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Disease-reducing effects of aqueous leaf extracts of *Annona glabra* and *Wedelia calendulacea* on Fusarium basal rot of shallot caused by *Fusarium oxysporum*

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ABSTRACT

Fusarium basal rot (Fusarium oxysporum) of shallot is a destructive disease, which constrains the production and quality of this crop in Vĩnh Châu (Sóc Trăng, Vietnam). This study aims at screening wild plants with antimicrobial activities to bio-control the disease. Among aqueous extracts of 49 commonly found plant species in Vietnam, the 4% (w/v) leaf extracts of Annona glabra and Wedelia calendulacea exhibited strongest inhibitory effects on the mycelial growth of F. oxysporum (up to 35.3% and 25.7%, respectively) in disc diffusion assays. Furthermore, incubating the conidia in A. glabra extract (4%) resulted in a significant suppression in germination (up to 95.3%). Under net house conditions, soil drenching of both extracts at 4% and 5% concentrations showed an equivalent reducing effect on both disease incidence and severity in shallot plants compared to the chemical treatment. Thus, A. glabra and W. calendulacea show their potentials for large-scale applications to sustainably control Fusarium basal rot of shallot.

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1 INTRODUCTION

Shallots (*Allium cepa* var. *aggregatum*, syn.: *A. ascalonicum*) have been extensively used for nutritional and aromatic values in cooking. They are also considered to be important in medical practices due to their anticancer properties and immune-enhancing effects (Mohammadi-Motlagh *et al.*, 2011). Currently, shallots are one of the major vegetative crops that are cultivated worldwide, particularly in the tropical lowlands (Shigyo and Kik, 2008). Large distribution of arenosols combined with tropical monsoon climate in Vĩnh Châu, a coastal town in Sóc Trăng province of Vietnam, provide favorable conditions for the propagation of this crop (Đặng Thị Cúc, 2008). Here, the shallot is a horticulture commodity of high economic and trading values to farmers. In 2012, the area for shallot growing totaled over 8,000 ha, accounting for nearly 70% of land use for vegetable cultivation in Vĩnh Châu. The average yield in 2012 was reported to be 14–15 tons/ha and was projected to increase up to 20 tons/ha in the near future (Dương Vĩnh Hảo, 2013).

During cultivation, harvesting and storage, shallots are under constant attacks by numerous foliar, bulb, and root diseases among which, Fusarium basal rot (FBR) caused by *Fusarium oxysporum* is one of the most destructive (Cramer, 2000; Sintayehu *et al.*, 2011a, 2011b, 2014; Conn *et al.*, 2012). In Vĩnh Châu, considerable yield and quality losses due to FBR occur when the crop is frequently planted with excessive use of fertilizers (Đặng Thị Cúc, 2008). Management of FBR can be made through fungicide application, host plant

resistance, crop rotation and biological control (Crammer, 2000; Sintayehu *et al.*, 2011a, 2011b, 2014; Conn *et al.*, 2012; Vu *et al.*, 2012). Chemical means have been overused by Vĩnh Châu's farmers, leading to several environmental and human health issues (Đặng Thị Cúc, 2008). The deployment of resistant cultivars is an alternative solution; in fact, the resistance in some onion cultivars against FBR has been reported. Unfortunately, no variety of shallots has been found to be highly resistant to FBR until now (Cramer, 2000; Sintayehu *et al.*, 2011a; Vu *et al.*, 2012). Long-term rotation with non-host crops may also help reduce losses. However, this strategy requires farmers' increased expertise since negligible implementation of a crop rotation plan may lead to imbalance in the soil nutrient composition and an accumulation of pathogens affecting a critical crop (Mohler and Johnson, 2009).

Recently, there has been a worldwide interest in applying natural compounds of plant origin as crude extracts or as pure bioactive compounds for biological control of many plant diseases due to easy availability, less toxicity to human and animals, and low environmental retention. Plants are able to synthesize a wide range of secondary metabolites, e.g., alkaloids, flavonoids, glycosides, quinones and phenolic compounds, some of which are known for antimicrobial activity (Fawcett and Spencer, 1970). There is evidence from many studies that various plant extracts possess antifungal properties against certain pathogens, e.g., the *Kalanchoe pinnata* extract exhibits inhibitory effects on *Fusarium moniliforme* causing bakanae of rice (Yasmin *et al.*, 2008). In addition, Masuduzzaman *et al.* (2008) reported that extracts from many species of the genus *Allamanda* were found to be effective in reducing symptoms of seedling damping off and seedling blight on eggplants caused by *Rhizoctonia solani*, *Phytophthora capsici*, *Sclerotium rolfsii* and *Phomopsis vexans*. The aqueous extract of *Chromolaena odorata* was demonstrated to greatly reduce severity of many important rice diseases, i.e. sheath blight (*R. solani*), blast (*Pyricularia oryzae*) and brown spot (*Bipolaris oryzae*). Interestingly, the extract does not have any direct antifungal effects on these pathogens (Khoa *et al.*, 2011).

In Vietnam, no attempts have been made so far to evaluate effects of plant extracts on FBR of shallots. This paper, therefore, presents the whole process of (1) screening aqueous extracts of 49 plants species commonly found in Vietnam for their antifungal activities against *F. oxysporum* under laboratory conditions, and (2) testing for the disease-reducing effects of *Annona glabra* and *Wedelia calendulacea* extracts on FBR of shallots under net house conditions. This research provides a solid basis for developing a new commercial biological-based agrochemical which could help subsistence farmers in Vĩnh Châu control FBR of shallots in a more sustainable way.

2 MATERIALS AND METHODS

2.1 The shallots and the pathogen isolate

The disease-free shallot sets (immature bulbs) of the susceptible cultivar, obtained via the Plant Protection Department of Sóc Trăng Province and the pathogen *F. oxysporum*, kindly provided by the Plant Pathology research group, Laboratory of Molecular Biology, Biotechnology Research and Development Institute, Can Tho University were used throughout this study.

2.2 Collection of plant materials and preparation of plant extracts

A total of 49 plant species which meet three following criteria, i.e. (1) they are commonly found in Vietnam, (2) they are usually used in herbalism, and (3) their extracts contain secondary metabolites that possess antimicrobial activities, were selected. Leaves or whole plants were collected from plants grown at the Campus 2 of Can Tho University. Healthy and mature parts of all plants were harvested at 7:00 AM to ascertain their physiologically comparable tissues.

Aqueous extracts were prepared as described by Ramaiah and Garampalli (2015) with some modifications, applying mass concentration (w/v). Initially, four grams of plant parts were washed with tap water, then sterile distilled water, rinsed and blotted dry. Next, they were subsequently cut into small pieces and ground thoroughly in sterile distilled water using a pestle and mortar. The volume of obtained solutions was then adjusted to 100 mL to achieve a 4% concentration. After soaking for 30 minutes, the mixtures were strained through a cheesecloth to remove debris, filtered through Whatman® qualitative filter paper No.4 and centrifuged twice at 10,000 rpm for 5 minutes. The obtained supernatants were used immediately as the 4% sterile plant extracts.

2.3 *In vitro* tests for direct antifungal activity of plant extracts against *F. oxysporum*

2.3.1 Inhibitory effects of plant extracts on the mycelial growth of F. oxysporum

The aim of this preliminary test is to screen for the plant extract(s) that exhibited highest inhibitory effects on the growth of *F. oxysporum* mycelium

for further study. The assay was carried out by disc diffusion method described by Harris *et al.* (1989) with minor modifications. At first, 600 µL of the plant extract at 4% was spread on agar plate containing potato dextrose agar (PDA) (1 liter of the medium contains 250 g of sliced washed unpeeled potatoes, 20 g of glucose and 20 g of agar powder). Subsequently, 6-mm discs from the actively growing 3-day-old PDA culture of *F. oxysporum* were placed in the center of separate PDA plates that had been already covered with the extracts. The PDA plate spread with sterile distilled water was used as the control. All the plates were incubated at 28 ± 2°C. The mycelial radii of all treatments were measured at 24-hour intervals during 2-7 days after incubation to calculate the percent inhibition on mycelial growth, using the formula:

Percent inhibition on mycelial growth = 100(C−T)/C (1)

where

C = the mycelial radius in the control treatment, and

T = the mycelial radius in the extract treatment

2.3.2 Inhibitory effects of plant extracts on the conidial germination of F. oxysporum

The method described by Rodríguez-Algaba *et al.* (2015) was followed in this study. A quantity of 50 µL of the conidial suspension at 10^8 conidia/mL made in sterile distilled water (from the 7-day-old PDA culture of *F. oxysporum*) was added to 450 µL of the plant extract at 4.5% (w/v) in a 1.5-mililiter Eppendorf tube. Thereby, 500 µL of the conidial suspension at 10^7 conidia/mL in 4% (w/v) plant extract was finally obtained. The tubes were then incubated and gently shaken in SH26.4 - Thermo shaker at 28 ± 2°C. For the control, the conidia were incubated in sterile distilled water. Percent germinated conidia (number of germinated conidia over total number of conidia) were recorded after incubating the conidia for 24 and 48 hours. The data were used to calculated percent inhibition on conidial germination, using the formula:

Percent inhibition on conidial germination = 100(C−T)/C (2)

where

C = the percent germinated conidia in the control treatment, and

T = the percent germinated conidia in the extract treatment

2.4 Disease-reducing effects of plant extracts on FBR of shallots under net house conditions

The extracts of two plant species, namely *A. glabra* and *W. calendulacea* were shown to possess highest antifungal activity against *F. oxysporym* in the preliminary tests. Thus, they were selected for this study. For each plant extract, three mass concentrations (3, 4 and 5%) were tested to investigate how low concentrations would still give an effect and to determine the maximum protection conferred by the extracts. The experiment also included a negative control using sterile distilled water and a positive control using the fungicide difenoconazole (commercialized as Score® 250SC, containing 250 g/L the active ingredient, Syngenta) which was finally diluted to 0.1% (w/v) concentration.

Soil preparation and shallot cultivation

Soil was initially amended by mixing with rice straws and husks (in mass ratio 2:2:1). The mixture was autoclaved at 121°C, 1 atm for 30 minutes, it was then transferred to a round pot (height 10 cm × diameter 17 cm). Prior to planting, all old roots were removed from the sets, and five sets were grown in each pot by pushing the bulbs into the ground so that their lower three-quarters was buried. The plants were watered daily and provided with recommended dose of fertilizers followed the guides of the Plant Protection Department of Sóc Trăng (Đặng Thị Cúc, 2011).

Inoculum preparation, pathogen inoculation and application of plant extracts

The conidial suspension (10^7 conidia/mL) of *F. oxysporum* (from the 7-day-old PDA culture) was prepared, following the methods developed by Prithiviraj *et al.* (2004) and Stankovic *et al.* (2007). The shallot bulbs were inoculated at 30 days after planting (DAP) by thoroughly spraying of the conidial suspension (5 mL/pot) at the basal plates. Application of plant extracts was carried out at 24 hours after inoculation by soil drenching, 5 mL of the plant extracts, sterile distilled water, or Score® 250SC were thoroughly sprayed in each pot. All the solutions were added with 0.1% polysorbate 20 (Tween® 20, Sigma-Aldrich) solution to facilitate their adhesions on the bulb surface.

Disease assessment

Disease incidence, i.e. percent infected bulbs (number of infected bulbs over total number of bulbs) and disease severity of FBR in each pot were recorded similarly to the method developed by Sintayehu *et al.* (2014). The data were recorded five times, at 37, 44, 51, 58 and 65 DAP to determine how long the extracts could reduce symptoms

of the disease. Per bulb, the disease severity was recorded on a scale of 5 scores, in which, 0 = no symptoms, 1 = up to 10% rotted roots, 2 = more than 10% rotted roots with up to 10% rotted basal plates, 3 = completely rotted roots with more than 10 % up to 30% rotted basal plates, and 4 = completely rotted roots with more than 30% rotted basal plates. Percent severity index (PSI) was then calculated using the formula:

$$PSI = Sum(a_i \times \text{number of infected bulb at the score } a_i) \times 100 / (4 \times \text{total number of bulbs}) \quad (3)$$

2.5 Data analysis

All experiments were arranged in completely randomized design, and each treatment had three replications. In net house experiment, the mean data for each replication were calculated from the data of 5 bulbs. The data of percent inhibition on mycelial growth, percent germinated conidia, percent inhibition on conidial germination, percent infected bulbs and PSI represented continuous variables by normality tests and were, therefore, analyzed by one-way analysis of variance (ANOVA) assuming a normal distribution. All data were analyzed by IBM SPSS Statistics version 22.0 (IBM Corporation), and all hypotheses were rejected at $P \leq 0.05$.

3 RESULTS AND DISCUSSION

3.1 Direct antimicrobial activity of plant extracts against *F. oxysporum*

3.1.1 Effects on the mycelial growth

Aqueous extracts at 4% (w/v) concentration of 49 plant species (not shown) that met all three criteria (Section 2.2) were tested for their direct antifungal activity against *F. oxysporum* using disc diffusion assay. Among 49 species, the extracts of 19 species inhibited the mycelial growth of *F. oxysporum* compared to the water control at all assessment time points (data not shown; the control was considered not to inhibit the mycelial growth, hence 0% inhibition). Furthermore, the extracts of 5 out of these 19 exhibited strongest effects with percent inhibition on mycelial growth over 10% at all time points (Table 1). Highest percent inhibition resulted from *A. glabra* extract (up to 35.3%), followed by *W. calendulacea* extract (up to 25.7%). In addition, both treatments gave significantly differed percent inhibition compared to other extracts at all time points, so they were selected to examine their effects on the conidial germination of *F. oxysporum*.

Table 1: Effects of 4% (w/v) aqueous extracts of five plant species on the mycelial growth of *F. oxysporum*

Plant extracts	Plant part used	Percent inhibition (%) on mycelial growth of *F. oxysporum*					
		2nd day*	3rd day	4th day	5th day	6th day	7th day
Annona glabra	Leaves	35.3 a	32.5 a	29.1 a	26.5 a	24.5 a	23.5 a
Boehmeria nivea	Leaves	16.0 c	17.3 c	15.7 c	11.4 c	11.2 c	10.1 c
Carica papaya	Leaves	16.0 c	15.2 d	14.3 d	11.9 c	11.6 c	10.3 c
Ocimum sanctum	Leaves	14.4 c	14.1 d	13.2 e	11.4 c	11.0 c	10.1 c
Wedelia calendulacea	Leaves	25.7 b	23.8 b	22.4 b	20.1 b	18.8 b	16.1 b

*In the same column, means from three replications followed by the same letters do not differ significantly by Duncan's multiple range test at $P \leq 0.05$. *Day after incubation*

3.1.2 Effects on the conidial germination

Only *A. glabra* extract was shown to have inhibitory effects on the conidial germination of *F. oxysporum*. Indeed, after 24- and 48-hour incubation, the 4% extract resulted in significantly lower percent germinated conidia and higher percent inhibition (up to more than 95%) compared to those of water control at both assessment time point (Table 2). In addition, the extract was found effective to restrict the hyphal growth of germinated conidia (Fig. 1A and 1C). In contrast, no effect was seen on the conidial germination of *F. oxysporum* in the *W. calendulacea* treatment since no differences in germination and percent inhibition were seen between treatment with 4% extract and treatment with water after soaking the conidia for 24 and 48 hours (Table 2). The extract, however, was shown to effectively suppress the hyphal growth of germinated conidia (Fig. 1B and 1C).

In a previous study, Bansal and Gupta (2000) reported a complete inhibition on mycelial growth and conidial germination *F. oxysporum* by the leaf extracts of *Azadiracta indica* at 100% (w/v). The authors also found that *Ocimum bacilicum* and *Lantana camera* extracts were toxic to the pathogen, inhibiting mycelial growth and conidial germination at all five concentrations tested (20, 40, 60, 80 and 100%). In a study conducted by Ramaiah and Garampalli (2015), three aqueous extracts, viz. *Solanum indicum*, *A. indica* and *Oxalis latifolia* (at concentration 10-60%) gave more than 50% inhibition on mycelial growth of *F. ox-*

ysporum. Furthermore, Dwivedi and Sangeeta (2015) showed the promising antifungal potentiality against *F. oxysporum* (up to 100%) of 75% aqueous extracts of three medicinal plants, namely *Tinospora cordifolia*, *Cymbopogon citratus* and *Moringa oleifera*.

Table 2: Effects of 4% (w/v) aqueous plant extracts of *A. glabra* and *W. calendulacea* on the conidial germination of *F. oxysporum* after incubation for 24 and 48 hours

Plant extracts	Percent (%) germinated conidia		Percent inhibition (%) on conidial germination	
	After 24 hours	After 48 hours	After 24 hours	After 48 hours
Annona glabra	3.89 [a]	18.3 [a]	95.3 [a]	80.5 [a]
Wedelia calendulacea	78.9 [b]	92.2 [b]	4.05 [b]	1.77 [b]
Water control	82.2 [b]	93.9 [b]	0.00 [b]	0.00 [b]

In the same column, means from three replications followed by the same letters do not differ significantly by least significant difference (LSD) test at $P \leq 0.05$.

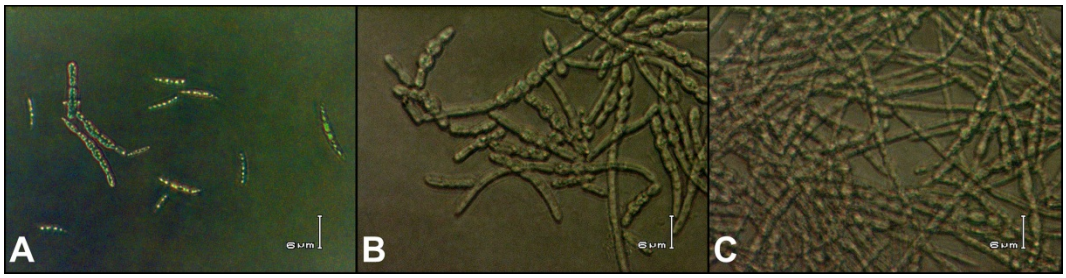

Fig. 1: Light microscopy (1000 ×) showing the inhibition on the hyphal growth of *F.* oxysporum after incubating the conidia in 4% (w/v) aqueous plant extracts of *A. glabra* (A) and *W. calendulacea* (B) at 28 ± 2°C for 48 hours. Sterile distilled water (C) was used as the negative control

The present study aims at finding plant extracts that could exhibit effective antifungal activities against *F. oxysporum* even though they are used at a low concentration. Thus, the 4% (w/v) was tested because this is generally a rather low concentration, which would consume fewer materials when it comes to large-scale practice. It was demonstrated that even at 4%, the *A. glabra* and *W. calendulacea* extracts still gave a significant inhibition on *F. oxysporum* (more than 25%). Interestingly, an 80.5% reduction in conidial germination was also recorded when the conidia was soaked in the 4% extract of *A. glabra* for 48 hours. This inhibitory effect is higher than those of *A. indica*, *O. bacilicum* and *L. camera* at 20-100% (Bansal and Gupta, 2000), and *Mentha arvensis* at 10% (Taskeen-Un-Nisa *et al.*, 2011).

3.2 Disease-reducing effects of *A. glabra* and *W. calendulacea* on FBR of shallots under net house conditions

To determine whether plant extracts could suppress FBR symptoms on shallots at their most susceptible stage, shallot plants were inoculated at 30 DAP. Three concentrations (3, 4 and 5%) of the two extracts (*A. glabra* and *W. calendulacea*) were tested by soil drenching at 24 hours after inoculation.

3.2.1 Disease incidence

This parameter reflects the proportion of diseased plants in a population, thus the percent infected bulbs were recorded. In general, the higher the concentrations of extracts applied, the lower the disease incidence achieved. All the extract treatments reduced mean percent infected bulbs compared to the water control, but only 4% and 5% concentrations of the two extracts gave similar effects as those of the chemical control at all time points, except for the 4% *W. calendulacea* treatment at 65 DAP. Treatment of shallot plants with *A. glabra* extract at 5% resulted in the most effective protection against the disease, where percent infected bulbs were reduced 100% at 37, 85% at 44, 76% at 51, 77% at 58 and 67% at 65 DAP compared to the water control (Fig. 2). At each time point, no differences were observed between the two extracts at the same concentration and between the 4% and 5% concentrations of the same extract, except for *W. calendulacea* treatments at 44 DAP where the 5% concentration gave better protection than the 4% one.

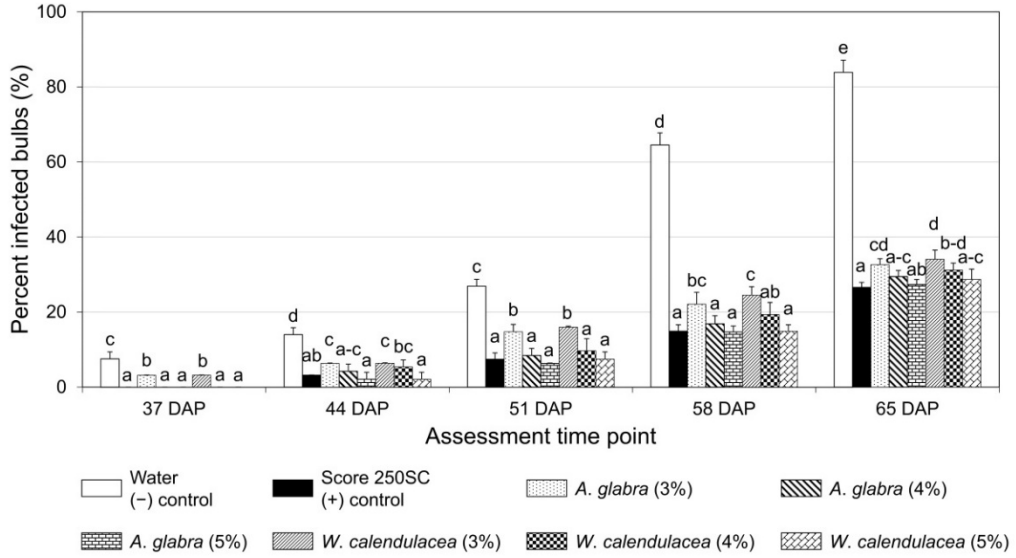

Fig. 2: Effects of aqueous leaf extracts of *A. glabra* and *W. calendulacea* at 3, 4 and 5% (w/v) concentrations on percent Fusarium basal rot-infected bulbs when shallots were inoculated with *F. oxysporum* at 30 days after planting. At the same time point, bars with same letters are not significantly different at P ≤ 0.05. DAP: Days after planting

3.2.2 Disease severity

Disease severity, i.e. the PSI reflects total area of plant tissues that is symptomatic by quantifying them into numerical ratings. The data on disease severity showed a similar tendency as those of disease incidence where both *A. glabra* and *W. calendulacea* were shown effective to restricting the spread of disease from infected to healthy tissues. Indeed, all the extract treatments reduced the PSI compared to the water control at all time points (up to 100% at 37, 87% at 44, 79% at 51 and 58, and 70% at 65 DAP). However, only 4% and 5% concentrations of the two extracts gave similar effects as those of the chemical control (Fig. 3). Similar to the data on disease incidence, no differences were seen between the two extracts at the same concentrations and between the 4% and 5% concentrations of the same extract, except for 4% and 5% *W. calendulacea* treatments at 44 DAP.

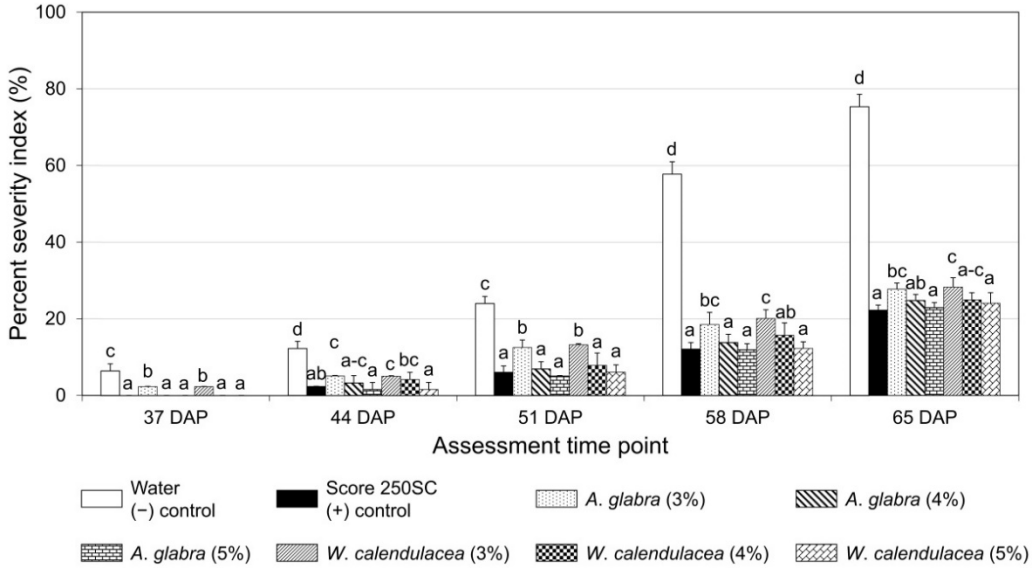

Fig. 3: Effects of aqueous leaf extracts of *A. glabra* and *W. calendulacea* at 3, 4 and 5% (w/v) concentrations on percent severity index of Fusarium basal rot when shallots were inoculated with *F. oxysporum* at 30 days after planting. At the same time point, bars with same letters are not significantly different at P ≤ 0.05. DAP: Days after planting

Previously, there appears to have only been a singular attempt by Sintayehu *et al.* (2014) to deploy Brassicaceae plant family as green manure amendments to bio-control FBR on shallots. Under laboratory conditions, extracts of macerated leaf tissues from mustard (*Brassica carinata*) and rapeseed (*B. napus*) showed highest inhibition (up to 77%) on the mycelial growth of *F. oxysporum*. Nevertheless, green manure amendments of the two species only gave moderate reduction in disease incidence (up to 30%) and severity (up to 29%) of FBR under net house conditions. Furthermore, members of Brassicaceae family are usually cultivated for food and oil production (Jahangir *et al.*, 2009). It would be, therefore, not an optimal choice for farmers to use extracts from plants of nutritional and economic importance to control FBR and other plant diseases.

Annona glabra (Bình bát nước in Vietnamese) is a tropical fruit tree belonging to the family Annonaceae. Through its introduction as a rootstock, *A. glabra* has escaped from cultivation and become an invasive species in many Pacific countries including Vietnam (Mai, 1995). *Wedelia calendulacea* (Sài đất in Vietnamese), belonging to the family Asteraceae is a slender, spreading herbaceous plant. It is largely distributed all over Vietnam and throughout tropical regions in Asia and is harvested for therapeutic purposes (Nguyen and Doan, 1989). In this study, the net house experiment clearly demonstrated that the aqueous extracts of these two plant species, despite being applied at low concentrations (4% and 5%), possessed an ability to protect shallots against FBR. The use of aqueous extracts from such commonly found plants offers a simpler, material-saving and less expensive solution for farmers to manage the disease. Indeed, farmers can easily find, collect leaves around their places and prepare the extracts using only water and tools such as pestle and mortar, and cheesecloths from households.

The disease-reducing effects on FBR of the two plant extracts are likely the result of direct antifungal activity against the pathogen as evidenced from results of *in vitro* assays. Chemical analysis of *A. glabra* leaf extract shows the presence of various bioactive compounds of three groups, i.e. steroid, diterpene and acetogenin. Among them, several compounds of diterpene and acetogenin exhibit promising pesticidal and antifungal activities (Matsumoto *et al.*, 2014). The whole *W. calendulacea* plant also contains high quantities of biologically active compounds (tannin, saponins, carotenes, flavonoids, isoflavonoids and wedelolactone) (Nguyen and Doan, 1989). Flavonoids and isoflavonoids are plant secondary metabolites which possess a number of important functions in plants including protection of plants against various bacterial and fungal pathogens (Mierziak *et al.*, 2014). Identification and structural elucidation of the exact bioactive compound(s) involving in the disease-reducing effects would provide insightful information to develop a new commercial biological-based fungicide as an alternative to already existing products. Such fungicides would be useful for farmers in areas where the two plants are not very abundant. Apart from antifungal activities, it could be speculated that other mechanisms, e.g., induced resistance, might be involved in the observable protection, but the exact mechanism(s) underlying the reducing effects has not been made in this study yet. Up to the presence, there has been no official document on the adverse effects of these two plant species to human health, suggesting that the plants could be tested and applied under field conditions although their toxicity need to be thoroughly investigated before widespread usage.

4 CONCLUSIONS

The aqueous extracts of 49 herbal plant species that are commonly found in Vietnam were screened for their antifungal activities against *F. oxysporum*. Under laboratory conditions, *A. glabra* and *W. calendulacea* extracts at 4% (w/v) showed the highest inhibition on the mycelial growth (up to 35.3% and 25.7%, respectively). In addition, the *A. glabra* extract was found to effectively suppress the conidial germination (up to 95.3%). Under net house conditions, the 4% and 5% of both extracts exhibited equivalent disease-reducing effects on FBR of shallots compared to the chemical control. These results suggest that *A. glabra* and *W. calendulacea* could possibly serve as environmental-friendly sustainable alternatives to the existing hazardous chemicals for the management of FBR of shallot.

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