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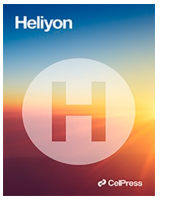

Research article

Exogenous inoculation of endophyte *Penicillium* sp. alleviated pineapple internal browning during storage

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ABSTRACT

Pineapple is ranked sixth in terms of global fruit production and the most traded tropical fruit worldwide. Internal browning (IB), a physiological disorder of pineapple fruit after harvest, limits the export and industry development of pineapple. Evidence confirmed that endophyte played a pivotal role in plant disease. This study investigated the relationship between endophyte fungi community structure, population abundance in healthy and IB pineapple fruit; as well as the effect of endophyte *Penicillium* sp. inoculation on pineapple IB. Intended to explore a new effective measure for controlling IB and reducing postharvest losses in pineapple by an economical and environmentally friendly approach. We found the abundance of endophyte fungi in healthy pineapple fruit was different from that in IB fruit by high-throughput sequencing. The results emphasized that the endophyte *Penicillium* sp. inoculation dramatically alleviated pineapple IB intensity and severity, delayed crown withering and fruit yellowing, and maintained the exterior quality traits during the postharvest period at 20 °C. *Penicillium* sp. retarded H_2O_2 accumulation and increased the total phenols level in pineapple. Application of *Penicillium* sp. also maintained the higher antioxidant capacity by increasing antioxidant enzyme activity and ascorbic acids levels, regulated of the homeostasis of endogenous hormones, and increased the abundance of *Penicillium* sp. in the fruit. In summary, *Penicillium* sp. retarded the occurrence of IB and enhanced the storability of pineapple at postharvest, and this economical and environmentally friendly technology is convenient to spread in agriculture.

1. Introduction

Pineapple (*Ananas comosus* (L.) Merr.) is a commercial tropical fruit which is ranking sixth in world-wide fruit production [1] and the most traded global tropical fruit [2]. It is also known for its good source of nutrition [1]. Internal browning (IB) is known as 'black heart', which is a physiological disorder of pineapple at postharvest [3]. Most pineapple varieties suffer from IB during postharvest storage periods [4,5], and IB often causes economic losses for the producers [5]. Food and Agriculture Organization of United Nations

Abbreviations: IB, internal browning; ROS, reactive oxygenspecies; TPC, total phenolic compounds; ABA, abscisic acid; GA, gibberellin; IAA, indoleacetic acid; ISR, induced systemic resistance; ACC, 1 - aminocyclopropane - 1- carboxylate; PDA, potato dextrose agar; PCR, the polymerase chain reaction; NCBI, the National Center of Biotechnology Information; H_2O_2, hydrogen peroxide; AsA, ascorbic acid; PPO, polyphenol oxidase; POD, peroxidase; SOD, superoxide dismutase; GR, glutathione reductase; CAT, catalase; GSH, glutathione; O_2^-, superoxide radical.

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indicated that the max loss of pineapple was 49% at postharvest in pineapple producing countries in 2021. Pineapple is often consumed fresh, while IB is a major problem faced by local growers during distant transportation of fresh pineapple and thereby limits the export potential of fruit [5].

Traditionally, the chemical agents and fungicides are high efficiency in controlling plant disease [6,7], whereas, many of them may lead pathogens to develop fungicide resistance [6,8]. Furthermore, the chemical agents and fungicides may created a significant threat to environment [7,9], animal and human health [9–11]. Biological control agent is an environmentally friendly and economical strategy alternative to alleviate plant diseases, including the application of endophytes [12].

Accumulating evidence established that certain endophytes play vital roles in controlling plant disease [13,14] and stress [15,16]. Endophyte control plant diseases and stress to induce an induced systemic resistance (ISR) of plant [12] and directly make an antagonistic effect on the pathogen [12,17]. The directly effects mainly include: producting secondary metabolites [15,17,18], such as antibiotics [19](Deng et al., 2011), enzymes [20], phytohormones [15,21], and alkaloids [22]; enhancing the resistance of host plants [23,24]; protecting against pathogens [18,25,26].

Previous studies found that chili endophyte bacteria could induce ISR and control bacterial wilt of chili [27]. Acuna-Rodriguez et al. [15]provided convincing evidence that the bacterial endosymbionts enhanced plant tolerance to cold and freezing stresses by impacting photosynthesis and trehalose and raffinose accumulation levels in the host. Tiryaki et al. [20] pointed out that endophytes in the leaf can increase tolerance of cold-sensitive crops by producing ACC (1-aminocyclopropane-1-carboxylate) and stimulating antioxidant enzymes. The endophyte *Streptomyces* strains protected wheat plants against the pathogen *Gaeumannomyces tritici*, which is a causative agent of take-all disease [25]. In recent decades, researches have emphasized that *Achnatherum inebrians-Epichloe* endophyte could enhance stress tolerances [22,28,29] and disease resistance [29,30] of drunken horse grass.

Interestingly, some endophytes exhibited broad spectrum antifungal activity [25,29,30]. For instance, *Bacillus velezensis* possess strong antagonistic activity against *Ralstonia solanacearum* and *Fusarium oxysporum* via lipopeptides compounds [17]. The growth of *Alternaria solani*, *Helminthosporium tritici-vulgaris*, *Curvularia lunata*, *Fusarium avenaceum*, *Fusarium solani* and *Trichoderma viride* were obviously inhibited by the extract of endophytes endosymbionts of *Achnatherum inebrians* [31]. However, up to now no studies have shown that plant endophytes can control plant physiological disorders by regulating the endophytes in plants. However, whether endophytes could control plant physiological disorders has not been reported except for that endophyte fungus *Aspergillus niger* can alleviate IB [32].

Harvested fruit and vegetables are a good source of water and nutrition content [1,2], and therefore, it is highly perishable causing great economic losses [2]. Postharvest loss of pineapple fruit is a global concern [2]. Various methods have been used to controlling pineapple IB [33–37]; however, there are a few reports of endophyte controlling IB except for endophyte *A. niger* [32]. Especially, whether the community structure and population abundance of endophytes in pineapple are related to IB has not been reported.

In order to solve the problems caused by chemical agents, find a new method to control pineapple IB reduce postharvest loss and explore the mechanism of controlling IB, we try to find endophyte fungi that can alleviate IB, and explore its mechanism.

In this study, we found that the endophyte *Penicillium* sp. can alleviate internal browning, a physiological disorder, and first proved that the relative abundance of *Penicillium* sp. in pineapple fruit was negatively correlated with the severity of IB. This result provides a new potential for developing effective and environmentally friendly measures for controlling IB in pineapple and reducing postharvest losses.

2. Materials and methods

2.1. Plant materials and treatments

2.1.1. Plant materials

Pineapple (*Ananas comosus* L. cv 'Comte de Paris') fruit at maturity of 70% [37] and size of about 500 g, free of disease or insect symptoms and physical injury, were harvested from a commercial plantation in Xuwen County, Guangdong Province, China.

2.1.2. Treatments of pineapple

Experiment 1, pineapple fruit were randomly divided into three groups, with 23 fruit for each group, then packed into polyethylene bags (50 μm) and stored for 12 days in darkness at 20 °C and 90–95% RH. Samples of pulp tissues, each sample consisting of a single pineapple from a replication were collected at 0 days (St.0d) and 12 days (St.12d), and each group were selected two fruits randomly. The twelve samples were then pooled, frozen in liquid nitrogen, and stored at −80 °C for high-throughput sequencing.

Experiment 2, *Penicillium* sp. was incubated for 11 days at 28 °C with darkness. Spore suspension of fungus endophyte *Penicillium* sp. (5×10^7 spores·ml^{-1}) was sprayed as fine mist to the pineapple fruit and crown until run off and air-dried. These were sprayed with sterile water as control. Each treatment was applied to three biological replicates, with 14 fruits for each replicate. Following inoculation, the pineapples were wrapped with polyethylene bags (50 μm) and kept in darkness at 20 °C and 95% RH for evaluation of severity of internal browning. Samples of pulp tissues, each sample consisting of a single pineapple from a replication were collected at 0 days (namely untreated), 3 days, 6 days, and 9 days and then pooled, frozen in liquid nitrogen, stored at −80 °C.

2.2. Internal browning severity assessment

2.2.1. Assessment of the internal browning index (IB index)

During storage, 1 pineapple fruit was randomly selected from each replicate every 3 days and cut along the longitudinal plane of the

fruit axis to observe IB incidence. When all 3 pineapple fruits in the control group had IB and there are at least two pineapples with IB index greater than 2, all pineapple fruits for observation were cut and IB index was analyzed. IB index was graded from 0 to 4 [38] based on the percentage of longitudinal section area with browning patches, 0 (<5% signs of longitudinal section area with browning patches), 1 (6–25%), 2 (26–50%), 3 (51–75%) and 4 (>75%). IB index = $\sum$ [($N_Y \times Y$)]/N, where Y represents browning severity (0–4), N_Y represents the number of pineapple fruit with the corresponding severity score and N represents the number of total fruits. 10 pineapple fruits were used for the determination of IB index in each treatment, with three replications.

2.2.2. Assessment of the internal browning incidence (IB incidence)

IB incidence was examined as we described previously [37].

2.3. High-throughput sequencing of pineapple pulp

We analyzed the composition of endophyte fungi in pineapple pulp by high-throughput sequencing. Total genome DNA from each sample in experiment 1 was extracted using CTAB method [39].

The nuclear ribosomal internal transcribed spacer region (ITS1-5F) was amplified by polymerase chain reaction (PCR) using the primer set for ITS5-1737F (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS2-2043R (5′-GCTGCGTTCTTCATCGATGC-3′). We constructed library using NEB Next® Ultra DNA Library Prep Kit. The constructed libraries were quantified by Qubit and q-PCR. After the qualified libraries were detected, NovaSeq 6000 (Illumina, San Diego, CA, USA)was used for high-throughput sequencing.

2.4. Endophyte fungal isolation and cultivation

Endophytic fungi were isolated from healthy pineapple pulp, as described previously with modification [40–42]. Pineapple pulp tissue was cut into pieces (5 cm × 5 cm) after fruit surface disinfection. Pieces was soaked with 5% sodium hypochlorite for 1 min, Then pieces were washed in sterilized distilled water three times. The segments were triturated with sterilized mortar and pestle, then soaked with sterilized distilled water for 10 min. The pineapple juice of gradient dilution was inoculated on potato dextrose agar (PDA) and incubated at 28 °C in dark. The organisms based on visual characteristics of the colonies were inoculated by triple re-streaking on fresh PDA until there is a single colony. All instruments being used for isolating and incubating endophytes are autoclaved (121 °C, 20 min). The strain was labeled B3.

The single colonies were transferred to fresh PDA for identification and maintained in paroline at 4 °C.

2.5. The endophye fungal stain identification

2.5.1. Morphology observations of the endophye fungal stain

The isolated endophye from pineapple was identified on the basis of their morphological characteristics including conidia and conidiophores mycelia structures using a biological microscope.

2.5.2. Molecular phylogenetic analyses of the endophye fungal stain

The B3 strain was grown on PDA for 11 days, mycelia were triturated with sterilized mortar and pestle. Approximately, 0.1 g of the mycelia powder was used for DNA extraction according to the method by Sim et al. [43] with slight modification. Characterization of fungus endophyte was performed based on the conserved nuclear ribosomal internal transcribed spacer region (ITS rDNA) sequence analysis [44]. The partial nucleotide base-pair fragment of the ITS rDNA gene from B3 was amplified using PCR with universal primers set for ITS1-F (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4-R (5′-TCCTCCGCTTATTGATATGC-3′). The PCR was carried out 2 × Tolo FastTaq Premix (TOLOBIO, 21401-2) according to the manufacturer's protocol. The amplified PCR product was detected by electrophoresis in a 1% agarose gel (TOLOBIO, China, #21401/21411), which was then stained with ethidium bromide (0.5 μg ml^{-1}), and the amplicon was visualized under a gel documentation system (BIO-RAD Gel Doc™ XR $^+$ Alpha Innotech, San Leandro, USA). The gel section with desired band was carefully excised under UV light and carried out extraction using an SanPrep Column DNA Gel Recovery Kit (SK8131, Sango Biotech) according to the manufacturer's instructions. After PCR, the PCR product was sent to a service provider for sequencing. The sequences obtained from B3 were discovered by making BLAST searches on the National Center of Biotechnology Information (NCBI) GenBank database. Subsequently, multiple sequences obtained were performed ClustalW analysis using MEGA 6.0 software for the phylogenetic analysis. We used maximum likelihood method by combining dataset of ITS regions to generate phylogenetic tree.

2.6. The relative abundance of Penicillium sp. evaluation

2.6.1. DNA extraction

The pineapple pulp samples came from experiment 1. The DNA from pineapple pulp was extracted by CTAB, following the manufacturer's instructions of DNA Extraction Kit (Nobleryder, China).

2.6.2. Real-time quantitative polymerasechain reaction (RT-qPCR)

RT-qPCR was used for the analysis of the relative abundance of *Penicillium* sp. in pineapple pulp [32]. The 5.8s gene (F, 5′-TCGAGTCTTTGAACGCACAT-3′, R, 5′-ATGTGACAAAGCCCCATACG-3′) was used as a reference gene (that is actin primers) to

correct the relative abundance. Primer set for RT-qPCR was F, 5′-TCCGAGGTCAACCTGGTTAAGA-3′ and R, 5′-TGCCTGTCCGAGCGTCATT-3'. And the reactions were run in CFX96™ Optics Module (Bio-rad, USA). Three replicates of each sample were analyzed. A 20 μl RT-qPCR reaction was prepared containing 1 μl of template DNA (from 2.6.1.), 1 μl of primer mix, 10 μl of the iTaq Universal 2 × SYBR green master mix (Bio-rad, USA), and 8 μl of water. The final primer concentration used for DNA and primers (actin primers and primers) was 20 ng, and 0.4 μM, respectively. All samples were amplified under the following conditions: 95 °C for 5 min for 1 cycle; followed by 40 cycles of 95 °C for 15 s, 57 °C for 30 s, and 72 °C for 20 s; final 72 °C for 10 min. Relative abundance level of *Penicillium* sp. in pineapple pulp was analyzed with the $2^{-\Delta\Delta T}$ algorithm by normalizing to the abundance levels of *Penicillium* sp. in the control day 0. The relative abundance of *Penicillium* sp. to reference gene was defined by the following equation: relative expression = $(E_{ref})^{Ct\ ref}/(E_{Ps})\ Ct_{target}$ [45,46], where E_{ref} and E_{Ps} are the efficiencies of the primers for the reference and *Penicillium* sp., respectively, and Ct_{ref} and Ct_{Ps} are the mean Ct value of reference and *Penicillium* sp., respectively.

2.7. Assays of total phenolic compounds (TPC)

The TPC content from pineapple were determined according to the procedures we described previously [36]. Pineapple pulp (1 g) were ground in liquid nitrogen to fine powder and then homogenized in 2 mL of HCl-methanol, extracted at room temperature for 2 h under sealed conditions, centrifuged at 12,000 rpm for 20 min, the supernatant was used for measure of TPC by Folin−Ciocalteu. The absorbance at 765 nm was determinated measured and used to determinate TPC using gallic acid (GA) as a standard. Results were determinated and expressed as milligrams of GA equivalent (GAE) per gram of fresh samples.

2.8. Assays of hydrogen peroxide (H_2O_2)

The H_2O_2 content from pineapple were determined according to the procedures we described previously [36]. Frozen pineapple pulp (1 g) were ground in liquid nitrogen to fine powder and then homogenized in 4 mL of precooled 0.05 mM potassium phosphate buffer, pH 7.8, containing 5% (w/v) polyvinylpyrrolidone. The homogenate was centrifuged at 15 000g for 20 min at 4 °C. The supernatant was used to assay the H_2O_2 content. 1 mL of hydroxylamine hydrochloride was reacted with 0.5 mL supernatant for 1h, then added 1 mL of paminobenzenesulfonic acid and 1 mL of α-naphthylamine, and the mixture was kept at 25 °C for 20 min. The absorbance at 530 nm was determinated, and the result was expressed as nmol·min^{-1}·g FW. The H_2O_2 content was calculated as a H_2O_2−titanium compound resulting from the reaction of tissue−H_2O_2 with titanium tetrachloride. Absorbance values at 415 nm were standardized to a standard curve generated employ known concentrations of H_2O_2.

2.9. Determination of ascorbic acid (AsA)

Pineapple pulp (100 g) was cut into small pieces, homogenized and filtered. The filtrate was used for was determining ascorbic acid level following Lu et al. [47]. 1 mL of pineapple juice, add 9 mL of 0.05 mol L^{-1} oxalate-EDTA (ethylenediaminetetraacetic acid disodium salt), the solution was centrifuged at 12 000g for 5 min at 4 °C, take the supernatant, added 1 mL metaphosphate-acetic acid, 2 mL 5% sulfuric acid, add 4 mL 5% ammonium molybdate, keep 30 min. Absorbance values at 415 nm were determinated to AsA content. It is used as mg of ascorbic acid per 100 mL of juice.

2.10. Measurement of enzyme activity

For assay of polyphenol oxidase (PPO), peroxidase (POD) and superoxide dismutase (SOD) activity were measured following the procedures described previously [36,48]. The contents of extracted enzyme reaction liquid is the same as the H_2O_2 reaction liquid (from 2.8.). For PPO measurement, a 3 mL reaction mixture contained 0.1 mL of enzyme extract, 0.9 mL of 10 mM pyrocatechol, and 2 mL of 50 mM phosphate bufffer, pH 7.8. PPO activity was defined as units of grams per fresh weight (FW). One unit was expressed as an increase in A_{398} of 0.01 min^{-1}. For POD determination, a 3 mL reaction mixture consisted of 0.1 mL of crude enzyme extract, 2.7 mL of 0.1 M potassium phosphate bufffer, pH 7.8, 0.1 mL of 0.46% H_2O_2, and 0.1 mL of 1% (v/v) guaiacol. The PDO activity was detected by following the change in absorbance at 470 nm due to guaiacol oxidation. One unit of POD activity was expressed as an increase in A_{470} of 0.01 min^{-1}. For SOD assay, a 3 mL reaction mixture consisted of 0.1 mL of enzyme extract, 2 mL of 0.2 M potassium phosphate bufffer, pH 7.8, 0.3 mL of 1.25 mM NBT (tetranitroblue tetrazolium chloride), 0.3 mL of 220 mM methionine, and 0.3 mL of 0.033 mM riboflavin. CAT activity was assayed by calculating the initial rate of H_2O_2 disintegration at 240 nm in a reaction of 10 mM H_2O_2, and its activity was expressed as unit g^{-1} on a fresh weight.

2.11. Assay of phytohormone contents

2.11.1. Assay of abscisic acid (ABA) and gibberellin (GA_4) contents

The contents of abscisic acid (ABA) and gibberellin (GA_4) were evaluated following Liu et al. [35]. A total of 1 g fresh pineapple sample was added with 80% (v/v) methanol, 2 mL of 1 mM butylated hydroxytoluene (BHT) and 0.1 g polyvinyl pyrrolidone (PVP), homogenated under ice bath, kept for 4 h at 4 °C, then centrifuged (15 min, 4000g), and collected the supernatant. The residue was extracted again for 1 h under 4 °C, centrifuged, and combined the supernatants and recorded the volume. Then the purified the extracted solution with a C18 plastic column, and concentrated with nitrogen gas. ABA and GA_4 was calculated using the ELISA kit manufacture by China Agricultural University.

2.11.2. Assay of indoleacetic acid (IAA) contents

IAA level was assayed in the same method described as ABA.

2.12. The crown withering index assessment

The crown withering index of pineapple was determined using the protocol of Liu [49] with minor modifications. The crown withering index was examined using the 0–8 scale based on the percentage of leaf area and leaf number with withering patches, 0, no leaf signs withering of patches; 1, only the crown tip is withering and the withering area is <25% of the total leaf area; 2, only the crown tip is withering and the withering area is 26–50%; 3, the withering patches of the crown tip is 25–50% at these leaves, and <25% area of crown stem is withering; 4, the withering patches of the crown tip and stem are both accounted for 26–50%; 5, 6 and 7, the withering patches of the crown tip are connected with stem, the withering patches are accounts for 26–50%, 51–75%, and 76–99% of the total leaves area, respectively; 8, leaves of crown are all withering. Crown withering index = $\sum [(N_Y \times Y)]/N$, where Y represents withering severity (0–8), N_Y represents the number of pineapple fruit with the corresponding severity score and N represents the number of total fruits. 10 fruits were used for determination of crown withering index in each treatment, with three replications.

2.13. Statistical analyses

The experiments were repeated two or three times with similar results in other experiments (those data were not published). There were three replications for analysis of each parameter. Data were analyzed by one way or two-way analysis of variance (ANOVA). Mean separations were executed using the least significant difference method (LSD test). Statistically significant differences were assumed when their *P* values were ≤0.05.

3. Results

3.1. Changes in internal browning and community structure and population abundance of endophyte fungi in pineapple during storage

During storage at ambient temperature, IB index and IB incidence of St.12d pineapple was significantly increased compared with

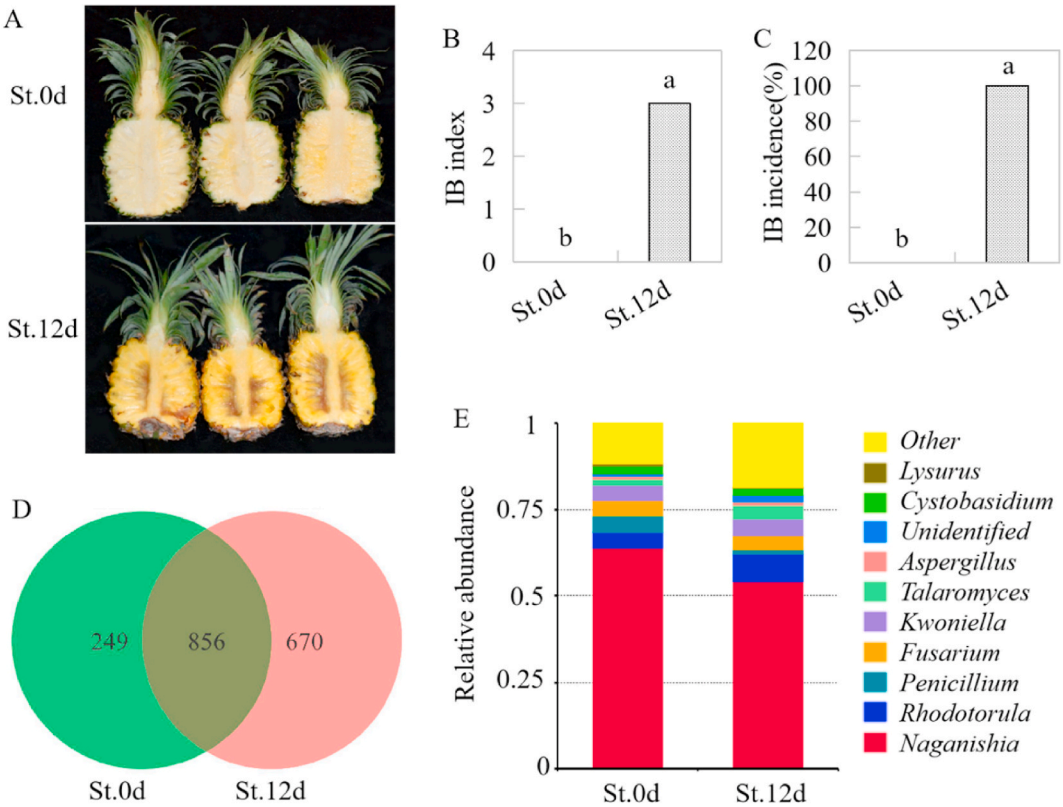

Fig. 1. A, changes in internal browning (IB) in pineapple during storage. B, IB index. C, IB incidence. D, venn diagram showing the shares and unique OUT number in pineapple by high-throughput sequencing. E, histogram showing the relative abundance of top 10 genus of endophyte in pineapple by high-throughput sequencing. St.0d, pineapple stored for 0 day, St.12d, pineapple stored for 12 day. Data from experiment 1.

those just harvested (stored 0 day, St.0d) (Fig. 1A, B, C).

To clarify whether internal browning severity is retaled to the endophyte fungi in pineapple, high-throughput sequencing based on ITS was analyzed. A total of 1175 OTUs were identified from all of the samples, and there were 856 shares OTUs in the two group samples (Supplementary material 1). What's more, the unique OUT number of St.12d was significantly higher than that St.0d (Fig. 1D). Following 12_d storage, relative abundance of top 10 genus of endophyte fungi in pineapple are differ from St.0d (Supplementary material 2). When compared with the St.0d, the relative abundance of *Rhodotorula* and *Talaromyces* are memorably higher in St.12d. While the relative abundance of *Naganishia*, *Penicillium*, *Cystobasidium* and *Lysurus* in St.12d are signally lower than that of St.0d (Fig. 1E).

3.2. Endophye fungal isolation, cultivation and identification

The endophytic fungus B3 was isolated the sterilized of fitness pulp tissue of pineapple. The colony is suble moss (Fig. 2A) and covered with pellucid light green and round spores (Fig. 2B) in late. It has a transparent sporangium in the shape of a broom (Fig. 2C). The ITS rDNA is 564 bp (Fig. 2D). The sequences of nearest eleven neighbors (Table 1) of the endophytic fungi B3 were retrieved from GenBank (NCBI) to construct the phylogenetic tree (Fig. 2E). Results from the alignment of the ITS rDNA regions, morphology, microscopic structures indicated that the strain endophyte fungus B3 is *Penicillium* sp..

3.3. Effect of Penicillium sp. on internal browning severity

In order to determine whether the endophyte strain *Penicillium* sp. can control pineapple IB, pineapple was spray inoculated with

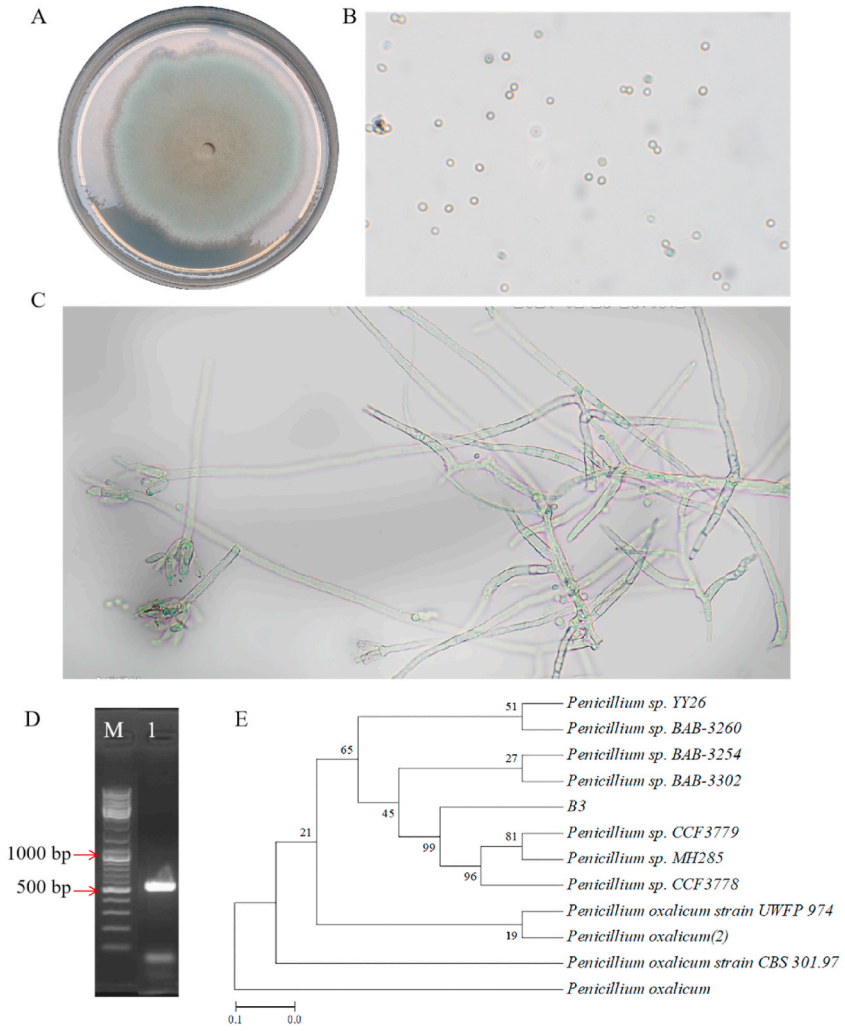

Fig. 2. Morphology and molecular phylogenetic analyses of B3. A, the colony of B3. B, spores, C, sporangium and hypha, D, ITS rDNA (M, DL 10000 Marker; 1, B3), E, phylogenetic tree.

Table 1
Analysis of homology between pineapple endophyte B3 of and other strains.

Description	Max score	Total score	Query cover	E value	Ident	Accession
Penicillium sp.	1007	1007	99%	0.0	99%	FJ430745.1
Penicillium sp. MH285	1003	1003	100%	0.0	99%	HE651149.1
Penicillium sp. CCF3779	1003	1003	100%	0.0	99%	HE651147.1
Penicillium oxalicum strain CBS 301.97	990	990	100%	0.0	98%	KF465777.1
Penicillium oxalicum	990	990	100%	0.0	98%	HE651145.1
Penicillium oxalicum (2)	990	990	100%	0.0	98%	HE651144.1
Penicillium oxalicum strain UWFP 974	990	990	100%	0.0	98%	AY213676.1
Penicillium sp. YY26	987	987	100%	0.0	98%	JF727885.1
Penicillium sp. BAB-3260	985	985	100%	0.0	98%	KU504312.1
Penicillium sp. BAB-3302	985	985	100%	0.0	98%	KU504303.1
Penicillium sp. BAB-3254	985	985	100%	0.0	98%	KU504300.1

spore suspension of *Penicillium* sp. (Ps). Following 9_d, the IB index and incidence of Ps-inoculated fruit were significantly lower than that of control (Fig. 3A, B, C). The result suggest that *Penicillium* sp. alleviated pineapple IB during storage.

3.4. Effect of Penicillium sp. on the relative abundance of Penicillium sp. in pineapple

To clarify the relationship between the relative abundance of *Penicillium* sp. in pineapple and IB, we determined the relative abundance of *Penicillium* sp. in pineapple fruit with time during storage. As showed in Fig. 3D, the time course changes of the endophyte *Penicillium* sp. relative abundance in control pineapple and Ps-inoculated were different, with the control rising to a peak on day 3 and then declining during the remaining time, while the Ps-inoculated fruit increased dramatically with time in the relative abundance of *Penicillium* sp. during the first 9 days. As a result, the Ps-inoculated fruit had markedly higher relative abundance of *Penicillium* sp. during the last 6 days of storage; especially on day 9, when those with *Penicillium* sp. inoculation fruit were 15.6 times of that of control.

3.5. Role of Penicillium sp on TPC contents and PPO activities

In order to determine the relationship between the accumulation of phenols and the control of IB by *Penicillium* sp., we determined

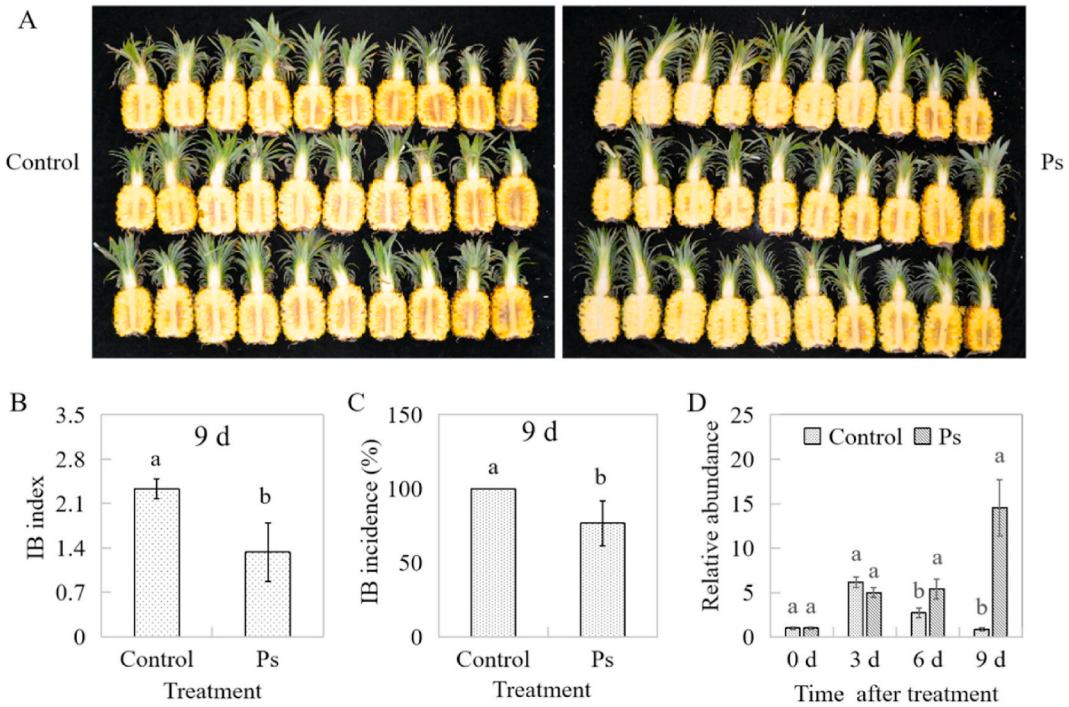

Fig. 3. Effect of *Penicillium* sp. inoculation on internal browning in pineapple fruit storage at 20 °C for 9 days. A, internal browning severity, B, IB index, C, IB incidence, D, relative abundance of *Penicillium* sp in pineapple. Control, spray with sterile water, Ps, spray inoculation of *Penicillium* sp spore suspension, data from experiment 2, the same to below.

the total phenol compounds (TPC) contents and polyphenol oxidase (PPO) activity in pineapple. TPC content of pineapple fruit decreased dramatically (Fig. 4A), whereas activities of PPO increased sharply (Fig. 4B) with time. Those of the Ps-inoculated fruit higher than control in TPC content and PPO activities during the first 9 days of storage.

3.6. Effect of *Penicillium* sp. inoculation on H_2O_2

As is shown in Fig. 4C, the level of H_2O_2 increased obviously in postharvest pineapple fruit during storage. In addition, compared with the control, *Penicillium* sp. treatment decreased H_2O_2 contents. In other words, *Penicillium* sp. inoculation impaired the accumulation of H_2O_2.

3.7. Effect of *Penicillium* sp. inoculation on AsA levels, SOD and POD activities

To determine whether *Penicillium* sp. alleviating IB is related to the antioxidant defense system of pineapple, we examined AsA levels, SOD and POD activities.

The content of AsA in pineapple fruit decreased gradually over time, and the Ps-inoculated fruit maintained higher levels of AsA than that of the control during the whole 9 d in storage (Fig. 4D).

The control decreased dramatically in activity of SOD and the Ps-inoculated fruit increased during the first 9 days (Fig. 4E). Furthermore, the Ps-treated fruit had much higher SOD activity during the last 3 days of storage, especially on 9 d (Fig. 4E).

The time-course changes of the POD activity rose to a peak on 6 d and then declined during the remaining time (Fig. 4F); inoculation of *Penicillium* sp. fruit showed a higher POD activity than that of the control during the first 6 days of storage (Fig. 4F).

3.8. Effect of *Penicillium* sp. on phytohormone contents

The ABA and GA_4 content of pineapple increased during the whole storage period; and those levels in the fruit of Ps-inoculated were always lower than that of the control (Fig. 5A and B). While Fig. 5C shows that the IAA level was much elevated compared with the control during storage, except for 6 d.

3.9. Effect of *Penicillium* sp. on crown withering index and yellowing index

In order to determine the effect of *Penicillium* sp. on the exterior quality of postharvest pineapple, the crown withering index and yellowing index were tested. Exogenous inoculation of endophyte *Penicillium* sp. significantly alleviated crown withering index

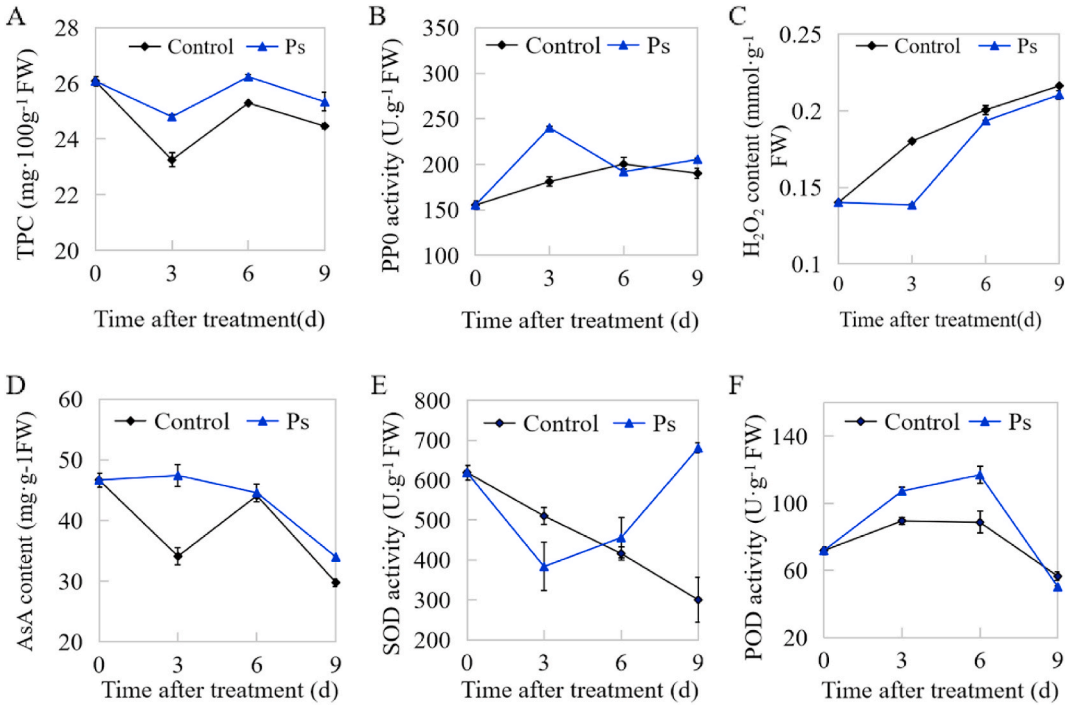

Fig. 4. Effects of *Penicillium* sp. on the TPC levels (A), PPO activity (B), H_2O_2 content (C), AsA contents (D), SOD activity (E), and POD activity (F) of harvested pineapple fruit.

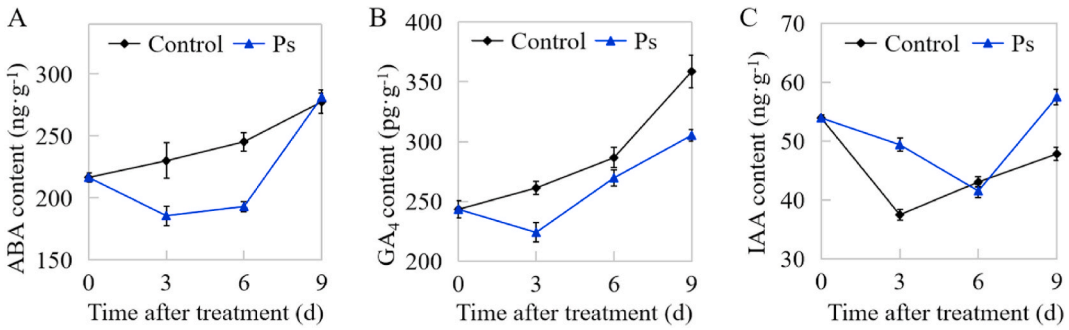

Fig. 5. Effects of *Penicillium* sp. on the ABA (A), IAA (B) and GA_4 (C) contents of harvested pineapple fruit.

(Fig. 6A and B) and yellowing index (Fig. 6A, C) of pineapple following 9 d storage, suggesting that Penicillium *sp.* can control crown withering and fruit yellowing.

4. Discussion

Pineapple is a highly perishable fruit and is susceptible to postharvest losses [47]. Internal browning is a major problem faced by the global pineapple industry [33]. Over the past decades, techniques to control IB has been a global concern. In this study, it provided convincing evidence that inoculation of endophyte *Penicillium* sp. effectively alleviated IB (Fig. 3A, B, C), inhibiting crown withering (Fig. 6A and B) and yellowing (Fig. 6A, C) during ambient temperature storage. This unexpected result highlights the vital roles of endophytes in physiological disorders and provides a new perspective for studying and unraveling the nature of internal browning.

So far, studies on endophyte controling plant diseases have focused on infectious diseases. Internal browning is a physical disorder of postharvest pineapple [33]. In our study, exogenous inoculation of endophyte *Penicillium* sp. alleviated pineapple IB during storage. This result indicated that endophytes could not only act as antagonists to control infectious diseases caused by pathogen, but also control the physiological disorder. During postharvest storage, the community structure and population abundance of endophyte fungi in pineapple were changed with IB degrees (Figs. 1 and 3D). The results implied that endophytes can regulate the physiological state of pineapple. The *Penicillium* sp. inoculated fruit had lower dramatically in IB index and IB incidence than that of the control during storage (Fig. 3A, B, C), while significantly elevated the relative abundance of *Penicillium* sp. in pineapple during the last 3 days (Fig. 3D). These proved that the physiological state of pineapple fruit is related to the abundance of endophytes.

Endophyte in plant affect plant health [50,51]. It has been shown that cross-talk between the intestinal microbiota and the human

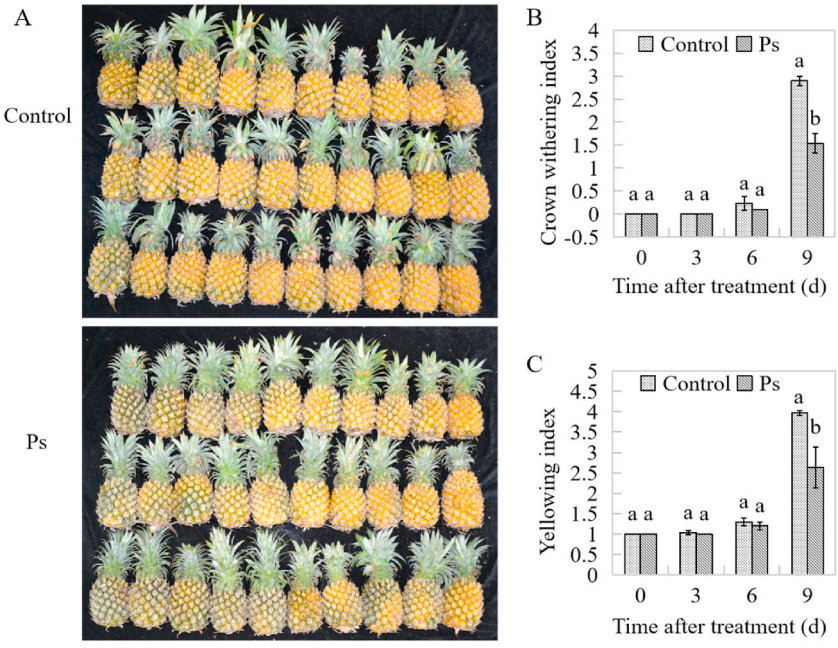

Fig. 6. A, effect of *Penicillium* sp. inoculation on the exterior quality traits in pineapple fruit storage at 20 °C for 9 days, B, crown withering index, C, yellowing index.

host is essential for human health, microbial dysbiosis associated with various human diseases [52–54]. Gut microbiota also affects therapeutic effect of human diseases [55,56]. For example, *Lactobacillus* could alleviate the intestinal flora imbalance and the trend of decreasing the diversity of intestinal flora that caused by chemotherapy, and had a good effect on improving diarrhea in Colorectal cancer patients [57]. In the present study we first found that the community structure and abundance of endophyte fungi in healthy pineapple fruit was different from that in IB fruit (Fig. 1). Furthermore, the higher the abundance of *Penicillium* in pineapple fruit, the lower severity of IB (Figs. 1 and 3). According to our current research, the increase of the abundance of endophyte *Penicillium* sp. in pineapple fruit is the direct reason for the decrease of IB incidence. And that, it may imply that *Penicillium* sp. is the beneficial endophyte fungus in alleviating pineapple internal browning. The higher relative abundance of *Penicillium* sp. in pineapple may serve as a potential biomarkers for alleviating IB. Nevertheless, whether there is a critical abundance of endophyte *Penicillium* sp. to control IB is still not well understood. If such a critical value exist, further studies are remains to be elucidated whether IB resistance and shelf life of pineapple can be predicted by detecting the abundance of *Penicillium* sp. after harvest. This may also provide a new method for nondestructive testing of postharvest pineapple. However, the causal relationship between endophyte abundance and fruit physiological status remains to be elucidated.

Pineapple internal browning results from enzymatic oxidation of phenolic compounds [58]. The most important factors that determine the rate of enzymatic browning are PPO active, phenolic compounds level e and the oxygen availability of the tissue [59]. An increase in PPO activity was directly contribute to the occurrence of IB symptoms, [60]. In our study, pineapple inoculated with *Penicillium* sp. were significantly increased in both PPO activity and total phenolic content (Fig. 4A and B), resulting in a lower IB index and incidence in Ps-treated pineapple than that of control (Fig. 3A, B, C). However, Youryon et al. [61] reported that higher IB incidence in pineapples was clearly associated with an increase in TPC and decreased PPO activity previously, this was inconsistent with our results. This might imply that the substrate of browning reaction was slower than the synthesis of total phenolic in *Penicillium* sp. treatment pineapple fruit. How inoculation of *Penicillium* sp. alleviated pineapple IB incidence by the biosynthesis and degradation of total phenolic remains to be elucidated.

Mittler [62] reported a strong oxidative burst leaded to cellular destruction and death. Plant diseases suppression was correlated with enhanced activity of antioxidant enzymes (POD, SOD, PAL, etc.) in the host plant [63]. The antioxidant capacity of pineapple coincided with IB tolerance [64]. Cells synthesized more ascorbic acids and antioxidants to balance ROS [64]. Under normal and healthy conditions, the ROS molecules were scavenged by various antioxidative defense mechanisms [65]. These included enzymatic antioxidant systems, such as SOD, POD, GR (glutathione reductase), and CAT (catalase), and nonenzymatic low molecular metabolites, such as ascorbic acids (AsA) and glutathione (GSH) [65–68]. Tiryaki et al. [20] pointed out that the inoculations of endophytes from various plants improved the resistance of bean seedlings. Jinal et al. [69] results clearly established that *Bacillus paralicheniformis* triggered ISR against *Fusarium oxysporum*-infected tomato plants and prevented oxidative damage by activating antioxidant defense enzyme (POD, SOD, PAL) that suppressed wilt diseases occurrence. In this study, *Penicillium* sp. significantly increased AsA contents (Fig. 4D) and elevated SOD and POD activities (Fig. 4E and F), demonstrating that *Penicillium* sp. strengthened the antioxidant capacity of postharvest pineapples. In Ps-inoculated pineapple, H_2O_2 contents was significantly lower than that of control (Fig. 4C), implying Ps efficiently detoxified ROS molecules. These results confirmed that the endophyte *Penicillium* sp. activated the antioxidative defense system to decrease ROS, which alleviated IB.

The reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2) and superoxide radicals ($O_2^{\cdot-}$), is identified as cytotoxic molecules destructive to cells [70]. Choi et al. [71] reported that the generation of ROS reduced cell viability and possibly triggered apoptosis [71]. Previous studies on postharvest pineapple showed that exogenous application of ABA decreased H_2O_2 and resulted in reducing IB incidence [36]. Evidence confirmed that endophyte bacteria isolation from leaf apoplast decreased ROS levels such as O_2^- and H_2O_2 to increase the cold resistance of plants [20]. In this study, *Penicillium* sp. inoculation pineapple had decreased H_2O_2 content (Fig. 4C), which contributes to alleviated IB (Fig. 3A, B, C). It implied that *Penicillium* sp. improved ability of scavenging ROS in pineapple.

Endogenous GAs levels are positively correlated to IB incidence [27,71]. Zhang et al. [72] reported that pineapple *AcGA2ox* gene was involved in control of GA biosynthesis and that the regulation of *AcGA2ox* expression could influence the occurrence of IB of pineapple fruits. Postharvest application of ABA reduced the IB index and incidence by up-regulating pineapple *AcGA2ox* expression, reducing endogenous GAs content and ingcreasing endogenous ABA level [72]. In this study, application of *Penicillium* sp. decreased endogenou GA_4 and ABA contents in pineapple (Fig. 5A and B), and decreased IB index and incidence (Fig. 3A, B, C). It suggested that *Penicillium* sp. controlling IB by reducing endogenous GA_4 and ABA content, *AcGA2ox* gene might regulate gibberellin content in pineapple. IAA could significantly inhibit the browning of explants in plant tissue culture [73]. Ge and Zhang [74] proved that *Rhodopseudomonas palustris* G5 induced systemic resistance in cucumber seedlings under salt stress by the production of IAA. Preharvest application of mixture of ABA and IAA to crown indicating good effects in controlling IB [49]. Our studies have revealed that *Penicillium* sp. alleviated IB (Fig. 3A, B, C) and increased endogenou IAA level in pineapple (Fig. 5C). We first found that the increased endogenous IAA in pineapple fruits was closely related to IB. While, whether the decrease of IAA level resulting in the occurrence of pineapple IB is still not well understood. Hormones affected IB incidence of pineapple [27,33,71]. Previous studies have demonstrated that endophytes controlled plant diseases and improved stress resistance by producting phytohormones [15,21]. Our results might imply that *Penicillium* sp. directly enhanced the disease resistance of pineapples to alleviate IB by regulating of the homeostasis of endogenous hormones.

Postharvest inoculation of *Penicillium* sp. in pineapple significantly reduced the crown withering index (Fig. 6A and B) and yellowing index (Fig. 6A, C), furthermore, it obviously improved the IAA accumulation (Fig. 5C) and decreased ABA level (Fig. 5A). ABA regulates the abscission of leaves and fruits [75]. The role of ABA in the promotion of leaf yellowing has been widely studied [76]; which might be one of the reasons that *Penicillium* sp. reduced crown withering and yellowing index (Fig. 6A, B, C). Evidence suggested

that bacterium product IAA, and IAA is a crucial player in stimulating and facilitating plant growth [77,78]. Furthermore, plant growth regulator treatments can reduce potted geranium plants' leaf yellowing [79]. These might account for *Penicillium* sp. application put off ripening. Liu et al. [35] pointed out that the higher IB occurrence in de-crowned pineapple might be related to the increased ripening. In our study, exogenous inoculation of *Penicillium* sp., together with the reduced IB index and incidence and put off ripening, which is concomitant with the findings of Liu et al. [35] indicated previously. In addition, in previous work, Worsley et al. [21] supported that *Streptomyces* antifungal activity was up-regulated about 2-fold in response to IAA, and its spores protected wheat seedlings against take-all disease. There was a strong correlation between *Bacillus amyloliquefaciens* JK6 significantly suppressed tomato bacterial wilt and JK6 produced IAA [80]. In conclusion, the *Penicillium* sp. treatment maintained the exterior quality traits and had a longer shelf-life of pineapple to a certain extent, which may be related to the regulation of endogenous hormones in pineapple fruit by *Penicillium* sp..

5. Conclusion

During postharvest storage, the community structure and population abundance of endophyte fungi in pineapple were different with IB degrees. Furthermore, the relative abundance of *Penicillium* sp. in pineapple fruit was negatively correlated with the severity of IB. Exogenous inoculation of endophyte fungus *Penicillium* sp. can control physiological disorders of plants. *Penicillium* sp. treatment alleviated internal browning and maintained the storability of pineapple during the storage period at atmospheric temperature. This technology is economical and environmentally friendly, as well as convenient to spread in agriculture. In this chapter, our results also demonstrated that the lower incidence of IB was closely related to increased the relative abundance of *Penicillium* sp. in pineapple, retarded ROS accumulation, enhanced the antioxidant capacity and homeostasis of endogenous hormones by inoculation *Penicillium* sp.. This research provides a useful and new information on endophyte controling plant disease and nondestructive testing of post-harvest pineapple, and the endophyte are potential to be used as a biological control agent to agricultural industry.

Author contribution statement

Fei Shen: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Guang Wang: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Xiaoyu Liu: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Shijiang Zhu: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Data availability statement

Data included in article/supp. material/referenced in article.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e16258.

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