2012	*Penicillium* spp., *B. cinerea*	Apple			-
Cháfer	2012	*Penicillium* spp.	Orange			-
Zahid	2012	*Colletotrichum* spp.	Banana, Papaya, Dragon	Yes		-
Feliziani	2013	*B. cinerea*, *Alternaria* spp., *Penicillium* spp.		Yes	Table grape	Chitinase
Wang	2013	-			Strawberry	β-1,3-Glucanase
Mohamed	2013	*Colletotrichum* spp.		Yes		-
Gao	2013	*B. cinerea*	Table grape			-
López-Mora	2013	*Alternaria* spp.	Mango	Yes		-

Romanazzi	2013	*Penicillium* spp., *B. cinerea*, *Rhizopus* spp.	Strawberry			-
Bill	2014	*Colletotrichum* spp.		Yes	Avocado	PAL, chitinase, β-1,3-glucanase
Ali	2014	*Colletotrichum* spp.		Yes	Dragon	Chitinase, β-1,3-glucanase
Wang	2014	*Penicillium* spp.	Jujube	Yes		-
Lu	2014	*Penicillium* spp.	Orange			-
Landi	2014				Strawberry	PAL, chitinase, β-1,3-glucanase
Edirisinghe	2014	*Colletotrichum* spp.	Bell pepper	Yes		-
Zahid	2015				Dragon	PAL
Feliziani	2015	*B. cinerea*	Strawberry			-
Waewthongrak	2015	*Penicillium* spp.		Yes	Citrus	PAL
Varela	2015	*Colletotrichum* spp.		Yes		-
Shao	2015	*Penicillium* spp.		Yes	Mandarine	PAL, chitinase, β-1,3-glucanase
Xing	2015	*Rhizopus* spp.	Jujube			-
Ali	2015	*Colletotrichum* spp.	Bell pepper	Yes		-
Song	2016				Loquat	PAL
El Guilli	2016	*Penicillium* spp.	Citrus			-
Zheng	2017	*B. cinerea*	Kiwi			-
Gutiérrez-Martinez	2017	*Colletotrichum* spp.	Mango, banana, soursop	Yes		-
Guo	2017	*Alternaria* spp.	Jujube			-
Shen	2017	-			Table grape	PAL, chitinase, β-1,3-glucanase

Jongsri	2017	-			Mango	PAL, chitinase, β-1,3-glucanase
de Oliveria	2017	*Colletotrichum* spp.		Yes		-
Kanetis	2017	*B. cinerea*	Table grape	Yes		-
Munhuweyi	2017	*B. cinerea*		Yes		-
Silva	2018				Guava	PAL
Gramisci	2018	*B. cinerea*, *Penicillium* spp.	Pear			-
Hajji	2018	*B. cinerea*	Strawberry			-
Kharchoufi	2018	*Penicillium* spp.	Orange			-
Flores	2018	*B. cinerea*		Yes		-
Ramos-Guerrero	2018	*Colletotrichum* spp.		Yes		-
Liu	2018	*Penicillium* spp.	Blueberry			-
Shi	2018	*Penicillium* spp.	Grapefruit			-
Xoca-Orozco	2018	*Colletotrichum* spp.		Yes		-
Obianom	2019	*Colletotrichum* spp.	Avocado			-
Madanipour	2019	*Penicillium* spp.		Yes		-

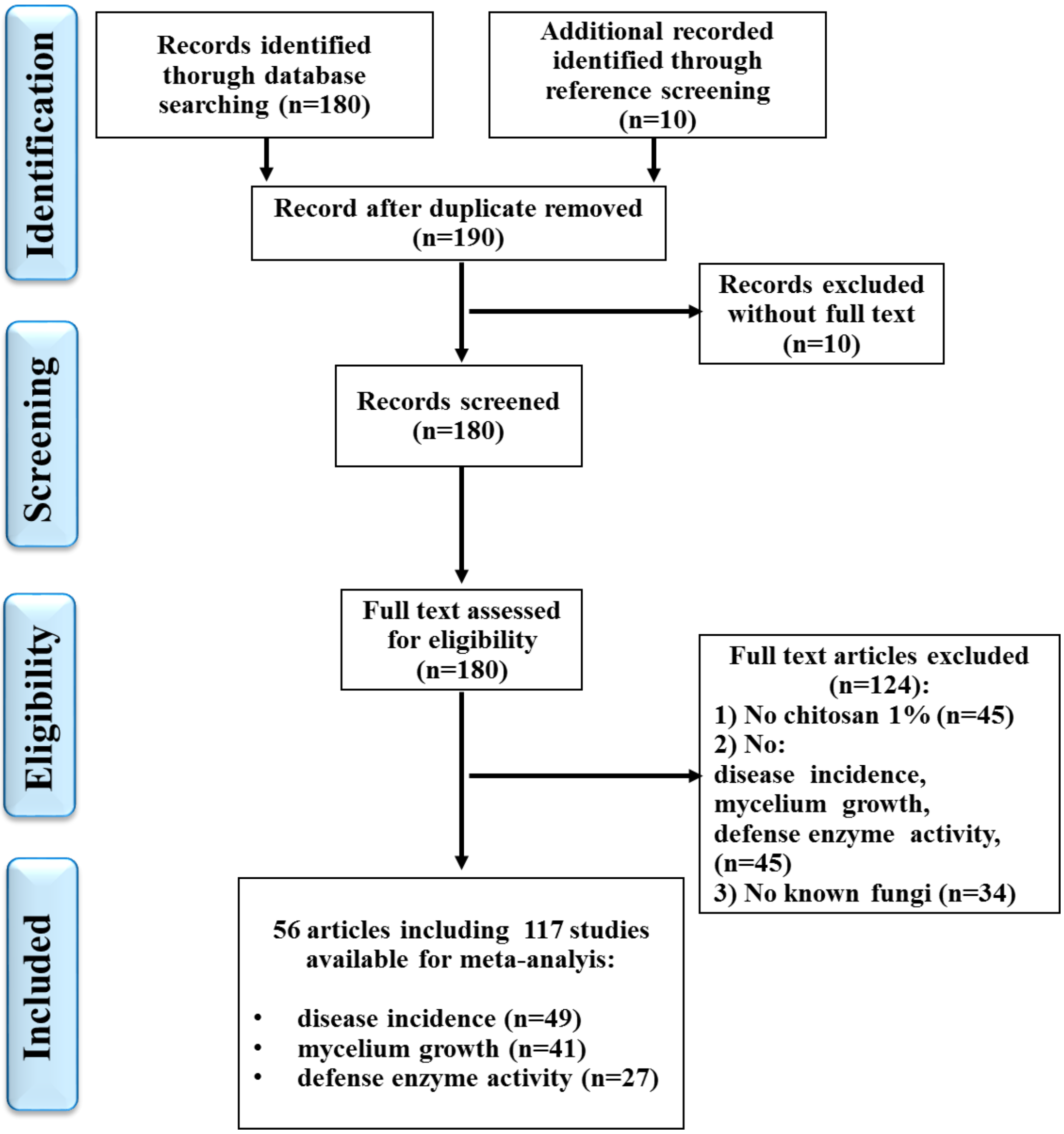
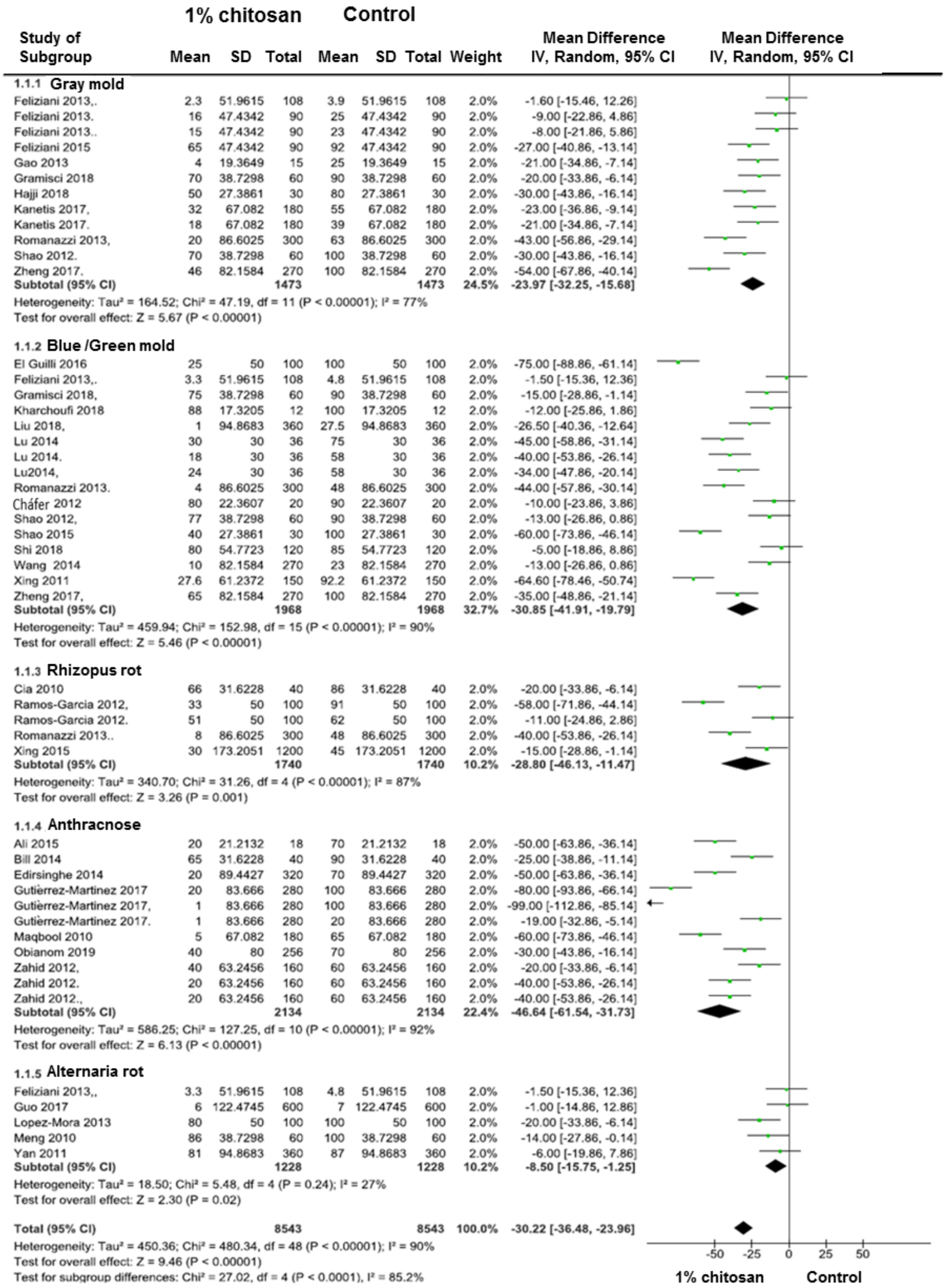
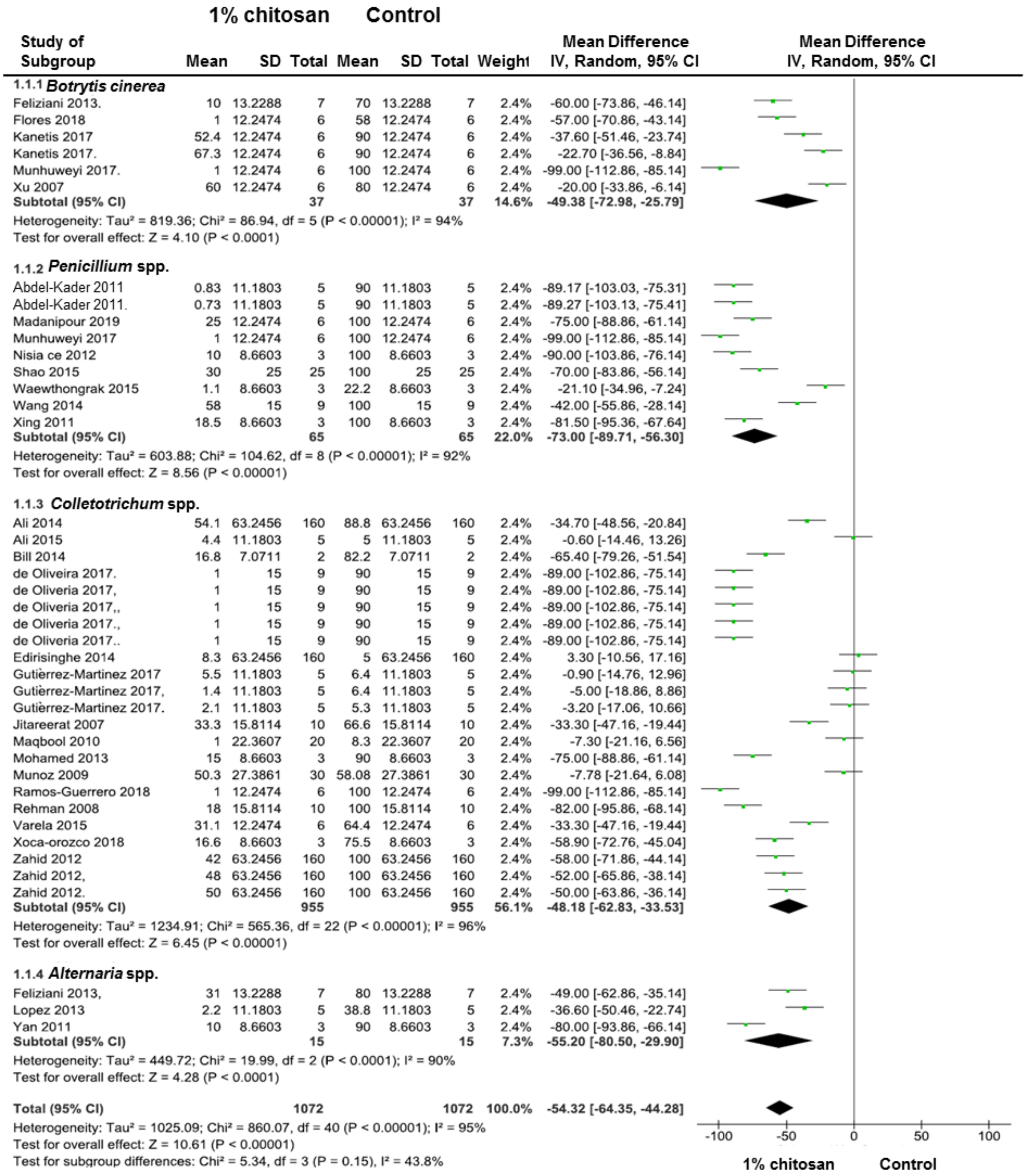


Figure 1. Flow chart exhibiting the selection process of eligible studies.



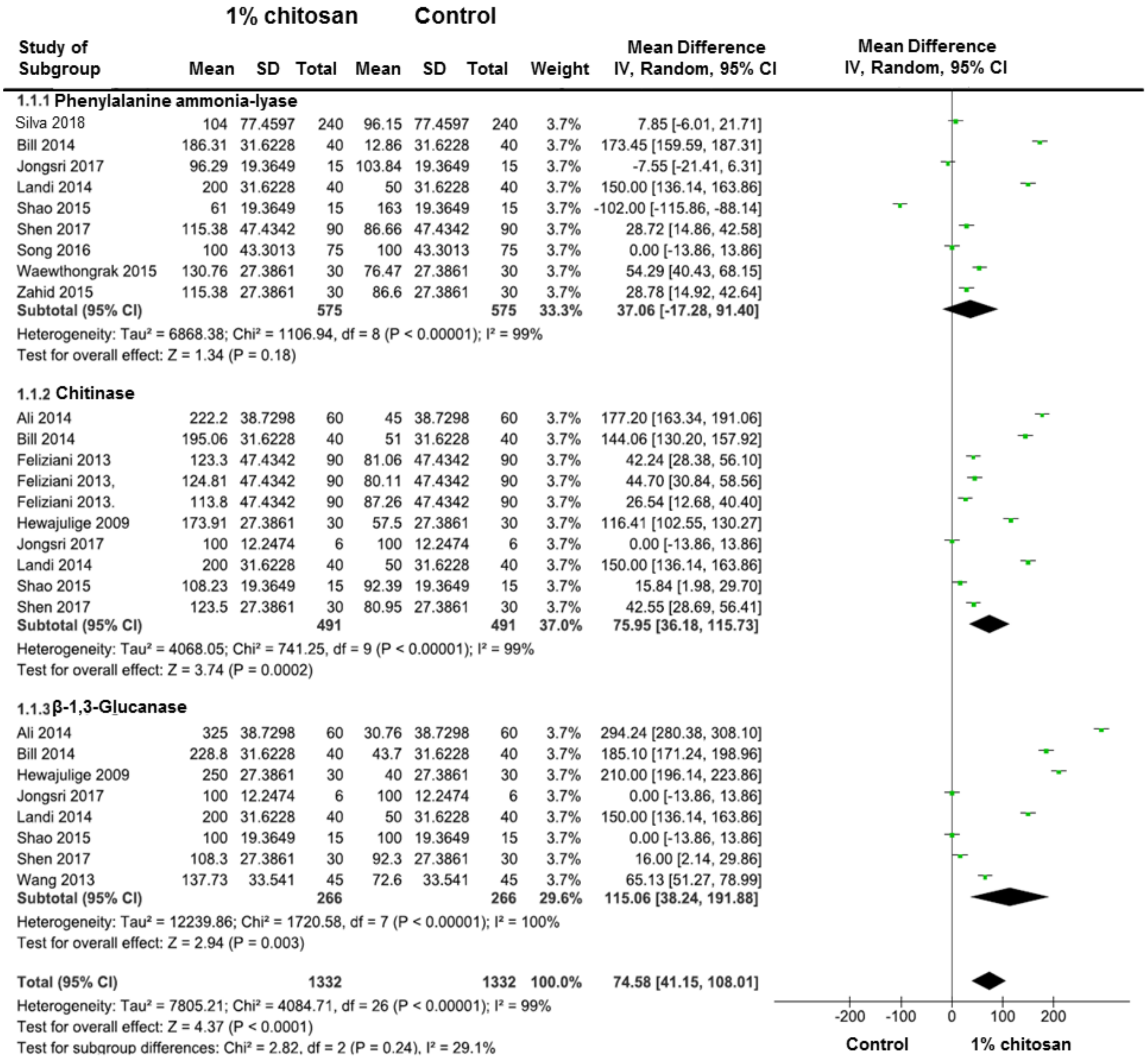
Study or Subgroup	1% chitosan			Control				Mean Difference
	Mean	SD	Total	Mean	SD	Total	Weight	IV, Random, 95% CI
1.1.1 Gray mold								
Feliziani 2013,.	2.3	51.9615	108	3.9	51.9615	108	2.0%	-1.60 [-15.46, 12.26]
Feliziani 2013.	16	47.4342	90	25	47.4342	90	2.0%	-9.00 [-22.86, 4.86]
Feliziani 2013..	15	47.4342	90	23	47.4342	90	2.0%	-8.00 [-21.86, 5.86]
Feliziani 2015	65	47.4342	90	92	47.4342	90	2.0%	-27.00 [-40.86, -13.14]
Gao 2013	4	19.3649	15	25	19.3649	15	2.0%	-21.00 [-34.86, -7.14]
Gramisci 2018	70	38.7298	60	90	38.7298	60	2.0%	-20.00 [-33.86, -6.14]
Hajji 2018	50	27.3861	30	80	27.3861	30	2.0%	-30.00 [-43.86, -16.14]
Kanetis 2017,	32	67.082	180	55	67.082	180	2.0%	-23.00 [-36.86, -9.14]
Kanetis 2017.	18	67.082	180	39	67.082	180	2.0%	-21.00 [-34.86, -7.14]
Romanazzi 2013,	20	86.6025	300	63	86.6025	300	2.0%	-43.00 [-56.86, -29.14]
Shao 2012.	70	38.7298	60	100	38.7298	60	2.0%	-30.00 [-43.86, -16.14]
Zheng 2017.	46	82.1584	270	100	82.1584	270	2.0%	-54.00 [-67.86, -40.14]
Subtotal (95% CI)			**1473**			**1473**	**24.5%**	**-23.97 [-32.25, -15.68]**
Heterogeneity: Tau^2 = 164.52; Chi^2 = 47.19, df = 11 ($P < 0.00001$); I^2 = 77%								
Test for overall effect: Z = 5.67 ($P < 0.00001$)								
1.1.2 Blue /Green mold								
El Guilli 2016	25	50	100	100	50	100	2.0%	-75.00 [-88.86, -61.14]
Feliziani 2013,.	3.3	51.9615	108	4.8	51.9615	108	2.0%	-1.50 [-15.36, 12.36]
Gramisci 2018,	75	38.7298	60	90	38.7298	60	2.0%	-15.00 [-28.86, -1.14]
Kharchoufi 2018	88	17.3205	12	100	17.3205	12	2.0%	-12.00 [-25.86, 1.86]
Liu 2018,	1	94.8683	360	27.5	94.8683	360	2.0%	-26.50 [-40.36, -12.64]
Lu 2014	30	30	36	75	30	36	2.0%	-45.00 [-58.86, -31.14]
Lu 2014.	18	30	36	58	30	36	2.0%	-40.00 [-53.86, -26.14]
Lu2014,	24	30	36	58	30	36	2.0%	-34.00 [-47.86, -20.14]
Romanazzi 2013.	4	86.6025	300	48	86.6025	300	2.0%	-44.00 [-57.86, -30.14]
Cháfer 2012	80	22.3607	20	90	22.3607	20	2.0%	-10.00 [-23.86, 3.86]
Shao 2012,	77	38.7298	60	90	38.7298	60	2.0%	-13.00 [-26.86, 0.86]
Shao 2015	40	27.3861	30	100	27.3861	30	2.0%	-60.00 [-73.86, -46.14]
Shi 2018	80	54.7723	120	85	54.7723	120	2.0%	-5.00 [-18.86, 8.86]
Wang 2014	10	82.1584	270	23	82.1584	270	2.0%	-13.00 [-26.86, 0.86]
Xing 2011	27.6	61.2372	150	92.2	61.2372	150	2.0%	-64.60 [-78.46, -50.74]
Zheng 2017,	65	82.1584	270	100	82.1584	270	2.0%	-35.00 [-48.86, -21.14]
Subtotal (95% CI)			**1968**			**1968**	**32.7%**	**-30.85 [-41.91, -19.79]**
Heterogeneity: Tau^2 = 459.94; Chi^2 = 152.98, df = 15 ($P < 0.00001$); I^2 = 90%								
Test for overall effect: Z = 5.46 ($P < 0.00001$)								
1.1.3 Rhizopus rot								
Cia 2010	66	31.6228	40	86	31.6228	40	2.0%	-20.00 [-33.86, -6.14]
Ramos-Garcia 2012,	33	50	100	91	50	100	2.0%	-58.00 [-71.86, -44.14]
Ramos-Garcia 2012.	51	50	100	62	50	100	2.0%	-11.00 [-24.86, 2.86]
Romanazzi 2013..	8	86.6025	300	48	86.6025	300	2.0%	-40.00 [-53.86, -26.14]
Xing 2015	30	173.2051	1200	45	173.2051	1200	2.0%	-15.00 [-28.86, -1.14]
Subtotal (95% CI)			**1740**			**1740**	**10.2%**	**-28.80 [-46.13, -11.47]**
Heterogeneity: Tau^2 = 340.70; Chi^2 = 31.26, df = 4 ($P < 0.00001$); I^2 = 87%								
Test for overall effect: Z = 3.26 ($P = 0.001$)								
1.1.4 Anthracnose								
Ali 2015	20	21.2132	18	70	21.2132	18	2.0%	-50.00 [-63.86, -36.14]
Bill 2014	65	31.6228	40	90	31.6228	40	2.0%	-25.00 [-38.86, -11.14]
Edirsinghe 2014	20	89.4427	320	70	89.4427	320	2.0%	-50.00 [-63.86, -36.14]
Gutièrrez-Martinez 2017	20	83.666	280	100	83.666	280	2.0%	-80.00 [-93.86, -66.14]
Gutièrrez-Martinez 2017,	1	83.666	280	100	83.666	280	2.0%	-99.00 [-112.86, -85.14]
Gutièrrez-Martinez 2017.	1	83.666	280	20	83.666	280	2.0%	-19.00 [-32.86, -5.14]
Maqbool 2010	5	67.082	180	65	67.082	180	2.0%	-60.00 [-73.86, -46.14]
Obianom 2019	40	80	256	70	80	256	2.0%	-30.00 [-43.86, -16.14]
Zahid 2012,	40	63.2456	160	60	63.2456	160	2.0%	-20.00 [-33.86, -6.14]
Zahid 2012.	20	63.2456	160	60	63.2456	160	2.0%	-40.00 [-53.86, -26.14]
Zahid 2012.,	20	63.2456	160	60	63.2456	160	2.0%	-40.00 [-53.86, -26.14]
Subtotal (95% CI)			**2134**			**2134**	**22.4%**	**-46.64 [-61.54, -31.73]**
Heterogeneity: Tau^2 = 586.25; Chi^2 = 127.25, df = 10 ($P < 0.00001$); I^2 = 92%								
Test for overall effect: Z = 6.13 ($P < 0.00001$)								
1.1.5 Alternaria rot								
Feliziani 2013,,	3.3	51.9615	108	4.8	51.9615	108	2.0%	-1.50 [-15.36, 12.36]
Guo 2017	6	122.4745	600	7	122.4745	600	2.0%	-1.00 [-14.86, 12.86]
Lopez-Mora 2013	80	50	100	100	50	100	2.0%	-20.00 [-33.86, -6.14]
Meng 2010	86	38.7298	60	100	38.7298	60	2.0%	-14.00 [-27.86, -0.14]
Yan 2011	81	94.8683	360	87	94.8683	360	2.0%	-6.00 [-19.86, 7.86]
Subtotal (95% CI)			**1228**			**1228**	**10.2%**	**-8.50 [-15.75, -1.25]**
Heterogeneity: Tau^2 = 18.50; Chi^2 = 5.48, df = 4 ($P = 0.24$); I^2 = 27%								
Test for overall effect: Z = 2.30 ($P = 0.02$)								
Total (95% CI)			**8543**			**8543**	**100.0%**	**-30.22 [-36.48, -23.96]**
Heterogeneity: Tau^2 = 450.36; Chi^2 = 480.34, df = 48 ($P < 0.00001$); I^2 = 90%								
Test for overall effect: Z = 9.46 ($P < 0.00001$)								
Test for subgroup differences: Chi^2 = 27.02, df = 4 ($P < 0.0001$), I^2 = 85.2%								

Figure 2. Forest plots using the RavMan 5.3 software for random effects analysis related to the effectiveness of 1% chitosan on disease incidence. Gray mold, blue/ green mold, *Rhizopus* rot., anthracnose and *Alternaria* rot were considered as subgroups. For Feliziani 2013, Kanetis 2017, Lu 2014, Shao 2012, Ramos-Garcia 2012, Gutièrrez-Martinez 2017 and Zahid 2012, several studies were included from each article into the subgroups. IV, inverse variance; CI, confidence interval.



	1% chitosan			Control				
Study of Subgroup	Mean	SD	Total	Mean	SD	Total	Weight	Mean Difference IV, Random, 95% CI
1.1.1 *Botrytis cinerea*								
Feliziani 2013.	10	13.2288	7	70	13.2288	7	2.4%	-60.00 [-73.86, -46.14]
Flores 2018	1	12.2474	6	58	12.2474	6	2.4%	-57.00 [-70.86, -43.14]
Kanetis 2017	52.4	12.2474	6	90	12.2474	6	2.4%	-37.60 [-51.46, -23.74]
Kanetis 2017.	67.3	12.2474	6	90	12.2474	6	2.4%	-22.70 [-36.56, -8.84]
Munhuweyi 2017.	1	12.2474	6	100	12.2474	6	2.4%	-99.00 [-112.86, -85.14]
Xu 2007	60	12.2474	6	80	12.2474	6	2.4%	-20.00 [-33.86, -6.14]
Subtotal (95% CI)			**37**			**37**	**14.6%**	**-49.38 [-72.98, -25.79]**
Heterogeneity: Tau² = 819.36; Chi² = 86.94, df = 5 (P < 0.00001); I² = 94%								
Test for overall effect: Z = 4.10 (P < 0.0001)								
1.1.2 *Penicillium* spp.								
Abdel-Kader 2011	0.83	11.1803	5	90	11.1803	5	2.4%	-89.17 [-103.03, -75.31]
Abdel-Kader 2011.	0.73	11.1803	5	90	11.1803	5	2.4%	-89.27 [-103.13, -75.41]
Madanipour 2019	25	12.2474	6	100	12.2474	6	2.4%	-75.00 [-88.86, -61.14]
Munhuweyi 2017	1	12.2474	6	100	12.2474	6	2.4%	-99.00 [-112.86, -85.14]
Nisia ce 2012	10	8.6603	3	100	8.6603	3	2.4%	-90.00 [-103.86, -76.14]
Shao 2015	30	25	25	100	25	25	2.4%	-70.00 [-83.86, -56.14]
Waewthongrak 2015	1.1	8.6603	3	22.2	8.6603	3	2.4%	-21.10 [-34.96, -7.24]
Wang 2014	58	15	9	100	15	9	2.4%	-42.00 [-55.86, -28.14]
Xing 2011	18.5	8.6603	3	100	8.6603	3	2.4%	-81.50 [-95.36, -67.64]
Subtotal (95% CI)			**65**			**65**	**22.0%**	**-73.00 [-89.71, -56.30]**
Heterogeneity: Tau² = 603.88; Chi² = 104.62, df = 8 (P < 0.00001); I² = 92%								
Test for overall effect: Z = 8.56 (P < 0.00001)								
1.1.3 *Colletotrichum* spp.								
Ali 2014	54.1	63.2456	160	88.8	63.2456	160	2.4%	-34.70 [-48.56, -20.84]
Ali 2015	4.4	11.1803	5	5	11.1803	5	2.4%	-0.60 [-14.46, 13.26]
Bill 2014	16.8	7.0711	2	82.2	7.0711	2	2.4%	-65.40 [-79.26, -51.54]
de Oliveira 2017.	1	15	9	90	15	9	2.4%	-89.00 [-102.86, -75.14]
de Oliveria 2017,	1	15	9	90	15	9	2.4%	-89.00 [-102.86, -75.14]
de Oliveria 2017,,	1	15	9	90	15	9	2.4%	-89.00 [-102.86, -75.14]
de Oliveria 2017.,	1	15	9	90	15	9	2.4%	-89.00 [-102.86, -75.14]
de Oliveria 2017..	1	15	9	90	15	9	2.4%	-89.00 [-102.86, -75.14]
Edirisinghe 2014	8.3	63.2456	160	5	63.2456	160	2.4%	3.30 [-10.56, 17.16]
Gutièrrez-Martinez 2017	5.5	11.1803	5	6.4	11.1803	5	2.4%	-0.90 [-14.76, 12.96]
Gutièrrez-Martinez 2017,	1.4	11.1803	5	6.4	11.1803	5	2.4%	-5.00 [-18.86, 8.86]
Gutièrrez-Martinez 2017.	2.1	11.1803	5	5.3	11.1803	5	2.4%	-3.20 [-17.06, 10.66]
Jitareerat 2007	33.3	15.8114	10	66.6	15.8114	10	2.4%	-33.30 [-47.16, -19.44]
Maqbool 2010	1	22.3607	20	8.3	22.3607	20	2.4%	-7.30 [-21.16, 6.56]
Mohamed 2013	15	8.6603	3	90	8.6603	3	2.4%	-75.00 [-88.86, -61.14]
Munoz 2009	50.3	27.3861	30	58.08	27.3861	30	2.4%	-7.78 [-21.64, 6.08]
Ramos-Guerrero 2018	1	12.2474	6	100	12.2474	6	2.4%	-99.00 [-112.86, -85.14]
Rehman 2008	18	15.8114	10	100	15.8114	10	2.4%	-82.00 [-95.86, -68.14]
Varela 2015	31.1	12.2474	6	64.4	12.2474	6	2.4%	-33.30 [-47.16, -19.44]
Xoca-orozco 2018	16.6	8.6603	3	75.5	8.6603	3	2.4%	-58.90 [-72.76, -45.04]
Zahid 2012	42	63.2456	160	100	63.2456	160	2.4%	-58.00 [-71.86, -44.14]
Zahid 2012,	48	63.2456	160	100	63.2456	160	2.4%	-52.00 [-65.86, -38.14]
Zahid 2012.	50	63.2456	160	100	63.2456	160	2.4%	-50.00 [-63.86, -36.14]
Subtotal (95% CI)			**955**			**955**	**56.1%**	**-48.18 [-62.83, -33.53]**
Heterogeneity: Tau² = 1234.91; Chi² = 565.36, df = 22 (P < 0.00001); I² = 96%								
Test for overall effect: Z = 6.45 (P < 0.00001)								
1.1.4 *Alternaria* spp.								
Feliziani 2013,	31	13.2288	7	80	13.2288	7	2.4%	-49.00 [-62.86, -35.14]
Lopez 2013	2.2	11.1803	5	38.8	11.1803	5	2.4%	-36.60 [-50.46, -22.74]
Yan 2011	10	8.6603	3	90	8.6603	3	2.4%	-80.00 [-93.86, -66.14]
Subtotal (95% CI)			**15**			**15**	**7.3%**	**-55.20 [-80.50, -29.90]**
Heterogeneity: Tau² = 449.72; Chi² = 19.99, df = 2 (P < 0.0001); I² = 90%								
Test for overall effect: Z = 4.28 (P < 0.0001)								
Total (95% CI)			**1072**			**1072**	**100.0%**	**-54.32 [-64.35, -44.28]**
Heterogeneity: Tau² = 1025.09; Chi² = 860.07, df = 40 (P < 0.00001); I² = 95%								
Test for overall effect: Z = 10.61 (P < 0.00001)								
Test for subgroup differences: Chi² = 5.34, df = 3 (P = 0.15), I² = 43.8%								

Figure 3. Forest plot using the RavMan 5.3 software for random effects analysis related to the effectiveness of 1% chitosan on *in-vitro* mycelium growth. *Botrytis cinerea*, *Penicillium* spp., *Colletotrichum* spp. and *Alternaria* spp. were considered as subgroups. For Kanetis 2017, Kader 2011, de Oliveria 2017, Gutièrrez-Martinez 2017 and Zahid 2012, several studies were included from each article into the subgroups. IV, inverse variance; CI, confidence interval.



Study of Subgroup	1% chitosan Mean	1% chitosan SD	1% chitosan Total	Control Mean	Control SD	Control Total	Weight	Mean Difference IV, Random, 95% CI
1.1.1 Phenylalanine ammonia-lyase								
Silva 2018	104	77.4597	240	96.15	77.4597	240	3.7%	7.85 [-6.01, 21.71]
Bill 2014	186.31	31.6228	40	12.86	31.6228	40	3.7%	173.45 [159.59, 187.31]
Jongsri 2017	96.29	19.3649	15	103.84	19.3649	15	3.7%	-7.55 [-21.41, 6.31]
Landi 2014	200	31.6228	40	50	31.6228	40	3.7%	150.00 [136.14, 163.86]
Shao 2015	61	19.3649	15	163	19.3649	15	3.7%	-102.00 [-115.86, -88.14]
Shen 2017	115.38	47.4342	90	86.66	47.4342	90	3.7%	28.72 [14.86, 42.58]
Song 2016	100	43.3013	75	100	43.3013	75	3.7%	0.00 [-13.86, 13.86]
Waewthongrak 2015	130.76	27.3861	30	76.47	27.3861	30	3.7%	54.29 [40.43, 68.15]
Zahid 2015	115.38	27.3861	30	86.6	27.3861	30	3.7%	28.78 [14.92, 42.64]
Subtotal (95% CI)			**575**			**575**	**33.3%**	**37.06 [-17.28, 91.40]**
Heterogeneity: Tau² = 6868.38; Chi² = 1106.94, df = 8 (P < 0.00001); I² = 99%								
Test for overall effect: Z = 1.34 (P = 0.18)								
1.1.2 Chitinase								
Ali 2014	222.2	38.7298	60	45	38.7298	60	3.7%	177.20 [163.34, 191.06]
Bill 2014	195.06	31.6228	40	51	31.6228	40	3.7%	144.06 [130.20, 157.92]
Feliziani 2013	123.3	47.4342	90	81.06	47.4342	90	3.7%	42.24 [28.38, 56.10]
Feliziani 2013,	124.81	47.4342	90	80.11	47.4342	90	3.7%	44.70 [30.84, 58.56]
Feliziani 2013.	113.8	47.4342	90	87.26	47.4342	90	3.7%	26.54 [12.68, 40.40]
Hewajulige 2009	173.91	27.3861	30	57.5	27.3861	30	3.7%	116.41 [102.55, 130.27]
Jongsri 2017	100	12.2474	6	100	12.2474	6	3.7%	0.00 [-13.86, 13.86]
Landi 2014	200	31.6228	40	50	31.6228	40	3.7%	150.00 [136.14, 163.86]
Shao 2015	108.23	19.3649	15	92.39	19.3649	15	3.7%	15.84 [1.98, 29.70]
Shen 2017	123.5	27.3861	30	80.95	27.3861	30	3.7%	42.55 [28.69, 56.41]
Subtotal (95% CI)			**491**			**491**	**37.0%**	**75.95 [36.18, 115.73]**
Heterogeneity: Tau² = 4068.05; Chi² = 741.25, df = 9 (P < 0.00001); I² = 99%								
Test for overall effect: Z = 3.74 (P = 0.0002)								
1.1.3 β-1,3-Glucanase								
Ali 2014	325	38.7298	60	30.76	38.7298	60	3.7%	294.24 [280.38, 308.10]
Bill 2014	228.8	31.6228	40	43.7	31.6228	40	3.7%	185.10 [171.24, 198.96]
Hewajulige 2009	250	27.3861	30	40	27.3861	30	3.7%	210.00 [196.14, 223.86]
Jongsri 2017	100	12.2474	6	100	12.2474	6	3.7%	0.00 [-13.86, 13.86]
Landi 2014	200	31.6228	40	50	31.6228	40	3.7%	150.00 [136.14, 163.86]
Shao 2015	100	19.3649	15	100	19.3649	15	3.7%	0.00 [-13.86, 13.86]
Shen 2017	108.3	27.3861	30	92.3	27.3861	30	3.7%	16.00 [2.14, 29.86]
Wang 2013	137.73	33.541	45	72.6	33.541	45	3.7%	65.13 [51.27, 78.99]
Subtotal (95% CI)			**266**			**266**	**29.6%**	**115.06 [38.24, 191.88]**
Heterogeneity: Tau² = 12239.86; Chi² = 1720.58, df = 7 (P < 0.00001); I² = 100%								
Test for overall effect: Z = 2.94 (P = 0.003)								
Total (95% CI)			**1332**			**1332**	**100.0%**	**74.58 [41.15, 108.01]**
Heterogeneity: Tau² = 7805.21; Chi² = 4084.71, df = 26 (P < 0.00001); I² = 99%								
Test for overall effect: Z = 4.37 (P < 0.0001)								
Test for subgroup differences: Chi² = 2.82, df = 2 (P = 0.24), I² = 29.1%								

Figure 4. Forest plots using the RavMan 5.3 software for random effects analysis related to the effectiveness of 1% chitosan on plant defence mechanism enzyme activities. Phenylalanine ammonia-lyase (PAL), chitinase and β-1,3-glucanase were considerd as subgroups. For Feliziani 2013 several studies were included from each article into the subgroups. IV, inverse variance; CI, confidence interval.

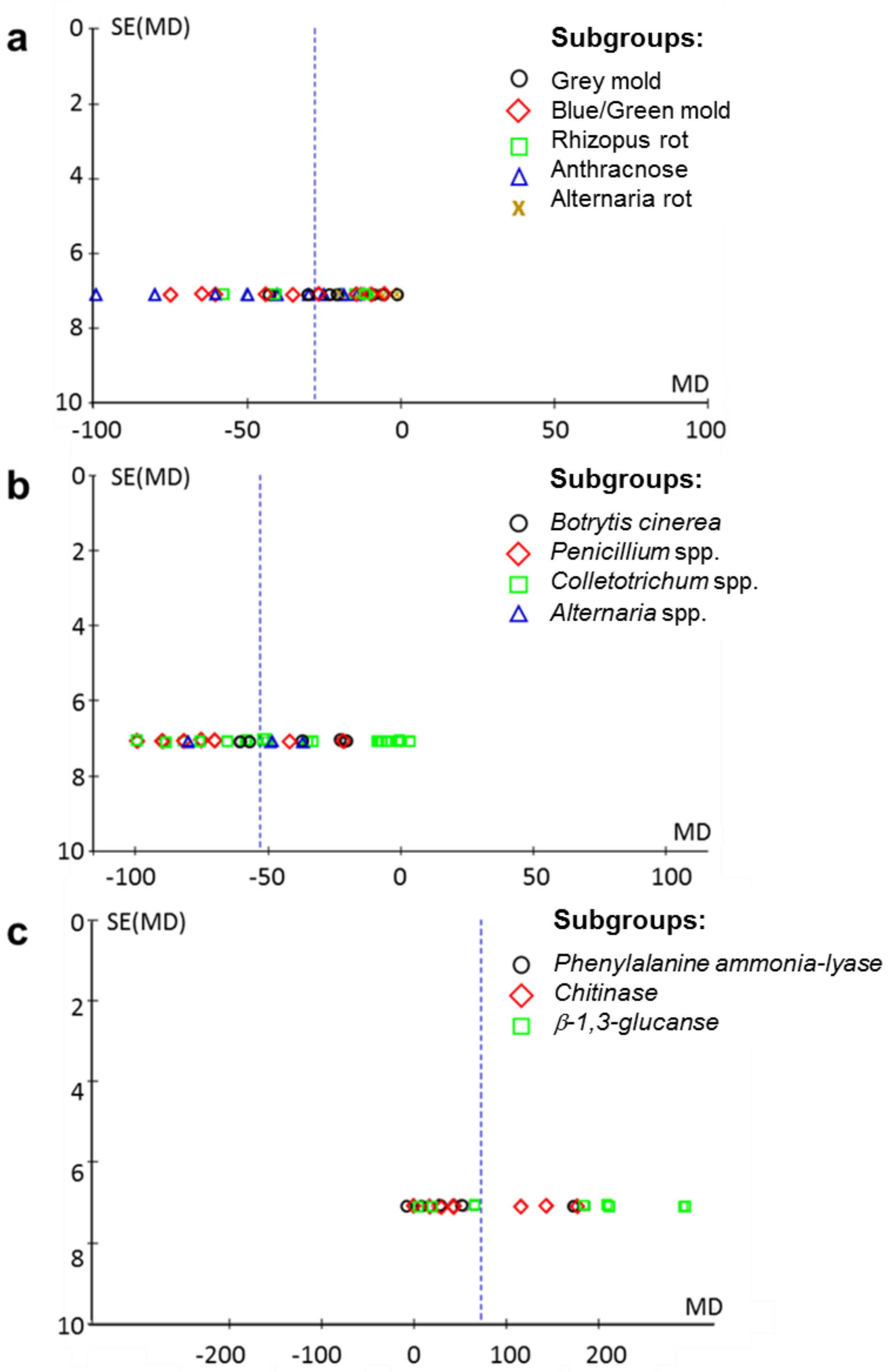


Figure 5. Funnel plots for the detect of publication bias in the studies, for the disease incidence **(a)**, mycelium growth **(b)** and defence enzyme activity **(c)** detected after 1% chitosan treatments, compared to the controls. SE(MD) = standard error (mean difference); MD = mean difference.