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Antifungal Activity of *Bacillus coagulans* TQ33, Isolated from Skimmed Milk Powder, against *Botrytis cinerea*

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Summary

Bacillus coagulans TQ33 is isolated from the skimmed milk powder and has a broad antifungal activity against pathogens such as *Botrytis cinerea*, *Alternaria solani*, *Phytophthora drechsleri* Tucker, *Fusarium oxysporum* and *Glomerella cingulata.* The characteristics of active antifungal substances produced by *B. coagulans* TQ33 and its antifungal effects against the growth of plant pathogenic fungi has been evaluated. The effect of pH, temperature and protease on the antifungal activity of *B. coagulans* TQ33 was determined. The results of partial characterization of the antifungal compound indicated that its activity is likely to be due to the production of a proteinaceous substance together with other substances. The greenhouse trials suggest that *B. coagulans* TQ33 has a great potential for the control of plant pathogenic fungi.

Key words: plant pathogenic fungi, antifungal activity, *Bacillus coagulans* TQ33, *Botrytis cinerea*

Introduction

Grey mould is an economically important disease (*1*). *Botrytis cinerea* is a ubiquitous fungal pathogen that causes grey mould in many fruits, vegetables and ornamental crops worldwide (*2*). It can infect flowers, stems, leaves and fruits of hundreds of plants and is the main hazard for the vegetables from Cucurbitaceae and Solanaceae families, together with bean, leek and onion. Overwintering sclerotia and mycelia of *B. cinerea* produce abundant conidia in the spring, which infect susceptible tissues (*3*).

Botrytis cinerea is a classical 'high-risk' pathogen. The chemical control and use of fungicides are the most effective way of preventing the occurrence of *Botrytis* disease (*4*). However, the use of chemicals is considered undesirable because of concerns over residues, following an increased public health concern and fast development of resistance to novel fungicides by fungi. Biocontrol has become an interesting alternative to conventional methods (*5*). The main principles of biological control are defined as the use of living organisms and their metabolites to control several different pathogens on fruits and vegetables. *Pichia membranifaciens* FY-101 was found to be antagonistic towards *B. cinerea*, possibly due to secretion of β-1,3-glucanase. It was confirmed by *in vitro* experiments that *P. membranifaciens* FY-101 can be used as a biological control organism against *B. cinerea* (*6*).

Many species of *Bacillus* are capable of producing biologically active substances that disintegrate fungal cell walls (*7*). *Bacillus*, a widespread non-pathogenic bacterium, can produce many different antimicrobial substances. The metabolites from *Bacillus* showed the activity against several kinds of fungi. *Bacillus* strains are capable of decomposing chitin, which is one of the main cell wall constituents of many plant pathogenic fungi (*8*). Wen *et al.* (*9*) demonstrated that *Bacillus cereus* QQ308 was an antifungal hydrolytic enzyme-producing strain. The antifungal lipopeptides produced by *Bacillus amyloliquefaciens* SH-B10 showed significant inhibitory activities against five plant fungal pathogens (*10*). *Bacillus subtilis* EDR4 produced an antifungal protein E2 which exhibited inhibitory activity against mycelium growth of *Fusa-*

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rium graminearum, *Macrophoma kuwatsukai*, *Rhizoctonia cerealis*, *Fusarium oxysporum* f. sp. *vasinfectum*, *Botrytis cinerea* and *Gaeumannomyces graminis* var. *tritici* (*11*). Antifungal compounds from *Bacillus licheniformis* N1 exhibited activity against grey mould disease of tomato and strawberry plants (*12*).

Bacillus coagulans is a lactic acid-forming and facultative anaerobic bacteria of the *Bacillus* genus. The main features of *B. coagulans* are acid and heat resistance and easy culture. Several investigations have been conducted to examine the antimicrobial substances of *B. coagulans*. Hyronimus *et al.* (*13*) reported a bacteriocin-like inhibitory substance, which is produced by *B. coagulans* I-4 strain and is a protease-sensitive antibacterial substance. *B. coagulans* was isolated from the industrial wastewater drainage and selected for its antimicrobial activity against Gram-positive and Gram-negative bacteria, and yeast strains (*14*). Lactosporin is a novel antimicrobial compound produced by *B. coagulans* ATCC 7050 with inhibitory activity against Gram-positive bacteria (*Micrococcus luteus* and *Listeria monocytogenes*), but not against Gram-negative bacteria. Its antimicrobial activity against pathogenic microorganisms indicates that it may have a potential for application in food and personal care products (*15*). However, little attention has been paid to greenhouse trials investigating the effect of *B. coagulans* in biological control of plant pathogenic fungi. In the present study, the main antifungal characteristics of *Bacillus coagulans* TQ33 against plant pathogenic fungi are investigated, and greenhouse trials studying its potential in biological control of *Botrytis cinerea* are performed.

Materials and Methods

Strains and culture conditions

Bacillus coagulans TQ33 was isolated from skimmed milk powder and preserved in the medium containing (in g/L of distilled water): peptone 20, yeast extract 3, glucose 2, agar 2; pH=7.2–7.4 (*16*). *B. coagulans* TQ33 slants were incubated at 37 °C for 48 h. *Bacillus subtilis* was isolated from naturally fermented congee from Inner Mongolia in China. It was preserved on nutrient agar plates containing (in %): peptone 0.5, beef extract 0.3 and agar 1.5; pH=7.0–7.2. *B. subtilis* cultures were maintained in the nutrient broth by subculturing with 1 % inoculum, incubated for 24 h at 37 °C, and were stored at 4 °C before use. The following plant pathogenic fungi were used: *Botrytis cinerea*, *Alternaria solani*, *Phytophthora drechsleri* Tucker, *Fusarium oxysporum* and *Glomerella cingulata*, obtained from the Institute of Plant Protection, Tianjin, PR China. All pathogenic fungi were stored at –70 °C in 15 % glycerol and subsequently grown on potato dextrose agar (PDA) plates at 28 °C for 5–7 days.

Production of antifungal substance

Bacillus coagulans TQ33 seed culture was prepared in a 100-mL flask with 20 mL of seed medium containing (in g/L of distilled water): peptone 20, yeast extract 3, glucose 2, $MgSO_4 \cdot 7H_2O$ 0.5; pH=7.2–7.4, and incubated at 38 °C and 140 rpm for 20 h. Fermentation was performed in a 500-mL flask with 100 mL of medium, and 5 % volume of seed culture was used as inoculum. The flasks were incubated at 38 °C and 140 rpm for 67 h. The fermentation medium was composed of (in g/L of distilled water): peptone 10, yeast extract 10, glucose 6, $MgSO_4 \cdot 7H_2O$ 1 and K_2HPO_4 2; pH=7.0. Well-developed *B. coagulans* TQ33 cultures were centrifuged (8000 rpm, 10 min) and subjected to sterile filtration (0.22 μm, Millipore, Billerica, MA, USA). Subsequently, the cell-free supernatant was stored at –20 °C and used for further characterization of antifungal compounds (*17*).

Antifungal activity assays

The antifungal activity of *Bacillus coagulans* TQ33 was determined by the following two methods. The first one was described by Wang *et al.* (*18*). PDA was used as the medium for all tested fungi. The media incorporating the tested compounds at a volume fraction of 10 % were inoculated with agar discs containing the tested fungi (5 mm) in the centre. Three replicate plates for each fungus were incubated at (27±2) °C. Control plates containing the medium mixed with sterile water (10 %, by volume) were included. Following a 2- to 6-day incubation period, the mycelial growth of fungi (in mm) in both treated (T) and control (C) Petri dishes was measured diametrically in perpendicular directions until the fungal growth in the control dishes was almost complete. The percentage of growth inhibition (I) was calculated using the formula:

$$I=[(C-T)/C]\cdot 100 \qquad /1/$$

The corrected inhibition (IC) was then calculated as follows:

$$IC=[(C-T)/(C-C_0)]\cdot 100 \qquad /2/$$

where C_0 is the diameter of the fungal agar discs (5 mm).

The second method is a well-diffusion assay with a few modifications. A volume of 10 mL of 2 % agar was poured into sterile Petri dishes and allowed to harden, then 10 mL of PDA were poured on the top of the hardened agar. Wells (7-mm diameter) were punched in the plates using a sterile stainless steel borer. Then the test dishes were filled with 200 μL of sample and sterile water was used as the control. The tested fungi were inoculated in the centre of the dishes. Three replicate dishes for each fungus were incubated at (27±2) °C. The diameters of the inhibition zones were measured in mm until the fungal growth in the control dishes was almost complete.

To determine the range of their inhibitory activity against five plant pathogenic fungi, *i.e. Botrytis cinerea*, *Alternaria solani*, *Phytophthora drechsleri* Tucker, *Fusarium oxysporum* and *Glomerella cingulata*, the supernatants were examined using the first method described above.

The design of the trials

The indicator strain used in these experiments was *Botrytis cinerea*. After each treatment, the residual antifungal activity was determined by the well diffusion method. All determinations were carried out in duplicates. The cell-free supernatant from the culture was subjected to different pH values: 3.0, 4.0, 5.0, 6.0, 7.0, 7.5 and 8.0. The pH of the cell-free supernatant was ad-

justed with 2 M HCl or 2 M NaOH, and the antifungal activity after each treatment was determined by the well diffusion assay with *B. cinerea* as the indicator strain. The antifungal activity test of each culture filtrate was repeated three times. Heat resistance of the filter-sterilised cell-free supernatant was tested by keeping it for 1 h at each of the following temperatures: 60, 80 and 100 °C, and then for 20 min at 120 °C. The antifungal activity after each treatment was determined as described above. The filter-sterilised cell-free supernatants were tested against the proteinase K (Amresco, Solon, OH, USA), trypsin (TianJin Nuoao Technology Development Co, Ltd, Tinjin, PR, China) and neutral protease (TianJin Nuoao). The enzyme (10 mg/mL in 20 mmol/L of sodium phosphate buffer, pH=7) and culture filtrates were incubated at 37 °C for 120 min before being tested against *B. cinerea*. A control sample containing only cell-free supernatant and 20 mmol/L of sodium phosphate buffer (pH=7) was also incubated under the same conditions and tested for antifungal activity (*19*).

HPLC analysis of lactic acid in the culture filtrate

Lactic acid was determined with high-performance liquid chromatography (HPLC) on Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, CA, USA) (*20*) using an ion exchange column (ZORBAX® SB-Aq, 4.6× 150 mm). The eluent was 1 % acetonitrile (ACN)/99 % 20 mmol/L of $NaHPO_4$ (pH=2), and the elution was monitored using a UV detector at 210 nm at a flow rate of 1.0 mL/min and the column temperature of 35 °C.

Greenhouse trials

To determine if *Bacillus coagulans* TQ33 has a potential application as antifungal agent in the agriculture, a trial was undertaken using 30 young tomato plants as a model and *Botrytis cinerea* as the indicator strain. The culture of *B. coagulans* TQ33 was freeze-dried and diluted in water 800 times. The experiment was performed in three treatments, each in three replicates: young tomato leaves were uniformly sprayed with bacterial suspension (10^8 CFU/mL) of freeze-dried *Bacillus coagulans* TQ33 culture, or with freeze-dried *Bacillus subtilis* culture (bacterial suspension 10^8 CFU/mL), and controls were sprayed with sterile water. All the tomato plants of each group were sprayed with *Botrytis cinerea* conidial suspension. The treated and the control plants were incubated in a greenhouse under natural daylight at (25±2) °C (day/night) with 90 % relative humidity for 8 days.

The severity of the disease was recorded by visual examination of the leaves using a 0–9 scale (*21*), where 0=leaves with no symptoms, 1=lesions on <5 % of the leaves, 3=lesions on 6–10 %, 5=lesions on 11–25 %, 7=lesions on 26–50 %, 9=lesions on >50 % of the leaves. The disease ratio, disease index and biocontrol effect were calculated according to the method described by Williamson *et al.* (*22*):

$$\text{Disease index} = \frac{\Sigma(\text{grade of disease severity} \times \text{no. of diseased plants of this grade})}{(\text{total no. of assessed plants} \times \text{the highest grade of disease severity})} \times 100 \quad /3/$$

$$\text{Biocontrol effect} = \frac{(\text{disease index of pathogen control} - \text{disease index of bacterial treatment})}{\text{disease index of pathogen control}} \times 100 \quad /4/$$

Results and Discussion

Activity of Bacillus coagulans TQ33 against Botrytis cinerea

The antifungal activity of *Bacillus coagulans* TQ33 against *Botrytis cinerea* is shown in Fig. 1. The results revealed that *B. coagulans* TQ33 had marked activity against *B. cinerea*, higher than 80 %. For a long time, the prevention of plant pathogenic fungi has mainly depended on the use of synthetic chemical fungicides, such as carbendazim, thiophanate-methyl and their mixtures. Bardas *et al.* (*3*) reported that pyraclostrobin[methyl *N*-{2-[1-(4-chlorophenyl)pyrazol-3-yloxymethyl]phenyl}(*N*-methoxy)carbamate] is a recently introduced unique outside inhibitor (QoI) fungicide that is highly effective against grey mould caused by *B. cinerea* and other serious fungal pathogens on a wide range of crops. However, continued use of these fungicides is problematical owing to the development of fungal resistance and increasing social and environmental concerns over chemical residues (*23*). Recently research has suggested that biological control of various plant pathogens, especially the selection and application of *Bacillus* has gradually been acknowledged. *Bacillus* is considered the main bacterial strain in biological control of plant pathogenic fungi. *B. coagulans*, a lactic acid-producing bacterium, can be used in the production of ethanol and lactic acid from acid hydrolysates of cellulose and hemicellulose. It also produces other commercially important chemicals like cyclodextrins and amylases, and has been used as a probiotic in animal feed. This investigation has shown for the first time the antifungal activity of *Bacillus coagulans* TQ33 against *Botrytis cinerea*.

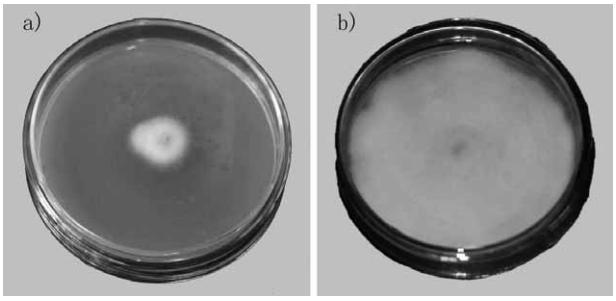

Fig. 1. The antifungal activity of *Bacillus coagulans* TQ33 against *Botrytis cinerea*: a) potato dextrose agar plate containing the supernatant of *Bacillus coagulans* TQ33 at a volume fraction of 10 %, inoculated with agar discs of the tested fungi (5 mm) in the centre, b) control plate containing PDA medium mixed with sterile water (10 %, by volume), inoculated with agar discs of the tested fungi (5 mm) in the centre

Spectrum of antimicrobial activity

The results of the antifungal acitivity assay indicate that *Bacillus coagulans* TQ33 had a wide spectrum of an-

tagonistic activities against *Botrytis cinerea*, *Alternaria solani*, *Phytophthora drechsleri* Tucker, *Fusarium oxysporum* and *Glomerella cingulata*. The antifungal activity against *P. drechsleri* Tucker was higher than 95 % and the inhibition of *F. oxysporum* was higher than 85 %, while the antifungal activity against *B. cinerea*, *A. solani* and *G. cingulata* was 80.9, 48.6 and 60 %, respectively. It has been reported that *Bacillus coagulans* 2–6 can produce an antagonistic compound that inhibits the growth of seven pathogenic species: *Bipolaris sorokiniana*, *Trichothecium roseum*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Fusarium oxysporum*, *Fusarium solani* and *Fusarium culmorum*. The addition of *B. coagulans* 2–6 to the culture of fungi resulted in the inhibition of ergosterol biosynthesis in the mycelium (*24*). This study has shown for the first time that the metabolites of *Bacillus coagulans* TQ33 had fairly high activity against *B. cinerea*, *A. solani*, *P. drechsleri* Tucker, *F. oxysporum* and *G. cingulata*.

Characterization of the active antifungal principles

Bacillus coagulans TQ33 was tested to determine whether the antifungal activity was stable after different treatments. The results showed that the cell-free supernatants of *B. coagulans* TQ33 were sensitive to the changes in pH, with the maximum antifungal activity at pH=6.0 (Fig. 2), which decreased when the pH was below 6 or

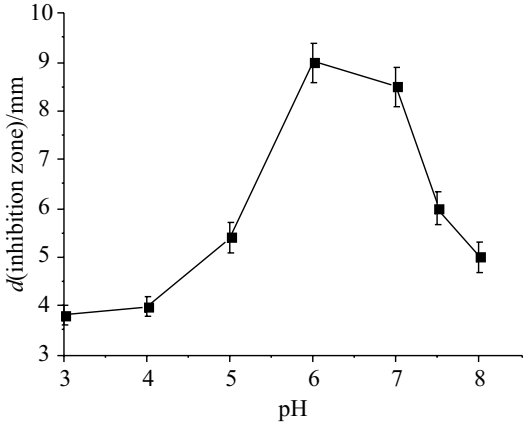

Fig. 2. Effect of pH on the antifungal activity of *Bacillus coagulans* TQ33

above 7. A decrease in the activity was observed when it was heated at 100 °C for 1 h or subjected to autoclaving at 120 °C for 20 min, but it was stable when heated at 80 °C for 1 h (Fig. 3). When the supernatant was tested for sensitivity to the neutral protease, proteinase K and trypsin, the antifungal activity was partly lost as compared to the original activity (Fig. 4). In another report, the antimicrobial activity of *B. coagulans* ATCC 7050 was no longer detected after the exposure to a number of proteolytic enzymes such as proteinase K and protease, thus indicating the proteinaceous nature of this inhibitory substance (*15*). Our results indicate that there are other substances, in addition to the proteinaceous substance, with the antifungal activity among metabolites of *B. coagulans* TQ33.

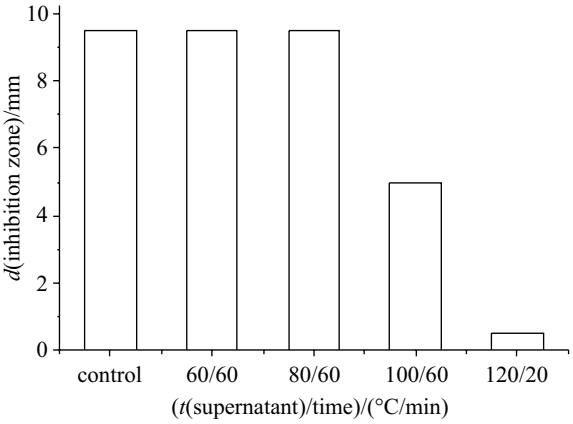

Fig. 3. Effect of temperature on the antifungal activity of *Bacillus coagulans* TQ33

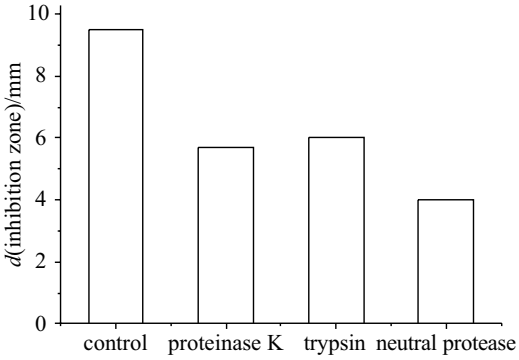

Fig. 4. Effect of proteolytic enzymes on the antifungal activity of *Bacillus coagulans* TQ33

HPLC analysis of lactic acid

To investigate whether the difference in the inhibition of pathogenic fungi was due to variations in lactic acid concentration, HPLC analysis of lactic acid in the cell-free supernatant of *B. coagulans* TQ33 was conducted. Fig. 5 shows the antifungal effects on *Botrytis cinerea* of the cell-free culture filtrate in relation to the concentration of lactic acid produced in the broth. After 67 h of

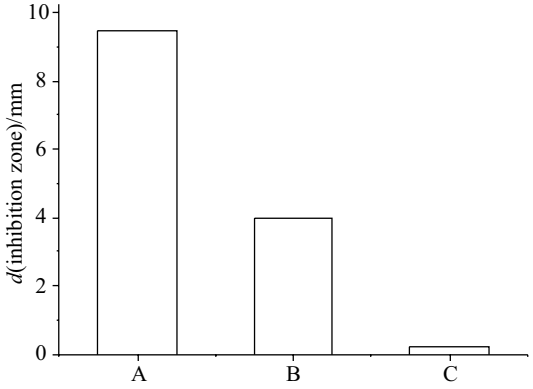

Fig. 5. Growth inhibition of *Botrytis cinerea*: A: cell-free supernatant of *Bacillus coagulans* TQ33; B: lactic acid (85 mmol/L) produced after 67 h of fermentation in the culture supernatant of *B. coagulans* TQ33; C: control (lactic acid was replaced by an equal volume of sterilised growth medium)

fermentation, the lactic acid concentration in *B. coagulans* TQ33 culture supernatant was 85 mmol/L, and its inhibition zone against *Botrytis cinerea* was 4 mm, whereas the inhibition zone of the cell-free supernatant of *B. coagulans* TQ33 against the growth of *B. cinerea* was 9.5 mm. The results indicated that lactic acid alone could not explain the antifungal activity of *Bacillus coagulans* TQ33 against *Botrytis cinerea* (Fig. 5).

Antifungal actitvity of B. coagulans TQ33

The effect of *Bacillus coagulans* TQ33 on *Botrytis cinerea* infection of tomato was evaluated under greenhouse conditions. On the untreated control plants, initial symptoms appeared on the leaves, accompanied by wilt of the entire plants. However, plants treated with *Bacillus coagulans* TQ33 culture exhibited a significant decrease in disease severity, unlike the plants sprayed with *Bacillus subtilis* or sterile water (Fig. 6). The disease index significantly decreased in plants treated with *B. coagulans* TQ33

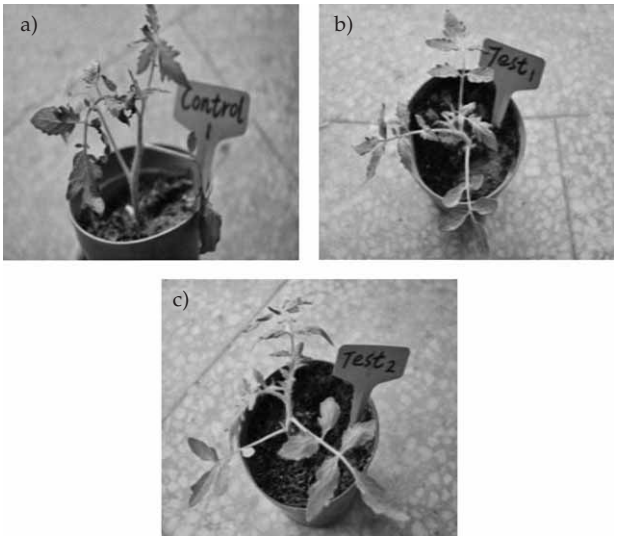

Fig. 6. Young tomato plants in the greenhouse trials sprayed with *Botrytis cinerea* conidial suspension and: a) sterilised water (control 1), b) *Bacillus subtilis* (test 1), and c) *Bacillus coagulans* TQ33 (test 2)

(Table 1). It revealed that *B. coagulans* TQ33 had the ability to suppress the growth of *Botrytis cinerea* and has possible application in agriculture. Zhang *et al.* (*25*) reported that 5-hydroxyl-5-methyl-2-hexenoic acid was the main antifungal compound in the culture of *Actinoplanes* sp. HBDN08, which showed strong *in vitro* activity of 71.42 % against *B. cinerea*. It was demonstrated under greenhouse conditions that 5-hydroxyl-5-methyl-2-hexenoic acid might also be a promising candidate as new antifungal agent. The antifungal activity of *Bacillus coagulans* TQ33 against *Botrytis cinerea* was higher than 80 %, but it is not known whether *Bacillus coagulans* TQ33 can produce 5-hydroxyl-5-methyl-2-hexenoic acid. Greenhouse trials showed that *B. coagulans* TQ33 caused a significant decrease in disease severity compared to the control. To the best of our knowledge, there has not been any report on the antifungal actitvity of *Bacillus coagulans* against *Botrytis cinerea* under greenhouse conditions so far.

Table 1. Biological control of *Botrytis cinerea* on tomato with *Bacillus coagulans* TQ33

Treatment	Disease index	Biocontrol effect
	%	%
Bacillus coagulans TQ33	54.2	43.7
Bacillus subtilis	37.8	19.9
sterile water control	96.3	–

Results are the mean values of three replications

A toxicological safety assessment was published in 2009 of a proprietary preparation of a novel probiotic *Bacillus coagulans* GanedenBC^{30}™. It was concluded that GanedenBC^{30} was safe for human consumption based on scientific procedures, supported by a safe history of use (*26*). A one-year chronic oral toxicity study combined with a one-generation reproduction study was conducted to further investigate the safety of its long-term consumption. The one-year study of GanedenBC^{30} administered to male and female Wistar Han rats in their diet showed no signs of toxicity at the highest dose tested. The conclusion of the reproduction toxicity study was that the administration of GanedenBC^{30} in the diet showed no signs of toxicity in the parental generation (male or female) or the F1 offspring (*27*). Therefore, *B. coagulans* TQ33 has a great potential in practical application for the control of plant pathogenic fungi, and compared with the applied fungicides on the market, it has many unparallelled advantages, as it is environmentally safe and cost-effective.

Conclusion

The metabolites of *Bacillus coagulans* TQ33 have high activity against *Botrytis cinerea*, and its antifungal potential is considerable due to its broad antifungal spectrum. Greenhouse trials show that *Bacillus coagulans* TQ33 is a potential biological control agent for reducing the impact of *Botrytis cinerea* on tomato. The results indicate that there are yet unknown substances, in addition to the proteinaceous substances, with antifungal properties. This means that further investigations of the nature of the inhibiting compounds and their structure, together with the development of suitable application, could add to the knowledge on the control of plant pathogenic fungi.

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