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Isolation and identification of the causal agent of gummy stem blight disease in *Cucumis sativus* caused by a bacterial pathogen in China

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Recently, a new bacterial disease was detected on cucumber stalks. In order to study the pathogenesis of this disease, the pathogenic bacteria were isolated and identified on the basis of morphological and molecular characteristics, and further analyzed for pathogenicity and antagonistic evaluation. Pathogenicity analysis showed that HlJ-3 caused melting decay and cracking in cucumber stems, and the strain reisolated from re-infected cucumber stalks was morphologically identical to HlJ-3 colonies, which is consistent with the Koch's postulates. The pathogenic strain HlJ-3 was identified as having similar morphological characteristics to *Bacillus subtilis*. Meanwhile, its internal transcribed spacer sequence (ITS) and DNA gyrase A subunit (gyrA) were both more than 99% homologous and clustered on the same branch with *B. subtilis*. Therefore, combined with morphological and molecular biological features, strain HlJ-3 was identified as *B. subtilis*. In addition, *B. subtilis*, which has a wide range of hosts, was able to infest other common crop species, including potato, tomato, pepper, melon, and radish. Furthermore, antagonistic evaluation confirmed that strain HlJ-3 strongly inhibited the mycelial growth of *Colletotrichum coccodes* and *Alternaria tenuissima* in vitro, with antagonistic effects of 69.92% and 68.08%, respectively. In conclusion, our results showed that strain HlJ-3 is *B. subtilis*, which is pathogenic to cucumber in vivo and can infect plants of *Solanaceae*, *Cucurbitaceae* and *Brassicaceae* with a wide range of hosts. In addition, this strain has good biocontrol effects against *C. coccodes* and *A. tenuissima* in vitro. The findings of this research will help to prevent and control the occurrence of this pathogen and regulate its use as a biocontrol agent.

Keywords *Cucumis sativus*, Gummy stem blight disease, *Bacillus subtilis*, Isolation, Identification, Pathogenicity, Antagonistic function

Cucumber (*Cucumis sativus*) is one of the world's top ten vegetable crops, belonging to the *Cucurbitaceae* family of annual climbing herbaceous plants[1], native to the Asian continent[2]. Due to its high nutritional and economic value[3–5], it has been planted in large quantities in China and its production scale is the first in the world[6]. Unfortunately, pests and diseases that arise during cultivation have severely impacted the productivity and quality of cucumbers as well as their various growth phases, even while the area under cultivation keeps growing[7]. According to the report, the main diseases causing great losses in cucumber are downy mildew caused by *Pseudoperonospora cubensis*[8], powdery mildew caused by *Sphaerotheca fuliginea*[9], gray mold caused by *Botrytis cinerea*[10], anthracnose caused by *Colletotrichum* spp[11]. and bacterial soft rot caused by *Pectobacterium carotovorum*[12], among others, which leads to a serious reduction in cucumber yield, thus causing significant losses to the agricultural economy[13].

Interestingly, *Bacillus* spp. is a common type of bacterium found in nature that has mostly been identified in earlier research as an excellent biocontrol agent that can control a wide range of plant fungi that cause disease[14]. *Bacillus* spp., however, is a plant pathogen that may also seriously harm a number of host plants, some of which have been recognized as phytopathogens. For example, *B. pumilus* causes the soft rot of potato tubers[15], leaf blight of *Mangifera indica*[16], and ginger rhizome rot[17]. *Sagittaria sagittifolia* black rot[18] and *Allium cepa* bacterial rot caused by *B. amyloliquefaciens*[19]. *B. megaterium* caused leaf spots of *Radermachera sinica*[20] and bacterial blight in *Lupinis termis*[21]. *Bacillus subtilis* is a species of *Bacillus* spp. commonus that has

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been shown in earlier research to be widely distributed in a range of different living environments, capable of producing endophytic spores, resilient to heat, and present on the surface of soils and plants in addition to being a common endophyte in plants[22]. As a result of these characteristics, *B. subtilis* is generally widely used as a biocontrol agent in production practice in most of the current studies, and previous antagonistic experiments have demonstrated that *B. subtilis* inhibited the development and colonization of *Rhizoctonia cerealis*[23], Pythium spp., *Cercospora* spp., and *Fusarium* spp[24,25]. Furthermore, studies have shown that *B. subtilis* possesses strong biocontrol capabilities because of its capacity to generate a wide range of secondary metabolites, or compounds that resemble glycosinophilic acid that can stimulate the synthesis of endogenous hormones and cooperate with the up-regulation of genes related to defense enzymes to fend off plant disease infections[26].

However, it has also been shown that *B. subtilis* is capable of resolving pectin and polysaccharides in plant tissues and that some strains of *B. subtilis* can infect potato tubers[27], tomatoes[28], sunflower[29], *Leucojum aestivum*[30], cabbage[31] and grape[32]. Nonetheless, *B. subtilis* is not only effective in infecting crops, fruits and vegetables but also in causing diseases in humans[33–35] and insects[36,37]. Hence, in the production process, *B. subtilis* does not only exist as a biocontrol microorganism but its pathogenic role should not be ignored.

Gummy stem blight disease in *Cucumis sativus* was recently discovered in a field in Baiyin City, Gansu Province, China. The symptoms were characterised by longitudinal cracks in the stems and loose whitish tissue. To identify the pathogen responsible for this disease, in this study, the collected diseased stalks were isolated and their pathogenicity was verified according to Koch's postulates. The pathogen was identified based on morphological characteristics and gene sequence analysis. In addition, its biocontrol potential and host plant range were tested. All of this will help to concretely identify the causal agent of cucumber gummy stem blight, resulting in more effective diagnosis, control and reduced economic losses.

Materials and methods

Test plant materials

Test plant seeds: The seeds of *Cucumis sativus Cucumis melo*, *Lycopersicon esculentum*, *Raphanus sativus* and *Capsicum annuum* were purchased from Herun Wanjia Seed Company. *Zea mays* from ZhangYe City, Gansu Province and *Solanum tuberosum* (original seed) from DingXi City, Gansu Province.

Pathogen isolation and purification

In October 2020, *Cucumis sativus* stems with typical bacterial disease symptoms were found in greenhouses in Baiyin, Gansu Province, China (maximum elevation 3321 m, minimum: 1275 m; geographic location. 103°33'-105°34'E, 35°33'-37°38'N) and were brought back to the laboratory for identification. Diseased and healthy stems were rinsed with sterile water and the tissue was cut with a sterilised knife and then sterilised with 75% alcohol for 30 s. The resulting stalk tissues were placed on beef extract peptone dextrose media (BPD), respectively. The inoculated plates were placed in an incubator at 28℃ and incubated under dark conditions for 48 h. After the colonies had grown, individual colonies of the same morphology, size and colour were separated by plate separation and numbered and transferred to test tubes and stored in a refrigerator at 4 °C for future use[38].

Pathogenicity tests

When the cucumber grows to about 30 days, the isolates obtained in the previous step were transferred to BPD plates and cultured in the dark at 28℃ for 24–48 h, 5–6 single colonies were picked and added into 50 ml of BPD culture fluid, and cultured for 36 h under the condition of 28℃ and 180 rpm with shaking.

In vivo surface wound inoculation tests

Cucumber stalks used for pathogenicity test were wiped with 75% alcohol and then wounded with a sterile insect needle, and each treatment was repeated 6 times. The inoculated isolates were spread evenly on the wounded stalks with sterile cotton and then placed in a plastic bucket, sprayed with sterile water and incubated at room temperature in a moisturised environment for 48 h before being grown under normal conditions. Disease symptoms on stalks were continuously observed and captured with a digital camera. BPD culture fluid not inoculated with isolates were used as a control.

In vivo surface wound-free inoculation tests

The bacterial liquid was sprayed on healthy cucumber stalks wiped with 75% alcohol for a total of six treatments, with BPD cultures not inoculated with the isolate as control. The cucumbers were then moisturised and placed under normal growing conditions after 48 h. Observations were continued to record disease symptoms and photographs were taken with a digital camera.

Subsequently, diseased cucumber stems with similar disease symptoms to those in the field were reisolated at the disease-health junction according to Koch's postulates to determine whether the inoculated isolates were pathogenic.

Identification with cultural and morphological characteristics

The pathogenic strains were inoculated onto BPD, Yeast Extract Tryptone culture Medium (LB) and beef extract peptone Nacl media (BPN) incubated at 28 ℃ in the dark for 48 h. The morphology of the colonies was observed, recorded and photographed. Then they were stained for Gram and Spore stain and 100 randomly selected single bacterium were measured for their size.

Identification with molecular characteristics

Ten single colonies of pathogenic bacteria were picked with a splice ring and incubated in 100 ml of BPD at 28°C and 180 r for 36 h. DNA was then extracted from the pathogenic bacteria using the TIANGEN Bacteria DNA

Kit (TIANGEN BIOTECH (Bei Jing) CO, LTD). 16S ribosomal RNA (16S rRNA) (27F: 5'-AGAGTTTGATCCT GGCTCAG-3')/1492R: 5'-GGTTACCTTGTTACGACTT-3') and DNA gyrase A subunit (gyrA) (gyrA-bs1: 5'-GCGATCCTTGACATGAGGCT-3' /gyrA-bs25'-AGACGCACACCTTGAGTAC-3') [39] primers were used for amplification.

The reaction system for 16 S rRNA was 25 uL, including 12.5 uL PCR Mix, 1 uL DNA, 1 uL 1492R, 1 uL 27 F, 9.5 uL ddH_2O; the reaction system for gyrA was 25 uL, including 12.5 uL Mix, 0.5 uL DNA, 1 uL gyrA-bs1, 1 uL gyrA-bs2, 10 uL ddH_2O.

The amplified condition for the 16s rRNA region was 5 min initial denaturation at 94 °C, and denaturation at 94 °C for 1 min, annealing at 48 °C for 30 s, extending at 72 °C for 1 min, with 40 cycles, a final extension step of 1 min at 72 °C and the preservation at 4 °C; the condition for the gyrA region was 3 min initial denaturation at 95 °C, the denaturation at 95 °C for 30 s, the annealing at 58 °C for 30 s, the extend at 72 °C for 1 min, the cycles of 32, a final extension step of 5 min at 72 °C and the preservation at 4 °C。.

Finally, PCR products were detected by 1% agarose gel electrophoresis, and the PCR products with obvious bands were sent to Lanzhou Tianqi Gene Biotechnology LTD for sequencing. The multilocus sequences were compared with the sequences previously deposited in the GenBank Database using the BLAST, and both 16s rRNA and gyrA amplified sequences were found to be *B. subtilis*, and then the phylogenetic tree was constructed by MEGA 7.0 adopted with 1000 bootstrap replications to clarify the taxonomic status of pathogenic bacteria.

Host range tests

The inoculation methods are the same as for pathogenicity tests, and the cultured bacterial solution was inoculated onto melon (*Cucumis melo*), potato (*Solanum tuberosum*), tomato (*Lycopersicon esculentum*), radish (*Raphanus sativus*), chilli (*Capsicum annuum*), and corn (*Zea mays*). Using the BPD culture fluid without the pathogen as a control, and then placed in a sterilized plastic bucket to moisturize and incubate, disease symptoms were continuously observed and photographed and recorded for a total of 6 treatments, each replicated 6 times.

In vitro antagonistic activity tests

The in vitro antagonistic activity of *B. subtilis* was determined by an antagonistic culture method. The following pathogens were selected from the laboratory: *Fusarium oxysporum*, *F. verticillioides*, *F. avenaceum*, *Alternaria tenuissima*, *A. solani*, *C. scovillei* and *C. coccodes* were tested for antagonistic activity. The above pathogens were inoculated onto potato-dextrose agar (PDA) media in a 25 °C incubator for 7 d in the dark, and then perforated with a hole punch at the edge of the colony to form a 5 mm mycelial discs. It was placed in the center of the plate and four test strains were attached at equal distances on a circumference 2.5 cm from the center (i.e., standoff method) (Fig. 1). The plates were then incubated in the dark in an incubator at 25 °C. After the control dish was full, the fungal colony diameter was measured by the cross method, and each treatment was repeated seven times to calculate the inhibition rate. The mycelium at the edge of the colonies in the treatment and control groups was picked and the changes in mycelial morphology were observed under a microscope and photographed.

Inhibition rate (%)=((diameter of control - diameter of treated pathogenic fungi)/(diameter of control − 0.5))×100, where 0.5 was the diameter of fungal plugs initially inoculated on the PDA plate.

Results

Field symptoms

During field investigations in Baiyin City, Gansu Province, China, it was found that cucumber gummy stem blight can occur both cucumber stems and fruits. Among them, the disease was most severe and obvious on the stalks, and the infested area was larger. The symptoms of old stalks are drying and cracking, with loose and whitish internal tissues (Fig. 2A and B). Newly grown stalks showed symptoms of wilting, yellowish-brown colour with a watery surface of the stalks (Fig. 2C, D and E). Cucumber fruits were cut open to a light yellowish-brown cracked appearance(Fig. 2F).

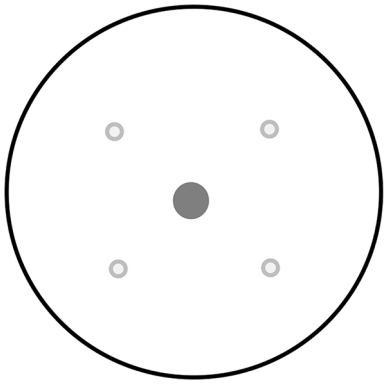

Fig. 1. In vitro antagonistic activity test method. Gray represents the pathogenic fungi tested for antagonistic activity

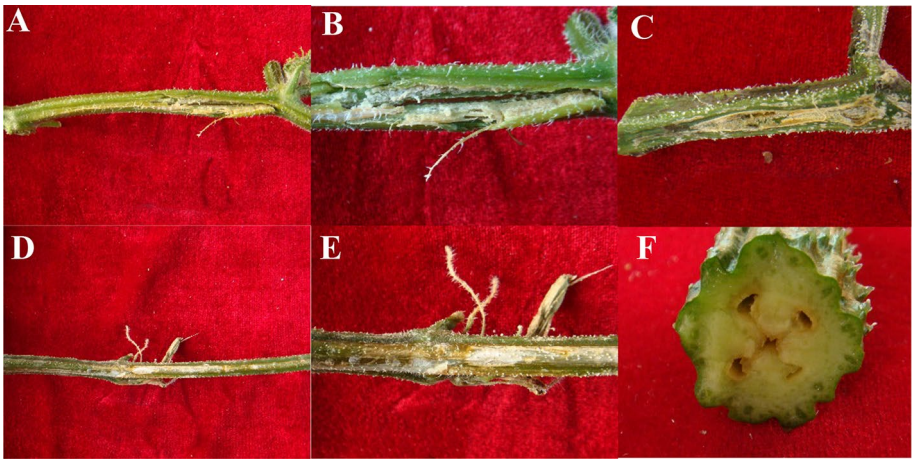

Fig. 2. A gelatinous secretion produced on the stalks and fruits of cucumber, found in the field. **A**–**E** symptoms on the surface of cucumber stems (the stems were wilted, yellowed and split). **F**, symptoms on the fruit of cucumber

Isolation of pathogen and pathogenicity tests

Typical symptomatic and healthy stalks were brought back to the laboratory for pathogen isolation. Three isolates, named HlJ-1, HlJ-2 and HlJ-3, were obtained from diseased stalks and no strain was isolated from healthy stalks. The three isolates were then inoculated into cucumber stalks and only HlJ-3 was found to cause pathogenicity in cucumber stalks, and when the diseased stalks were reisolated, the reisolates were identical to the isolates in terms of morphological characteristics.

Symptoms of HlJ-3 inoculation were as follows: on the fifth day of inoculation with isolate HlJ-3 under needling conditions, small yellow-brown cracks appeared on the cucumber stalks (Fig. 3f, g, and h). As the disease expanded, the aboveground cucumber leaves turned yellow and withered (Fig. 3b, c, d, n, o and p), on the 10th day after inoculation, the stalks cracked and brown secretion appeared at the cracks (Fig. 3j, k, l, r, s t and supplementary Fig. 3), whereas the control did not develop the disease (Fig. 3a, e, i, m and q). Under non-needling conditions, the onset symptoms were consistent with the above description, with the difference that visible symptoms could be observed on day 15 after inoculation (Fig. 4). By reisolating the onset site, colonies consistent with isolate HlJ-3 were obtained (Fig. 5g and h). Thus, pathogenicity tests showed that isolate HlJ-3 is the pathogen responsible for cucumber stem disease.

Identification of morphological characteristics

The pathogenic bacterium HlJ-3 was inoculated onto BPD, BPN and LB media by plate scribing method, and incubated for about 48 h at 28 °C under dark conditions (Fig. 5a-d). Single colonies were found to be dirty white, rough and opaque on the surface, with central folds and spreading irregular edges, and a ring of light red pigment near the edge of the colony (Fig. 5g, h, i, and j). Single colonies were picked out for Gram staining and found to be Gram-positive with rod-shaped bodies, size (0.33–0.60) μm × (1.92–5.47) μm. It was found by Spore stain that bacteria were purplish red when stained, and endospores were colorless or light green (Fig. 5e and f). According to morphological observation, HlJ-3 colony morphology was found to be consistent with the morphology of *B. subtilis* in the *Bergey's manual of determinative bacteriology*, which was initially identified as *B. subtilis*[27].

16s rRNA and gyrA sequence analysis

16 S rRNA, 16 S ribosomal RNA gene; gyrA, DNA gyrase A subunit. HlJ-3 was the pathogen of the study.

The DNA of strain HlJ-3 was amplified by 16 S rRNA and gyrA. The 1444 bp and 999 bp amplified fragments were obtained by 1% agarose gel electrophoresis, respectively. The 16 S rRNA (OP341449.1) and gyrA (OQ803359.1) sequences of strain HlJ-3 showed 100% homology with the *B. subtilis* sequences, respectively. The results of phylogenetic tree indicated that strain HlJ-3 clustered with *B. subtilis* with a bootstrap value of 99% and 100% (Fig. 6A and B). Based on the morphological and molecular characteristics, strain HlJ-3 was identified as *B. subtilis*.

Host range assays

In order to further investigate the pathogenicity of the HlJ-3 strain, the host range of the pathogen HlJ-3 was assessed by inoculating six common plants, namely Melon, Potato, Tomato, Corn, Radish and Chilli. Under pinprick conditions, the stems of all five plants, except maize, were infested (Table 1). Symptoms appeared on the 3rd day after inoculation in melon, radish and chilli, on the 7th day after inoculation in potato and on the 5th day after inoculation in tomato. We observed that the disease symptoms were evident on the 14th day after inoculation on the affected stalks (Fig. 7a to f). Cracking and brownish-yellow gelatinous secretion appeared on the surface of the stalks of melon and radish, whereas ulceration, melting and rotting appeared on the surface of the stalks of tomato, potato, and chilli, while none of the inoculated control plants developed the disease.

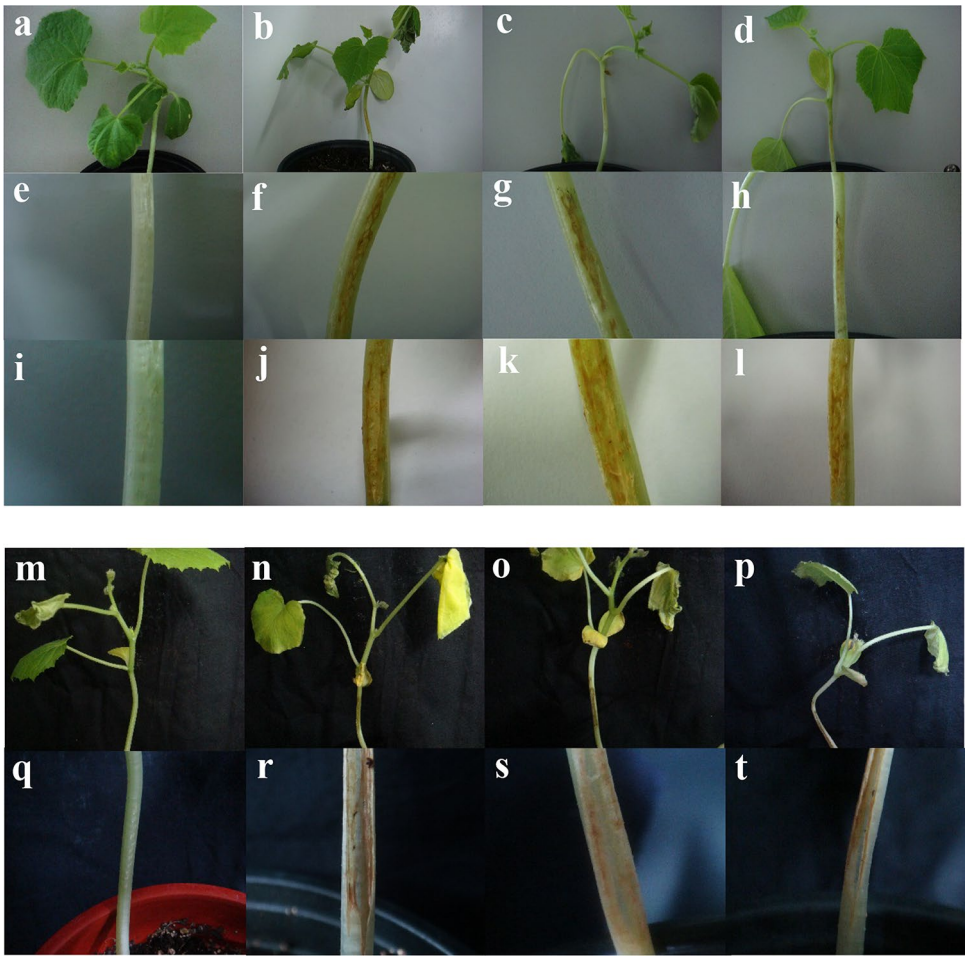

Fig. 3. Pathogenicity of isolate HlJ-3 in cucumber stalks with surface wound inoculation (vivo inoculation tests). **a**, **e**, **i**, **m** and **q** represent in vivo controls with surface wounds that showed no disease symptoms; **b**, **c**, **d**, **n**, **o** and **p** represent yellowing and wilting of foliage, splitting of stalks, and yellowish brown of cucumber when cucumber stalks were inoculated with HlJ-3 by pinprick inoculation 10 days after inoculation; and **j**, **k**, **l**, **r**, **s** and **t** represent localised symptoms of cucumber stalks inoculated with HlJ-3 10 days after inoculation, w stand for the third pathogenicity, the leftmost stalk is CK.

Fig. 4. Pathogenicity of isolate HlJ-3 in cucumber stalks with surface non-wound inoculation (vivo inoculation tests). **a** and **e**, the control in vivo pathogenicity about surface non-wound inoculations, no disease symptoms appeared. **b**, **c** and **d** represent the disease status of the whole cucumber plant 15 days after inoculation with HlJ-3 under non-needling conditions; **f**, **g** and **h** represent the disease status of the inoculation site.

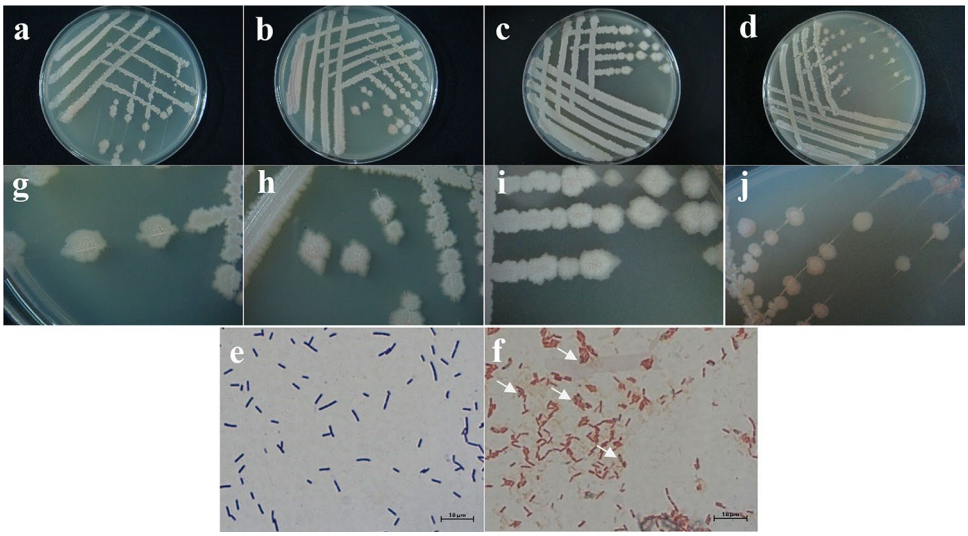

Fig. 5. Morphological characteristics of strain HlJ-3. **a** and **g**, represent morphological characteristics of the proto-bacteria isolated from field samples and inoculated into NA medium. **b** and **h**, Morphological characteristics of the pathogenic bacteria obtained after isolate HlJ-3 was inoculated onto Cucumber stalks and caused disease in *Cucumber* and then re-inoculated into NA medium. **c** and **i**, represent the morphological characteristics of the colonies obtained after inoculating the pathogen on LB plates. **d** and **j** represent the morphological characteristics of colonies inoculated on NB plates. **e** represents the characteristics of the pathogenic bacteria HlJ-3 after Gram staining. **f** represents the characteristics of the pathogenic bacteria HlJ-3 after Spore stain, and the direction of the arrow is the stained bacteriophage.

Under non-needling conditions, 14 days after inoculation, stalks of maize, melon, and radish did not show any disease symptoms (Fig. 7g, j and k), whereas potato, tomato, and chilli were not as severe as under needling conditions, with only 1–2 plants showing localised tissue lysis on the surface of the stalks (Fig. 7h, i and l), and none of the inoculated control plants developed any disease. In the Fig. 7, m and n represent different treatment models for different crop varieties. Finally, reisolation of the above-diseased plants yielded strains consistent with the inoculated strain HlJ-3, indicating that HlJ-3 is the pathogen. The above results indicated that among these six hosts, maize plants were not infested under both needling and non- needling conditions, suggesting that the pathogen is not pathogenic. For the remaining five plants, on the other hand, it was strongly pathogenic under needling conditions.

In vitro antagonistic activity test of HlJ-3

Since *B. subtilis* is a good biocontrol agent in the majority of studies, we tested the pathogen for antagonistic activity in vitro in order to verify whether it also functions as a biocontrol agent. The results of the plate standoff assay showed that strain HlJ-3 had different levels of antagonistic activity against the seven pathogenic fungi tested: *F. oxysporum*, *A. tenuissima*, *A. solani*, *C. scovillei*, *F. verticillioides*, *F. avenaceum* and *C. coccodes.*

Among them, the greatest inhibitory effect was observed on *A. tenuissima* and *C. coccodes* with obvious circles of inhibition (Fig. 8) whose inhibition rates were 69.92% and 68.08%, respectively; the inhibitory effect on *C. scovillei* and *A. solani*, the inhibitory effect was also more evident, with inhibition rates of 60.83% and 62.49%, respectively; while *F. oxysporum*, *F. verticillioides* and *F. avenaceum* had the worst inhibitory effect, with inhibition rates of 53.59%, 47.84%, and 44.29%, respectively (Table 2).

Effect of strain HlJ-3 on mycelial growth of pathogenic fungi: Mycelia at the junction of strain HlJ-3 and pathogenic fungi were picked and observed under the microscope. As can be seen in Fig. 8, compared with the control, the mycelium of *C. coccodes*, *A. tenuissima*, *A. solani* and *C. scovillei* showed different degrees of breakage, disappearance of septa, curved expansion and protoplasmic spillage after treatment with HlJ-3, among which the mycelium of *C. coccodes* had the greatest degree of ring-breaking, and the vesicles appeared in the middle of the mycelium. The effects on the mycelium of *F. oxysporum*, *F. verticillioides* and *F. avenaceum* were not significant.

The above results showed that HlJ-3 had strong teratogenic and bacteriostatic effects on four fungi (i.e., *C. coccodes*, *A. tenuissima*, *A. solani*, *and C. scovillei*), which suggests the potential biocontrol function of HlJ-3 (Fig. 8).

Discussion

A popular vegetable in China, cucumbers are produced for their anti-aging, diuretic, and oedema properties, as well as for their ability to dissipate heat and ease coughs. China is the world's largest producer of cucumbers, with the largest planted area[40]. Although cucumbers are highly sought after in China, their quality and output have been negatively impacted by a number of diseases[41]. In this study, a cucumber disease was discovered in the field in Baiyin City, Gansu Province, China, which primarily infested cucumber stalks, its main symptoms were

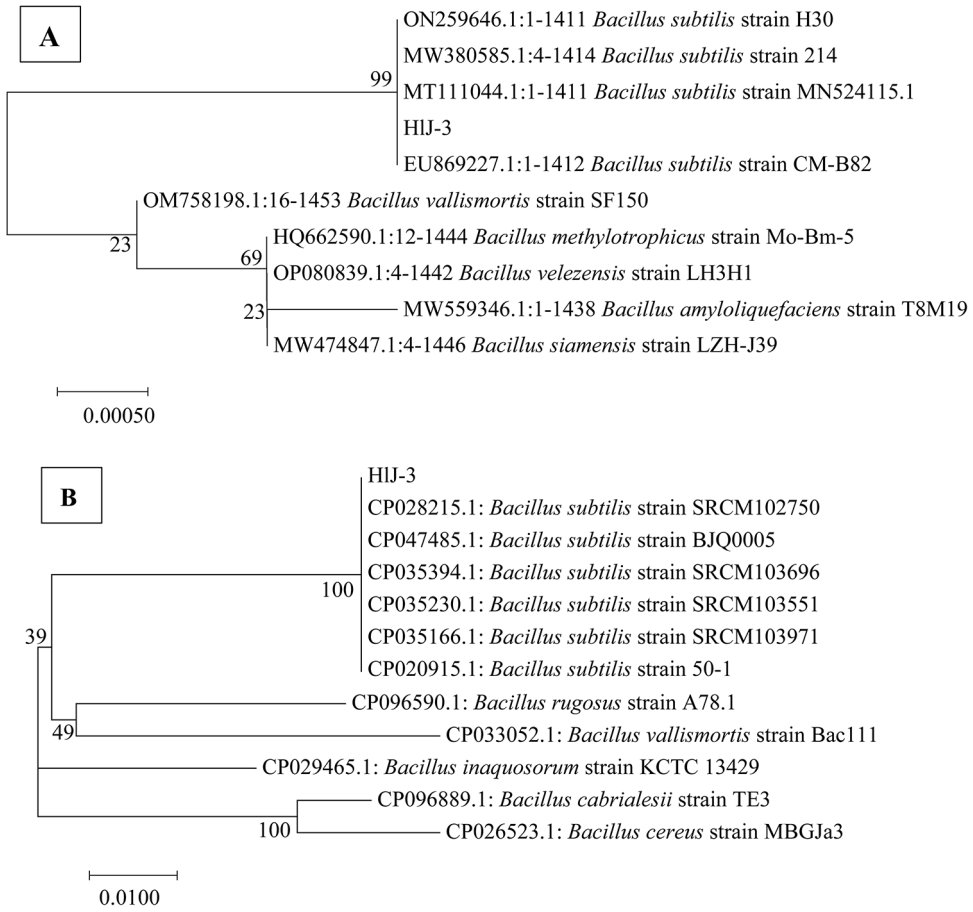

Fig. 6. Phylogenetic tree of *B. subtilis* strain HlJ-3 combined 16 S rRNA with gyrA. **A** amplifed by 16 S rRNA Primer; **B** amplifed by gyrA Primer.

longitudinal surface cracking and drying, and its tissues were loose and whitish. The strain HlJ-3 was verified as the causative agent by Koch's postulates, and was identified as *B. subtilis*. *B. subtilis* is generally considered non-pathogenic, and most of the laboratory cultures are considered contaminating or endophytic bacteria[42]. Since it is not generally pathogenic, three subsequent pathogenicity assays were performed in this study (The third pathogenicity results are shown in Supplementary Fig. 3), and all tests revealed that *B. subtilis* HlJ-3 was the pathogenic agent.

After inoculating cucumber stalks with HlJ-3, the disease firstly appeared as water-soaked, depressed onset site with a large amount of white to light yellow pus overflowing when humidity was high; later, the disease progressed to a middle stage where the stalks cracked and rotted longitudinally, leaving white scars at the onset site when humidity was lowered. Finally, the disease showed symptoms of soft-rotting and eventually caused the entire plant to wilt. The symptom was similar to the symptoms of the disease in the field while the control did not have the disease, which is similar to the symptoms of Melting Decay of 'Red Globe' Grapes[32], and leaf mottle of sunflower reported in California and Northern Kazakhstan[29], and the pathogen of both diseases is *B. subtilis*. However, compared with the symptoms in the field, the disease conditions were slightly different, which might be due to the different environmental conditions, host resistance, and the period when the pathogen infested the hosts, and the exact causes need to be further investigated.

Morphological culture and molecular identification are essential for differentiating complicated species within a genus for identifying diseases[43,44]. Since molecular biology technology has advanced recently, PCR and multigene identification techniques have become commonplace for species identification. These techniques allow for the quick identification of species based on their affinities[45–47]. However, it is hard to reliably discriminate between some subspecies and relatives of *Bacillus* spp. due to the variability of the 16s rRNA gene. To quickly identify species and analyze the evolutionary and genetic links between bacterial species, phylogenetic analyses have been used to 16s rRNA, gyrB, ropB, cheA, and gyrA gene sequences or gene fragments. The housekeeping genes gyrB, ropB, cheA, and gyrA, on the other hand, exhibit significantly higher genetic divergence[48,49] because they are highly conserved and only exist in a single copy in all bacteria[50]. The gyrA gene may be utilized as a phylogenetic marker to differentiate between the *B. subtilis* group and related relatives because it encodes the DNA promoter enzyme A subunit, which regulates DNA superhelix and replication start[51]. This gene compensates for the limitations of the 16s rRNA gene. As a result, the gyrA gene gives a greater degree of differentiation compared to the gyrB, rpoB, and cheA genes [39]. Following this, HlJ-3 morphology was found to be

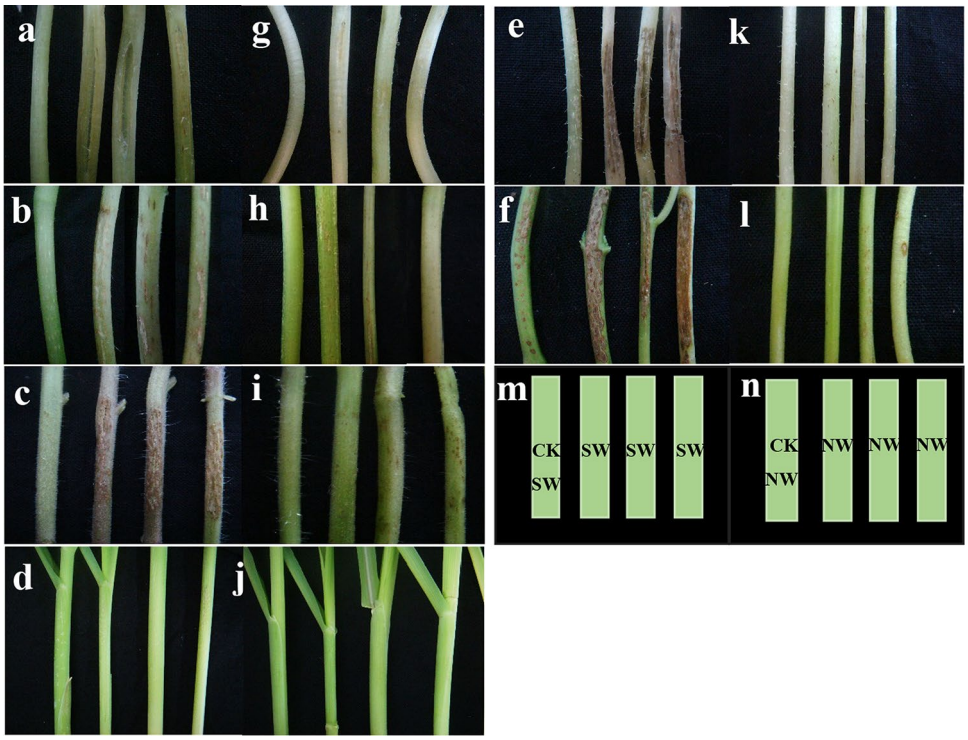

Fig. 7. Lesions of infection by *Bacillus subtilis* HlJ-3 on melon, potato, tomato, corn, radish and chilli stems 14 days after surface wound and non-wound inoculation (a to f for needle inoculation, g to l for non-needle inoculation). m and n, models of different treatments for different crop species are in this figure. SW; Surface wound inoculation *Bacillus subtilis* HlJ-3. NW; Surface Non-wound inoculation *Bacillus subtilis* HlJ-3, CK (SW and NW) was inoculated with blank cultures.

Crop name	Scientific name	Family	Inoculation tissue	Degree of incidence [g]	
				SW [e]	NW [f]
Melon	*Cucumis melo*	*Cucurbitaceae*	Stem	+++	-
Potato	*Solanum tuberosum*	*Solanaceae Juss*	Stem	+++	+
Tomato	*Lycopersicon esculentumMill.*	*Solanaceae Juss*	Stem	+++	+
Corn	*Zea mays*	*Poaceae*	Stem	-	-
Radish	*Raphanus sativus*	*Brassicaceae*	Stem	+++	-
Chilli	*Capsicum annuum*	*Solanaceae Juss*	Stem	+++	+

Table 1. The pathogenicity of *B. subtilis* HlJ-3 in 6 crops 14 days after surface wound and non-wound inoculation. [e] SW; Surface wound inoculation. [f] NW; Surface Non-wound inoculation. [g] Strain HlJ-3 infection host ability. + Represents 1–2 plants with disease, ++ represents 3–4 plants with disease, +++ represents 5–6 plants with disease, -represents no plant disease.

essentially comparable with that of *B. subtilis*, as documented in Berger's manual of bacterial identification[27,52]. 16s rRNA and gyrA were utilized in this work to differentiate the taxonomic status of pathogenic bacteria to more precisely identify pathogenic bacteria. With bootstrap values greater than 99%, it was discovered that both gene sequences were grouped in the phylogenetic tree and were highly similar to *B. subtilis*, with more than 99% homology. Therefore, based on morphological characteristics and molecular biology, HlJ-3 was identified as *B. subtilis* causing cucumber gummy stem blight disease. To the best of our knowledge, this is the first report of *B. subtilis* causing cucumber gummy stem blight.

Six popular crops from four families—*Cucurbitaceae*, *Solanaceae*, *Brassicaceae*, and *Poaceae*—were chosen for the current study in order to further investigate the pathogenicity of HlJ-3 due to the paucity of references on the disease-causing potential of *B. subtilis*. Under needling conditions, HlJ-3 infected every inoculated crop in the research, except for maize. Three *Solanaceae* crop varieties—potato, tomato, and chilli—were shown to be infected in both wounding and non-wounding environments, suggesting that *B. subtilis* is a potent *Solanaceae* plant pathogen. HlJ-3 infested radish and melon under needling conditions, but strain HlJ-3 did not infest

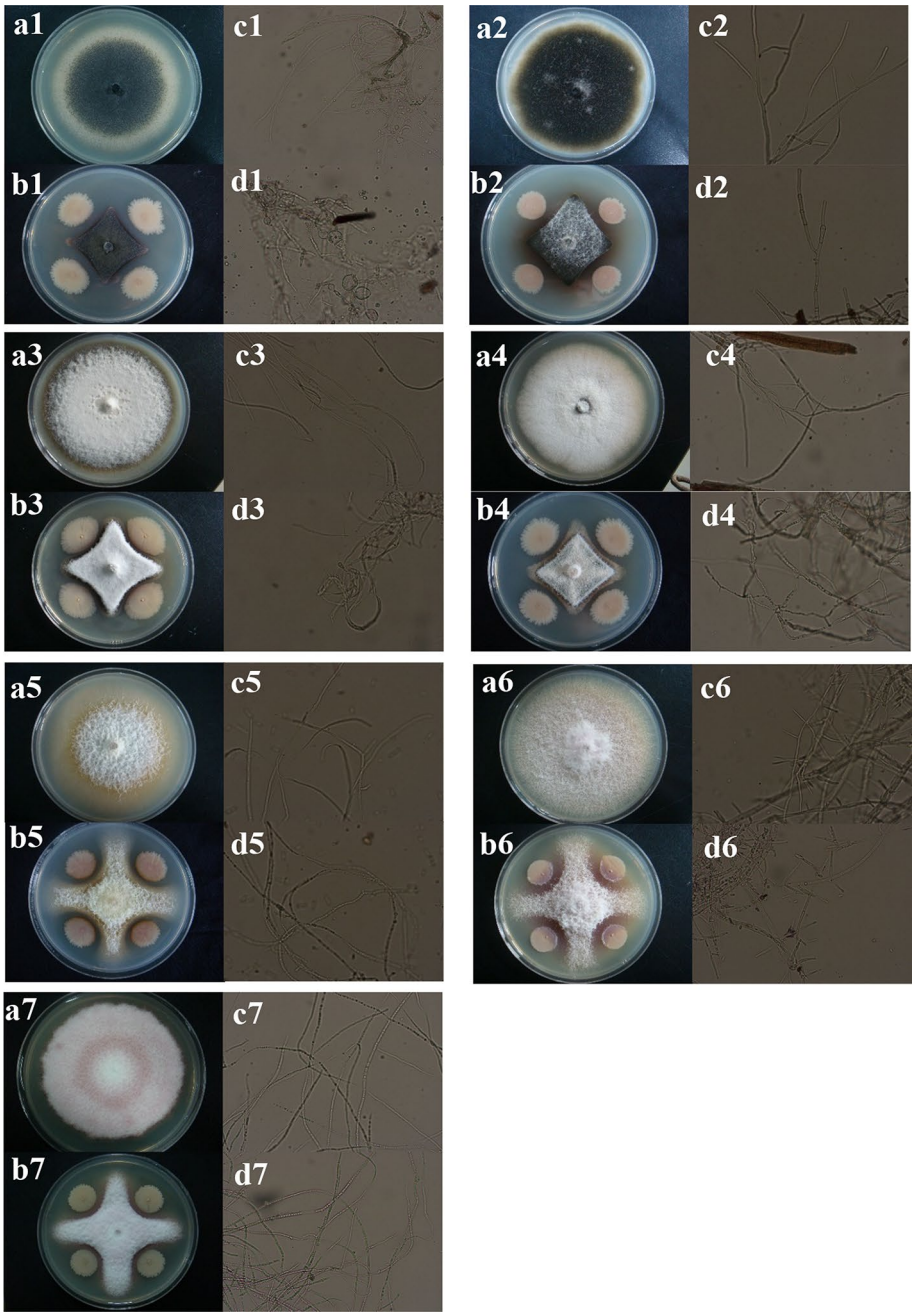

Fig. 8. In vitro biocontrol of fungi by *B. subtilis* strain HlJ-3. The effects of HlJ-3 on *C. coccodes* (a1), *A. tenuissima* (a2), *A. solani* (a3), *C. scovillei* (a4), *F. avenaceum* (a5), *F. verticillioides* (a6), *F. oxysporum* (a7) for biological control. Pathogenic fungi and HlJ-3 were double-cultured on the same potato dextrose agar plate with a distance of 2.5 cm between them, and in the image, the pathogenic fungi are in the center, and HlJ-3 is around; a1 to a7 are negative controls, and the plates were cultured with a single pathogenic fungus. d1 to d2 are the effects of HlJ-3 on the mycelium of seven pathogenic fungi after standoff culture, respectively. c1 to c7 are mycelial controls, respectively.

Brassicaceae radish and *Cucurbitaceae* melon under non-needling conditions, suggesting that strain HlJ-3 is an opportunistic pathogen. Among the six crops studied above, HlJ-3 (*B. subtilis*) infestation of potato and tomato has been reported, but not in chilli, radish and melon.

As *Bacillus subtilis* is often considered as in vitro biocontrol agent against a wide range of diseases including *Colletotrichum spp*[53]. , *Fusarium spp*[54], *Alternaria spp*[55] , *Botrytis spp*[56]. and *Sclerotium spp*[57]. Therefore, in the present study, strain HlJ-3 (*B. subtilis*) was evaluated using in vitro dual culture method against pathogenic fungi *Fusarium oxysporum*, *F. verticillioides*, *venaceum*, *A. tenuissima*, *A. solani*, *C. scovillei* and *C. coccodes*. The average antagonism value was greater than 58.15%, indicating that strain HlJ-3 possessed broad-spectrum antimicrobial activity, and HlJ-3 showed the greatest inhibition of *A. tenuissima* and *C. coccodes*, which was

Pathogenic fungus	Strain HlJ-3 [c]		
	Diameter of CK colony(cm)	Diameter of treatment colony(cm)	Inhibition rate(%)[l]
C. scovillei (Capsicum annuum)[d]	7.32 ± 0.08b	3.17 ± 0.04c	60.83 ± 0.52b
A. solani (Solanum tuberosum)[e]	8.08 ± 0.09a	3.34 ± 0.03c	62.49 ± 0.39b
F. verticillioides (Zea mays)[f]	8.27 ± 0.02a	4.83 ± 0.20a	44.29 ± 2.5e
F. avenaceum (S. tuberosum)[g]	8.18 ± 0.03a	4.06 ± 0.03b	53.59 ± 0.42c
A. tenuissima (C. melo)[h]	8.17 ± 0.08a	2.81 ± 0.05d	69.92 ± 0.64a
C. coccodes (S. tuberosum)[i]	7.37 ± 0.02b	2.69 ± 0.03d	68.08 ± 0.43a
F. oxysporum (Allium tuberosum)[j]	6.95 ± 0.24c	3.86 ± 0.08b	47.84 ± 1.19d
Average [k]	7.76	3.54	58.15

Table 2. The inhibition rate of *B. subtilis* against pathogenic fungi (%). [c] Strain HlJ-3 was the pathogen of the study. [k] Average represents the overall antibacterial status of HlJ-3 against the 7 pathogenic fungi in the table. [d~j] Host of pathogenic fungi. [l] Data are presented as the means ± SE of seven replications. Different letters indicate the results of Duncan's multiple range te ($P < 0.05$).

more than 68%. In contrast, lower inhibition rates were reported in the studies of Wu and Lee[58,59]. Meanwhile, we observed a significant effect of HlJ-3 on the mycelial growth of *A. tenuissima* and *C. coccodes*, as evidenced by mycelial rupture, vesicles, curling, and swelling (Fig. 8), which is in agreement with the effects of *B. subtilis* on the mycelium of pathogenic fungi reported by Khan and Zhang[60,61]. These results suggest that strain HlJ-3 has special biocontrol properties and broad-spectrum antimicrobial effects against various common pathogenic fungi.

Generally, this is the first report of *B. subtilis* induced cucumber gummy stem blight disease in China. It offers a theoretical foundation for the disease's diagnosis and comprehensive control, but more research is still needed to determine the pathogenic mechanism. In the meantime, its virulence to host plants should not be disregarded when *B. subtilis* is employed as a biological microbe for agricultural development, based on the pathogenicity of *B. subtilis* on cucumber in this work. Therefore, in order to lessen the drop in agricultural output brought on by the employment of *B. subtilis* as a biological agent, it is imperative to further validate the pathogen's host range. Additionally, owing to the broad-spectrum antimicrobial activity of *B. subtilis*, effective antagonistic substances can be extracted from it for further bioanalysis in crop production.

Data availability

Sequence data that support the findings of this study have been deposited in the NCBI repository with the GenBank accession numbers: 16 S rRNA, OP341449; gyrA, OQ803359.

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Author contributions

CY, and MJ conceived and designed the study. YW performed most of the experiments.FC, TM, CW and NQ helped carry out experiments.CY and RO revised the manuscript and provided critical discussions.All authors contributed to the study and approved this submission.

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Declarations

Competing interests

The authors declare no competing interests.

Research involving plants

The plant material collected for this test, as well as the plants for the test, complied with the relevant laws and regulations.

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Additional information

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