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Assessing Cold Plasma's Impact on Banana Growth and Fusarium Wilt Control

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Bananas (*Musa* spp.), which serve millions of people worldwide, face a serious threat from Fusarium wilt (FW) disease caused by *Fusarium oxysporum* f. sp. *cubense* (*Foc*). Developing disease-resistant varieties particularly through breeding is challenging due to banana's seedless nature (parthenocarpic). As an alternative, cold plasma (CP) technology, has the potential to be used for crop improvement. Our study demonstrates a favourable impact of CP on the growth performance of banana (Berangan cultivar, AAA) in terms of height, leaf number and stem diameter. CP-treated plants also displayed delayed disease progression as well as lower disease severity indicated by slightly lower value of leaf symptoms index and rhizome discoloration index compared to the control plants. Additionally, quantitative real-time polymerase chain reaction analysis revealed differential expression of several defence (*PR1*, *WRKY22*, *PAL*, and *CEBiP*) and growth (*Cytochrome P450*, *NAC68*, and *CAT*) related genes in CP-treated plants, particularly in conjunction with *Foc* infection. These findings shed light on the potential use of CP in managing FW in banana and offer insights into possible mechanism behind improved traits.

Keywords **:** banana, cold plasma, gene expression, *Musa* sp., plant improvement

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Banana (*Musa* spp.) is a tropical cash crop cultivated in more than 130 countries, playing a pivotal role in ensuring food security (Alzate Acevedo et al., 2021). Renowned as the world's most important fruit crop in terms of production volume and global trade, bananas ranked as the second most produced fruit following citrus (Dita et al., 2018). They contribute to nearly 16% of the world's fruit production and it is the fourth most important food crop following rice, wheat and corn (Alzate Acevedo et al., 2021). Despite its prominence, banana plants are facing serious threat by a destructive disease called Fusarium wilt (FW).

FW is a fungal disease caused by *Fusarium oxysporum* f. sp. *cubense* (*Foc*). Differential host and pathogenicity determine the three physiological races of *Foc*, namely race 1 (R1), race 2 (R2), and race 4 (R4), in which R1 and R4 are the most important races that have led to greater yield loss. R1 affects Gros Michel (AAA) and Silk (AAB), R2 affects the hybrid triploid cultivar cooking bananas (Bluggoe, ABB) while R4 affects all cultivars in Cavendish (AAA) varieties in addition to R1- and R2-susceptible varieties (Dita et al., 2018). Since its discovery in Australia in 1876,

FW has caused significant banana yield losses (Dong et al., 2020). By the mid-twentieth century, FW's impact on the Gros Michel banana industry led to the adoption of the *Foc* R1-resistant Cavendish cultivar, which has since dominated production and exports (Dita et al., 2018). Around 47% of global banana production relies on Cavendish, with approximately a third for export (Viljoen et al., 2020). However, the emergence of *Foc* tropical race 4 (TR4) threatened Cavendish regardless of environmental conditions (Thangavelu et al., 2020), raising concerns about production deficits and food security.

Long-term control of FW remains a challenge for smallholder farmers due to knowledge-intensive, laborious, costly recommended practices (Tinzaara et al., 2018) and limited availability of environmentally safe chemicals (Ismaila et al., 2023). Besides, *Foc*-infected banana plants are hard to detect without expressed symptoms. Thus, developing *Foc*-resistant cultivars is a promising strategy against FW, but limited availability hampers public access despite efforts in genetic engineering (Dale et al., 2017) and breeding (Wang et al., 2021). Moreover, challenges like polyploidy, reduced reproductive fertility, and complete sterility significantly lower the success rate of breeding programs (Xu et al., 2020).

An eco-friendly agricultural method called cold plasma (CP) treatment has gained attention in scientific circles due to its speed and convenience (Konchekov et al., 2023). Plasma, the fourth state of matter is produced via energizing a gas mixture, leading into ionization and the formation of active components such as ultraviolet radiation. CP shows promise in boosting plant growth and improving disease resistance. Recent studies on Hemp (*Cannabis sativa* L.) found that CP increased the expression of the *WRKY1* transcription factor and genes related to cannabinoids production, enhancing plant defence mechanisms (Iranbakhsh et al., 2020). CP has also been demonstrated to increase resistance against bacterial wilt disease in tomato (Jiang et al., 2014). Besides, CP treatment has improved yield of oilseed rape by enhancing their permeability, wettability, and capacity to uptake water (Ling et al., 2018). Unlike traditional control methods, CP offers a quicker and more environmentally friendly solution as an alternative to produce superior plant materials.

However, despite its promising applications across various crops, the utilization of CP to enhance growth and disease tolerance against FW is not well studied especially on vegetatively propagated crops such as banana. Here, we aimed to evaluate CP effect on the growth performance and tolerance against FW (*Foc* TR4) in *M. acuminata* cultivar (cv.) 'Berangan'. In addition, we attempted to elucidate the mechanism of action underlies CP in FW disease control through expression study of growth- and defence-related genes.

Materials and Methods

Plant material. Rooted stage of tissue culture-derived plantlets of *M. acuminata* cv. 'Berangan' with green leaves (~5 leaves), height and stem diameter of ~7 cm and ~0.5 cm, respectively were selected for this study. In total there were 80 plantlets categorised into four groups (20 plants each): (1) CP treatment followed by *Foc* inoculation, (2) untreated (non-CP) followed by *Foc* inoculation, (3) CP treatment without *Foc*, and (4) no treatment, including CP and *Foc* inoculation.

CP treatment. CP treatment was carried out on the banana plantlets within 2 days after their removal from Murashige & Skoog media. Plantlets were rinsed prior to CP treatment. A non-thermal plasma jet generated via a pin electrode housed in a quartz tube and ground ring electrode was employed for the CP treatment. The discharge was produced under atmospheric air at 15 kV, 20 kHz for 15 s and targeted at corm of the banana plantlets. Plantlets were immediately placed in a closed container with some water after the treatment to prevent dehydration.

Plantlets acclimatization. Plantlets were maintained in hydroponic system supplied with AB medium (containing essential macro and micronutrients for plant growth, provided by UH Agroponics, Malaysia) for 2 months to allow rooting and to reach minimum size suitable for further analysis including the bioassay experiment. Rooted plants were then transferred to polybag (10″ × 10″) containing black soil, cocopeat, and compost in the ratio of 10:5:3. Plantlets were then acclimatized in a greenhouse with controlled temperature of 25 ± 2°C and 12-h photoperiod following average natural light exposure in Malaysia. Plants were watered regularly every 2 to 3 days and fertilized once a week with N:P:K (15:15:15) fertilizer Please provide manufacturer information in the format (Gardem-Well, YMWOO Corporation, Selangor, Malaysia).

Fungal isolates. *Foc* TR4 isolate used in this study was obtained from an infected *M. acuminata* cv. 'Cavendish's inner stem in Kuala Terengganu, Terengganu, Malaysia and was maintained as pure culture on potato dextrose agar (PDA). Prior to bioassay, the identity of the *Foc* TR4 isolate was verified by polymerase chain reaction using *Foc* TR4 specific primers (Supplementary Table 1, Supplementary Fig. 1).

Fungal suspension preparation. Fungal suspension culture was prepared as previously described by Mohd-Yusuf et al. (2019). The fully grown *Foc* TR4 mycelia on PDA were cut into five to ten pieces of approximately 1 cm × 1 cm plug using sterile scalpel and inoculated into 1 liter of potato dextrose broth (Oxoid, Basingstoke, UK). The inoculated suspension culture was incubated at room temperature (26 ± 2°C) and was manually swirled twice a day. On the 7th day post-inoculation, spore concentration was determined using haemocytometer (Weber, Teddington, UK) under microscope (Leica, Wetzlar, Germany). Final concentration of suspension culture was adjusted to 1×10^6 spores/ml using sterile distilled water.

FW bioassay. FW bioassay was carried out on healthy plants of 2 months old after CP treatment using a double tray method as previously described (Mohd-Yusuf et al., 2019). Plants of group 1 (treated (CP) + *Foc* inoculated) and group 2 (untreated (non-CP) + *Foc* inoculated) were subjected to FW bioassay. The plants were carefully uprooted and soil were removed without damaging their roots. Prior to inoculation, roots of plants were rinsed with tap water to remove any soil residues. Then, plants were soaked into the *Foc* TR4 suspension culture (1×10^6 spores/ml) for 2 h before re-planting into their original soil and were labelled accordingly. The plants were then transferred into a double-container apparatus containing a smaller tray with holes (43 × 29 × 9 cm) that fits into a bigger tray at the bottom (46 × 31 × 20 cm). On the other hand, plants of groups 3 and 4 (non-*Foc* inoculated) were soaked with sterile distilled water and served as controls. All plants were continued to be watered and fertilized.

Observation of plant growth parameters. Plant height, number of leaf, and stem diameter was observed and recorded for plants from groups 3 and 4 (non-*Foc* inoculated) for a duration of 3 months following CP treatment. Data was presented as percentage of increment based on the readings measured on day 1 (plasma treatment) and day 90.

Disease progression observation. Response of both *Foc* inoculated (groups 1 and 2) and non-inoculated plants (groups 3 and 4), towards *Foc* infection was observed from 1st until 5th week post-inoculation. The number of yellowing and wilting leaves was recorded for each group and difference in disease progression indicated by clear yellowing of leaves between CP-treated and untreated plants were recorded.

Disease scoring using leaf symptom index and rhizome discoloration index. Severity of *Foc* TR4 infection on banana plants was determined based on the final disease score examined at the end of 5th week post-inoculation as previously described (Mohd-Yusuf et al., 2019). Number of wilting and yellowing leaves were observed and recorded. Then, all the plants were carefully uprooted from soil. After that, vertical dissection of their rhizomes was done for internal symptoms observation and to record the various level of brownish discolorations. Plants' responses against FW were determined based on leaf symptoms index (LSI) and rhizome discoloration index (RDI) based on LSI and RDI scores are shown in Table 1. Then, disease severity index (DSI) was calculated for both LSI and RDI from the scores obtained using the formula:

$$\text{DSI: } \frac{\Sigma(\text{Number on scale} \times \text{Number of seedlings in that scale})}{\Sigma(\text{Number of treated seedlings})}$$

Then, the DSI values were translated into four designations such as resistant, tolerant, susceptible, and highly susceptible referring to guidelines (Table 1). If the LSI and RDI is tolerant and susceptible respectively, the cultivar will be considered susceptible. If the LSI and RDI is tolerant and resistant respectively, the plant will be considered tolerant. Only when both LSI and RDI scores fall in resistant category, the plant will be considered as a resistant individual.

Gene selection and primer design. Primers for six defence genes: *Pathogenesis related-1* (*PR1*), *WRKY transcription factor-22* (*WRKY22*), *WRKY transcription factor-50* (*WRKY50*), *Phenylalanine ammonia lyase* (*PAL*), *Chitin elicitor binding protein* (*CEBiP*), *Putative Chitinase* (*ChiH*) and three growth-related genes: *Cytochrome P450 protein* (*Cytochrome P450 714B3-like*), *NAC domain containing protein 68-like* (*NAC68*), *Catalase* (*CAT*) were selected to study their expression profile following CP treatment and *Foc* TR4 inoculation. *RPS2* gene was used as housekeeping gene (Chaurasia et al., 2016). All primers

Table 1. Translation of DSI scales

DSI scales for LSI	DSI scales for RDI	Translation
1	1	Resistant
Between 1.1 and 2	Between 1.1 and 3	Tolerant
Between 2.1 and 3	Between 3.1 and 5	Susceptible
Between 3.1 and 4	Between 5.1 and 8	Highly susceptible

DSI, disease severity index; LSI, leaf symptoms index; RDI, rhizome discoloration index.

were designed based on DH Pahang genome sequences from Banana Genome Hub database (https://banana-genome-hub.southgreen.fr) and Phytozome (https://phytozome-next.jgi.doe.gov) using Primer 3 software (https://primer3.ut.ee) and listed in Supplementary Table 2.

RNA extraction, DNase treatment, and cDNA synthesis. Total RNA was extracted from root samples collected from all four groups of plants at 2-, 24-, 48- and 72-hours post-inoculation (hpi) using cetyl trimethylammonium bromide (Kistner and Matamoros, 2005). For each time point, there were three replicates. Total RNA extracted were then subjected to DNase Treatment using DNase I (RNase-free) kit (New England Biolabs, Ipswhich, MA, USA), according to manufacturer's description. Subsequently, quantity and quality assessments of RNA were carried out using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and 1% (w/v) agarose gel. cDNAs were synthesized using Viva cDNA synthesis kit (Vivantis, Malaysia) according to manufacturer's description.

Real time - quantitative polymerase chain reaction and data analysis. The real-time expression profiles of all the selected defence and growth-related genes were analyzed in all the cDNA samples using gene-specific primers of all the target genes using Applied Biosystems ViiA 7 real time system (Thermo Fisher Scientific). Real-time analysis was done using the qPCRBIO SyGreen Blue Mix Separate-ROX kit (PCR Biosystems, Wayne, PA, USA) according to manufacturer's description. Gene expression analysis was performed for three technical replicates from the pool of three biological replicates. Relative expression levels of all the target genes were calculated via the Ct value using $2^{\Delta\Delta Ct}$ formula derived from Pfaffl method (Pfaffl, 2007). Then, all the triplicated data were subjected to statistical analysis and normality test using two-way analysis of variance (ANOVA) followed by Tukey's test (*post-hoc*). Statistical Package for Social Sciences (SPSS) software version 20 (IBM Corp., Armonk, NY, USA) was used for all the analysis at the level of 5%.

Results

Effect of CP on plant growth. Average percentage of increment in terms of plant height, number of leaves, and stem diameter in CP-treated plants was higher (128.91%, 52.08%, and 88.22%, respectively) compared to untreated plants (72.74%, 27.86%, and 55.42%, respectively) (Fig. 1). While it is apparent that plasma treatment had a favorable effect on plant growth, the outcomes did not attain statistical significance. Nonetheless, it's important to note that the plasma's impact on plant growth is at least neutral or even potentially beneficial.

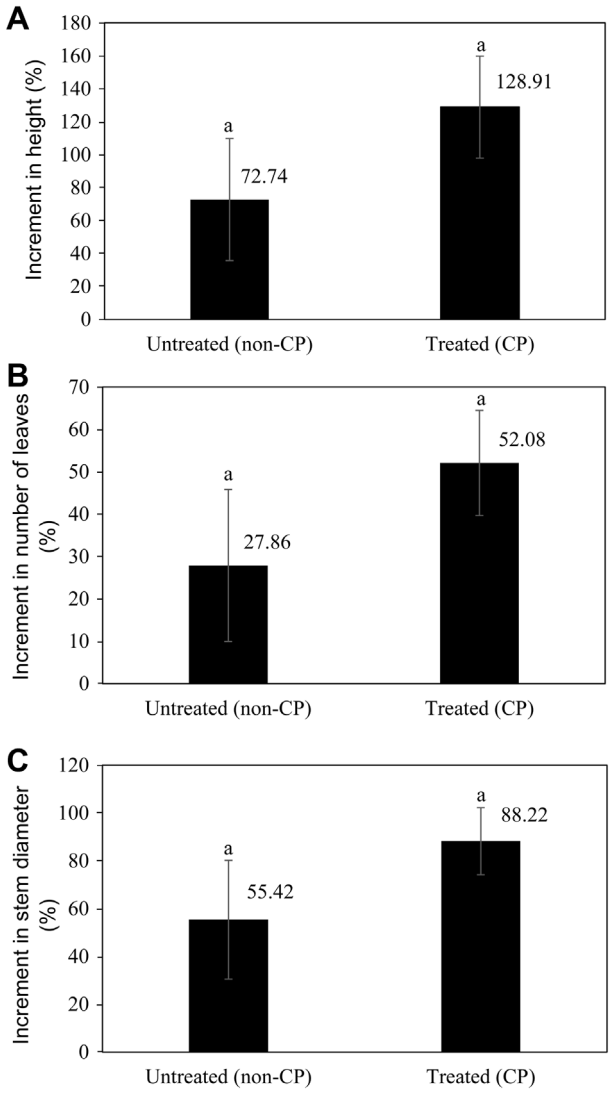

Fig. 1. Effect of plasma treatment on the growth of *Musa acuminata* cv. 'Berangan'. Percentage of increment in plant height (A), number of leaves (B), and stem diameter (C) observed for the duration of 3 months. Data represents the mean ± standard error ($n = 4$). Same alphabet on top of error bars of mean represents no significant differences as determined by T-test ($P > 0.05$).

CP treatment delayed disease progression. By the end of the 5th week post-inoculation, all *Foc*-inoculated plants (CP-treated and untreated) displayed severe wilting symptoms which initially manifested as light streaking and yellowing of lower leaves and later affected entire leaves.

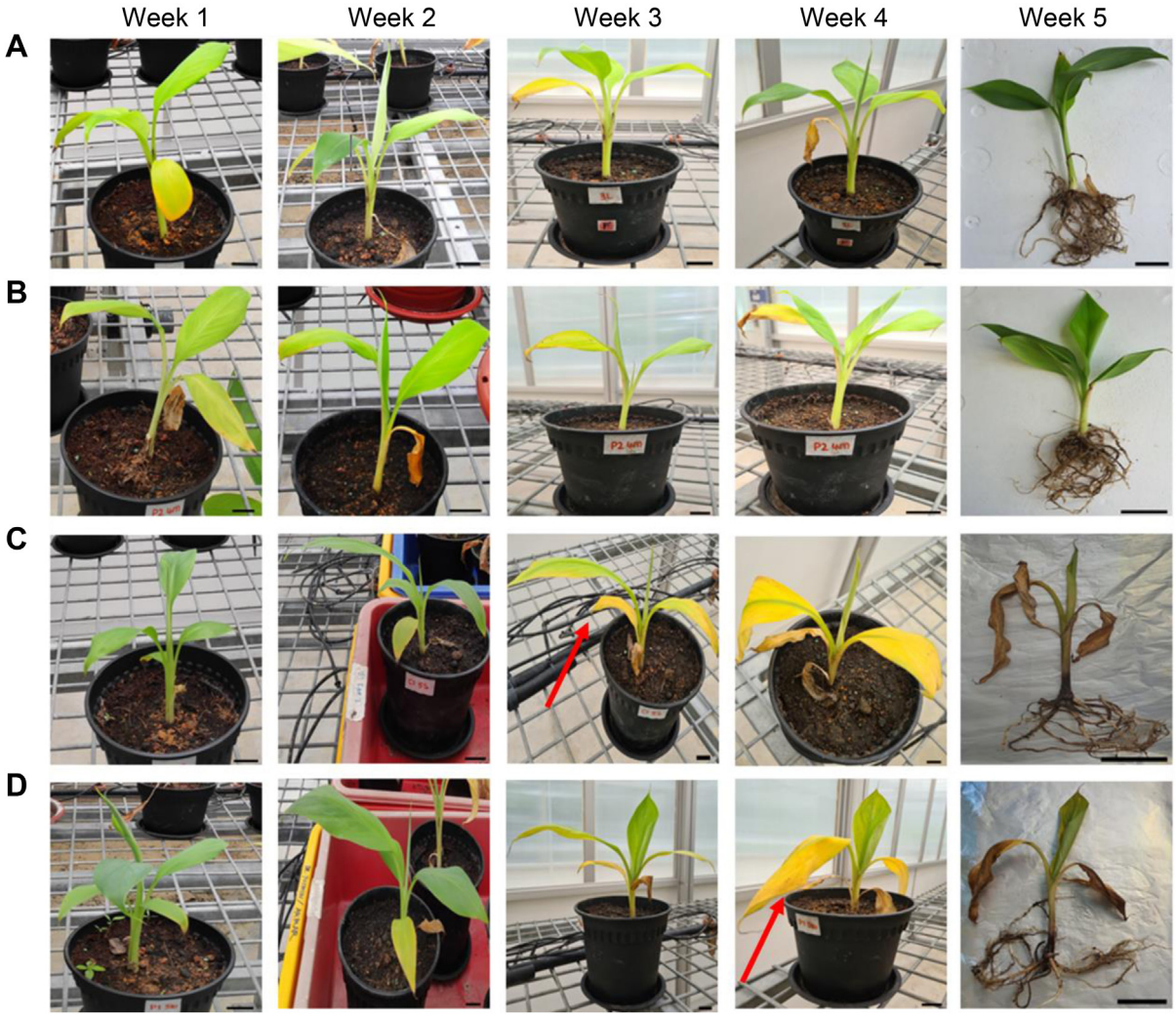

Fig. 2. Disease progression in plasma-treated and untreated *Musa acuminata* cv. 'Berangan' plants following *Foc* inoculation. Images of representative plants of untreated (non-CP) + non-*Foc* inoculated (A), treated (CP) + non-*Foc* inoculated (B), untreated (non-CP) + *Foc* inoculated (C), and treated (CP) + *Foc* inoculated (D) groups from week 1, 2, 3, 4 and 5 post-inoculation were shown above. *Foc*, *Fusarium oxysporum* f. sp. *cubense*; CP, cold plasma. Scale bars = 5 cm. Red arrows indicate the onset of the symptom.

Notably, CP-treated plants (group 1) exhibited delayed disease progression (4th week post-inoculation) (Fig. 2D) compared to CP-untreated plants (group 2) in which extensive streaking and yellowing symptoms progressed to the mid-rib area a week earlier (3rd week post-inoculation) (Fig. 2C). Non-*Foc* inoculated plants from both CP-treated (group 3) and untreated (group 4) groups remained healthy with no visible symptoms (no wilting and yellowing) at the end of 5th week post-inoculation (Fig. 2A and B). These observations demonstrate that plasma treatment delayed disease symptoms in 'Berangan' plants inoculated with *Foc*.

No rhizome discoloration was observed at the stellar regions and surrounding tissues of non-*Foc* inoculated plants from both CP-treated (group 3) and untreated (group 4) plant groups (Fig. 3A and B). Following *Foc* inoculation, CP-treated plants (group 1) and untreated (group 2) plants exhibited rhizome discoloration covering 21% to 50% (Fig. 3D) and more than 50% of stele region (Fig. 3C), respectively. Despite demonstrating delayed disease progression, CP-treated plants were finally evaluated as highly susceptible to *Foc* infection with LSI and RDI scores of 4.0 and 6.0, respectively. The untreated plants showed slightly higher DSI with LSI and RDI scores of 4.2 and 6.6, respectively (Table 2) following *Foc* inoculation. This observed data indicates that the application of plasma treatment did not confer complete resistance to *Foc* in the plants (Fig. 3D) but reduced the severity of disease slightly

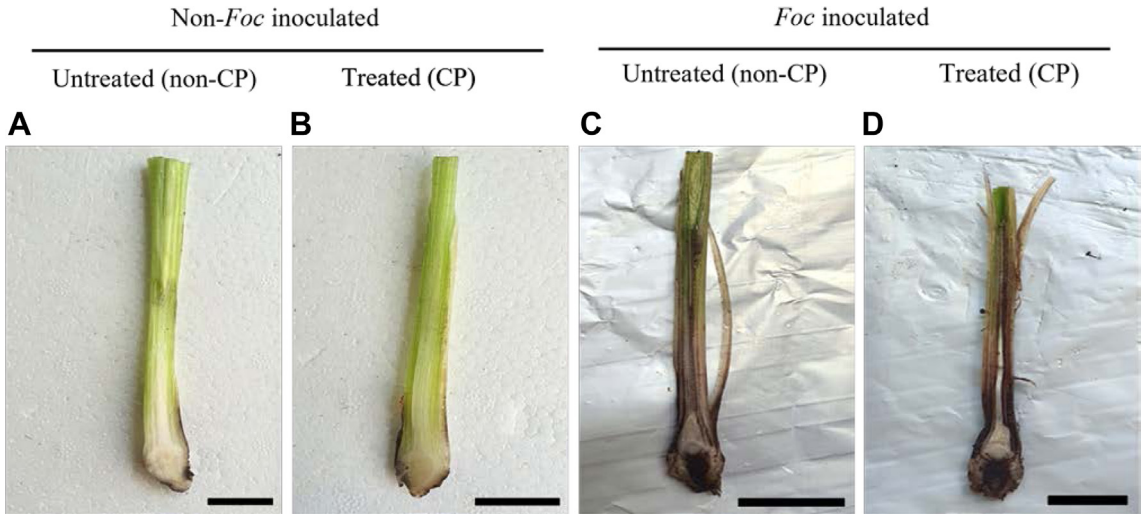

Fig. 3. Internal disease symptom observation in rhizomes of untreated (non-CP) + non-*Foc* inoculated (A), treated (CP) + non-*Foc* inoculated (B), untreated (non-CP) + *Foc* inoculated (C) and treated (CP) + *Foc* inoculated (D) *Musa acuminata* cv. 'Berangan' plants (n = 4). CP, cold plasma; *Foc*, *Fusarium oxysporum* f. sp. *cubense*. Scale bars = 2 cm.

Table 2. DSI showing LSI and RDI from disease scoring of *Musa acuminata* cv. 'Berangan' plants (n = 4)

Treatment		DSI scales for LSI	DSI scales for RDI	Translation
Foc	Plasma			
Non-inoculated	Untreated	1.5	1	Tolerant
	Treated	1.5	1	Tolerant
Inoculated	Untreated	4.2	6.6	Highly susceptible
	Treated	4.0	6.0	Highly susceptible

DSI, disease severity index; LSI, leaf symptoms index; RDI, rhizome discoloration index; *Foc*, *Fusarium oxysporum* f. sp. *cubense*.

as observed in the LSI and RDI scores.

Expression analysis of growth and disease response genes. In response to plasma treatment and *Foc* TR4 infection, the relative expression profiles of nine genes—*PR1*, *WRKY22*, *WRKY50*, *PAL*, *CEBiP*, *ChiH*, *Cytochrome P450*, *NAC68*, and *CAT* genes across the three groups, treated (CP) + *Foc* inoculated (group 1), untreated (non-CP) + *Foc* inoculated (group 2) and treated (CP) + non-*Foc* inoculated (group 3) demonstrated differential expression pattern within 3 days upon *Foc* inoculation when normalized against *RPS2* gene (Fig. 4, Supplementary Table 3). Generally, CP-treated plants showed higher expression levels in most genes, particularly *PR1* and *WRKY22*, which exhibited significant upregulation at 72 hpi, with a notable 63 and 18-fold change, respectively, in group 1 distinguishing it from the other treatment groups. *PAL* gene also displayed a significant increase, exceeding 9-fold in CP-treated plants, regardless of *Foc* inoculation status, notably peaking at 24 hpi compared to a later increment at 48 hpi in untreated plants (group 2). Furthermore, *CEBiP* showed early expression at 2 hpi in CP-treated plants following *Foc* inoculation (group 1). Interestingly, *WRKY50* expression did not exhibit significant differences due to CP treatment; however, it was observed to be highest at 72 hpi in *Foc*-inoculated plants (groups 1 and 2). As for growth-related genes, *Cytochrome P450* and *CAT* demonstrated elevated expression in CP-treated plants post-*Foc* inoculation (group 1). Conversely, *NAC68* displayed a later upregulation (7-fold) at 72 hpi in CP-treated plants post-*Foc* inoculation (group 1). *ChiH* expression remained relatively unchanged across time points in most groups, except for significant increase (35-fold) in untreated plants (group 2).

Discussion

Effectively managing *Foc* TR4 for sustainable banana production poses a significant challenge. Prioritizing strategies to prevent *Foc* entry or delay its spread is crucial (Staver et al., 2020). This study explored CP, an eco-agricultural ap-

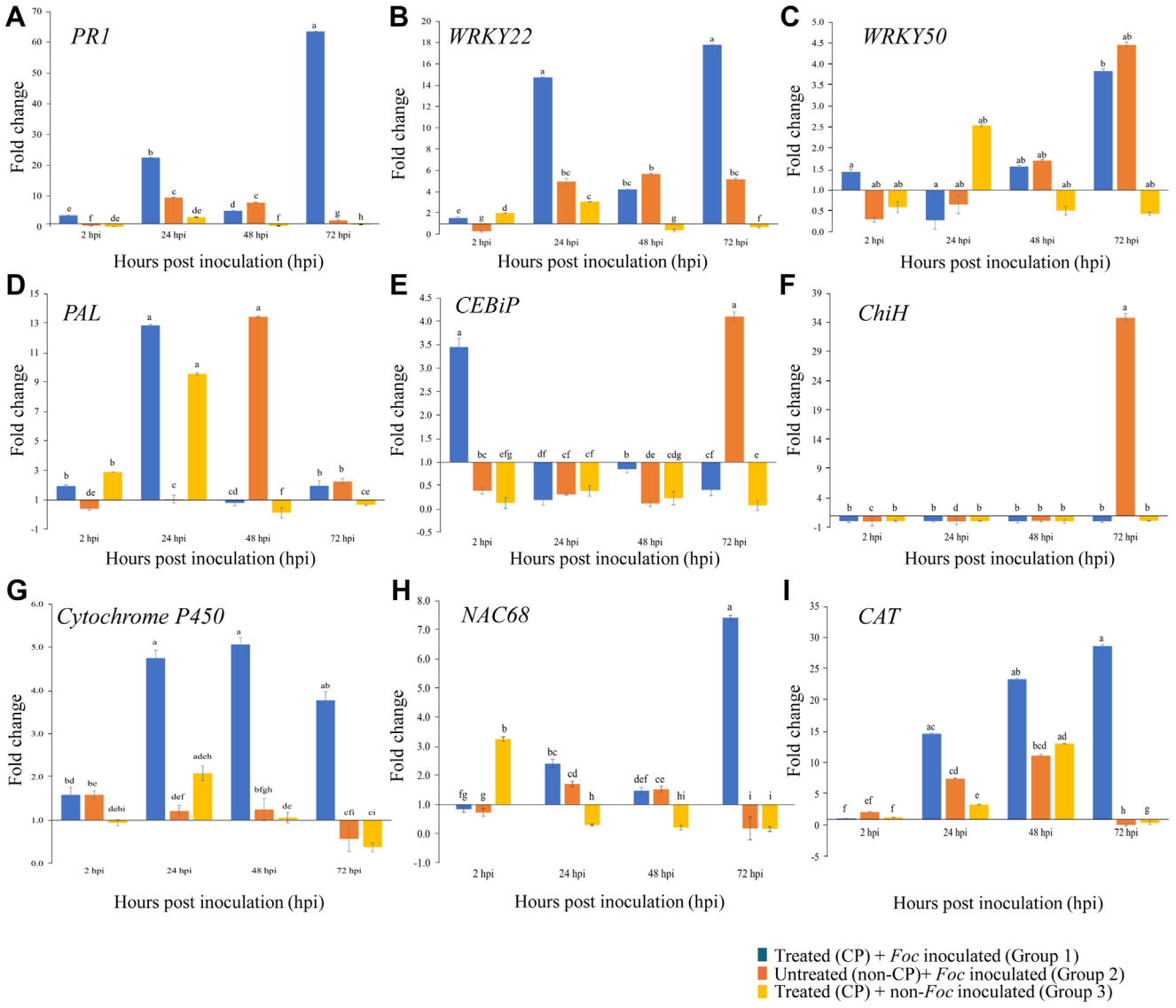

Fig. 4. Relative expression value of *PR1* (A), *WRKY22* (B), *WRKY50* (C), *PAL* (D), *CEBiP* (E), *ChiH* (F), *Cytochrome P450* (G), *NAC68* (H) and *CAT* (I) genes by qPCR in three different groups; treated (CP) + *Foc* inoculated (group 1), untreated (non-CP) + *Foc* inoculated (group 2) and treated (CP) + non-*Foc* inoculated (group 3). Target gene expression level was normalized to *RPS2* reference gene. Relative expression value of the target genes was calculated based on the fold change value of 'untreated (non-CP) + non-*Foc* inoculated' (group 4) plants at 2 hpi. qPCR, quantitative polymerase chain reaction; CP, cold plasma; *Foc*, *Fusarium oxysporum* f. sp. *cubense*; hpi, hours post-inoculation. The bars represent the fold changes of the expression levels. All data were subjected to two-way ANOVA. The data shown are expressed as the mean (%) of three technical replicates from the pool of three biological replicates ± standard error. Error bar represents standard error; same letter denotes not significant ($P > 0.05$) by Tukey's test.

proach, showing potential to improve banana plants' tolerance against *Foc*.

The first aspect examined was the potential tissue damage caused by CP. Given the delicate nature of the tissue culture plants used, a mild CP treatment was employed in this study. Banana plantlets exhibited no apparent damage after CP treatment and remained healthy throughout the study demonstrating that the selected parameters are not detrimental to the plant tissues. Furthermore, CP treatment had no adverse effects on growth. Although statistical significance was not achieved, CP-treated plants showed a favorable impact on growth, displaying increases in height, leaf number, and stem diameter (Fig. 1). This improved growth performance could be attributed to enhanced water uptake, crucial for healthy growth (Ling et al., 2018), and possibly increased nutrient uptake (Jiang et al., 2014). Sim-

ilar observations were reported in various plants, including tomatoes (Adhikari et al., 2020; Jiang et al., 2014), oilseed rape (Ling et al., 2018), and peanuts (Li et al., 2016).

The impact of CP on plant tolerance against *Foc* was observed in which CP-treated plants exhibited delayed disease progression (Fig. 2) and slightly reduced disease severity (Fig. 3). Studies have reported that the external and internal disease observation often do not associate with each other, and thus inconsistent results are produced. Infected plants might have no rhizome discoloration but with severe yellowing symptoms. This might be due to external factors such as lack of nutrient and unfavorable environment but not due to pathogen infection (Czembor et al., 2015). Thus, to overcome this misinterpretation issue, this study correlates external and internal observations to produce a reliable disease scoring scale.

Based on these results, it was demonstrated that CP treatment delayed the disease progression by one week (external observation) (Fig. 2) and slightly reduced the FW disease severity (internal observation) (Fig. 3) although the treatment did not confer complete resistance to *Foc* TR4 in the banana plants (Table 2). This finding is similar to the previously reported study on tomato where plasma-treated plants showed slower disease progression with lesser disease severity compared to untreated plants (Jiang et al., 2014).

To understand the molecular mechanisms underlying these observations, we assessed the expression of several pivotal genes related to defence and growth in banana plant roots at 2-, 24-, 48-, and 72-h post *Foc* inoculation. These timepoints were carefully chosen to identify early *Foc*-responsive genes and investigate their expression profiles following CP treatment, crucial for combating Fusarium wilt. Understanding the expression of these early *Foc*-responsive genes is vital as they initiate the early defence mechanism in banana plants upon pathogen infection through the roots. This aids banana plants in combating *Foc* more effectively by limiting pathogen spread, delaying disease progression, and reducing disease severity, as demonstrated in our current study. Strengthening the resilience of *Foc*-infected banana plants enables them to endure until fruiting, thereby supporting farmers in achieving better harvests.

CP treatment upregulated most of the genes analyzed in this study, with a more pronounced effect observed in CP-treated plants after *Foc* inoculation. Notably, *Cytochrome P450* and *CAT* exhibited a similar trend of expression and were significantly increased in CP-treated plants following *Foc* inoculation (group 1). Cytochrome P450s are known as multifunctional heme-thiolate proteins responsible for both growth and defence in plants (Niu et al., 2018). Previous study by Zhang et al. (2019) has shown similar upregulation of *Cytochrome P450* following *Foc* TR4 attack. Plasma treatment induces early upregulation of this gene, starting from 24 hpi, suggesting it may enhance the plant's ability to produce defence compounds like lignin (Dong et al., 2020), contributing to delayed disease progression and lower DSI.

Meanwhile, *CAT* encodes an antioxidant enzyme which serves as the main reactive oxygen species (ROS) scavenger. This enzyme involves in ROS detoxification and helps in minimizing the host cell damage thus improving plant health. *CAT* expression was reported to be upregulated within 96 h following *Foc* inoculation indicating its role in early defence response (Mohd-Yusuf et al., 2019) which is in accordance with current study. In this study, *CAT* gene expression was significantly higher in CP-treated plants following *Foc* inoculation within 72 hpi, consistent with findings by Mujahid et al. (2020) in plasma-treated grape plants. Plasma treatment alone did not elevate *CAT* gene expression as effectively as the combination of plasma treatment and *Foc* inoculation, indicating a synergistic effect between plasma treatment and pathogen presence. The CAT enzyme, known for scavenging ROS, likely plays a crucial role in enhancing plant health and reducing cellular damage under these conditions.

NAC, a plant-specific transcription factor was also found to be upregulated at later timepoint following CP and *Foc* inoculation, is involved in the regulation of plant development and stress responses (Zhang et al., 2019). In *Foc*-inoculated plants without plasma treatment (group 2), *NAC68* was initially upregulated from 24 to 48 hpi, indicating its role in defence against *Foc* TR4. Interestingly, in the absence of *Foc* inoculation, *NAC68* remained elevated in plasma-treated plants (group 1), highlighting plasma's role in sustaining *NAC68* induction. CP-treated *Foc*-inoculated plants (group 1) exhibited higher *NAC68* expression compared to untreated plants (group 2), suggesting that plasma enhances *NAC68* induction possibly through additional stress factors like ROS release during *Foc* TR4 attack. Early upregulation in group 3 at 2 hpi suggests plasma treatment may initiate a stress response triggering *NAC68* gene expression.

Gene expression analysis of selected defence-related genes revealed heightened expression primarily in CP-treated plants after *Foc* inoculation (except *WRKY50* and *ChiH*), demonstrating plasma's role in enhancing plant defence responses and potentially improving plant tolerance against FW. Among the genes examined, *PR1* displayed the highest expression level and showed the earliest increase in CP-treated plants after *Foc* inoculation.

PR1 is known for its antifungal activity and is important for a plant's innate immunity (Zhang et al., 2019). *Foc*-inoculated plants despite CP treatment exhibited higher *PR1* expression compared to non-inoculated plants over 72 hours post *Foc* TR4 infection. This aligns with findings by Van den Berg et al. (2007), linking *PR1* upregulation to banana tolerance against FW through early activation of cell wall-strengthening genes in roots. In group 2 (untreated with CP), *PR1* expression peaked at 24 hpi and then decreased, consistent with findings by Li et al. (2017), which indicate early induction followed by a decline during *Foc* TR4 infection. Conversely, group 1 (CP-treated) showed sustained upregulation of *PR1*, reaching its peak at 72 hpi with a significant 63-fold increase. This prolonged upregulation could potentially account for the observed delayed disease progression and lower DSI in plasma-treated plants during the bioassay experiment. The persistent elevation of *PR1* expression in CP-treated *Foc*-inoculated plants suggests its role in enhancing plant tolerance against *Foc* infection, supported by the pivotal function of PR proteins in plant innate immunity (Niu et al., 2018). Overall, PR1-encoded proteins are crucial for defending banana roots against Fusarium wilt, highlighting their importance in plant defence mechanisms.

Another early defence gene, *WRKY22*, was also upregulated. WRKY transcription factors (TFs), play crucial role in the regulation of defence-related gene expression and disease response pathways such as salicylate and jasmonate. *WRKY* TFs were upregulated in banana plants despite genotypes following *Foc* TR4 attack suggesting its participation in general response towards *Foc* in banana (Niu et al., 2018). In this study, *WRKY22* exhibited upregulation with higher expression in *Foc*-inoculated plants (groups 1 and 2) compared to non-inoculated plants (group 3) starting from 24 h post *Foc* TR4 infection. This finding underscores the involvement of *WRKY22* in the early defence response mechanism against Fusarium wilt. In *Foc*-inoculated plants untreated with plasma (group 2), *WRKY22* expression increased from 24 hpi but slightly decreased by 72 hpi. However, a significant 18-fold increase in *WRKY22* expression was noted at 72 hpi in plasma-treated plants inoculated with *Foc* (group 1) compared to other treatment groups. This indicates that plasma treatment may similarly influence the upregulation of *WRKY22* as observed with *PR1* expression during the early stages of *Foc* TR4 infection contributing to the improved disease severity and tolerance observed in CP-treated plants. In group 3, non-*Foc* inoculated plants showed initial upregulation of *WRKY22* at 2 hpi and 24 hpi following plasma treatment, indicating its induction in response to stress. However, *WRKY22* expression decreased at 48 hpi and 72 hpi, suggesting that sustained upregulation of this gene may require both plasma treatment and pathogen presence, as observed in group 1.

Moreover, gene encoding a vital enzyme in the phenylpropanoid pathway, *PAL*, was also upregulated. PAL governs the synthesis of phenolic compounds and phytoalexins with antimicrobial properties. The increased production of *PAL* may contribute to the better tolerance against *Foc* (Mohd-Yusuf et al., 2019). In this study, *PAL* expression was found to be upregulated in *Foc*-inoculated plants (groups 1 and 2), with earlier upregulation observed in CP-treated plants compared to untreated ones, irrespective of *Foc* inoculation. *PAL* expression peaked at 48 hpi in untreated, *Foc*-inoculated plants (group 2), consistent with previous findings by Mohd-Yusuf et al. (2019) and Li et al. (2013), indicating a similar expression pattern following *Foc* R4 attack. CP-treated plants (groups 1 and 3) showed *PAL* upregulation regardless of *Foc* inoculation, suggesting *PAL* induction beyond pathogen attack, possibly due to plasma treatment effects.

Furthermore, *CEBiP,* encoding a critical component in the plant signaling pathway to induce pathogen-triggered immunity (Thangavelu et al., 2020) showed interesting expression pattern. The role of *CEBiP* in helping combat disease was also demonstrated where the gene was reported to be highly expressed in resistant banana cultivar compared to susceptible banana cultivar following *Foc* infection (Thangavelu et al., 2020). *CEBiP* was significantly upregulated at an early stage of *Foc* inoculation (2 hpi) in CP-treated plants (group 1), whereas untreated plants (group 2) showed delayed upregulation of the gene at 72 hpi. No upregulation of *CEBiP* was observed at any time point in non-*Foc* inoculated plants treated with plasma (group 3), suggesting that *CEBiP* induction is primarily dependent on *Foc* TR4 infection rather than plasma treatment. This response may be attributed to the presence of chitin oligosaccharides, which act as pathogen elicitors triggering plant-associated molecular pattern-triggered immunity (De Jonge et al., 2010). The late upregulation of *CEBiP* in *Foc*-inoculated plants untreated with CP (group 2) at 72 hpi aligns with findings from previous study (Bai et al., 2013), which reported similar late inductions of *CEBiP* in susceptible banana cultivars at 5- and 10-days post *Foc* TR4 inoculation.

It's noteworthy that the growth observations, independent of *Foc* infection, suggest plasma treatment alone could have induced early upregulation of growth genes immediately after CP treatment, aligning with the favourable growth observed. This is supported by the upregulation of growth

genes observed in non-*Foc* inoculated plants treated with plasma. It's possible these genes were initially induced shortly after CP treatment, with their expression decreasing later during the *Foc* challenge phase.

Overall, current study elucidates the intricate relationship between growth and defence-related genes in banana plants exposed to *Foc* TR4 infection and plasma treatment. Plasma treatment synergistically enhanced the expression of these crucial genes, which play dual roles in bolstering both growth and defence mechanisms. Notably, plasma treatment alone did not markedly induce the expression of *Foc*-responsive genes; however, when combined with pathogen infection, it potentiated these responses. This suggests that plasma treatment primes plants to better withstand pathogenic stress by activating systemic immune responses and modifying stress-related gene expression, like mechanisms described by Adhikari et al. (2020) in tomato plants. This finding parallels a study on tomatoes (Jiang et al., 2014), where plasma-treated plants showed improved growth and better resistance to bacterial wilt caused by *Ralstonia solanacearum*, likely due to increased hydrogen peroxide (H_2O_2) production activating natural defence mechanisms. Similarly, in our study on banana plants treated with CP, this mechanism likely contributed to enhanced tolerance against FW (*Foc*). CP treatment may induce H_2O_2 and ROS, priming plants to mount stronger defences upon *Foc* infection, leading to improved disease tolerance and delayed progression. Thus, plasma-induced stress appears to prepare plants for future pathogen encounters, triggering robust defence mechanisms even before actual infection occurs. The heightened expression of specific defence and growth-related genes under the dual stress of plasma treatment and *Foc* TR4 infection likely contributes to the observed delays in disease progression and the potential reduction in DSI scores during phenotypic assessments. These findings underscore the potential of plasma treatment as a proactive approach to enhancing plant resilience against pathogens, highlighting its role in stimulating both growth and defence mechanisms crucial for sustainable agriculture and food security.

In summary, the study highlights CP's potential in improving banana tolerance against *Foc*. The delayed disease progression observed in this study holds promise for farmers seeking to maximize their banana yields and profits, thus addressing the pressing issue of global food security issue and improving economic outcomes. However, our study had certain limitations. The CP treatment (15kV for 15 s - using plasma jet) on the plant tolerance was limited, suggesting the need to refine parameters such as exposure time, types of discharges, and types of plasma treatment (e.g., plasma-activated water) which better suits the plant materials. Extended exposure to high voltage should be avoided as it can lead to chlorophyll degradation and negatively impact growth (Yagual et al., 2023). Additionally, only a small number of plants were used in this study due to the constraint of the CP setup, which could only accommodate one plant at a time. This required meticulous manual treatment, making the process both time-consuming and labor-intensive which limited the number of experimental repeats. Thus, to enhance the robustness of findings, future investigations should encompass repeated experiments with a larger number of banana plants, thus facilitating a more comprehensive examination of plasma treatment effects. Additionally, exploring alternative CP methods such as corona discharges, dielectric barrier, and plasma-activated water could allow more plants samples to be treated in shorter period and more significant to induce tolerance against FW. It is also important to note that this is the pioneering study that uses plantlets as starting materials for CP treatment. Commonly, seeds were used in many studies on plasma effects on plants and significant increase was observed in the growth parameters tested (Adhikari et al., 2020; Jiang et al., 2014). Since this study used tissue culture plantlets, the influence of CP treatment might not be the same and a lot of optimizations need to be done to obtain a significant result. Nevertheless, this study has shed some light on the potential use of plasma on the banana plantlets instead of the regular use of seeds which is not possible to obtain from cultivated banana (such as 'Berangan') as the cultivated banana are sterile and do not produce seeds.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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Electronic Supplementary Material

Supplementary materials are available at The Plant Pathology Journal website (http://www.ppjonline.org/).

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