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Identification and characterization of a stem canker and twig dieback disease of pear caused by *Neofusicoccum parvum* in Chinese mainland

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Abstract

Pear (*Pyrus* spp.) is one of the most consumed fruits in China, but the pear production has to confront the growing threat from fatal diseases. In this study, we report two incidences of stem canker and twig dieback disease on pear plants, which led to death of pear seedlings (approximately 10% of total plants) in Guangxi and Jiangsu provinces. Using a combination of morphological and molecular diagnoses, along with pathogenicity test, the causal agent of the disease in these two locations was identified to be the fungus *Neofusicoccum parvum*. However, the isolates were divided into two clades: CY-2 isolate and other four isolates including ZL-4, BM-9, BM-10 and BM-12 might split into two groups of *N. parvum*. Two representative isolates (CY-2 and ZL-4) were selected for further investigation. We observed that the optimal temperature for in vitro infection on pear trees of these two isolates was at round 25 °C. Both CY-2 and ZL-4 could infect different sand pear varieties and other horticultural plants in vitro, while CY-2 had a higher virulence on several pear varieties including Nanyue, Lvyun, Qiushui and Ningmenghuang. Furthermore, the eficacy of fungicides against these two isolates was evaluated, and carbendazim and flusilazole were found to be the most effective fungicides in inhibiting the growth of these fungal pathogens. Taken together, these findings redefine the *N. parvum* species and provide potential strategies for the future management of this disease.

Keywords: Pear, *Neofusicoccum parvum*, Pathogenicity, Susceptibility

Background

Pear, belonging to the Rosaceae family, is the third most produced fruit in China. Pear trees are cultivated in 32 provinces and regions in China (Silva et al. 2014), with a wide varieties of pear species including *Pyrus serotina*, *P. ussuriensis*, *P. bretschneideri* and *P. communis* (Teng 2017). To increase productivity, pear trees are grown as a monoculture crop in most orchards in China, which facilitates adaption of pathogens to a specific plant host and increases the chance of disease epidemics. Several pathogens have been reported to cause canker diseases on pear trees. For examples, *Phomopsis fukushii*, *Diaporthe eres* and *Phomopsis amygdali* cause shoot canker (Bai et al. 2015); *Valsa pyri* causes Valsa canker (Wang et al. 2014); and *Dickeya fangzhongdai* causes bleeding canker (Zhao et al. 2018). These canker diseases have caused massive yield reduction and impaired fruit quality in the last several decades. The incorporation of new pear species/varieties into China as well as an increase in destructive new pathogens results in new diseases that were not previously observed in the country. Furthermore, many of these new diseases are caused by pathogens with a broad host range.

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Neofusicoccum parvum, belonging to the family Botryosphaeriaceae, is a fatal fungal pathogen that affects multiple crop species worldwide. Symptoms caused by this pathogen often appear as deadly trunk or stem cankers which are commonly found in young trees. The pathogen can infect a broad range of hosts. Susceptible hosts discovered so far include *Eucalyptus* spp. (Li et al. 2018), *P. pyrifolia* (Shen et al. 2010), *Rhododendron* spp. (Yang et al. 2015), *Dendrobium oficinale* (Liu et al. 2017), *Punica granatum* (Riccioni et al. 2017) and *Santalum album* (Wang et al. 2016). Moreover, the pathogen may successfully evolve into new races after adapting to various host environments. Due to these factors, there is a dire need to limit the spread of this pathogen in the field. In China, *N. parvum* has been shown to cause stem canker disease on different landscape plants including *Rhododendron* (Yang et al. 2015), *Eucalyptus* (Li et al. 2018) and *S. album* (Wang et al. 2016). Since this pathogen can invade the xylem vessels of plant stems or trunks, resulting in canker lesions, the damage caused by this pathogen is dificult to manage. The effective ways to control this disease include breeding for host resistance, crop rotation and the application of fungicides. So far, there are no related reports on pear stem canker and twig dieback diseases in Chinese mainland.

In this study, we report *N. parvum* as a pear pathogen causing canker and twig dieback disease in two geographically distinct orchards in Guangxi and Jiangsu provinces in China. The pathogen produced similar symptoms with severe disease outbreaks on pear trees in these two distinct locations. The *N. parvum* isolates from diseased samples of these locations exhibited diverse types of colony morphologies. Furthermore, sequence analysis indicated that these isolates belong to two clades of *N. parvum*. Characterization of pathogen growth conditions, host susceptibility and pathogen's sensitivity to different fungicides pointed to potential management strategies. This study reveals a newly found pear disease caused by *N. parvum* in Chinese mainland, and provides potential management strategies for the disease.

Results

Symptoms and damages of a newly emerged disease on pear trees in Guangxi and Jiangsu provinces

Two disease incidences, one in Guilin, Guangxi Province in 2016, and the other in Nanjing, Jiangsu Province in 2018, exhibited different levels of damages. Infections in Guilin were more sporadic, and did not significantly affect the whole orchard, while infections in Nanjing were more severe, killed more than 100 Cuiguan trees (account for approximately 10% of the total plants) in one orchard. In our survey, the symptoms are hard to be identified at early stages, because small black lesion is similar to other pear stem canker diseases. Moreover, the disease symptoms were generally more severe during the rainy season (from the end of April to June). The dieback symptoms began to occur on seedlings of pear trees (<3 years old) especially on one or two-year-old trees. The stem formed long strip lesions with brown to black color. The inside of the infected parts changed to brown color (Fig. 1a, b). Canker lesions with clear edge initially collapsed and then produced crack between the healthy tissue and the lesion part (Fig. 1c). With development of the lesions, twigs and shoots wilted and showed dieback symptoms, and leaves discolored (Fig. 1d, e), which led to tree death within two to three months.

Morphological and cultural features of the causal fungal isolates

The diseased stems were collected from pear orchards and the pathogens were isolated. Five fungal isolates were obtained from distinct samples showing typical canker symptoms, which we named CY-2, ZL-4, BM-9, BM-10 and BM-12. Among those isolates, CY-2 and ZL-4 were isolated from two pear orchards in Guilin, and BM-9, BM-10 and BM-12 were from Nanjing. For the observation of cultural characteristics, the strains were cultured on PDA plates at 25 °C in darkness. After two days, the white mycelia of these five isolates covered the whole plate and the aerial mycelia were generated. However, the aerial mycelia of the isolate CY-2 were flocculent, joining the mycelium together. Thus, CY-2 differed from the other four isolates, forming small spherical floc (Fig. 2a). The mycelia color of all the isolates changed to grey after they were cultured on PDA for 7 days (Fig. 2). One month later, the whole petri plate including the mycelia and the medium turned black and aerial mycelia were dissolved on the plates of the ZL-4, BM-9, BM-10 and BM-12 isolates. Whereas, in the case of CY-2, the colony appeared as white aerial mycelia (Fig. 2a). The CY-2 isolate showed a unique growth phenotype compared with the other four isolates, and whether there are differences in their morphological features of pycnidia and conidia were further investigated. It was observed that very few pycnidia were produced on the plates inoculated with the five isolates, and the size of their pycnidia was different (4–14, 4–11, 1.5–2, 1.5–5.0 and 1–2 mm for CY-2, ZL-4, BM-9, BM-10 and BM-12, respectively). The pycnidia of isolate CY-2 were close to knurl, but others were flat humps (Fig. 2b). The ascosporic morphology of the five isolates were similar as all were single celled with a smooth surface and contained a spindle with round apices at the top. However, the size of CY-2 conidia was significantly bigger than that of the others (Fig. 2c and Additional file 1: Table S1). For CY-2, the conidia size was 18.81～23.49 μm × 7.699～8.95 μm, whereas those for ZL-4, BM9,

Fig. 1 Symptoms of pear stem canker and twig dieback disease in the field. **a** Stem canker on pear seedling. **b** Longitudinal section of stem canker. **c** Typical symptoms of stem canker in the field. **d** Twig symptoms on pear. **e** A dead tree caused by the disease. Red arrow indicates the typical flaw

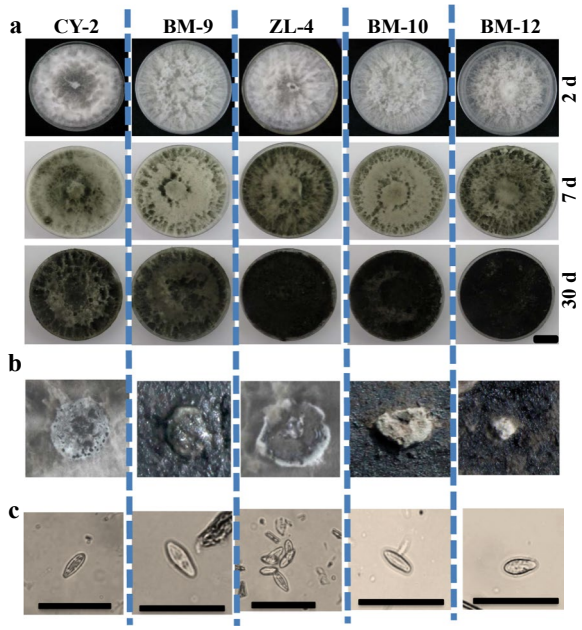

Fig. 2 Morphological and cultural features of *N. parvum* isolates. **a** Cultural features. Five isolates were cultured on PDA medium at 25 °C for 2, 7 and 30 days. Bar = 10 mm. **b** Pycnidia of the isolates. The pycnidia were induced at room temperature for 30 days under 16/8 light/dark. **c** Conidial features of the isolates. Conidia were obtained from broken pycnidium. Images were obtained using a Zeiss microscope, with the sizes of the conidia measured using the image capturing software

BM10 and BM12 were 14.01–14.8 µm × 7.16–8.86 µm, 11.68–20.59 µm × 5.95–9.76 µm, 10.95–16.56 µm × 5.9–8.33 µm and 10.91–17.32 µm × 5.84–8.96 µm, respectively. These results demonstrate that conidial features of the isolates are similar to that of *N. parvum* and CY-2 might vary from the other four isolates.

Phylogenetic analysis

To further identify these isolates, we performed the phylogenetic analysis. The phylogeny tree based on ITS sequences showed that ZL-4, BM-9, BM-10 and BM-12 were clustered more closely to those of the reported *N. parvum* isolates, sharing a nucleotide sequence identity of 100%; by contrast, CY-2 shared a slightly lower level of identify (99%) (Additional file 2: Figure S1). Since the similarity among the *N. parvum* isolates is extremely high, the phylogeny tree based on ITS sequences alone is not always reliable to identify the isolates accurately. Therefore, a phylogeny tree based on the combined sequences consisting of ITS, *RPB2*, *β-tubulin*, *BotF15* and *EF-1a* was constructed using the five isolates from this study in relation with other isolates from different host species isolated from China and other countries. The results showed that the five isolates were clustered into two main clades (Fig. 3). Among them, isolates including ZL-4, BM-10, BM-9 and BM-12 were clustered in the same clade, but the isolate CY-2 was similar to *N. parvum* CMW14143 and was grouped into another clade. The data revealed that all isolates from the pear stem canker and dieback tissues could be identified as *N. parvum*, while CY-2 is

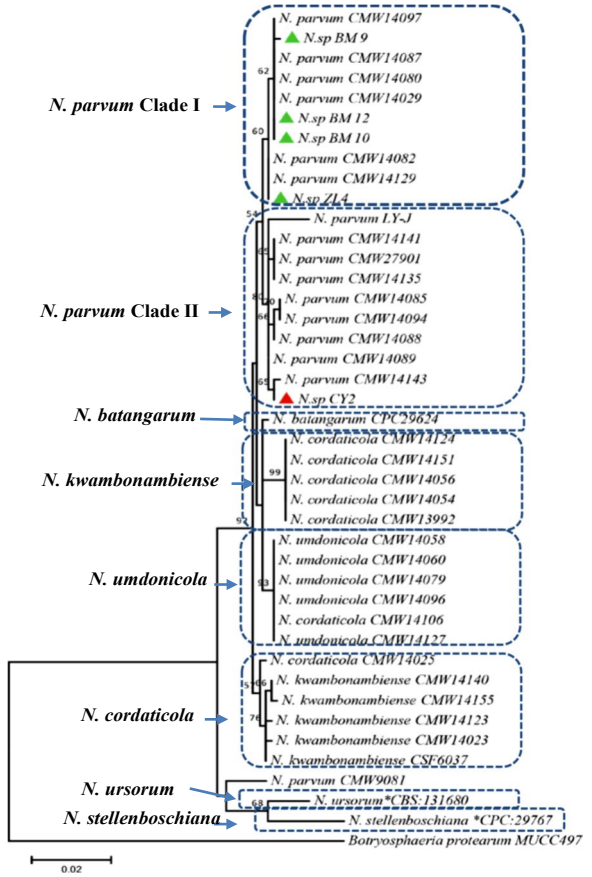

Fig. 3 Phylogenetic analysis of *N. parvum* isolates. Phylogeny of *N. parvum* isolates associated with stem canker and twig dieback using MEGA 7.0 with the Maximum Likelihood analysis of the combined sequences consisting of the internal transcribed spacer (ITS), *EF-1α*, *RPB2*, *BotF15 and β-tubulin*. Analysis was performed with the bootstrap value of 1000 replications, and pairwise deletion option. Values greater than 50% bootstrap are shown on the pictures. Reported sequences from different isolates were downloaded from the GenBank database. Distance scale is shown in the graph

different from the other four isolates we obtained, which implies that the *N. parvum* species should be divided into two groups.

Pathogenicity assays

To deduce whether the disease symptoms were caused by these isolates, a pathogenicity assay was performed. We chose a field in Nanjing, where most of the diseased pear plants were found, and two-year-old "Fengshui" pear seedlings were used in the pathogenicity test. Mycelial plugs of 2-day-old cultures of five isolates were inoculated on wounded sites and wrapped tightly with parafilm for sustaining the humidity and preventing contamination. Pear seedlings inoculated with the mycelial plugs of 2-day-old cultures of sterile PDA agar were used as negative controls. After 7 days, disease symptoms started to appear as black lesions, which kept on migrating from the wounded sites of inoculation (Fig. 4). Moreover, leaf dieback was observed on the seedlings inoculated with the five *N. parvum* isolates (Fig. 4). After 21 days, the infected sites that were inoculated with the five isolates produced typical canker lesion symptoms, similar to those observed in pear orchards (Fig. 4). However, in the case of PDA agar-treated control treatments, dead bark appeared only around the wounded areas and recovered later without any external manipulation. The association between disease symptoms and the pathogens was further confirmed after the same five isolates were recovered from the inoculated plant parts. Based on these observations, we conclude that these five isolates are the causal agents of the pear stem canker and twig dieback disease.

The optimum temperatures for growth of *N. parvum* isolates and infection on pear trees

The stem canker and twig dieback disease produced by *N. parvum* was most prevalent in southern and eastern China around April to June (18–27 °C). We also observed that disease incidence reduced significantly during late July to late August when the temperature was 33–40 °C. Here, we deduced that the environmental temperature might be one of the key factors that affect *N. parvum* growth and virulence. Thus, we selected five temperatures (12, 18, 25, 30 and 37 °C) that mirror existing environmental temperatures in pear-growing areas of China and examined the pathogen's growth and infection development at these temperatures. CY-2 and ZL-4, two representative isolates in different clades, were selected to test the hypothesis. We discovered that CY-2 and ZL-4 have similar growth behavior when exposed to the five different temperatures and showed maximum colony diameter when they were cultured at 23.95 °C and 23.4 °C, respectively (Fig. 5a). The virulence of these two isolates was also tested under the same temperatures and they showed similar disease patterns. The largest lesion length was observed at around 25 °C, and temperatures below 20 °C or above 35 °C yielded reduced lesion length (Fig. 5b), which is consistent with our field observations that disease progression reduced during late July to late August. Based on these results, we conclude that 25 °C may be the optimal temperature for infection in vivo.

The horticultural host plants of *N. parvum* isolates

According to the previous reports, many *N. parvum* isolates were isolated from horticultural plants, such as *Prunus pseudocerasus*, *Prunus persica*, *Latanus acerifolia*, *R. simsii* and *Vaccinium ashei*, which are widely cultivated in the field or along the road in China. To learn more about where *N. parvum* originates, two representative isolates,

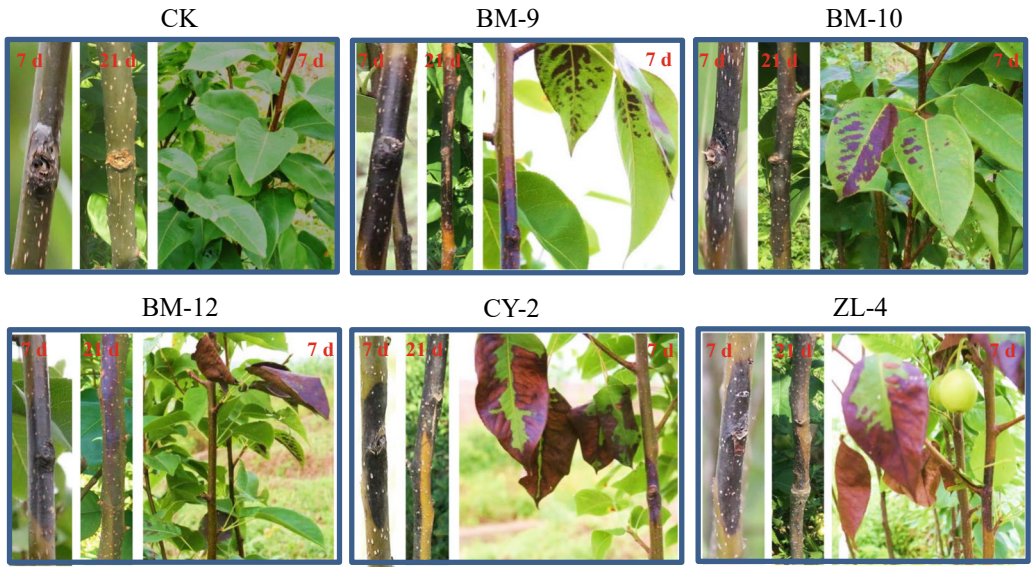

Fig. 4 Pathogenicity of five *N. parvum* isolates on pear trees in the field. The PDA plugs with *N. parvum* mycelia were placed on the wounded pear branches, using PDA plugs without fungi as the control. The photos were taken at 7 and 21 days post-inoculation (dpi)

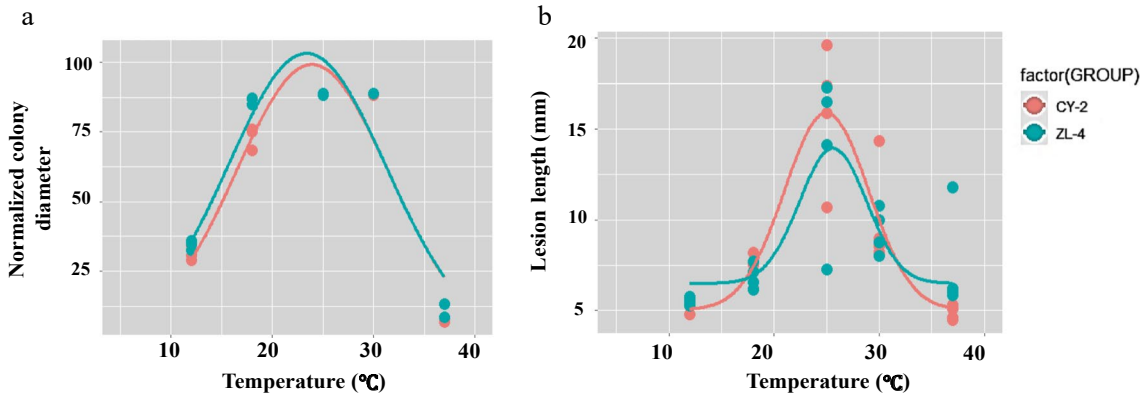

Fig. 5 The optimal temperature for growth and infection. **a** Optimum temperature for growth. Fungal plugs of CY-2 and ZL-4 isolates were inoculated on PDA plates and incubated at different temperatures as indicated. Colony diameters were measured at 2 dpi. The column graph was formatted using the GraphPad prism software based on the lesion length. **b** The optimum temperature for infection on pear. Fungal plugs of both isolates were used to inoculate the pathogens onto pear branches and incubated under different temperatures. Two days later, the lesion length was measured, and photos were collected. The column graph was formatted using the GraphPad prism software based on the lesion length. Each treatment replicates three times, and data were analyzed using ANOVA. $^{**}P<0.01$

CY-2 and ZL-4, were inoculated on the shoot of the five plants previously mentioned. We observed that both isolates can infect these plants (Fig. 6), but the susceptibility of the plants varies. *V. ashei* is the most susceptible species to these two isolates, followed by *L. acerifolia*, and *P. persica* is the most resistant species (Fig. 6). Moreover, ZL-4 caused larger lesions than CY-2 on *P. pseudocerasus*, *L. acerifolia*, *R. simsii* and *V. ashei* (Fig. 6). These results demonstrate that the isolates from the pear trees may emerge from some horticultural plants, and have the potential to spread to other horticulture plants, causing major threats to these plants in the south of China.

Susceptibility of different sand pear varieties to *N. parvum* isolates

Since the disease resulted in the death of a substantial number of pear trees, finding resistant pear varieties is an effective way to control the disease. To evaluate the

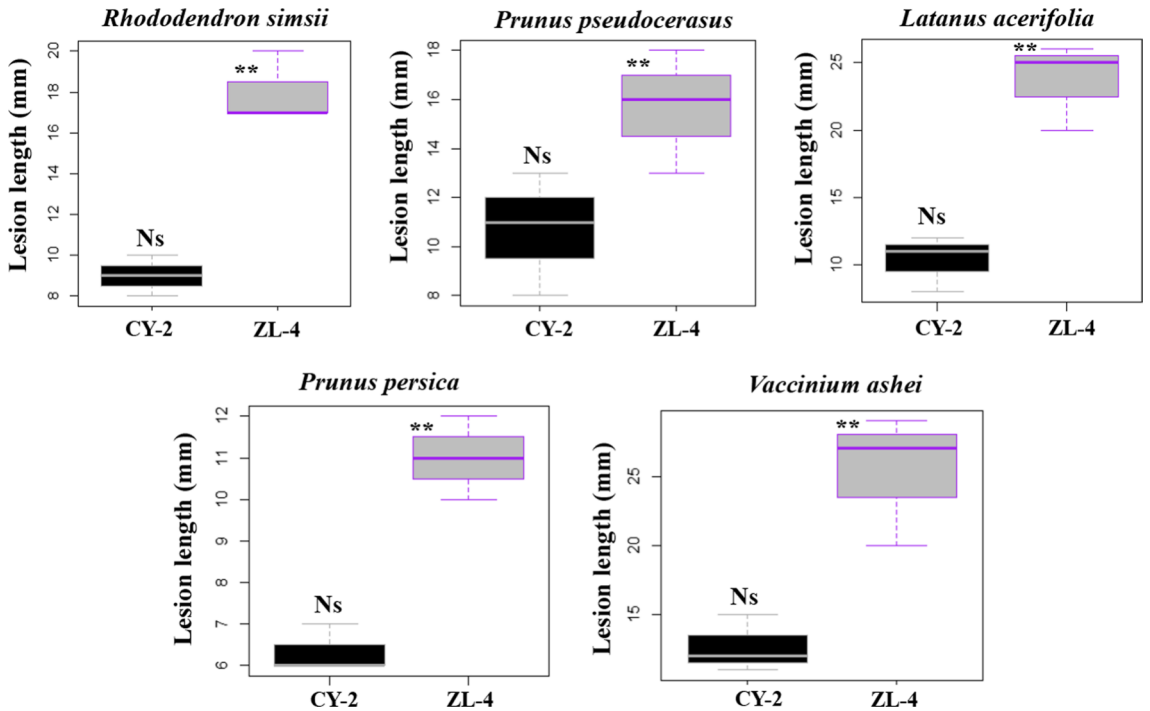

Fig. 6 Host ranges of the two *N. parvum* isolates. Lesion sizes caused by the two isolates on *Prunus pseudocerasus*, *Prunus persica*, *Latanus acerifolia*, *Rhododendron simsii and Vaccinium ashei* were measured at 2 dpi. Graphs were generated using R ggplot2. Data were analyzed using ANOVA. $^{**}P<0.01$

susceptibility of different sand pear varieties to stem canker pathogen, we tested the virulence of two representative isolates, CY-2 and ZL-4, on eight major sand pear cultivars in southern China. All pear varieties tested were found to be infected by these two isolates, but the susceptibility levels of different pear varieties varied. Aidang was shown to be the most susceptible pear variety, whereas Nanyue was the most resistant pear variety to both isolates (Fig. 7). Additionally, CY-2 showed stronger virulence than ZL-4 on all seven pear varieties excluding the Xinxue variety (Fig. 7). In contrast, Nanyue, Lvyun, Qiushui and Ningmenghuang showed significantly higher resistance to isolate ZL-4, but less resistance to isolate CY-2 (Fig. 7). These results not only confirmed that different sand pear varieties display various levels of susceptibility, but also suggested that the isolate genotype may also affect the virulence of the pathogen.

Sensitivity of *N. parvum* isolates to different fungicides and HSAF

In addition to screening of resistant plant cultivars, chemical control using fungicides is another effective way to control the spread of fungal diseases. To investigate which is the most effective fungicide against *N. parvum*, we compared the inhibitory activities of seven fungicides that have already been registered on pears in China and HSAF, one biocontrol agent, on two representative *N. parvum* isolates (CY-2 and ZL-4). We found that all fungicides could effectively inhibit the growth of CY-2 and ZL-4 at all concentrations tested. The minimum colony diameter of the two isolates was found at 1.0 μg/mL concentrations of carbendazim and flusilazole, and their concentrations for 50% of maximal effect (EC50) were found to be less than 0.05 μg/mL (Additional file 1: Table S2). The colonies on plates with 1.0 μg/mL tebuconazole and prochloraz were also very small (Additional file 1: Table S2), and the EC50 values were below 0.2 μg/mL. However, the isolates could still grow well on plates with 40 mg/L chlorothalonil (EC50: 5.639 for CY-2, 2.604 for ZL-4) and 40 μg/mL mancozeb (EC50: 3.925 for CY-2, 2.429 for ZL-4) (Additional file 1: Table S2), which suggests that these two fungicides are not ideal for controlling the disease. These results suggest that the fungicides including carbendazim, flusilazole, tebuconazole and prochloraz will be beneficial to disease management in the field.

Discussion

Sand pears are extensively cultivated in eastern and southern China. Recently, the increasing number of diseases on sand pears has raised social concerns due to the possibility of severe outbreaks, which can seriously affect

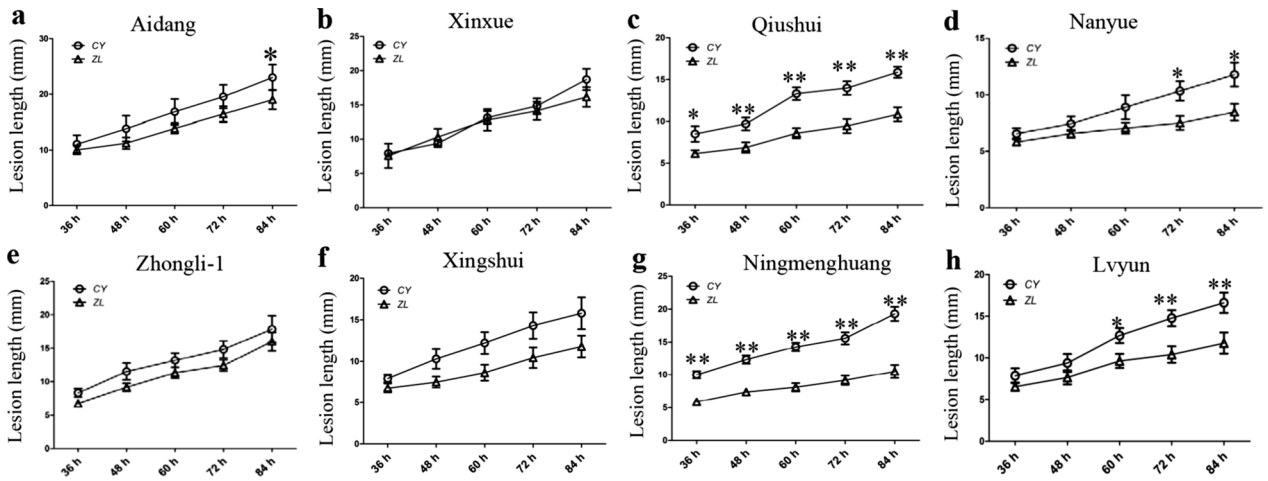

Fig. 7 Pathogenicity of CY-2 and ZL-4 isolates on eight sand pear varieties. The virulence of the two isolates on eight sand pear varieties, including Aidang (**a**), Xinxue (**b**), Qiushui (**c**), Nanyue (**d**), Zhongli-1 (**e**), Xingshui (**f**), Ningmenghuang (**g**) and Lvyun (**h**), was examined. Fungal plugs were placed on the wounded fresh branches. Lesion length was measured at 36, 48, 60, 72 and 84 h post-inoculation (hpi). The column graph was generated using the GraphPad prism software based on these data. The photos were collected at 84 hpi. CY indicates CY-2 isolate, ZL indicates ZL-4 isolate. Bar = 10 mm. Every isolate was inoculated on five branches, and data were analyzed using ANOVA. *$P<0.05$, **$P<0.01$

pear production. In this study, we performed pathological studies of a new canker disease on pears using combined morphological, molecular and pathological analyses, which allowed us to identify *N. parvum* as the causal agent of pear stem canker and twig dieback in China. To our knowledge, the pathogen was frequently reported to cause the disease in other hosts, but the observed symptoms that occur on pears are not well documented. Furthermore, phylogenetic analyses suggested the existence of two lineages of *N. parvum* isolates in pear orchards in China. To identify potential cultural and chemical control options, host susceptibility of various pear cultivars to this disease was tested and fungicide sensitivity tests were performed.

ITS sequences of *Botryosphaeria* species are similar to that of *N. parvum*, sharing 93% similarity. This suggests that the similarity among *N. parvum* species may be higher. The five *N. parvum* isolates from this study were classified into two groups based on the ITS sequence (Additional file 2: Figure S1). The CY-2 isolate was found to be similar to the isolate m110805-5-2, which was previously reported to infect mangoes in China (not published). ITS sequences of these five isolates shared 99% identity. However, characterization of phylogeny of pear isolates combining ITS, *RPB2*, *β-tubulin*, *BotF15* and *EF-1a* sequences classified different *N. parvum* isolates into two main clades, which is consistent with earlier classifications of *N. parvum* (Pavlic et al. 2009; Sakalidis et al. 2011; Aiello et al. 2020). Interestingly, the CY-2 isolate showed unique colony, pycnidia and spore features compared with the other four *N. parvum* isolates. Moreover, CY-2 also exhibited, in general, higher virulence on most pear varieties than ZL-4, a representative isolate from another clade. These results indicate that CY-2 is an isolate that differs from other isolates. For this reason, the study of the evolution and distribution of this isolate should be of particular interest.

The stem canker and dieback disease caused by *N. parvum* is destructive to many agricultural and landscape plants worldwide including *Eucalyptus* spp. (Li et al. 2018), *Pyrus pyrifolia* (Shen et al. 2010), *Rhododendron* spp. (Yang et al. 2015), *Dendrobium oficinale* (Liu et al. 2017), *Punica granatum* (Riccioni et al. 2017) and *Santalum album* (Wang et al. 2016). Since the pathogen has a broad range of hosts, it can survive in many woody plants, which is why there are many obstacles in controlling this disease in fields. In this work, we evaluated the eficacy of fungicides of different categories against the pear canker pathogen *N. parvum*, and found that carbendazim and flusilazole could inhibit both *N. parvum* isolates at low EC50 (Additional file 1: Table S2). However, the inhibitory effect of chlorothalonil and mancozeb against *N. parvum* isolates was less effective (Additional file 1: Table S2), which is consistent with previous studies on *N. parvum* isolated from walnut hosts (Yin et al. 2016). Moreover, we found that the antifungal agent HSAF (heat-stable activity factor) exhibited good inhibitory activities against this pathogen in vitro (Additional file 1: Table S2). However, *N. parvum* can infect xylem of woody plants and the penetration of these fungicides on woody tissues in the field is still unclear. Therefore, fungicide application may be more effective in preventing the

spread of this disease rather than curing trees that have already been infected.

The plant diseases may develop under complicated environment, like the humidity, temperature, nutrients and growth status of the pear trees. One of the key factors is temperature. The optimum temperatures for fungal growth and infection, as well as the disease occurrence in relationship with rain have been characterized in this study. Our findings have many practical implications and should be applied to disease management. For instance, farmers can scout for the onset cankers and twig diebacks during the rainy season when the temperature is near the prime infection temperature, especially on pear trees younger than 2 years old. Once the canker tissues are identified, the diseased shoots and branches can be pruned off, removed from the orchard and destroyed. If the entire tree is infected, that specific tree needs to be removed from the orchard. Such practices may be beneficial for ceasing the spread of the pathogen, which can prevent it from killing the entire tree and spreading to nearby trees in the orchard.

The emergence of this new disease, as well as the source of the pathogens in young pear trees is unclear. Since *N. parvum* was found on weeping cypress in 2010 (Li et al. 2010), an increased number of diseases caused by *N. parvum* have been reported on various hosts including landscape plants in China (Li et al. 2010, 2019, 2020; Yang et al. 2015; Chen et al. 2019; Gao et al. 2019; Song et al. 2019; Du et al. 2021), most of which are widely distributed in Southern China. Because our results showed that the two isolates can also infect five landscape plants (Fig. 6), it is possible that landscape woody plants are the source of pear canker pathogen *N. Parvum.* Pathogens can shift between hosts and quickly spread to healthy plants by people and natural sources such as wind, rain or insects. Therefore, pear canker pathogens may be transferred from ornamental plants that are infected by *N. Parvum* to the pears. This suggests that one of the disease management strategies for pear cankers is to eliminate potential reservoirs of *N. parvum* near pear orchards.

Since *N. parvum* can infect many different fruit plants, such as blueberries, strawberries, grapes, mangoes, pomegranates and pears, we can predict that more diseased fruit plants will be found in future in China. For pear plants, the disease was found in Guangxi and Jiangsu provinces, but it may also exist in pear orchards of other parts of China. Diseases caused by *N. parvum* on pear trees were observed in May and June. Accordingly, we observed that 25 °C is the optimum temperature for the growth and virulence of *N. parvum* isolates. We have screened several resistant sand pear varieties that are widely cultivated in southern China, which will assist pear production. Thus, our findings provide interesting insights that can help avoid possible *N. parvum* infections during pear production. However, to reduce the impact of the disease on pear plants, we should focus on field management practices before May. There is a dire need to develop resistant pear varieties, to further classify the *N. parvum* species, and to evaluate their virulence in the future.

Conclusions

In this study, we found a canker and twig dieback disease caused by *N. parvum* on pear plants. Five *N. parvum* isolates were isolated from the diseased pear branches. According to the morphological and molecular analyses, the isolate CY-2 was classed in a clade different from the other one containing the four isolates (ZL-4, BM-9, BM-10 and BM-12), indicating that two groups of *N. parvum* existed in fields. Additionally, we evaluated the optimal growth and infection temperatures, host range and host susceptibility of the representative isolates (CY-2 and ZL-4). The eficacy of fungicides against these pathogens was also assessed. The resulting data provided several ideas that would control this disease in pear orchards. Taken together, this study found a new pear disease in Chinese mainland, provided new clues for classification of the causal agent, and explored several ways to control this disease.

Methods

Survey, sample collection and isolation of pathogens

From 2016 to 2018, around 20 field surveys were performed to determine the incidence of pear stem cankers in 11 provinces of China. Two cases in Guilin, Guangxi and Nanjing, Jiangsu were found. The stem canker and twig dieback diseases dominantly occurred on pear seedlings in a new planting site that were less than 3 years old. In Guilin, black cankers were progressively spreading on one-year-old Cuiyu pear stems, which finally led to twig dieback. In Nanjing, small black cankers were found on infected two-year-old Cuiguan pear trees, although some appeared as atypical dots at the early stage of infection. Then, the canker lesions expanded along the stems forming necrotic cracked cortexes and a clear rip around the lesions. Samples with typical canker symptoms were collected from branches of infected pear trees.

Pathogen isolation was performed as described in previous study (Bai et al. 2015). The surface cuticle from infected tissues neighboring the asymptomatic regions was removed, and internal tissue was excised and surface-sterilized with 75% ethanol for 1 min, and rinsed with sterilized water three times. The excised tissues were placed onto a sterilized filter paper for drying and subsequently transferred to potato dextrose agar (PDA) plates supplemented with 50 mM ampicillin. The inoculated

plates were incubated at 25 ℃ for 2–4 days until colony formation occurred. Five isolates of *N. parvum* were obtained which were further purified and stored at 4 ℃ for further studies. For long-term storage, fresh cultures of purified isolates were also stored in 30% glycerol at -70 ℃. Pycnidia production was induced by exposing the isolates to continuous light for 16 h and darkness for 8 h for 30 days at 25 ℃. The conidia were acquired from the broken pycnidia. The morphological characteristics of conidia and pycnidia were observed under a reverse microscope (Zeiss).

Amplification of target sequences

Total DNA from each isolate was extracted using the CTAB (Cetyltrimethyl Ammonium Bromide) method. A polymerase chain reaction (PCR) was performed based on the total DNA with the primers ITS1 (5' TCC GTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCC GCTTATTGATATGC 3'), containing 50–200 ng of genomic DNA, 0.25 μM each primer, 10 μL 2 × *Taq* mix kit (Vazyme Biotech, China) (total volume: 20 μL). The reaction conditions were as follows: 94 ℃ for 5 min; followed by 30 cycles consisting of denaturation for 30 s at 94 ℃, annealing for 30 s at 56 ℃, and extension for 1 min at 72 ℃; and a final extension at 72 ℃ for 5 min. The PCR products were sequenced by Sanger sequencing (Genescript, Nanjing). Target sequences of *RPB2*, *β-tubulin*, *BotF15* and *EF-1a* were PCR amplified following the method reported in previous study (Pavlic et al. 2009). The sequences were blast against the NCBI Nr database and deposited into the GenBank database with accession number MW266960-MW266979, MT229071-MT229075.

Phylogenetic analysis

The ITS sequences of reported *N. parvum* or *B. protearum* were acquired from the NCBI Nr database, and assembled using UltraEdit software. Sequences of four genes (*RPB2*, *β-tubulin*, *BotF15* and *EF-1a*) in *N. umdonicola, N. parvum, N. kwambonambiense* and *N. cordaticola* were also downloaded from NCBI Nr database. The ITS and *β-tubulin* sequences from *B. protearum* were set as rooted species. Multiple sequence alignments were performed by BlastW, and the phylogenetic tree was constructed by MEGA 7.0 (Kumar et al. 2016). The robustness of the inferred evolutionary relationships was assessed by 1000 bootstrap replicates.

Pathogenicity tests

Isolates of *N. parvum*, which showed similar molecular characteristics, were used for the pathogenicity test on both pear trees and detached pear branches. The isolates were sub-cultured on PDA petri plates at 25 ℃ for 2 days before inoculation. Later, the agar plugs (5 mm diameter) of five *N. parvum* isolates cultured on PDA medium were inoculated on wounded shoots of two-year-old Cuiguan pear trees. PDA agar plugs without the fungi were put on the same pear varieties in same conditions to serve as the negative control. One seedling was inoculated at three different sites. For the wounding treatment, pear plants were washed with 75% ethanol and dried on sterilized filter paper. The bark (0.5 cm length) of each shoot was wounded with a sterilized knife. The high humidity was maintained by wrapping the inoculated sites with parafilm. Canker formation was investigated on a weekly basis. This experiment was performed three times with three biological repeats of each treatment. The lesion length recorded at 7, 14 and 21 dpi.

For the pathogenicity tests on different pear species, two isolates of *N. parvum*, ZL-4 and CY-2, were put on detached pear branches of wounded one-year-old shoots of eight different sand pear varieties. The shoots were disinfested with 75% ethanol before inoculation, washed with sterilized water, and dried on filter paper. Bark was wounded with a sterilized iron bar (2 mm diameter). A 5-mm agar plug of each *N. parvum* isolate was placed on the wound, and a PDA agar plug without the pathogen was used as a negative control. The end of each branch was wrapped with a wet cotton ball to maintain high relative humidity. All the treated branches were placed in plates with a layer of wet filter paper and covered by a preservative film. Four branches were inoculated for each treatment and plates were maintained at 25 ℃. Lesion size resulting from each treatment was measured at 36, 48, 60, 72 and 84 hpi. This experiment was performed three times with four technical repeats in each treatment. Statistical analysis was performed using two-way ANOVA, GraphPad Prism 7.0.

For testing virulence on different hosts, two isolates of *N. parvum*, ZL-4 and CY-2, were used to inoculate detached branches of wounded one-year-old shoots of five different potential host plants including *P. pseudocerasus*, *P. persica*, *L. acerifolia*, *R. simsii* and *V. ashei*. Many stem canker diseases were found on these five horticulture plants in China. The shoots were treated according to the methods above. Three branches were inoculated for each isolate and lesion size resulting from each treatment was measured at 2 dpi. Statistical analysis was performed using two-way ANOVA, GraphPad Prsim 7.0.

Identification of the optimal temperature for growth and infection

The CY-2 and ZL-4 isolates were cultured on the PDA petri plates and kept at five different temperatures (12, 18, 25, 30 and 37 ℃). Mean colony diameter was measured after two days. This experiment was repeated three

times with three technical repeats in each treatment. To identify the optimum temperature for infection, a virulence test was also performed with five different temperatures (12, 18, 25, 30 and 37 °C). One-year-old healthy Cuiguan pear branches, obtained from a field in Baima, Nanjing, were inoculated with CY-2 and ZL-4 isolates and incubated separately at 12, 18, 25, 30 and 37 °C. Four curves were calculated using the mathematical formulas $f(x) = a \times \exp -\frac{1}{2}\frac{x-x_0}{b}^2$. The pictures were drawn using R based on the temperatures and the size of colony or diameters.

Sensitivity of *N. parvum* to various fungicides and antifungal compound HSAF

In order to demonstrate the sensitivity of isolates to fungicides, we tested seven commercial fungicides (chlorothalonil, mancozeb, carbendazim, tebuconazole, flusilazole, thiophanate methyl and prochloraz, bought from Meilinxuehai, Nanjing, China) registered to be used on pears, and one antifungal compound HSAF. After preliminary treatment using these fungicides, we selected various concentrations for each fungicide to perform the further assay. The PDA petri plates amended with chlorothalonil, mancozeb at five different concentrations (0, 10, 20 and 40 mg/L), along with PDA plates amended with the 0, 1, 2 and 4 mg/L of carbendazim, tebuconazole, flusilazole, thiophanate methyl, prochloraz or HSAF were used to sub-culture with CY-2 and ZL-4 isolates. Colony diameters were measured and photographed at 36 h. Each treatment was performed three times with three technical repeats in each treatment. Statistical analysis was performed using two-way ANOVA, compare cell means regardless of rows and columns, GraphPad Prism 7.0. Additionally, EC50 was also calculated using Nonlinear regression (curve fit) based on the inhibitory data (Additional file 1: Table S2), GraphPad Prism 7.0.

Abbreviations

dpi: Days post-inoculation; EC50: Concentration for 50% of maximal effect; hpi: Hours post-inoculation; HSAF: Heat-stable activity factor; *N. parvum*: *Neofusicoccum parvum*.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42483-022-00111-7.

Additional file 1: Table S1. Spore size of *N. parvum* strains. **Table S2.** Control effect of several fungicides against *N. parvum* isolates.

Additional file 2: Figure S1. Phylogenetic tree of *N. parvum* isolates based on ITS sequences.

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Authors' contributions

FL conceived the project. FH, JY, YJ and BL performed most of the experiments with assistance from the others listed in author board. FH analyzed the data and wrote the manuscript. PL, QZ, AS, SB and ZQF revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Not applicable.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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