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Bicontrol of *Pythium aphanidermatum* and Damping-off Disease of Papaya Seedlings (*Carica papaya* cv. Tainung No.2) by Different Smoke-water

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Abstract. **Two key trends of sustainable agriculture are reducing the amount of inputs such as pesticides, fungicides, or fertilizer and finding ways to reduce or reuse agricultural waste. Leafy plant waste can be burned to produced smoke-water extracts that have effective antimicrobial and germination properties. Damping-off disease caused by *Pythium* spp. leads to significant losses at the papaya seedling stage and is usually managed with fungicides. Five smoke-water extracts derived from burning different plant residues—namely, rice straw smoke-water (R-SW), wheat straw smoke-water (W-SW), pangola grass smoke-water (P-SW), cornstalk smoke-water (C-SW), and bamboo leave smoke-water (B-SW)—were prepared. These were mixed into the V8 media used for culture of *Pythium aphanidermatum*. In vitro treatment with 5% P-SW, C-SW, or B-SW reduced mycelial growth rate significantly, whereas 5% B-SW inhibited mycelial growth completely. All 1% smoke-water preparations reduced zoospore production significantly, but the inhibition rate of 3% R-SW, 3% W-SW, 1% P-SW, 1% C-SW, and 1% B-SW reached 100%. For in vivo experiments, *P. aphanidermatum* was inoculated in 1 kg of potting soil and mixed with B-SW in concentrations of 1% to 5%. The papaya seedlings treated with 2% to 5% B-SW maintained the growth parameter without damping-off symptoms.**

Papaya (*Carica papaya* Linn), in the family Caricaceae, is native to the lowlands of eastern Central America and is a semiherbaceous tropical tree. The main cultivar grown in Taiwan is 'Tainung No.2', which accounts for about 90% of the total papaya cultivation area. Papaya trees must be cultivated in an insect-blocking screen net to prevent aphids from transmitting viral diseases, which shorten the longevity of orchards. Because papaya orchards are under pressure from numerous diseases, the number of seedlings required is more than 3 million every year in Taiwan. Damping-off disease is considered to be the most serious problem during the seedling stage and it causes great economic losses. Damping-off disease is a soil-borne or seed-borne disease that inhibits seed germination or seedling growth and causes symptoms such as seedling rot and lodging. Damping-off is a disease complex that can include *Fusarium* spp., *Pythium* spp., *Phytophthora* spp., *Rhizoctonia solani*, and *Sclerotinia* spp., with *Pythium* one of the more common pathogens causing damping-off (Lamichhane et al. 2017). Chemical fungicides, especially those containing copper, have been used to manage this disease widely (Vawdrey et al. 2015). However, chemical fungicides have many negative impacts on the environment and human health, popularizing the application of effective and nonchemical fungicides.

Forest fire as well as smoke from burning plants can stimulate seed germination (De Lange and Boucher 1990). In horticultural operations, smoke can be used in three forms: gaseous smoke, smoke-water extracts, and smoke-derived active compounds such as the karrikins (KARs) (Kulkarni et al. 2011). KAR1 has been identified as a key agent promoting germination (Flematti et al. 2004), but trimethyl-butenolide (TMB) acts a negative role in seedling growth (Light et al. 2010). Recently, the low concentration of plant-derived smoke has been extended to stimulating crop seed germination, promoting seedling growth, and controlling crop diseases in both agricultural and horticultural plant species (Kulkarni et al. 2011; Stevens et al. 2007) on the one hand, but the high-concentration smoke water (> 5%) showed herbicidal activity in weeds such as *Amaranthus viridis*, *Raphanus raphanistrum*, and *Urochloa decumbens* (Garrido et al. 2023) on the other. Smoke can protect seeds and seedlings against microbial activity, and grains treated with smoke showed less microbial contamination by endophytic species (Roche et al. 1997). Wood vinegar, made from the bark residues of balsam fir and white spruce, contains organic acids, phenols, phenol derivatives, benzenediol, quinones, and furans, and can inhibit two brown rot fungi (*Postia placenta* and *Gloeophyllum trabeum*) and two white rot fungi (*Irpex lacteus* and *Trametes versicolor*) (Stevens et al. 2007). Several compounds including phenols, carbonyls, organic acids, and acetic acids are regarded as antimicrobial. Phenolic compounds can increase the permeability of the microbial cell membrane, which causes damage to the microbial cell membrane and the growth of fungal cells (Greenberg et al. 2008). Organic acids or carbonyls are involved in antimicrobial properties by changing intracellular pH values (Hirshfield et al. 2003). Smoke-water derived from rice straw inhibited significantly both the mycelial growth in vitro and damping-off diseases on papaya seedlings in vivo (Lin et al. 2012). Although the feedstock from the family Poaceae can produce phenolic compounds and organic acids during the pyrolysis process (Hlavsová et al. 2016), the antimicrobial function of smoke-water extracts for crop protection is less studied.

Because the different levels of antimicrobial compounds depend on different materials and the pyrolysis process (Lingbeck et al. 2014), agricultural waste—namely, wheat straw, pangola grass, cornstalks, and bamboo leaves—is considered to have high potential antimicrobial activity because of its high phenol content and organic acids (Arifani et al. 2021; Hlavsová et al. 2016; Menzel et al. 2020). The use of different smoke-water is expected to reduce the levels of chemical fungicides, and an effective concentration for an application in horticultural cultivation is recommended. Therefore, the goal of this study was 1) to evaluate the effect of vie different smoke-waters derived from the family Poaceae, 2) to determine the critical concentration to inhibit *Pythium* mycelial growth and zoospore production, and 3) to apply smoke-water with a critical concentration to control damping-off disease and maintain papaya seedling vigor.

Materials and Methods

Smoke-water production. The agricultural waste from five species in the family Poaceae was used as material for preparation: rice straw (*Oryza sativa* L.) and wheat straw (*Triticum aestivum* L.), pangola grass (*Digitaria decumbens* Stent), cornstalks (*Zea mays* L.), and bamboo leaves (*Dendrocalamus hamiltonii*). The smoke-water extracts were modified from the procedure described by Baxter et al. (1994). The dry plant material (1 kg) was placed in a 20-L stainless steel barrel and burned, and the smoke was bubbled continuously through 500 mL water for 45 min by compressed air to extract compounds from the smoke (Guan Jun Agriculture Technology Develop Co., Ltd, Xiluo Township, Taiwan). After cooling, the smoke-water extract was filtered through Whatman No. 1 filter paper and stored at 1 °C until later study. The different plant-derived smoke-water extracts were named: rice straw smoke-water (R-SW), wheat straw smoke-water (W-SW), pangola grass smoke-water (P-SW), cornstalk smoke-water (C-SW), and bamboo leaves smoke-water (B-SW). These

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smoke-water preparations were used as stock and were diluted to different concentrations with distilled water.

Plant pathogenic fungi isolation, purification, and preservation. Pythium aphanidermatum was originally isolated from papaya plants exhibiting symptoms of seedling damping-off in a greenhouse of the Horticultural Experiment Station of National Chung Hsing University (Taichung City, Taiwan). Symptomatic parts of stems and roots were removed, washed in 1% sodium hypochlorite (NaOCl) for 30 s, rinsed twice in sterile distilled water, and blotted dry with a paper towel. Diseased parts of the plants were cut into 1-cm pieces, placed on water agar, and cultured in a 25 °C incubator. After two to three subcultures on V8 medium [100 mL V8 juice, 2 g calcium carbonate ($CaCO_3$), 20 g agar, and distilled water filled to 1 L], a single mycelium was cut out to purify a single strain. Each strain was numbered and then subcultured at 25 °C for 2 d. The agar containing mycelium was cut into several pieces, placed in a sterilized tube containing sterile distilled water, and preserved at room temperature.

Effects of five different preparations of smoke-waters on mycelial growth. To study the mycelial growth inhibition of the five different smoke-water preparations, each 5-mL of smoke-water (filtered through a 0.22-μm membrane) was mixed with V8 medium (10 mL V8 juice, 85 mL distilled water, 2 g agar, and 0.2 g $CaCO_3$ autoclaved at 121 °C for 20 min) after the medium had cooled to around 45 °C. The five 5% smoke-water preparations were dispensed in 9-cm petri dishes as the treatment group (Fig. 1). The 6-mm-diameter plugs of mycelium containing agar from young and pure cultures of *P. aphanidermatum* were inoculated with various smoke-water treatments at 25 °C for 3 d. The V8 medium without the addition of smoke-water was also inoculated with *P. aphanidermatum* as the control. The radial growth of the mycelia was measured at 12-h intervals for 3 d, with four replications of each treatment. Bamboo smoke-water was tested with a wider range of concentrations, from 1% to 5%. (Fig. 2). The mycelial growth inhibition rate was calculated as (Average diameter of mycelia in control group – Average diameter of mycelia in treatment group) ÷ Average diameter of mycelia in control group × 100%.

Effect of different preparations of smoke-water on zoospore production of *P. aphanidermatum.* After the isolated *P. aphanidermatum* was cultured on V8 agar for 2 d, 10 6-mm-diameter plugs were punched from the colony margin using a sterile steel borer. These plugs were placed in a petri dish, rinsed twice with sterile distilled water, and then mixed with 25 mL of different concentrations (0, 1%, 3%, or 5%, v/v) of five smoke-water extracts and distilled water. After 20 h in a 25 °C incubator, four 2-μL smoke-water suspensions were placed on a glass slide and observed under a light microscope at 40× magnification. The number of zoospores was estimated using a mechanical counter, with four replications of each treatment. The B-SW preparation was further tested against *P. aphanidermatum* zoospore production at concentrations of 0.1%, 0.5%, 1%, 3%, and 5%.

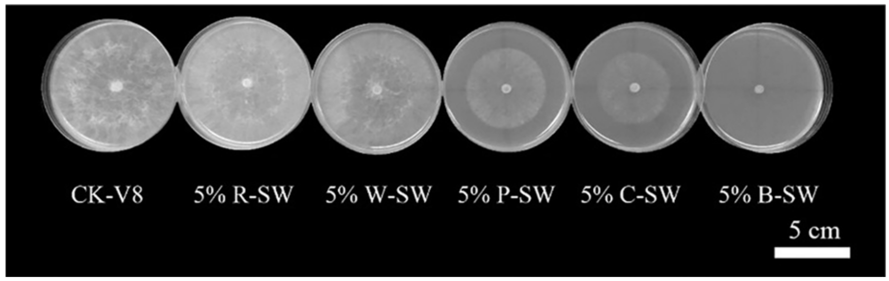

Fig. 1. Effect of 5% smoke-water preparations from different Poaceae sources on mycelial growth of *Pythium aphanidermatum* after 36-h inoculation. B-SW = bamboo leaves smoke-water; CK-V8 = control- only V8 medium; C-SW = cornstalk smoke-water; P-SW = pangola grass smoke-water; R-SW = rice straw smoke-water; W-SW = wheat straw smoke-water.

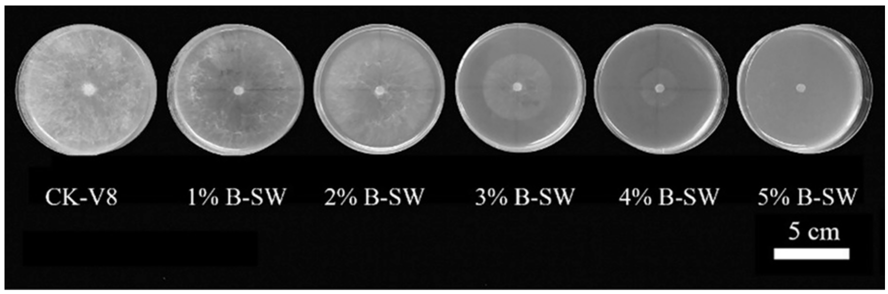

Fig. 2. Effect of 1% to 5% bamboo leaves smoke-water (B-SW) on mycelial growth of *Pythium aphanidermatum* after 36-h inoculation. CK-V8 = control- only V8 medium.

Effects of B-SW preparation on damping-off in pot-grown papaya seedlings. One kilogram of potting soil (Australia; pH, 5.5; electrical conductivity, 2.5 $mS \cdot cm^{-1}$; organic matter content, 85%; total nitrogen, 160 $mg \cdot g^{-1}$; phosphorus, 78.66 $mg \cdot g^{-1}$; potassium, 166 $mg \cdot g^{-1}$; and magnesium, 80 $mg \cdot g^{-1}$) was mixed with 200 mL distilled water and autoclaved at 121 °C for 1 h as the negative noninoculated control (CK-N) medium. The potting mixture was inoculated with 10 mL *P. aphanidermatum* zoospore suspension with a concentration of 10^4 spores/mL. The zoospore suspension was initiated with 10 6-mm plugs from 2-d-old V8 agar cultures in a petri dish containing 25 mL sterile distilled water. The cultures were incubated for 24 h at 25 °C, and the concentration of zoospores was counted with a hemacytometer and adjusted to 10^4 spores/mL. After incubation at 25 °C for 7 d, the 200-mL B-SW extracts in a range of concentrations (1%–5%) were mixed with 1 kg potting soil. After the B-SW treatment for 14 d, pots were filled with 120 g potting soil. Three papaya seedlings with two true leaves were transplanted into each pot. The experiment included both inoculated (CK-I) and CK-N potting soil, and the treatment with distilled water was used as a control. Each B-SW concentration (1%, 2%, 3%, 4%, or 5%, v/v) was applied to four pots, with three replications. Pots were placed in a greenhouse and maintained for 3 weeks. Plants were observed for typical damping-off symptoms as well as plant growth traits (plant height, stem diameter, and leaf number). The damping-off rate was determined as a percentage of the total number of seedlings: Damping-off rate of papaya seedlings was calculated as (Number of seedlings with damping-off symptoms ÷ Total number of seedlings) × 100%.

Statistical analysis. The experimental data were analyzed using CoStat software Version 6.45 (CoHort Software, USA) and subjected to analysis of variance. Mean values within each smoke-water preparation were compared by the least significant difference test at the 5% ($P \leq 0.05$) level of significance.

Results

Antimicrobial activity of five smoke-water extracts on mycelial growth of P. aphanidermatum. The mycelium growth rate was significantly less in all treatments when growing in the five smoke-extract treatments except R-SW (Table 1). The mycelial growth inhibition rates of 5% B-SW, P-SW, and C-SW were 100%, 41.61%, and 41.54%, respectively (Table 1). Because of the significant antimicrobial activity of 5% B-SW, the B-SW was tested over a range of concentrations (1%–5%) in the same assay. The mycelial inhibition rates at 3%, 4%, and 5% (v/v) were 51.04%, 83.23%, and 100%, respectively, reflecting significantly less growth than the control group (Table 2). Although only the highest concentration of B-SW (5%) could inhibit mycelial growth completely, the critical concentration was between 4% and 5% (v/v), because the mycelial inhibition rate

Table 1. Effect of 5% smoke-water extracts from different sources on the mycelial growth of *Pythium aphanidermatum*.

Smoke-water[i]	Mycelial growth (mm) 12 h	24 h	36 h	Growth rate (mm/h)	Mycelial growth inhibition rate (%)
CK-V8	20.31 a[ii]	52.86 a	79.00 a	2.44 b	—
5% R-SW	15.34 b	46.24 b	79.00 a	2.68 a	0.00 d
5% W-SW	13.60 c	42.82 c	75.4 1b	2.58 a	4.54 c
5% P-SW	5.63 d	23.80 d	46.13 c	1.71 d	41.61 b
5% C-SW	3.52 e	21.78 e	46.18 c	1.83 c	41.54 b
5% B-SW	0.00 f	0.00 f	0.00 d	0.00 e	100.00 a

[i] B-SW = bamboo leaves smoke-water; CK-V8 = control- only V8 medium; C-SW = cornstalk smoke-water; P-SW = pangola grass smoke-water; R-SW = rice straw smoke-water; W-SW = wheat straw smoke-water.
[ii] Values in each column with the same letter are not significantly different at $P \leq 0.05$ by the least significant difference test.

Table 2. Effect of 1% to 5% bamboo leaves smoke-water on the mycelial growth of *Pythium aphanidermatum*.

Smoke-water concn[i]	Mycelial growth (mm) 12 h	24 h	36 h	Growth rate (mm/h)	Mycelial growth inhibition rate (%)
CK-V8	21.45 a[ii]	54.90 a	79.00 a	2.40 a	—
1% B-SW	15.00 b	45.80 b	79.00 a	2.67 a	0.00 e
2% B-SW	7.76 c	35.75 c	65.43 b	2.40 a	17.18 d
3% B-SW	0.00 d	13.98 d	38.68 c	1.61 b	51.04 c
4% B-SW	0.00 d	2.80 e	13.25 d	0.55 c	83.23 b
5% B-SW	0.00 d	0.00 e	0.00 e	0.00 d	100.00 a

[i] B-SW = bamboo leaves smoke-water; CK-V8 = control- only V8 medium.
[ii] Values in columns followed by the same letter are not significantly different at $P \leq 0.05$ by the least significant difference test.

Table 3. Effect of 1%, 3%, and 5% smoke-water extracts from different Poaceae sources on *Pythium aphanidermatum* zoospore production.

Concn (%)	Smoke-water[i] R-SW	W-SW	P-SW	C-SW	B-SW
0	25.3 a[ii]	25.3 a	25.3 a	25.3 a	25.3 a
1	7.8 b	2.0 b	0.0 b	0.0 b	0.0 b
3	0.0 c	0.0 c	0.0 b	0.0 b	0.0 b
5	0.0 c	0.0 c	0.0 b	0.0 b	0.0 b

[i] B-SW = bamboo leaves smoke-water; C-SW = cornstalk smoke-water; P-SW = pangola grass smoke-water; R-SW = rice straw smoke-water; W-SW = wheat straw smoke-water.
[ii] Values in columns followed by the same letter are not significantly different at $P \leq 0.05$ by the least significant difference test.

increased from 83% to 100% in this treatment range compared with the control (Table 2).

Effect of smoke-water on zoospore production of P. *aphanidermatum.* All concentrations of the five smoke-water extracts reduced zoospore production significantly. The zoospore production at 1% R-SW and 1% W-SW was significantly less than the control group (Table 3). The 1% P-SW, C-SW, and B-SW extracts showed stronger negative effects on zoospore production than 1% R-SW and W-SW (Table 3). Zoospore production was inhibited completely by 3% R-SW and W-SW, and by 1% P-SW, C-SW, and B-SW (Table 3). Zoospore production at 0.1% and 0.5% B-SW was significantly less than the control group and was inhibited completely when the concentration was greater than 1% (Fig. 3). Therefore, the critical concentration was between 0.5% and 1% B-SW.

Suppression of Pythium *damping-off disease by B-SW in vivo.* In the preinoculated media without a smoker-water treatment, damping-off occurred in 75% of the papaya seedlings. Treatment with 1% B-SW decreased the rate of damping-off significantly to 8.33% (Table 4). The damping-off rate of seedlings treated with 2% to 5% B-SW was 0%, which indicated that treatment of the growing media with a greater than 2% concentration of B-SW could prevent damping-off diseases completely. The stem diameter and plant height of the untreated seedlings in CK-I were significantly less than those of both the CK-N group and all B-SW treatment groups (Table 4). There was no significant difference in growth traits, including stem diameter, leaf number, and plant height, among the groups treated with 1% to 5% B-SW and

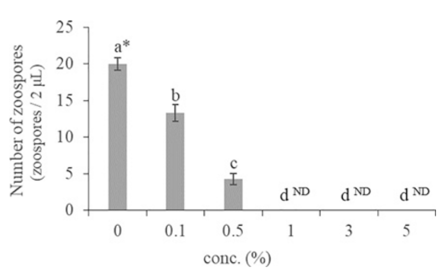

Fig. 3. The effect of 0.1% to 5% bamboo leaves smoke-water (B-SW) extracts on zoospore production of *Pythium aphanidermatum*. *Means followed by the same letter are not significantly different at $P < 0.05$ by the least significant difference test. The vertical bars represent ±*SE* of the mean (n = 4). ND = No zoospore production.

the CK-N group, indicating that B-SW not only enhanced disease resistance, but also maintained similar seedling vigor in the untreated and healthy plants (Table 4 and Fig. 4). After treating with 2% to 5% B-SW, the papaya seedlings did not show damping-off diseases. Moreover, the plant height of the seedlings at a low concentration (2% B-SW) was greater than that in the high concentrations (4% to 5% B-SW), indicating the 2% B-SW was the better concentration in the in vivo experiment.

Discussion

The results showed that 5% P-SW, C-SW, and B-SW had the strongest effect on inhibiting the mycelia growth of *P. aphanidermatum* (Table 1), and 5% B-SW could inhibit mycelia growth completely (Table 2). The 3% R-SW and W-SW inhibited *P. aphanidermatum* zoospore production completely, but the 1% P-SW, C-SW, and B-SW showed the same effect (Table 3). The smoke from Douglas fir sapwood inhibited both *Staphylococcus aureus* and *Aeromonas hydrophila*, whereas smoke from mesquite or lodge pole pines showed a less suppressive ability to these pathogens (Sofos et al. 1988). Therefore, the complex compounds showed quantitative and qualitative variations in smoke-water derived from different materials, which in turn may explain the different suppressive effects from the five different materials in our experiment. The smoke-water derived from rice straw showed significant disease suppression against *Pythium* sp. For example, a medium with more than 1.5% R-SW inhibited *Pythium* sp. zoospore production completely (Lin et al. 2012). A 5-kg rice straw was used to prepare the 500-mL smoke-water extracts in experiment of Lin et al. (2012). Lower volumes were used in our experiment— namely, a 1-kg dry biomass to prepare the 500-mL smoke-water extracts. As a result, the greater than 3% R-SW extract showed complete inhibition of zoospore production in our study (Table 3). Because the B-SW showed greater efficacity than R-SW, bamboo leaves can support the alternative material to replace R-SW when rice straw is not available.

Although all plant materials could be converted to smoke-water (Jäger et al. 1996), and different plant-diverse smoke showed a positive effect on seed germination and seedling growth (Elsadek and Yousef 2019), there may be thousands of unknown compounds in smoke (Smith et al. 2003), and the effects of smoke may depend on the plant species (Ren et al. 2017). For instance, the active compound KAR1, which can stimulate seed germination, was absent in smoke derived from legume materials such as alfalfa, but was present with prairie hay and wheat straw (Smith et al. 2003). The growth parameters in snap beans was better at the 1.5% level as a foliar spray combined with soil drenching than 2% smoke-water derived from *Casuarinas* leaves (El-Nour 2021). The high concentration of smoke-water extracts influenced the growth of snap beans negatively (El-Nour, 2021),

Table 4. The effect of 1% to 5% smoke-water extracts from bamboo leaves (B-SW) on growth parameters of papaya seedlings inoculated with damping-off diseases.

Smoke-water concn[i]	Papaya seedling damping-off rate (%)	Stem diam (mm)	Leaf no.	Plant ht (cm)
CK-N	0.00 b[ii]	3.56 ab	8.53 a	12.95 ab
CK-I	75.00 a	2.84 c	7.56 b	9.36 c
1% B-SW	8.33 b	3.75 a	8.56 a	12.58 ab
2% B-SW	0.00 b	3.78 a	8.19 ab	14.70 a
3% B-SW	0.00 b	3.54 ab	8.33 a	13.50 ab
4% B-SW	0.00 b	3.31 b	8.36 a	12.32 b
5% B-SW	0.00 b	3.49 ab	8.31 ab	12.33 b

[i] B-SW = bamboo leaves smoke-water; CK-I = inoculated control; CK-N = noninoculated control.
[ii] Values in columns followed by the same letter are not significantly different at $P \leq 0.05$ by the least significant difference test.

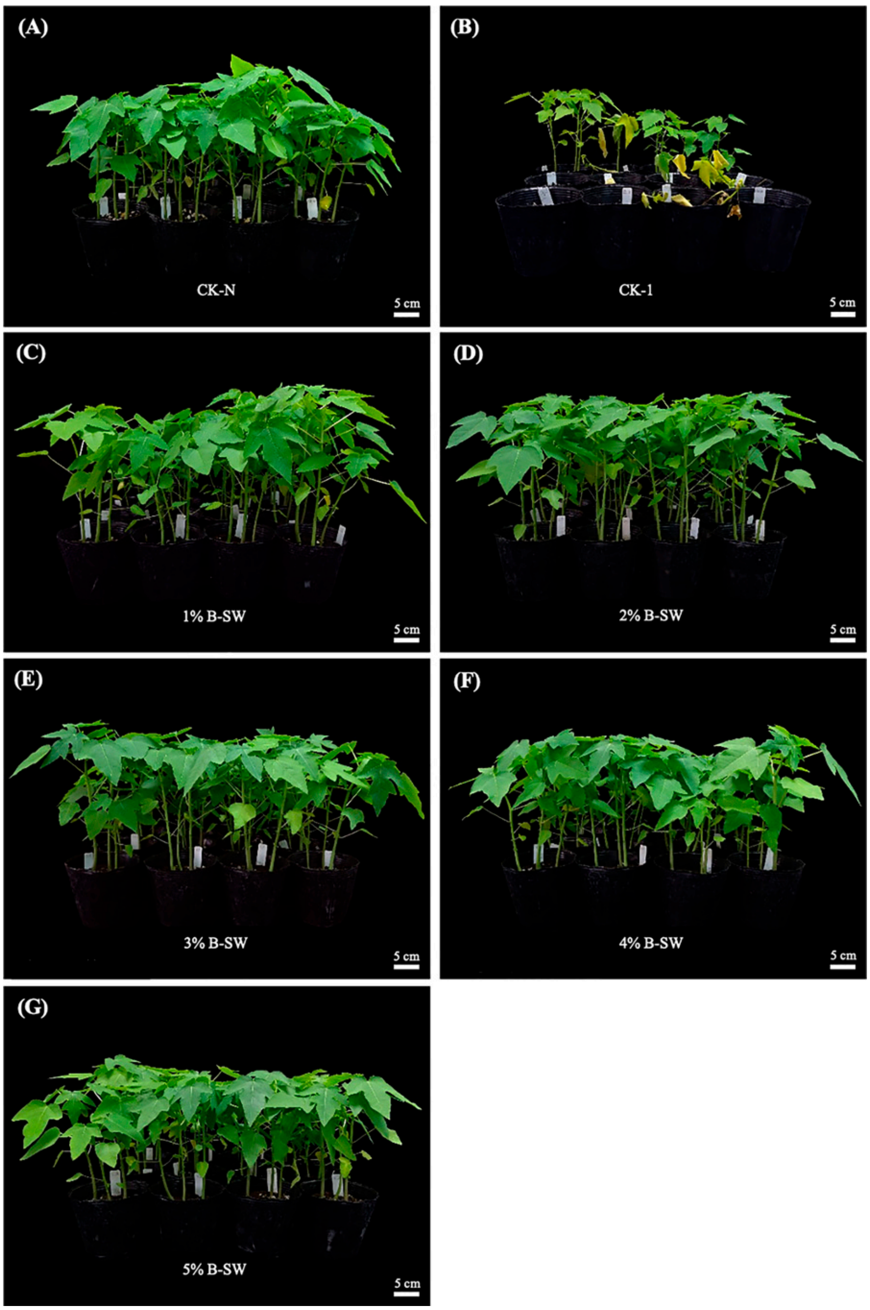

Fig. 4. The appearance of papaya seedlings in transferred noninoculated control (**A**), in potting soil with of *Pythium aphanidermatum* (**B**), or in potting soil inoculated with *P. aphanidermatum* and 1% to 5% bamboo leaves smoke-water (**C–G**) for 3 weeks in a greenhouse. B-SW = bamboo leaves smoke-water; CK-I = inoculated control; CK-N = noninoculated control.

indicating some compounds may have an inhibitory effect on seedling growth, such as TMB, as isolated and confirmed (Light et al. 2010; Papenfus et al. 2015). Although the TMB in smoke-water plays a negative role in regulating seed germination of lettuce seeds (Gupta et al. 2019), papaya seedlings treated with 5% B-SW had a similar performance with regard to plant parameters compared with the CK-N group (Table 4). In agreement with our results, the height of papaya seedlings was not significantly different when treated with 1% to 5% R-SW (Lin et al. 2012). However, plant height in seedlings treated with 2% B-SW extract was greater than with the 4% to 5% B-SW extracts, inferring that the different concentration of KARs or inhibitory compounds influenced seedling growth. Therefore, 2% B-SW was recommended for application to seedlings because of the better growth traits and no symptoms of damping-off disease. Further studies are essential, not only to explore the compounds, but also to investigate the dilution effect of smoke from different plant materials on growth parameters and disease control.

According to our in vivo experiment, the growth stem diameter, leaf number, and plant height showed no significant difference between the CK-N group and the 2% B-SW treatment (Table 4). Compared with soil stem sterilization, application of B-SW to combat pathogens showed a similar result, but soil stem sterilization is relatively costly (Dietrich et al. 2020). Because organic acids could suppress significantly soil-borne pathogens such as *Fusarium oxysporum*, *Ralstonia solanacearum*, and *Ralstonia solani* (Huang et al. 2015), applying the smoke-water containing organic acids may be an alternative way to protect the plants from pathogens when soil stem sterilization is unavailable. An antagonistic effect between smoke-water and *Bacillus licheniformis* as plant growth–promoting rhizobacteria was observed in okra growth (Papenfus et al. 2015). Applying smoke-water to protect plants from pathogens may also influence the balance of growth-promoting rhizobacteria due to antimicrobial activity. This aspect requires further investigation to figure out a better application schedule.

Conclusion

Our study showed there can be large variations in antimicrobial activity of smoke-water from different agricultural waste products. Of five different smoke-waters, B-SW showed the strongest inhibition of *P. aphanidermatum* in our in vitro experiments compared with the other smoke-water extracts. Although the soil was contaminated with *P. aphanidermatum*, the seedlings treated with 2% B-SW could maintain their growth traits without damp-offing symptoms. Therefore, this concentration shows great potential for agricultural application and may replace the partial use of chemical fungicides for the control of damping-off diseases during the early cultivation of papaya seedlings.

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