Evaluation of the Antagonistic Effect and Influencing Factors of *Bacillus subtilis* against Wood Stain Fungi: A Systematic Literature Review and Meta-analysis Approach

Congxun Huang, Lihai Wang,* Guoqi Xu,* Pengwei Zhao, and Chuhong Wang

Wood sapstain is a serious problem caused by the wood stain fungus, and it has a great influence on the international wood industry. The utilization of biological methods has good prospects for wood conservation. The objective of this study was to systematically estimate the antagonistic effect and influencing factors of *Bacillus subtilis* against wood stain fungus by using meta-analysis of literature data. Through report retrieval, a total of 992 references on *B. subtilis* related to wood were obtained. After strict screening, 163 data items from 7 articles were integrated. Estimated by the random-effects model, the combined effect Odds Ratio of the overall antagonistic effect was 0.15 (95% confidence interval [0.06, 0.34]). The results showed that *B. subtilis* could produce significant antagonistic effects against wood stain fungi. The inhibitory effect of wood stain fungi was affected by the strains of *B. subtilis*, species of wood stain fungi, the *B. subtilis* dosage, the type of mixed reagent, and the amount of mixed reagent on different wood stain fungi. The results of this study may provide a reference for biological control experiments, field tests, and practical applications of wood conservation.

Keywords: Bacillus subtilis; Wood stain fungus; Biological control; Meta-analysis

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INTRODUCTION

Serious attack by sapstain fungi has caused great economic loss to the global wood industry (Schmidt 2007; Velmurugan *et al*. 2009). The process of preventing wood sapstain begins with the development of physical control, chemical control, and low-toxicity environmental protection by chemical reagents. Recently, biological control for non-toxicity environmental protection has become a key development direction in the field of wood protection, and there is more attention to health and environmental protection (Jin and Shi 2004; Sun *et al*. 2009; Susi *et al*. 2011; Teng *et al*. 2018; Zhao *et al*. 2019). *Bacillus subtilis*, a member of the genus *Bacillus*, has been a concern of both domestic and foreign scholars and has been developed into a series of products for various purposes because of its high temperature resistance, acid and alkali resistance, strong stress resistance, and ability to produce a variety of secondary metabolites (Feio *et al*. 2004; Adandonon *et al*. 2006; Harish *et al*. 2008; Ongena and Jacques 2008; Alfonzo *et al*. 2009; Castillo-Reyes *et al*. 2015; Du *et al*. 2020). In addition, it has strong antibacterial and antifungal abilities (Melent'ev *et al*. 2006; Sajitha and Dev 2016; Sa *et al*. 2018). It has the advantage of being able to serve in the role of a biocide.

Many studies have reported that *B. subtilis* has a certain antagonistic effect against wood stain fungi (Zhang *et al*. 2014; Sajitha *et al*. 2018). Stein (2005) mentions that *B. subtilis* has an average 4 to 5% genomes used for antibiotic synthesis and may produce more than 20 antibacterial compounds with different structures. Feio *et al*. (2004) screened *B. subtilis* 355 and indicated that there are at least two active compounds against the blue-stain fungus *Cladosporium cucumerinum* (Moita *et al*. 2005). In the research by Melent'ev *et al*. (2006), the antagonistic effect of *B. subtilis* on wood stain fungus is reported and the possible antagonistic mechanism between the metabolite lipid peptide of *B. subtilis* and other fungi, such as wood stain fungus, is discussed. Velmurugan *et al*. (2009) screened from *B. subtilis* and *Bacillus licheniformis* the antifungal lipopeptide with high activity to *Ophiostoma flexuosum*, *Ophiostoma tetropii*, *Ophiostoma polonicum*, and *Ophiostoma ips* in wood stain fungus. The effects of different bacteria and fungi in the microenvironment are complex, and individual studies inevitably have certain limitations (Johnston *et al*. 2016). It is necessary to systematically estimate and analyze the overall antagonistic effect and influencing factors of *B. subtilis*, which is of great significance to further improve the efficiency and stability of bacterial antagonism against wood stain fungus.

Meta-analysis refers to the statistical analysis of a large collection of results from individual studies, such as experimental studies, opinion surveys, and random models, with the purpose of integrating these research results (Glass 1976). In addition, it is a valuable method to aggregate different experimental data sets that are inadequate or unconvincing (Verstraete 2002). Meta-analysis can overcome the limitations of fuzzy bibliographic retrieval strategies, low literature recall rate, and subjectivity of research conclusions in traditional review literature retrieval and provide reference suggestions for practical wood conservation issues and research directions in the future. As the main content and research method of evidence-based medicine, meta-analysis has been applied in many fields. In this study, the antagonistic effect of *B. subtilis* against wood stain fungi and its main influencing factors were comprehensively analyzed by meta-analysis to estimate whether *B. subtilis* can be further applied.

This study considers the average antagonism effect of *B. subtilis* against wood stain fungi and answers the following questions: (i) whether different *B. subtilis* for wood stain fungi have different ability of antagonism, (ii) whether the species of *B. subtilis* will affect its antagonism effect, and (iii) whether *B. subtilis* can achieve consistent inhibitory effect on different wood stain fungus and other reagent mixes with *B. subtilis* inhibitory effects.

EXPERIMENTAL

Reports and Researches Date Collection

In this study, the convention of Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) was followed to collect relevant research data for meta-analysis (Moher *et al*. 2009). One English-language database (Web of Science) and three Chinese-language databases (CNKI, Wanfang Database, and CQVIP) were selected for relevant scientific reports. The following search strategy was applied for collecting potentially relevant publications from English-language database: (wood) AND (*B. subtilis*). The general format for retrieving three Chinese-language databases was: (wood) AND (*B. subtilis*); all terms were used in the Chinese language. The start and end times are from building the database to August 2, 2020. Then, the research data were screened by the following criteria: (1) The review literature was excluded because it might lead to

duplicate data; (2) a control group of *B. subtilis* is required; (3) irrelevant research literatures are not included; and (4) can only keep one if there are duplicate literature items including data duplication. The collection was performed by using EndNote Citation Management Software (Clarivate Analytics, X8, Philadelphia, USA)

Data Extraction

In this study, the final selected literature items were included in the meta-analysis, and the following data information was extracted: the species of *B. subtilis* and stain fungus, the number of colonies, types of mixed reagent, the volume ratio of the mixture to *B. subtilis*, and the data sets from the treatment group with and without *B. subtilis*. The data were extracted and edited independently by two of the authors (C-X. Huang, C-H. Wang) and listed in Microsoft Excel (Microsoft Corp., version16.44, Redmond, WA, USA) for further analysis. The unit of colony quantity was cfu/mL. The extracted data was further divided into five subgroups: species of stain fungus, *B. subtilis* species, *B. subtilis* dosage, mixed reagent type, and mixed reagent on different wood stain fungi. Data from tables and articles in the report were extracted directly, while images were extracted using Getdata Graph digitizer (http://getdata-graph-digitizer.com, version 2.24, by S. Fedorov, Russia).

Meta-analysis

The sampling method, experimental environment, and methodologies varied from study to study, resulting in different findings from each study (Gonzales-Barron and Butler 2011; Gonzales-Barron *et al*. 2013). When the results of different studies were different, there was heterogeneity. Describing the heterogeneity between different studies is the key to meta-analysis (Higgins *et al*. 2003; Liu *et al*. 2020). Due to the diversity of biological systems and different research schemes, a fixed effects model may not be suitable, so a random effects model was selected for this study (Gonzales-Barron and Butler 2011; DerSimonian and Laird 2015). Inspection and metrology systems can choose a Q or I^2 test, but an I^2 test is better for measuring multiple results between the heterogeneous degrees of size (Higgins and Thompson 2002; Gonzales-Barron and Butler 2011). The counting data was presented by the Odds Ratio (OR: the ratio of the exposed to the unexposed in the case group divided by the ratio of the exposed to the unexposed in the control group) and a 95% confidence interval (CI), while measurement data was presented by a mean difference (MD) or standardized mean difference (SMD) and 95% CI. Subgroup analysis was performed according to the difference of the antagonistic test of *B. subtilis* against wood stain fungus in the research data. All items were included in the results of the study of heterogeneity using the I^2 test. If $I^2 = 0$ (if negative, it is still set to 0), it showed no heterogeneity. If I^2 is larger, the heterogeneity is greater. The I^2 value (the percentage was 25%, 50%, and 75%) represented low, medium, and high heterogeneity (Martinez-Rios and Dalgaard 2018). When there was statistically significant homogeneity ($P > 0.1$, $I^2 < 50\%$) among the results, a fixed effect model was used for meta-analysis. If there was statistical significance of heterogeneity among the study results ($P < 0.1$, $I^2 > 50\%$), the source of heterogeneity was analyzed. When there was heterogeneity between the two study groups without clinical heterogeneity or the difference was not statistically significant, a random-effects model was used for meta-analysis. If the heterogeneity between results was too large, descriptive analysis was used. If a sufficient of literature items were available, inverted funnel plot analysis was performed to test for the presence of publication bias. Additionally, a forest plot or funnel plot was constructed according to

the analysis results. The meta-analysis was performed by using RevMan software (The Cochrane Collaboration, version5.0.2, London, UK)

Sensitivity Analysis

Sensitivity analysis is an analysis method used to estimate the stability and reliability of a certain meta-analysis. During the process of sensitivity analysis, if there was no essential change in the results of meta-analysis, the analysis results were highly reliable. If it led to different conclusions, it meant that caution should be taken in the interpretations and conclusions of meta-analysis results. In this research, Revman 5.0.2 (The Cochrane Collaboration, version 5.0.2, London, UK) was used to analyze the sensitivity of the included literature. The included literature items were removed in the Revman software to observe the heterogeneity changes. If the I^2 and P values changed greatly after excluding literature, the literature items were likely to be the main source of heterogeneity. The literature should be read again; if the I^2 and P values did not change much, the documents will not be ruled out easily. The sources of heterogeneity should be further analyzed.

RESULTS

Collection of Literatures and Data

The detailed flowchart of the literature search is shown in Fig. 1.

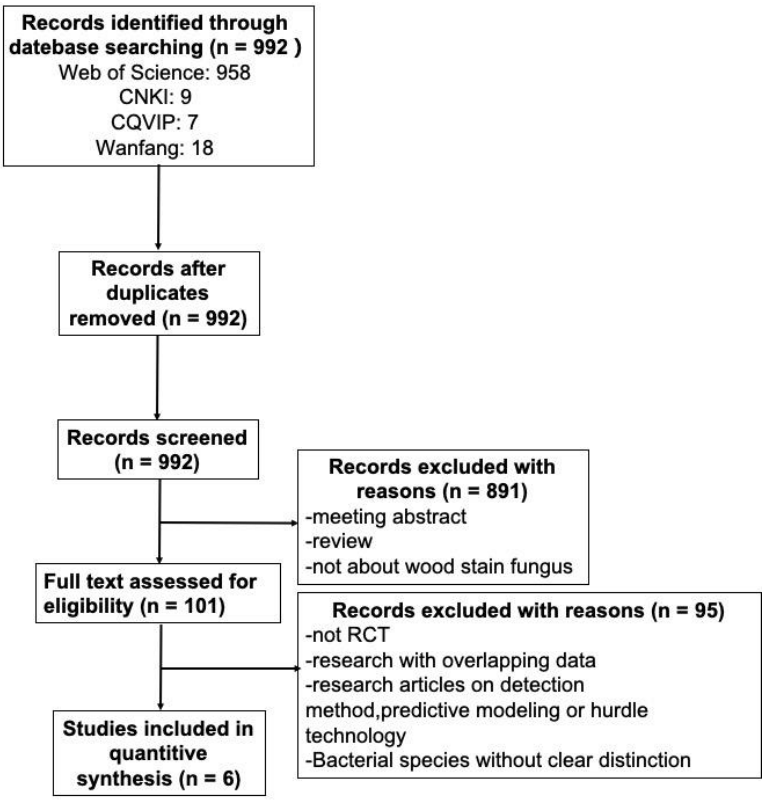

Fig. 1. The flowchart of the literature searching and collecting

The amount of research data collected was as follows: 958 articles on Web of Science, 9 articles on CNKI, 18 articles on Wanfang Database, and 7 articles on CQVIP. A total of 992 articles were initially retrieved from the four databases according to the search strategy. After importing the EndNote Citation Management Software (Clarivate Analytics, X8, Philadelphia, USA) to remove duplicate references, manual filtering was performed. Through reading the literature, research without a control group, research with unserious literature experiment designs, and literature or conference papers repeatedly published were excluded. Finally, 163 data that met the requirements were included for evaluation. The data of four of the papers concerned the diameter of mycelia and the data of two of the papers were about inhibition zone diameters. The mycelia growth effect value was treated as a dichotomous variable, and the diameter of the inhibition zone was treated as continuous variable. Although the sampling period selected was longer, the remaining reports were all published between 2013 and 2016.

The Overall Antagonistic Effect of *B. subtilis* Against Wood Stain Fungi

Four literature pieces report that the growth effect of mycelia treated by *B. subtilis* and its total effectiveness is relatively high (Han *et al*. 2013; Sun *et al*. 2013; Wu *et al*. 2013; Sajitha *et al*. 2014). The treatment group was found to be better than that of the control group, and the difference was statistically significant. The RevMan 5.0.2 software could be used for calculation. The mycelia growth effect value was treated as a dichotomous variable. As shown in Fig. 2, the total combined effected amount was Z = 4.46 (P < 0.00001), and the combined effect OR = 0.15, 95% CI [0.06, 0.34]. It could be considered that there was a statistical difference in the treatment of the wood stain fungus by *B. subtilis* and that *B. subtilis* had a good antagonistic effect against the wood stain fungus.

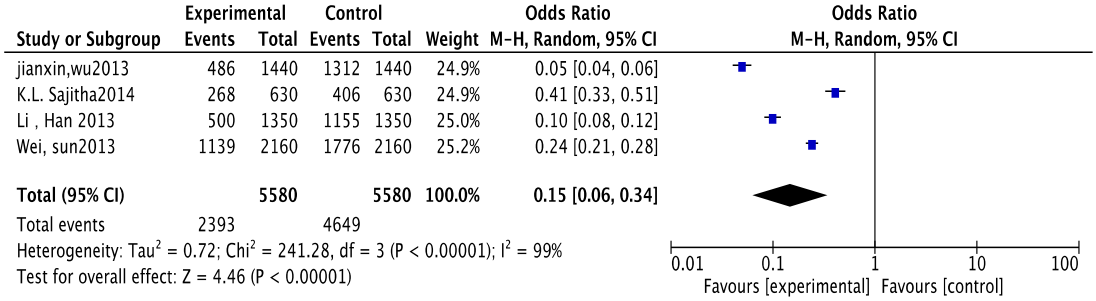

Fig. 2. The overall growth effect value of wood stain fungus treated by *Bacillus subtilis*

Influence of Species of Stain Fungus on the Antagonistic Effect of *B. subtilis* Against Wood Stain Fungus

As shown in Fig. 3, the OR values of *B. subtilis* on mycelium growth inhibition of *Lasiodiplodia theobromae*, *Ceratocystis fimbriata*, *Curvularia lunata*, Alternaria, *Ceratocystis* sp. were 0.07, 0.09, 0.05, 0.29, and 0.18, and the 95% CI was [0.02, 0.24], [0.04, 0.19], [0.04, 0.07], [0.10, 0.86], and [0.13, 0.24], respectively. The combined effected size OR was used in the random effect model. The total combined effected amount was Z = 7.14 (P < 0.00001). The combined effected OR was 0.11, and the 95% CI was [0.06, 0.20]. The values of mean effect and confidence intervals of the five species were

significantly different, indicating that *B. subtilis* had significantly different antagonistic effects against different wood stain fungi. The confidence intervals of the five tested strains did not overlap with 1, indicating that *B. subtilis* had certain effects on all the five tested strains. The data showed that *B. subtilis* had a significant inhibitory effect on the growth of mycelia of *L. theobromae*, *C. fimbriata*, and *C. lunata*, among which *C. lunata* had the strongest inhibition effect, while the inhibition effects on *Alternaria* and *Ceratocystis* sp. were general.

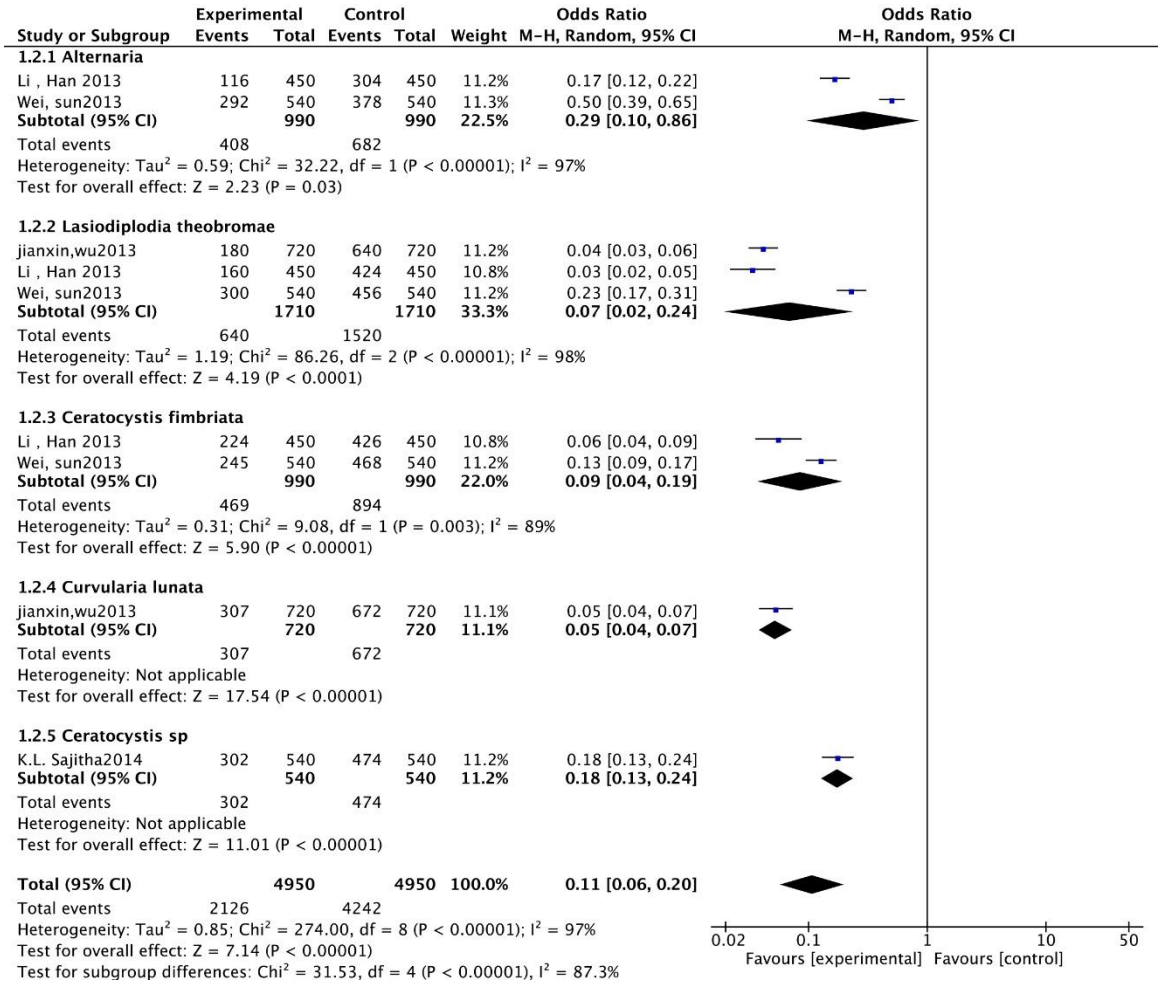

Fig. 3. Mycelia growth effect value of different strains treated by *Bacillus subtilis*

The Influence of *B. subtilis* on the Antagonistic Effect

The average effect values and confidence intervals of the 17 of strains are shown in Table 1. The species number could not be listed here because it involved the species of bacteria. The combined effected amount OR adopted the random effect model. The total combined effected amount was Z = 8.78 (P < 0.00001) and the total combined effect OR = 0.13, 95% CI = [0.08, 0.20]. The values of the mean effect and confidence intervals of the 17 of subgroups varied greatly, indicating that different *B. subtilis* strains had significantly different antagonistic effects against wood stain fungi. Among them, 12 of the *B. subtilis* strains had significant antagonistic effects against wood stain fungi. The average effect values and confidence intervals of three *B. subtilis* strains overlapped with one, indicating that the antagonistic effects were not significant.

Table 1. Average Effect Values and Confidence Intervals

Subgroup	Odds Ratio	95% CI
B26	0.10	[0.08, 0.12]
B26-10	0.24	[0.21, 0.28]
B19	0.01	[0.01, 0.03]
B35	0.03	[0.02, 0.06]
B37	0.04	[0.02, 0.07]
B38	0.08	[0.04, 0.14]
B41	0.05	[0.03, 0.09]
B66	0.11	[0.06, 0.20]
B175	0.02	[0.01, 0.04]
B215	0.13	[0.07, 0.23]
A3	0.55	[0.30, 1.00]
A8	0.5	[0.28, 0.92]
B1	0.17	[0.09, 0.32]
B2	0.19	[0.10, 0.36]
B4	0.75	[0.41, 1.38]
C3	0.39	[0.21, 0.70]
C4	0.63	[0.35, 1.15]
Total (95% CI)	0.13	[0.08, 0.20]
Test for overall effect: Z = 8.78 (P < 0.00001)		

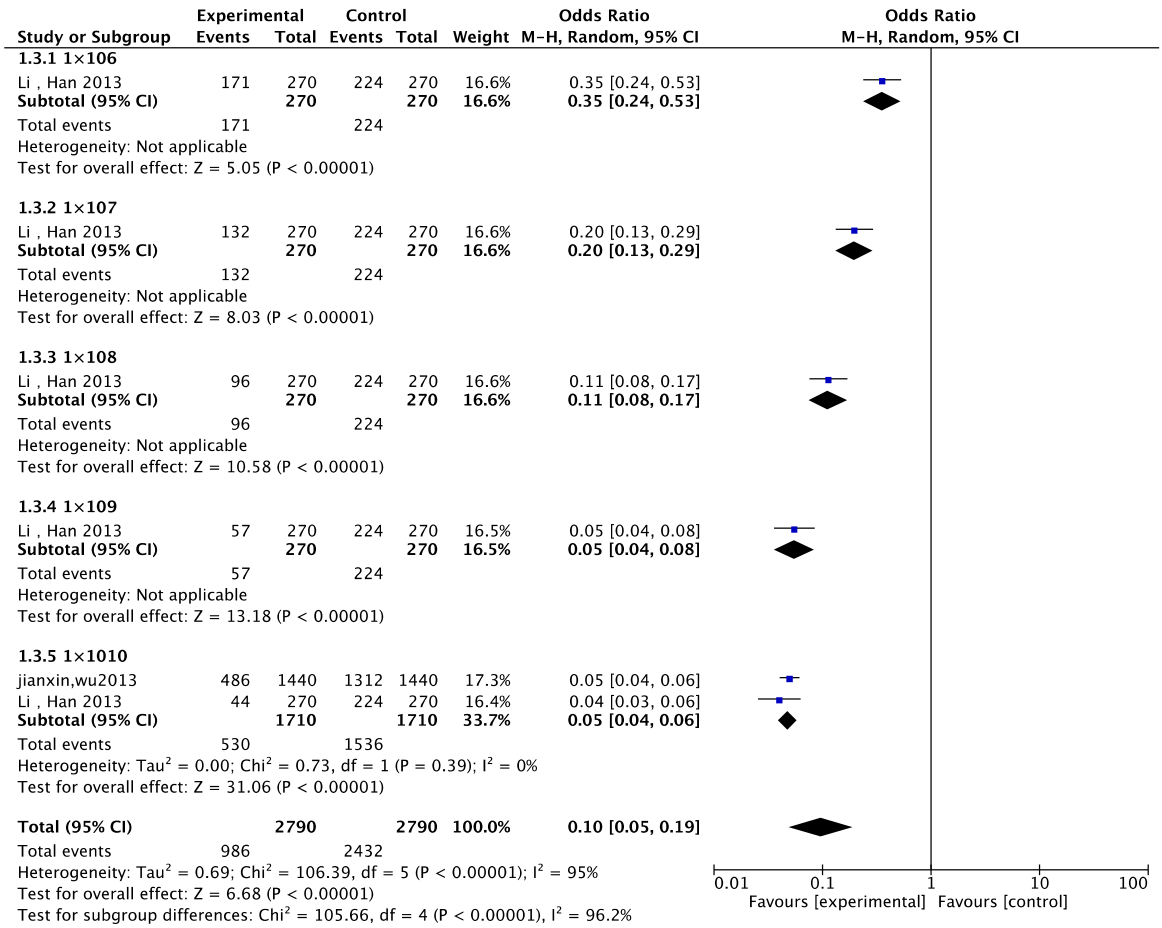

Fig. 4. Mycelia growth effect value of wood stain fungus treated by different dosages of *Bacillus subtilis*

Effect of *B. subtilis* Dosage on the Antagonistic Effect of *B. subtilis* Against Wood Stain Fungus

The calculation results are shown in Fig. 4. The OR values of different doses from 1 × 106 to 1 × 1010 were 0.35, 0.20, 0.11, 0.05, and 0.05, and the 95% CI was [0.24, 0.53], [0.13, 0.29], [0.08, 0.17], [0.04, 0.08], and [0.06, 0.06], respectively. The combined effected amount OR adopted the random effect model. The total combined effected amount was Z = 6.68 (P < 0.00001) and the total combined effected OR = 0.10, 95% CI = [0.05, 0.19]. Among them, the average effected values and confidence intervals of the four gradients (1 × 106 to 1 × 109) were significantly different, and only the intervals of the two gradients (1 × 109 to 1 × 1010) were similar. This indicated that different application doses had significant influences on the antagonistic effect of *B. subtilis*. The data showed that different doses of *B. subtilis* on the wood stain fungus were significant for the antagonistic effect. With the gradient of dosage of *B. subtilis* increased from 1 × 106 to 1 × 1010, the antagonistic effect of *B. subtilis* on the wood stain fungus strengthened.

Effects of Mixed Reagent Type on Antagonistic Effect

The diameter of the inhibition zone was a continuous variable, which was calculated *via* inverse variance method using the RevMan software. The measurement units of the two papers were consistent. For this aspect, MD value is selected to measure the outcome index. As shown in Fig. 5, Han *et al*. (2013) used a tebuconazole reagent to mix with *B. subtilis* and the MD value was 22.84 [22.14, 23.54]. Wang *et al*. (2012) and Wang *et al*. (2016) used Leyland Cypress Needle oil to mix with *B. subtilis* and the MD value was 13.65 [13.50, 13.80]. The comprehensive MD value was 18.24 [9.23, 27.24] (P < 0.0001). The confidence interval of the two subgroups did not overlap with 0, which indicated that both the tebuconazole mixed reagent and the Leyland Cypress Needle oil mixed reagent were effective against the wood stain fungus. However, the average effect value and confidence interval of the two subgroups were significantly different, and the mixture of tebuconazole and *B. subtilis* had a better inhibitory effect on wood stain fungi than the mixture of Leyland Cypress Needle oil and *B. subtilis*.

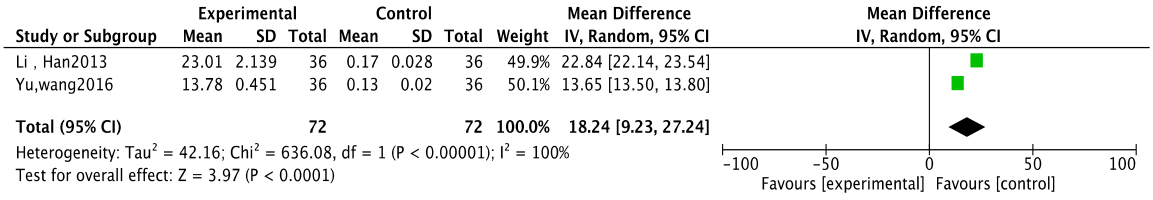

Fig. 5. Effect value of inhibition zone diameters of different mixed reagents

Effects of Mixed Reagent on Different Wood Stain Fungi

Similarly, in the RevMan software, the inverse variance method was used to calculate the diameter of the inhibition zone, and the MD value was selected to measure the outcome index. As shown in Fig. 6, the Alternaria, *L. theobromae*, and *C. fimbriata* were treated with a mixture of *B. subtilis* and tebuconazole reagent. The MD values were 14.53 [13.46, 15.60], 17.36 [15.84, 18.88], and 19.58 [18.01, 21.15], respectively. *Trichoderma viride* was treated by a mixture of *B. subtilis* and Leyland Cypress Needle oil with an MD value of 13.61 [13.46, 13.76]. The comprehensive MD value was 16.18 [13.71, 18.65]; however, the MD value changed to 17.11 [14.10, 20.13] when *T. viride* was

removed. The values of the mean effect and confidence intervals of the four subgroups were significantly different, indicating that the mixed reagents still had different effects on different wood stain fungi. The results showed that tebuconazole mixed reagent had the best effect on *C. fimbriata*, followed by *L. theobromae* and Alternaria. However, the Leyland Cypress Needle oil mixture reagent to *T. viride* was generally effective.

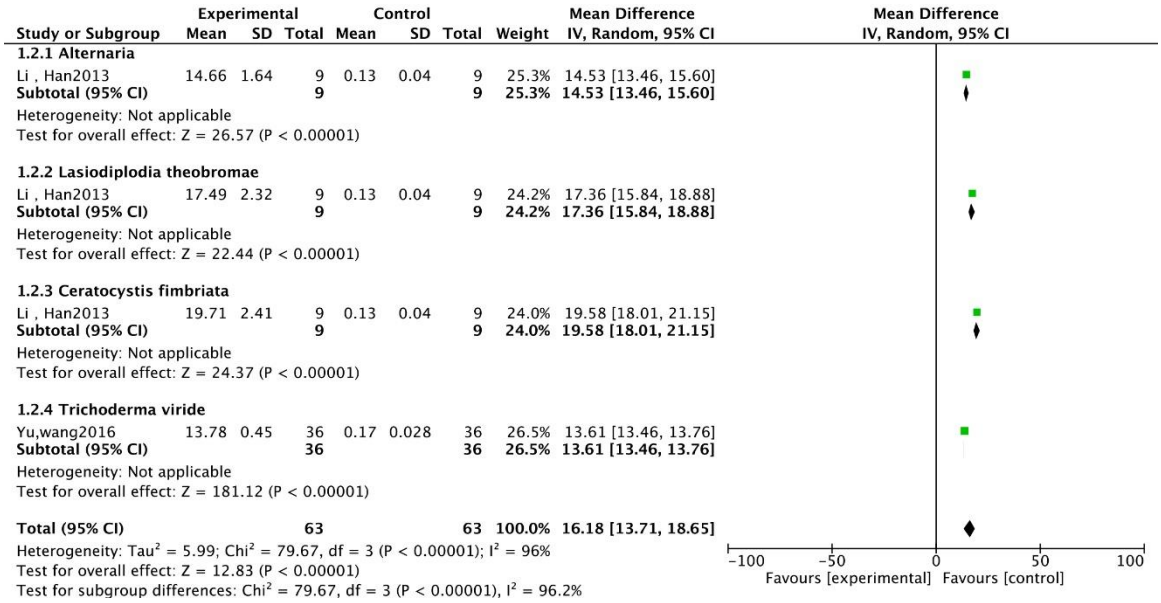

Fig. 6. Effect value of inhibitory zone diameters of different wood stain fungi treated with mixed reagent

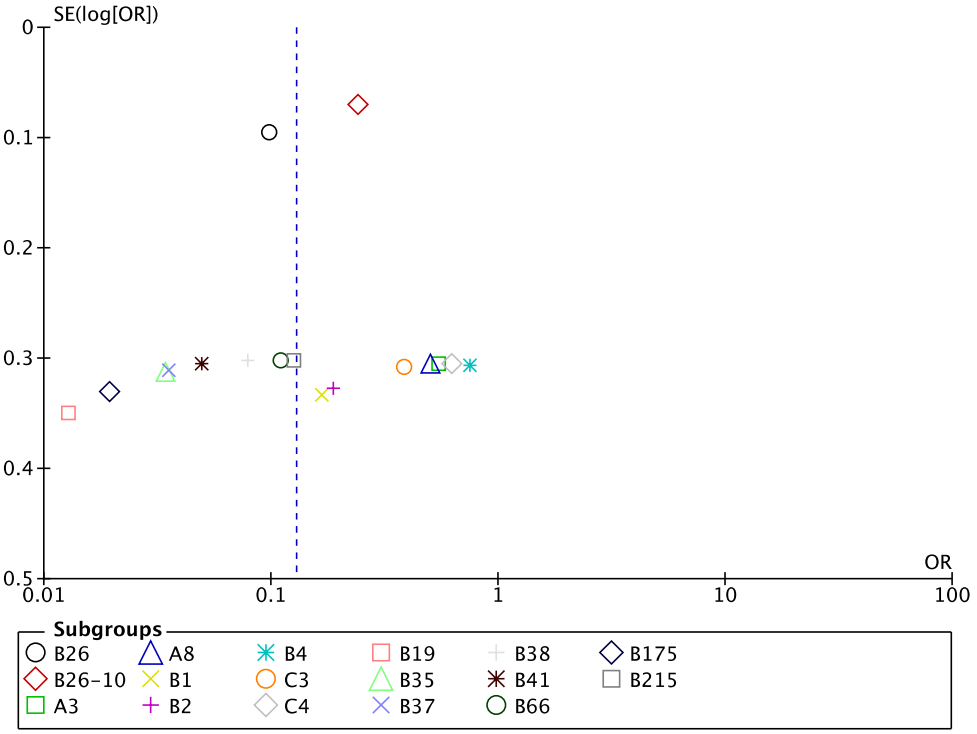

Fig. 7. Funnel plot regarding the influence of *Bacillus subtilis* for the antagonistic effect

Selection and Publication Bias

In this study, the mycelium diameter and inhibitory zone diameter of wood stain fungi were used as outcome indices to analyze the inhibitory effect of *B. subtilis* on wood stain fungus and the influencing factors. A total of 112 data were included. As shown in Fig. 7, the references included in the analysis of the influence of *B. subtilis* species on the antagonistic effect were selected as funnel plots. The distribution of subgroups of funnel plot indicated publication bias or low methodological quality, and it is related to the sample size of the included experiment.

DISCUSSION

Analysis of the Integral Antagonistic Effect of *B. subtilis*

The quantitative and comprehensive estimation of systematic analysis and the analysis of single outcome index showed that the application of *B. subtilis* had a significant inhibitory effect on wood stain fungi. Although this conclusion was basically consistent with the conclusions of the other six reports, the antagonistic effect of *Bacillus subtilis* was affected by a variety of factors. This result showed that both single and mixed uses of other reagents had a good inhibitory effect, and the inhibitory effect of mixed use of other reagents might be better than that of single use. This suggested that more attention should be paid to the use of biological control in the process of wood preservation. At the same time, not only biological control alone, but also biological control combined with chemical means could be adopted (Xing 2004). Many laboratory tests showed that *B. subtilis* had a good antagonistic effect on wood stain fungi. In evidence-based modern science, it is still difficult to provide convincing evidence that *B. subtilis* can effectively prevent wood sapstain. Therefore, rigorous, large sample size, multicenter, randomized controlled outdoor trials are needed to clearly establish the role of *B. subtilis*. This will provide sufficient basis for *B. subtilis* to be used as a means of biological control of wood preservation.

Limitations of this Systematic Review

Selection of test objects

Bacillus subtilis has been shown to have different antagonistic effects against different wood stain fungi. There were few strains mentioned in the literature included in this study and many effective strains had not been found. Screening efficient antagonistic strains is basic work that needs to be continued. In most reports, the screening strains are mostly confined to soil, but the screening range can be expanded, *i.e.*, to leaves and tree trunks, to broaden the source of biocontrol strains. Antagonistic bacteria are obtained from the isolation and culture of poplar and leaf spot of three plants (Zhao *et al*. 2007). Rezgui *et al*. (2016) isolate *B. subtilis* B6 from non-necrotic wood tissues with grapevine trunk diseases in Tunisia. It is possible to use biotechnology to mutate and genetically modify known strains to obtain new strains. Sun *et al*. (2011) previously isolated B26 with good antagonistic effect for ultraviolet mutagenesis and screened the mutant strain B26-10 with stronger antibacterial activity.

Methodological quality of the relevant tests

Based on the available evidence of randomized controlled trials of *B. subtilis* against wood stain fungi, most of the trials were conducted in the laboratory. There were

few field trials of *B. subtilis* regarding its products against wood stain fungi. The low quality of the published experimental evidence methodology is widespread. Almost all the included reports only mentioned randomized control, and there is a possibility of bias. The quality of the included reports is generally low, which affects the evidence strength of the systematic review. In addition, the success of the biological control depends on the comprehensive ability of biocontrol. It follows that a set of reliable test methods and selection parameters are needed to evaluate the comprehensive ability of biocontrol and to define the applicable scope of biocontrol (Sun *et al*. 2009). However, some data cannot be measured by unified indices because many studies have different estimation indices. In future experiments, the estimation indexes of the species or their products on wood and environment can be added to form a perfect evaluation system of biocontrol strains.

The Necessity of Using Meta-analysis in Forestry

The volume of data generated by wood conservation research has been growing over the past decade. Advances in information technology are likely to accelerate this growth further. Meta-analysis in the area of wood conservation could be used to identify, evaluate, and synthesize results. By such means, decision makers could obtain effective and concise information and researchers could better describe the prevention and protection effects of interventions on wood. The application of meta-analysis in wood conservation research has not been utilized yet. In principle, meta-analysis could be used to solve the problem of extensive research on protection of wood, such as storage effectiveness of the intervention, the degree of wood sapstain, regional prevalence of rot fungus, and wood stain fungus. The discovery of the independent meta-analysis could provide valuable information about the best intervention and could provide a data input risk assessment model.

CONCLUSIONS

1. Based on the meta-analysis for the integrated analysis and estimation of the two outcome indicators, the antagonistic effect of *B. subtilis* against wood stain fungi showed that *B. subtilis* had a significant inhibitory effect on wood stain fungus in general. The combined effect OR of the overall antagonistic effect was 0.15 (95% CI [0.06, 0.34]). Compared with the six studies upon which the analysis is based, the conclusion is consistent on the inhibition effect of *B. subtilis*.
2. The inhibition of wood stain fungi was influenced by the species of *B. subtilis*, the species of wood stain fungi, *B. subtilis* dosage, and the species of mixed reagent. The effect of value was OR = 0.13 (95% CI [0.08, 0.20]), OR = 0.11 (95% CI [0.06, 0.20]), OR = 0.10 (95% CI [0.05, 0.19]), and MD = 18.24 (95% CI [9.23, 27.24]).
3. *Bacillus subtilis* had different inhibitory effects for wood stain fungi. *Bacillus subtilis* had significant inhibitory effects on the growth of mycelia of *L. theobromae*, *C. fimbriata*, and *C. lunata*, among which *C. lunata* had the strongest inhibitory effects. The inhibitory effects on *Alternaria* and *Ceratocystis* sp. were general.
4. Good inhibitory effects were obtained for mixed with different reagents. The tebuconazole reagent was better for mixing with *B. subtilis* than the Leyland Cypress Needle oil.

5. *Bacillus subtilis* can be judged as being suitable for inhibiting wood stain fungi and some factors were controlled to maximize the inhibition effect. The results of this study were expected to provide a reference for wood protection biocontrol tests, field tests, and practical applications.

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