ASIAN JOURNAL OF PHARMACOGNOSY *Research Article*
Asian J. Pharmacogn 3(4):14 - 22

eISSN-0128-1119

Catharanthus roseus extract as bio-fungicide for controlling *Fusarium oxysporum* on selected vegetable seedlings

Rozihawati Zahari[1], *Razak Terhem[1], Normala Halimoon[2], Siti Zulaikha Rusmadi[2], Mohd Farid Ahmad[3] and Norhayati Abdullah[4]

[1]*Laboratory of Forest Pathology, Faculty of Forestry, Universiti Putra Malaysia, 43400 Serdang, Malaysia.*
[2]*Department of Environmental Sciences, Faculty of Environmental Studies, Universiti Putra Malaysia, 43400 Serdang, Malaysia.*
[3] *Division of Biodiversity, Forest Research Institute Malaysia, 52109 Kepong, Selangor, Malaysia.*
[4]*Division of Natural Product, Forest Research Institute Malaysia, 52109 Kepong, Selangor, Malaysia.*

*For correspondence: razakterhem@upm.edu.my

Abstract: *Catharanthus roseus* extracts were studied to evaluate their potentials replacing chemical fungicide which give a bad impact on human health and cause environmental pollution problems. The samples of *C. roseus* were extracted with different extraction solvents including dichloromethane (DCM), acetone, ethanol, and methanol at 5, 10, 15 and 20 mg/mL concentrations. The solvents without the plant extract were used as the controls. After 6 days of incubation, the inhibition zone of the fungal pathogen on PDA media was measured. The extracts were significantly effective ($p \leq 0.05$) in limiting the antifungal activities. The DCM extract of *C. roseus* was the most effective against *Fusarium oxysporum* with 8.06 mm compared to acetone (0.055 mm), ethanol (0.15 mm), methanol (0.41 mm), and water (0.06 mm). Among of the concentrations, 20 mg/mL gave the best effect to control the fungal pathogen compared to 5, 10 and 15 mg/mL. The *C. roseus* extract was also effective in controlling *F. oxysporum* on the selected vegetable seedlings based on leaf number and disease suppression (%) results. However, without treated with the extract; mortality due to *F. oxysporum* increased. The *C. roseus* extract was effective and may to be developed as a bio-fungicide agent to control *F. oxysporum* in the field.

Keyword: Plant solvent extract, Extract concentration, Antifungal activity, Pathogenicity test and Plant extract application

INTRODUCTION

Fusarium oxysporum is a fungal pathogen of most vegetable crops and well known as *Fusarium* wilt disease (Bogale et al., 2006). It has various range of host plants. The infection-causing diseases such as vascular wilt disease (Nelson, 1981), damping-off problems (Nelson et al., 1981; Armstrong & Armstrong, 1981), crown and root rot (Armstrong & Armstrong, 1981). The plants may wilt and die soon after the symptoms appear at the seedling stages (John & Brett,

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2006). *Fusarium* wilt results in reducing yield and decreased quality of tomato (Wokoma, 2008), chili (Ehteshamul-Haque, 2006) and corn (Saremi et al., 2011) productions. The fungal pathogen also causes serious damage to eggplant (Negin et al., 2013) and okra (Frederick et al., 2013) crops.

Chemical fungicides such as fludioxonil and bromuconazole are used to control the fungus on the crops (Jahanshir & Dzhalilov, 2010) and effective in enhancing seedling growth and protecting seedling fungi. However, the application of fungicide causes negative effects on humans and the environment (Lee et al., 2007). Most of the researchers focused on medicinal plants that have been used as important sources of antifungal agents and in the development of bio-fungicides production (Baraka, 2011). One of the locally available plants with abundant active compounds present in the extract is *Catharanthus roseus*. This species is containing compounds with antifungal activity (Eufrocinio et al., 2001; Balaabirami et al., 2012). Phenolics as well as 2, 3-dihydroxybenzoic acid (Moreno, 1994a), and phenylpropanoids well as cinnamic acid derivatives, flavonoids, and anthocyanins (Natali & Robert, 2007) are found in the *C. roseus* extract. Our aim is here to assess the efficacy of *C. roseus* extracts against *F. oxysporum* on corn, chili, tomato, eggplant, and okra seedlings. We also include the *in-vitro* and *in-vivo* studies using the extract against the fungal pathogen.

MATERIALS AND METHODS

Antifungal activity: C. roseus stems were collected in Terengganu, Malaysia. The samples were air-dried and ground to powder form. The plant materials were soaked for three days in different solvents; dichloromethane (DCM), acetone, ethanol, methanol, and water. The solvents were removed from the extracts by using a rotary evaporator. The extracts were diluted with the solvents to give concentrations at 5, 10, 15 and 20 mg/mL. *F. oxysporum* culture was obtained from Forest of Research Institute Malaysia (FRIM), Kepong, Selangor, Malaysia. The fungal pathogen was cultured in potato dextrose agar medium (PDA) for six days. Whatman no. 1 filter paper with 5 mm in diameter was soaked in different extract concentrations. The filter papers with extracts were placed at the surface media. Filter papers soaked with solvent without the plant extract were used as the controls. All the treated plates were incubated at 25±2°C for six days. The experiment was conducted in Complete Randomized Design (CRD) with five replicates. The growth inhibition zone of the fungus growing on the surface of the PDA after six days of incubation was measured. The mean values of the treatments were separated using Tukey HSD at $p \leq 0.05$.

Preparation of selected vegetable seedlings: One week old germination of chili, tomato, corn, okra, and eggplant seedlings were potted and kept in a greenhouse. A total of 20 of each seedling with healthy and uniform size sampling were used in the experiment. Each pot contained sterilized mixed soil (top soil : sand : peat soil at ratio of (3 : 2 : 1 v/v/v). The potting size used was 10 x 5 cm. The potted seedlings were kept under greenhouse conditions at 73 - 75% relative humidity, air temperature around 31ºC and 75% light intensity.

Preparation of plant extract and fungal conidia: C. roseus extracts were prepared as described by Sharif et al. (2010). The plant extracts were dissolved in 5% dimethyl sulfoxide (DMSO) and mixed with Tween 20 (200 µg/mL), diluted with sterilized water at 1000, 1500 and 2000 µg/mL concentrations, and stirred at 35°C for one hour using the hotplate magnetic stirrer. *F. oxysporum* pathogens were cultured on Potato dextrose agar (PDA) and incubated in a culture room (25±2°C) for ten days. Ten day old of *F. oxysporum* were used. The fungal conidia were mixed with 10 mL of sterilized water in conical flasks. The conidia were shaked at 2000 rpm for one hour. The samples were filtered through three layers of muslin cloth to remove mycelia fragments. The fungal conidia were mixed with 10 mL of sterilized water in test tubes. The

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eISSN-0128-1119

fungal conidia were counted using hemocytometer. The fungal conidia were standardized at 1.0 x 10^8 spores/mL (Sharif et al., 2010).

Pathogenicity test and application of C. roseus extract for controlling F. oxysporum on seedlings: Twenty five of three-week-old healthy chili and tomato seedlings were used. The root seedlings were immersed in 25 mL of *C. roseus* extract for 24 hours. After one day treatment, the root seedlings were immersed in 25 mL of *F. oxysporum* conidia (1.0 x 10^8spores mL^{-1}). The root seedlings were immersed in 25 mL of sterilized water containing 5% DMSO used as the control. The root seedlings which untreated with the plant extract were used also as the control (water + fungus). Twenty four hours after immersion, the seedlings were planted in sterilized potting medium used was 3:2:1 v/v/v top soil, river sand and peat, and the potting size used was 10 x 5 cm. The seedlings were watered everyday in morning and evening. The treatment and control experiments were arranged five replicates. The seedling mortality and leaf number was also recorded daily for two weeks duration. In addition, the percentage of disease incidence based on leaves symptoms in every day for 2 weeks was also recorded as described according to Askarne et al. (2012). The percentage disease was calculated as follows: Disease incidence (%) = [(number of wilt seedlings / number of total seedlings)] x 100 (Askarne et al., 2012).

RESULTS AND DISSCUSSION

Antifungal activity: In-vitro results, antifungal activity of *C. roseus* extracted with DCM, acetone, ethanol, methanol, and water solvents at 5, 10, 15 and 20 mg/mL concentrations against *F. oxysporum* are summarized in Figure 1. There were significant differences ($p \leq 0.05$) between solvent extracts and concentrations. *C. roseus* extracted with DCM showed the highest inhibition zone against *F. oxysporum* with a value of 8.06 mm compared to acetone (0.055 mm), ethanol (0.15 mm), methanol (0.41 mm), and water (0.06 mm). Besides, 20 mg/mL concentration showed the most effective inhibition zone to control the growth of the fungal pathogen compared to 5, 10, and 15 mg/mL. According to Oumadevi et al. (2007), DCM extract of *Michelia champaca* and *Antidesma madagascariense* were effective to control the growth of *Cladosporium cucumerinum.* Besides, at 20 mg/mL of *Melaleuca leucadendron* extract was an effective as antifungal (Ayme et al., 2008). *C. roseus* extract at 20 mg/mL concentration was also effective to control *Candida albicans* (Kratika & Sharmita, 2013). The DCM extract of *Senna didymobotryo* also had good antifungal activities against *Trichophyton mentagrophyte* and *Microsporum gypseum* (Korir et al., 2012). The results are also consistent with Yurima et al. (2014), which DCM extract of *Ceramium rubrum* inhibited against *Saprolegria parasitica* fungus. The possibility of the effect of antifungal compounds from the extracts on spore germination leading to its inhibition or may be due to the effect of the compounds on cell wall altering its permeability (William, 2008). The DCM extract of *C. roseus* was the most effective antifungal perhaps to the presence of active compounds in the extract. As reported by Maria (2007), *C. roseus* species containing various types of phenolics and some of them active compounds act as an antifungal activity. For examples, pyrocatechol, vanillin, *p*-hydroxybenzoic, *p*-coumaric, ferulic, and caffeic acids, and the compounds are antifungal (Farah et al., 2007). Besides phenolics, alkaloids also have antifungal properties. According to Cantrell et al. (2005), flindersine, anhydroevoxine, and haplamine have antifungal activity against *Colletotrichum acutatum, C. fragariae,* and *C. gloeosporioides.* The stems of *C. roseus* extract contained an alkaloid, effective against *Candida albicans* (Kratika & Sharmita, 2013).

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eISSN-0128-1119

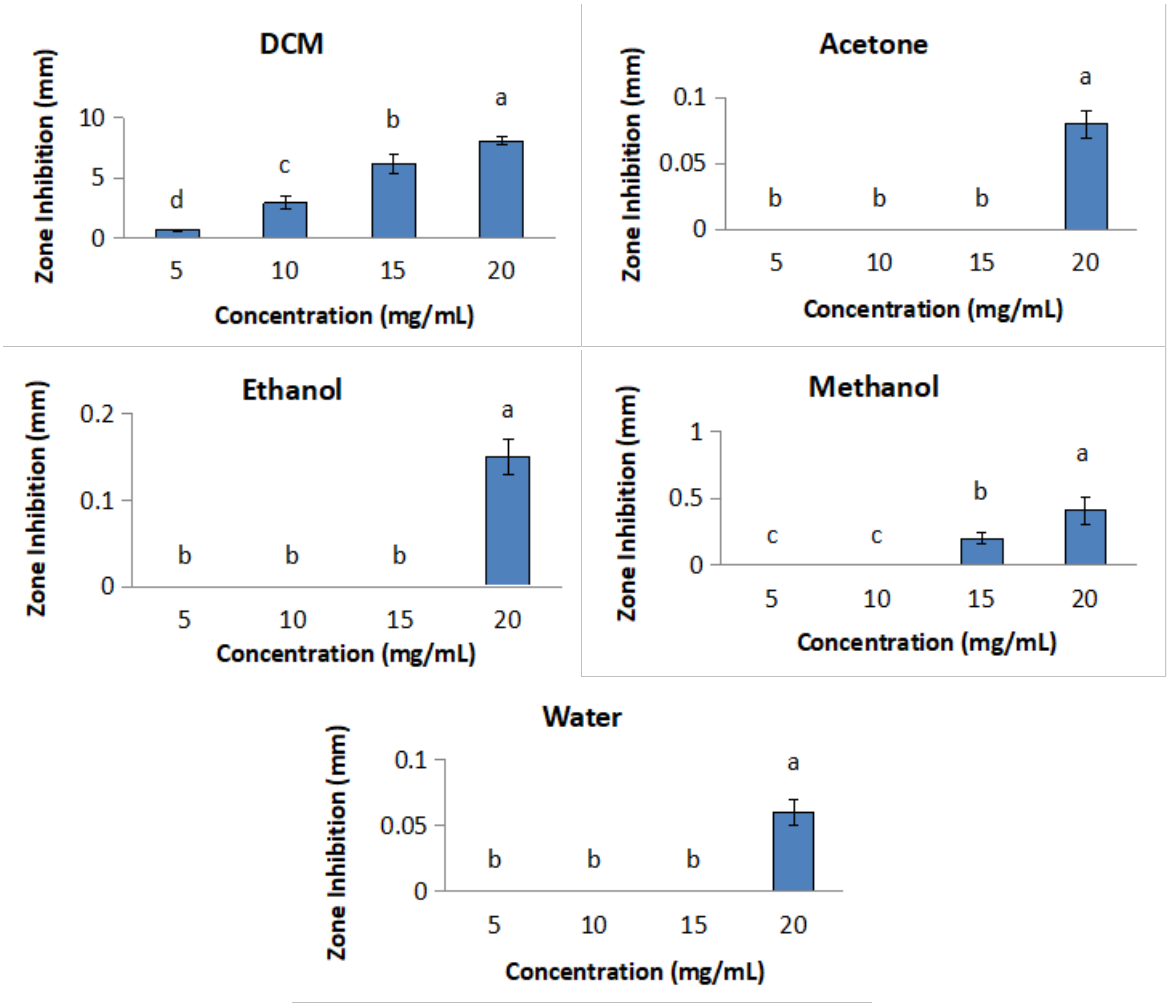

Figure 1. Zone inhibition in millimeter of *Catharanthus roseus* extract against *F. oxysporum* in different solvent extracts and concentrations. Mean of each the figure followed by the same letter are not siginificantly different according to Tukey HSD test ($p \leq 0.05$). Vertical bars indicate standard error (±).

Mortality of seedlings Figure 2 shows the mortality percentage of chili, tomato, corn, eggplant, and okra seedlings caused by *F. oxysporum* daily for two weeks. Corn and tomato seedlings showed high mortality compared to eggplant, chili, and okra seedlings. The seedlings were 100% mortality on day eight. Meanwhile, chili and okra seedlings showed 100% mortality at day 10 and eggplant seedlings at day 11. The control seedlings did not show any mortality. Early mortality caused by the fungal pathogen occurred at day six of corn, tomato, and eggplant seedlings. Meanwhile, dead of chili and okra seedlings caused by the fungus was first observed on day 7.

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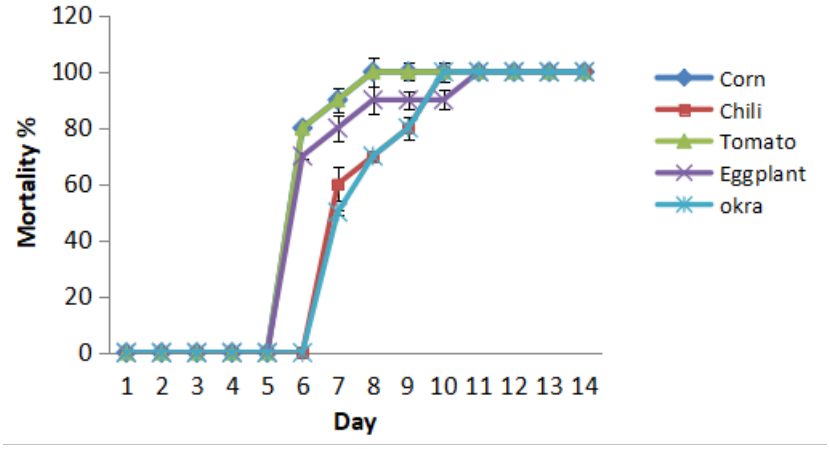

Figure 2. Mortality (%) of several vegetable seedlings with treated by *F. oxysporum.* The mortality percentage values are average of five replicates standard error (±).

The high mortality of seedlings infected with *F. oxysporum* was in line with Elizabeth (1994): the infected tree will defoliate or wilt due to the fungal grows out from the vascular tissue into the bark and finally, the tree dies. The fungi infect a range of host plants including vascular wilt, yellow, root rot or damping-off (Shilpi et al., 2011). This causes xylem vessels to be blocked. The blockage is due to gels composed of neural sugars commonly found in the host plant's cell wall (Van der Molen et al., 1986). Chili, eggplant and okra seedlings showed less mortality and may due to having more active chemical compounds to act as antifungal compared to corn and tomato seedlings. As reported by Soumya and Bindu (2012), the fruits and leaves of chili extract are effective against seed-borne fungal pathogens. Furthermore, the chili fruit extract is also very effective to control *Aspergillus* sp. (Morrine et al., 2014). The chili fruit extract has also been used in traditional treatments because the plant contains various chemicals and act as antimicrobial activity (Cichewicz & Thorpe, 1996). Among the chemicals, saponin is highly efficient against *Aspergillus fumigatus* and *Cryptococcus neoformans* (Renault et al., 2003).

Leaf number and disease suppression (%) of seedlings: The leaf number of the selected vegetable seedlings is shown in Figure 3. Among the seedlings, chili and tomato seedlings showed the highest leaf number compared to corn, eggplant, and okra seedlings. The control seedlings did not show any effect on leaf number. However, the control seedlings of treated with fungal pathogen exhibited less leaf number of all the vegetable seedlings. Possibility, in the study, less of leaf number was caused by the infection of roots seedlings. According to Booth (1971), the fungus from roots of trees followed by observed infection of stems is invaded by pinhole borers at advanced stages. Besides that, the typical symptoms of infection are yellowing or wilting of leaves and finally, it was dieback on one side of the tree (Elizabeth, 1994).

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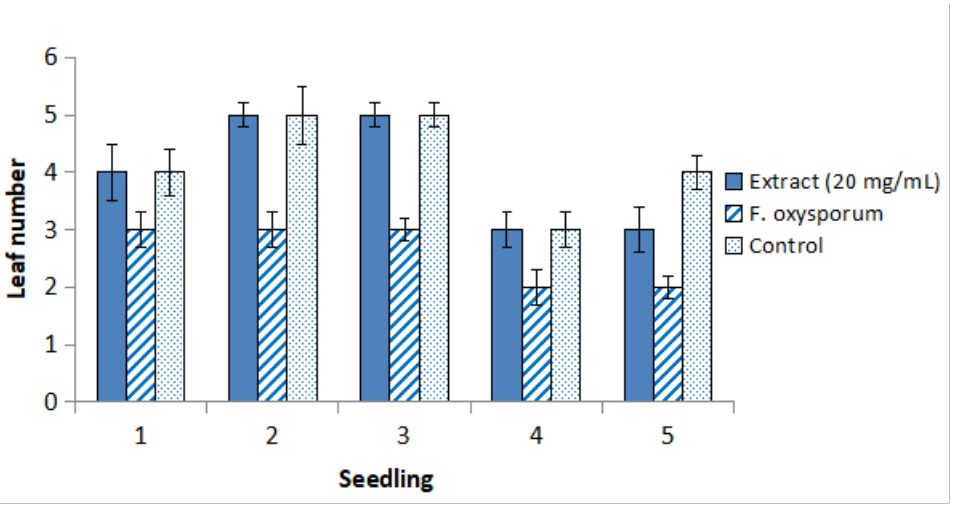

Figure 3. Leaf number of several vegetable seedlings (1: corn, 2:chili, 3:tomato, 4:eggplant, 5:okra) treated with *C. roseus* extract to control *F. oxysporum* after 14 days of observation. The leaf number values are average of five replicates standard error (±).

Disease suppression (DS %) of the selected vegetable seedlings was observed based on their healthy effects percentage by assessing on the symptoms of leaves after two weeks (Table 1 and Figure 4). The % DS results showed the extract concentration of 20 mg/mL with the value of 100% was effective to control *F. oxysporum,* except okra seedlings with a value of 90% DS. The control treatment (water + Tween 20) had 100% of the fungus treated on seedlings showed DS % with value of 0.0%. The effects of leaf symptom of seedlings caused by *F. oxysporum* attack resulted in the leaves to wilt, dieback, and defoliation. The leaf symptoms effect of seedlings due to root cells become damaged by the fungal pathogen. *F. oxysporum* blocks the xylem vessels that serve as food transport in plant cells (John and Brett, 2006). At the stage, control treatment on the growth of the fungus is may not effective. Therefore, early treatment is proposed in the study to ascertain the effects of the extract were more effective to control *F. oxysporum* on the seedlings.

Table 1. Percentage of disease suppression efficacy (%) of selected vegetable seedlings for comparison of *C. roseus* extract against *F. oxysporum* after 14 weeks. The disease suppression percentage values are average of five replicates standard error (±).

Seedling	Treatment	Mean (%)
Corn	Control	100 ± 0.0
	F. oxysporum	0 ± 0.0
	Extract (20 mg/mL)	100 ± 0.0
Chilli	Control	100 ± 0.0
	F. oxysporum	0 ± 0.0
	Extract (20 mg/mL)	100 ± 0.0
Tomato	Control	100 ± 0.0
	F. oxysporum	0 ± 0.0
	Extract (20 mg/mL)	100 ± 0.0
Eggplant	Control	100 ± 0.0
	F. oxysporum	0 ± 0.0
	Extract (20 mg/mL)	100 ± 0.0
Okra	Control	100 ± 0.0
	F. oxysporum	0 ± 0.0
	Extract (20 mg/mL)	90 ± 0.8

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eISSN-0128-1119

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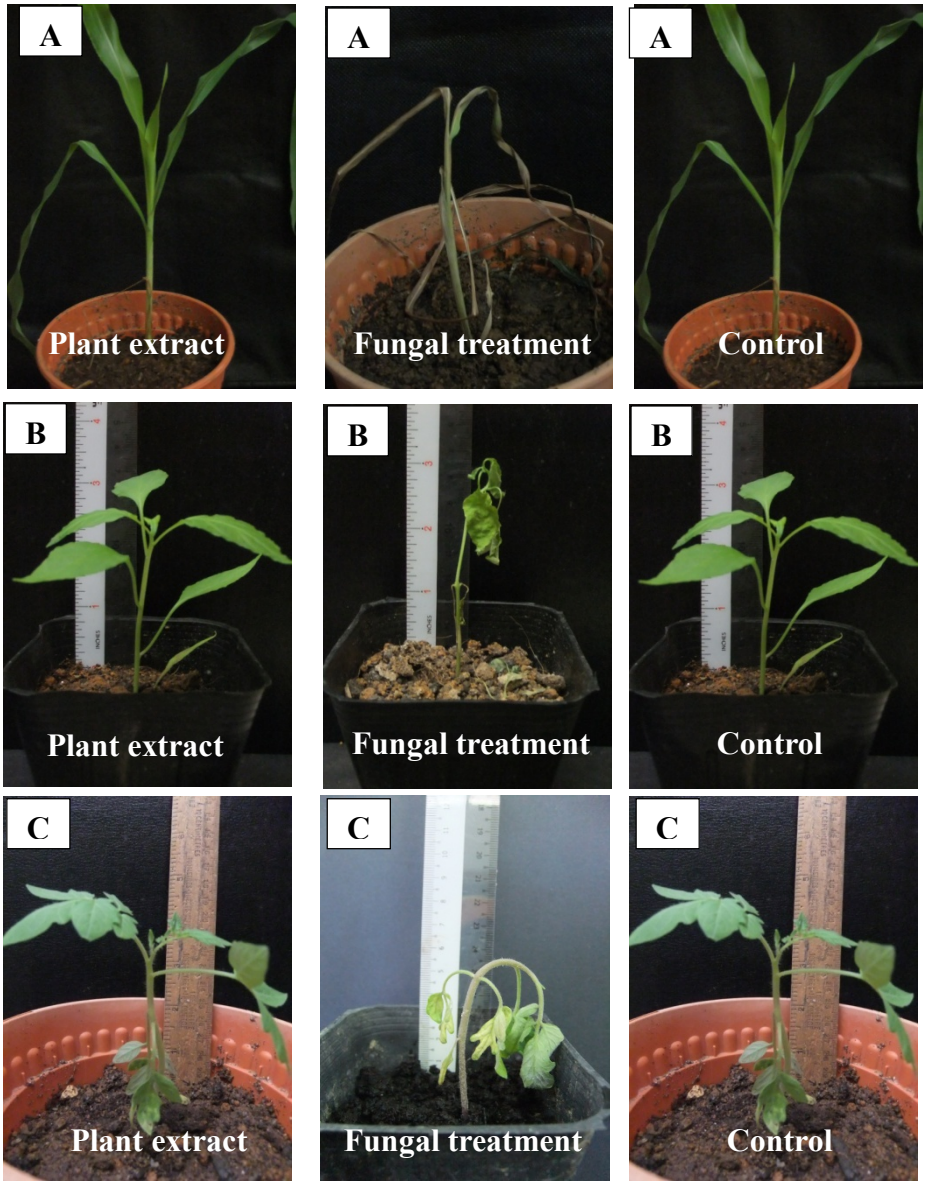

Figure 4. Selected vegetable seedlings treated with C. roseus extract to control F. oxysporum after two weeks. Note: A; Corn, B; Chilli, and C; Tomato seedlings.

CONCLUSION

Disease suppression (DS %) of the selected vegetable seedlings was observed based on their healthy effects percentage by assessing the symptoms of leaves after two weeks (Table 1 and Figure 4). The % DS results showed the extract concentration of 20 mg/mL with the value of 100% was effective to control *F. oxysporum,* except okra seedlings with a value of 90% DS. The control treatment (water + Tween 20) had 100% of the fungus treated on seedlings showed DS % with a value of 0.0%. The effects of leaf symptom of seedlings caused by *F. oxysporum* attack resulted in the leaves to wilt, dieback, and defoliation. The leaf root cells were damaged by the fungal pathogen. *F. oxysporum blocks* the xylem vessels that serve as food transport in plant cells. At the stage, control treatment on the growth of the fungus is may not effective. Therefore, early

eISSN-0128-1119

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DECLARATION OF CONFLICT OF INTEREST

No conflict of interest to declare.

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