



## Original Research Article

### Bio-organic fungicide of *Catharanthus roseus* stems extract inhibit the growth of *Fusarium oxysporum* *Capsicum annum* seedling

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#### A B S T R A C T

#### Keywords

*Catharanthus roseus*, stems extract, antifungal activity, bio-organic fungicide, *Fusarium oxysporum*, *Capsicum annum*, seedling

*Fusarium oxysporum* is a well-known fungus that causes a major commercial plant disease in the world. Due to the issue, the antifungal activity of *Catharanthus roseus* stems extract as bio-organic fungicide against *F. oxysporum* *Capsicum annum* seedling was studied. The spore suspensions of *F.oxysporum* and *C. roseus* stems extract were prepared to study the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) tests in the laboratory. The application of *C. roseus* stems extract at the concentrations of 100, 500, 1,000, 1,500 and 2,000 µg/mL against *F. oxysporum* were included in the *in-vitro* study. For *in-vivo* test, *F. oxysporum* on *C. annum* seedlings were applied with the plant extract at concentrations of 1,000, 1,500 and 2,000 µg/mL in greenhouse study. The result showed that the extract with 2,000 µg/mL has higher significant difference ( $p < 0.05$ ) inhibited the growth of *F. oxysporum* plant fungal compared to other concentrations of MIC tested. The MFC test indicated that day nine was proven to have high negative impact of the fungal than day six. In *in-vivo* study, the antifungal activity showed 100% effect of disease injury for the growth of *C. annum* plant species in green house. The result also showed that the concentration of *C. roseus* stems extract at 2,000 µg/mL has significantly higher ( $p < 0.05$ ) activity against *F. oxysporum* on the seedlings compared to other concentrations. Thus, the study indicated that *C. roseus* stems extract has novelty of bio-organic compounds that contribute to the development of new antifungal agents to protect crop plants from fungal disease which also safe to environmental ecology compared to other commercial chemical fungicide which is highly used nowadays.

## Introduction

*Fusarium* spp. is ubiquitous pathogens which causes a variety of diseases in many agricultural, horticultural and forestry crops (Logrieco *et al.*, 2002; Desjardins, 2006). *Fusarium* spp. of fungi also an economically important disease in worldwide, where the

disease causes tuber rot of plant, with severe reductions in crop yield and estimated about 25% annually in production (Lui and Kushappa, 2002; Slininger *et al.*, 2004). *Fusarium* is the most common fungi species found associated with *Capsicum annum* crop

disease in Pakistan (Ehteshamul-Haque and Ghaffar, 1994; Ehteshamul-Haque, 2006; Qureshi *et al.*, 2004). The diseases can be described with a significant impact on *C. annuum* production and present a formidable challenge of the plant producers.

Plant infected with the disease may wilt and die soon after symptoms appear at the seedling stage. In older plants, leaf will downward dropping and vein clearing are often followed by stunting, yellowing of the lower leaves, formation of adventitious roots, wilting of leaves and young stems, defoliation, marginal necrosis of remaining leaves, and finally death of the entire plant. As reported by Agrios (1988), browning of vascular tissue is a strong evidence of *Fusarium* wilt in *C. annuum* and causes loss of post harvest in world crops about more than 12% in developing countries (Agrios, 1997). Furthermore, it can reduce the shelf life and market values of food commodities. The infected product also unfit for human consumption because the undesirable effects on human health. Moreover, a serious health problem caused by the fungi is about 4.5 billion people in underdeveloped countries are exposed to the deleterious effects of *Fusarium* and *Aspergillus* spp. pathogens (Williams *et al.*, 2004).

The chemical pesticides which are highly used nowadays causes significant drawbacks involving cost, pesticide residues, handling hazards and threats to human health and environment ecosystem (Paster and Bullerman, 1988). Numerous years, a variety of different synthetic chemicals such as aromatic hydrocarbons, benzimidazoles and sterol biosynthesis inhibitors have been used as antifungal agents to inhibit the growth of plant fungal diseases. However, the fungi have developed resistance due to series of problems against the effective use of the chemicals in areas (Brent and

Hollomon, 1988). Thus, to solve the problem, the higher concentration of chemicals was used which increased the contribution high-level risk of toxic residues of the products and also increased the resistance of fungi. In addition, some synthetic pesticides also cause environmental pollution (Barnard and Padgitt, 1997).

For this reason, a new innovation of bio-organic fungicide with biodegradable alternative is currently needed to replace the commercial synthetic chemical of antifungal. Hence, the possible use of natural products such as plant extract might be less damaging to control the pests and fungal diseases (Costa *et al.*, 2000). Based on previous study, it indicated that *C. roseus* possible as one of the potential plants that can be applied as a natural product. The plant extract of *C. roseus* has high potential of varieties medicinal properties such as antidiabetic (Srivinaset *et al.*, 2003), anticancer (Karet *et al.*, 2003; Jaleel *et al.*, 2009), antioxidant (Wei and Shiow, 2001; Abdul *et al.*, 2006), antimicrobial (Prajakta and Jai, 2010), antibacterial (Goyal *et al.*, 2008; Muhammad *et al.*, 2009; Balaabirami and Patharajan, 2012) and antifungal (Balaabirami and Patharajan, 2012). The compound of 2,3-dihydroxybenzoic acid which present in the plant extract also has been used to control fungal of *Phytium aphanidermatum* (Moreno *et al.*, 1994a). The extract also contains vinblastine and vincristine, which belongs to anti-cancer drugs category as reported by Paulo *et al.* (1995). The plant roots also are the important components in medicines to control high blood pressure and other types of cardiovascular maladies, which contain ajmalicine and serpentine compounds in the extract (Tikhomiroff and Jolicoeur, 2002). Due to the potential of *C. roseus* extract as antifungal agent, it might be caused by their

biochemical active compounds against *F. oxysporum* of plant fungus. The purpose of the study was to test the possibility of *C. roseus* stems extract to inhibit wilt disease caused by *F. oxysporum* on *C. Annum* seedling. The *in vitro* and *in vivo* tests of antifungal activity were also studied to determine the efficiency of *C. roseus* stems extract as antifungal agent. Medicinal plants are possible to be applied as bio-fungicide in agricultural activity based on the presence of bio-organic active compounds in their extract which effectively inhibit the growth of plant fungal disease to plant seedling.

## **Materials and Methods**

### ***Fusarium oxysporum* culture**

The spore suspension of *F. oxysporum* in sterile distilled water was obtained from Forest Research Institute Malaysia (FRIM). The ten days old cultures was used in the study. The spore suspension was collected and centrifuged to separate the culture from solution. A hemocytometer was used to obtain a homogenous spore suspension of  $1 \times 10^8$  spores/mL (Sharif *et al.*, 2010). The suspension was used as spore culture in the study.

### **Preparation of *Catharanthus roseus* stems extract**

The plant samples of *C. roseus* stems were collected from Peninsular Malaysia. The free disease and insect pest of plant samples were selected for the study. The specimens of samples were cleaned with 1% of sodium hypochlorite and distilled water. The samples were air dried at room temperature then ground to powder. The crude extracts were weighed at 400  $\mu$ g and dissolved in 1.3 mL of 100% dimethyl sulfoxide (DMSO). The DMSO plant extract was used as a stock extract in the study.

### ***In-vitro* study of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) tests**

The minimum inhibitory concentration (MIC) of organic extract against fungal pathogens was determined using agar dilution method as described by Sharif *et al.* (2010). The appropriate concentrations of *C. roseus* stems extracts were prepared from stock of DMSO plant extract. The different concentrations of plant extract were prepared by dilution with their respective 5% DMSO and added to potato dextrose broth (PDB) medium to produce the final concentrations of 100, 500, 1,000, 1,500 and 2,000  $\mu$ g/mL. The final concentration of dimethyl sulfoxide (DMSO) in the assay did not exceed 2%. A 100  $\mu$ L spore suspension ( $1 \times 10^8$  spores/mL) of each test strain was inoculated in the test tubes which contained PDB medium with different concentrations of plant extract and incubated for six days at 28°C. The control tube which contained PDB medium without plant extract was inoculated with fungal spore suspension. The tubes were prepared in triplicate for each treatment and conducted for two times.

For the minimum fungicidal concentrations (MFC) test, the cultures were compared at day six and nine after incubation. The concentrations of plant extract were prepared similar as procedure above in the range of 100 to 2,000  $\mu$ g/mL. The MFC test was observed where there was no visible mycelia growth after the period of incubation. There were three replicated for each concentration and the experiments were conducted twice. The MIC and MFC without visible growth were observed and defined the value, expressed in  $\mu$ g/mL. After six days of incubation, mycelia dry weight was determined. Tubes containing mycelia were filtered through Whatman filter paper no.1 then washed with distilled water. The mycelia were dried at 60°C for 1 hour. The

filter paper containing dry mycelia was weighed and the mean value was obtained (Fillipe *et al.*, 2011).

### ***In-vivo* study of pathogenicity test on *Capsicum annum* seedlings**

In the *in-vivo* study, the tested of three weeks old of *C. annum* seedlings with possessing an average of 4 to 5 leaves were kept under the following conditions of greenhouse. The optimum relative humidity was arranged at 73 - 75% and air temperature was around 31°C using data logger (Model Skye Lynx Deluxe). The required higher light intensity was also maintained. The seedlings were planted in the pot containing the sterilized mixture media (soil: river sand: peat with the ratio of 3:2:1 v/v/v). A total 10 seedlings of each *C. annum* were used in the experiment. A total of 6 mL of fungal spore suspension ( $1.0 \times 10^8$  spores/mL) of *F. oxysporum* were watered onto each pot. For control treatment, the seedlings were watered with water only and without treated with fungal spore. Five replicates of treatment and control on the seedlings were arranged. The percentage of mortality was also calculated in the study.

### **Effect of *C. roseus* stems extract on infected *C. annum* seedlings**

Twenty five of three weeks old of *C. annum* seedlings with healthy plant samples were prepared in the experiment. The seedlings were planted in the pots containing sterilized soil mixture (top soil:sand: peat at the ratio of 3:2: 1 v/v/v). They were kept under greenhouse conditions with the relative humidity at 73 - 75% and air temperature around 31°C using data logger (Model Skye Lynx Deluxe). The required higher light intensity was also maintained.

The preparation of bio-fungicide from the extract plant to control fungal disease on

seedlings was modified from Sharif *et al.* (2010) method. The tested solutions at the concentrations of 1,000, 1,500 and 2,000 µg/mL were dissolved in 5% dimethylsulfoxide (DMSO) followed by dilution with water containing surfactant Tween 20 (200 µg/mL), where the final concentrations of dimethylsulfoxide and Tween 20 were 0.5% and 0.1%, respectively. For the application of different concentrations of plant extract, 5 mL of the solution was watered onto each pot at the same time. Then, 6 mL of fungal spore suspension ( $1.0 \times 10^8$  spores/mL<sup>-1</sup>) of *F. oxysporum* was watered onto each pot. For controls, the seedlings were watered with dimethylsulfoxide and Tween 20 solutions, where the final concentrations of DMSO and Tween 20 were 0.5% and 0.1%, respectively. On the other hand, the seedlings were watered with fungal spore suspension only for the second control. The seedlings were also watered with the tap water in the morning and evening every day. All treatments were conducted in five replicates. The effects of antifungal efficacy of the seedlings on disease were evaluated in between 14 days of observation for *C. annum* seedlings based on the average of leave number calculation. The incidence of disease percentage based on wilt host symptoms was also calculated as follows:

Disease incidence (%) = [(number of wilt seedlings / number of total seedlings)] x 100

### **Data analysis**

The organic extracts were assayed for antifungal activities of MIC and MFC tests in the *in-vitro* study. Five concentrations of plant extract at 100, 500, 1,000, 1,500 and 2,000 µg/mL were used in the study. The experiment was run in triplicate, and the mean values were calculated. While, for the *in-vivo* study, the percentage of mortality (%), leaves number and disease infection

percentage of *C. annum* seedlings were observed after treatment application for two weeks of observation. There were three concentrations of plant extract at 1,000, 1,500, 2,000 µg/mL and two controls with five replicates were conducted. Data from repeated experiment for the *in-vitro* and *in-vivo* studies were subjected to variance analysis (ANOVA) for each concentration using SPSS programme and computed for the statistical significance at  $p < 0.05$  of the results. Tukey's range test was also used to compare the treatment means in this experiment.

## Results and Discussion

### *In-vitro* study of MIC and MFC tests

According to the results given in Figure 1, there were significant data ( $p < 0.05$ ) obtained in term of their treatment. The MIC tested of *C. roseus* stems extract at 2,000 µg/mL concentration showed significantly ( $p < 0.05$ ) more effective against *F. oxysporum*, followed by 1,500, 1,000, 500 and 100 µg/mL with the value of 90, 173, 300 and 390 µg, respectively. The seedling with 2,000 µg/mL of plant extract resulted no fungal growth after six days of observation compared to other concentrations. While, for the minimal fungicidal concentration (MFC) tested at nine days of treatment resulted more significantly ( $p < 0.05$ ) effective at 2,000 µg/mL concentration of plant extract against *F. oxysporum* than 1,500, 1,000, 500 and 100 µg/mL concentrations with the values of 61, 150, 210 and 280 µg, respectively (Figure 2). For the control seedlings, the fungal was well growth in the MIC and MFC tested. The correlation analysis between the yield (µg) of *F. oxysporum* incidence and the final concentration of plant extract in MIC and MFC treatments at day 6 revealed a negative correlation with  $R^2$  values of - 0.99 and - 0.89, respectively. The results proved that the

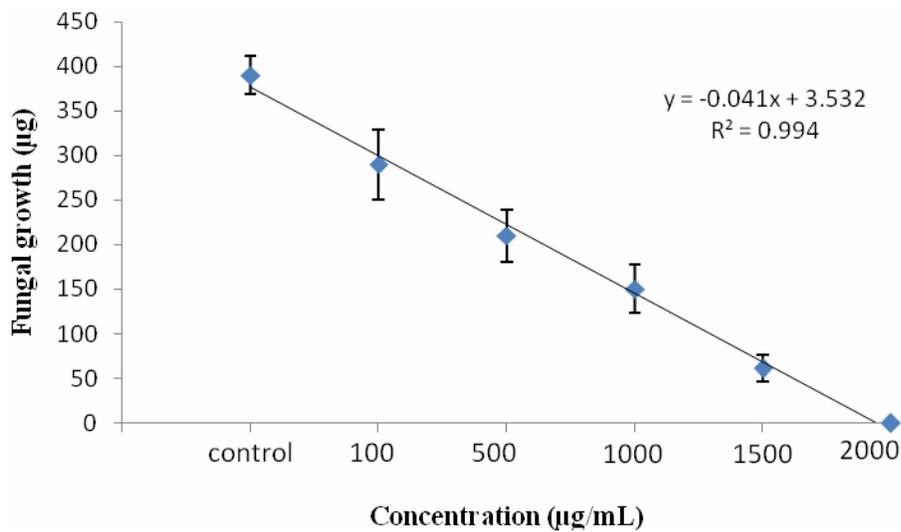
negative impact occurred to the *F. oxysporum* plant fungal when exposed with the *C. roseus* stem extract. However, comparison between day six and nine of MFC treatment indicated that day nine indicated the highest negative correlation ( $R^2 = - 0.99$ ) than day six with  $R^2$  values of - 0.89.

Based on the previous study, the MIC result of *Cestrum nocturnum* chloroform extract showed that high antifungal activities were obtained against *F. oxysporum*, *Phytophthora capsici*, *Sclerotinia sclerotiorum* and *Colletotrichum capsici* with the concentrations ranged from 500 to 1,000 µg/mL (Sharif *et al.*, 2010). However, the MIC and MFC values of *Cymbopogon winterianus* essential oil against *Trichophyton rubrum* were displayed at the concentration of 312 µg/mL (Fillipeet *al.*, 2011). Thus, the MIC and MFC values of plant extracts to control fungal growth might be depended on the resistance of the fungal species.

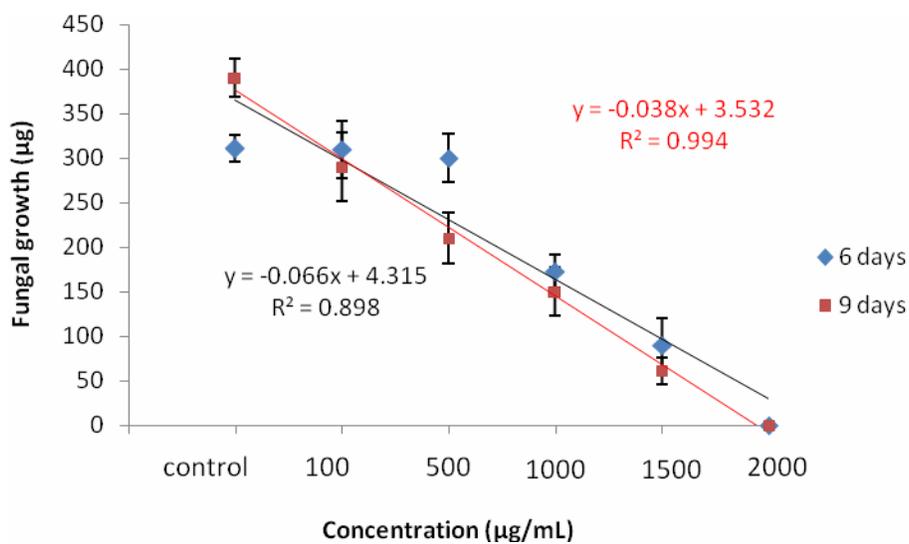
### *In-vivo* study of infected *Capsicum annum* seedlings mortality

The mortality of *C. annum* seedlings were 100% occurred at day 14 after applied with *F. oxysporum* fungal, but no mortality presented for the control (Figure 3). According to the observation in the study, the seedlings mortality was caused by the fungal activity with early dead at day 8 after fungal application. The high mortality of *C. annum* seedlings infected with *F. oxysporum* showed that the fungal has aggressively attacked the plant hosts. This statement proved by Elizabeth (2008), the infected tree will defoliate or wilt due to the fungal grows out from the vascular tissue into the bark and finally the tree become die. The fungal infected at various range of host plants including vascular wilt, yellow, root rot or damping-off (Shilpiet *al.*, 2011).

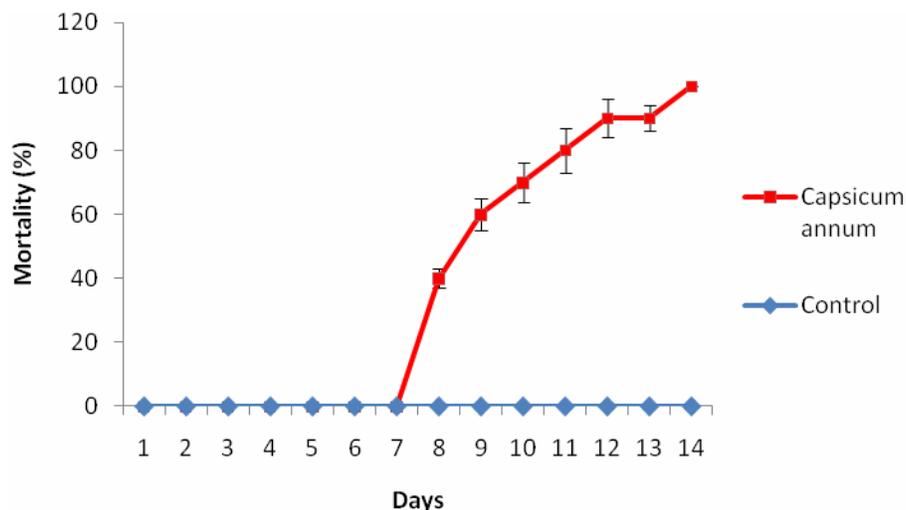
**Figure.1** Correlation between the different concentration of *C. roseus* extract and fungal growth of minimum inhibition concentration (MIC) at six days of observation



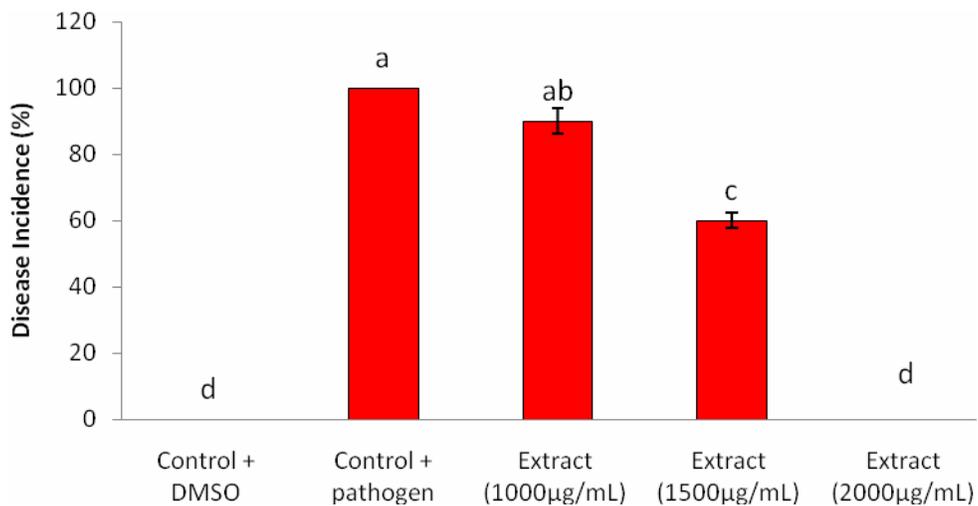
**Figure.2** Correlation between the different concentration of *C. roseus* extract and fungal growth of the minimum fungicidal concentration (MFC) at six and nine days of observation



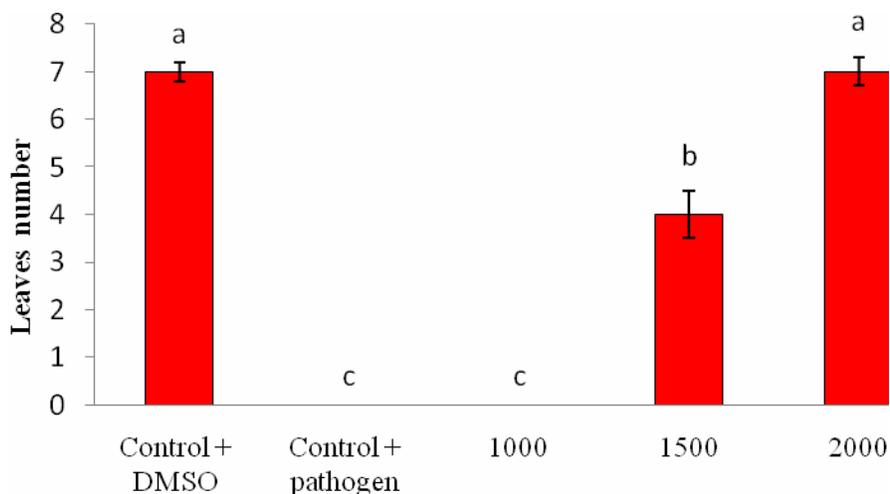
**Figure.3** The mortality of *C. annuum* seedlings with treated by *F. oxysporum*



**Figure.4** The leaves number of *C. annuum* seedlings treated with *C. roseus* extract against *F. oxysporum* for 14 days of observation. Note: The means showed significant difference at  $p < 0.05$  level by Tukey HSD



**Figure.5** Disease incidence percentage of *C. roseus* extract effect against *F. oxysporum* of *C. annum* seedlings at 14 days of observation. Note: The means were significantly difference at  $p < 0.05$  level by Tukey HSD



#### Leaves number of treated *Capsicum annum* seedlings

The *C. roseus* extract at concentration of 2,000  $\mu\text{g/mL}$  showed significantly ( $p < 0.05$ ) high effectiveness against *F. oxysporum* compared to 1,500 and 1,000  $\mu\text{g/mL}$  with the high leaves number of *C. annum* seedlings as shown in Figure 4. The average number of leaves of seedling at the concentration of 2,000  $\mu\text{g/mL}$  was 7, while for 1,500 and 1,000  $\mu\text{g/mL}$  have 4 and 0 leaves number, respectively. For the controls involved, the number of leaves of seedling treated with DMSO plant extract was 7 and the seedling applied with fungal was 0 leaf number. Furthermore, the control with DMSO solvent without plant extract and fungal application exhibited no negative effect on the growth of *C. annum* seedlings in the experiment. The possibility loss of leaves in the study caused by the infection of roots seedlings. The reason proved by Booth (1971), which have isolated fungal from roots of Angsana trees followed by observed

infection of stems are invaded by pin hole borers at advanced stages. Beside that, the typical symptoms of infection are yellowing of leaves and finally, it was dieback on one side of the tree (Elizabeth, 1994).

#### Disease incidence percentage of treated *Capsicum annum* Seedlings

The efficacy of *C. roseus* stems extract in reducing the incidence of infected *C. Annum* seedlings is shown in Figure 5. There were significant ( $p < 0.05$ ) results presented in term of controlled and treated of infection *C. annum* seedlings. The incidence of infection *C. annum* seedlings treated by *C. roseus* stems extract at 1,500 and 2,000  $\mu\text{g/mL}$  concentrations and controlled seedlings with DMSO solvent showed significantly ( $p < 0.05$ ) lower percentage than 1,000  $\mu\text{g/mL}$  concentration and controlled seedlings with fungal infection. Indeed, the percentages of wilt seedlings were 60% for 1,500  $\mu\text{g/mL}$  concentration and 0% for

the concentration of 2,000 µg/mL, respectively, after 14 days of treatment. The result showed that the infected seedlings applied with 2,000 µg/mL *C. roseus* stems extract exhibited high effectiveness to control the growth of plant fungal disease. Based on the previous study, many researchers stated that certain plant extracts and their constituents are found to have antifungal properties. For example, mono and sesquiterpene hydrocarbons and their oxygenated derivatives are the major components of essential oil of plant origin and it has enormous potential with strongly inhibited the growth of microbial pathogens (Cakiret *et al.*, 2004). Other studies demonstrated that phenolic acids such as caffeic (Ravn *et al.*, 1989), ferullic, cinnamic (Fernandez *et al.*, 1998), and salicylic acids (Tawata *et al.*, 1996) presented in the plants also have antifungal activities.

The study can be concluded that 2,000 µg/mL of *C. roseus* stems extract showed highly significant ( $p < 0.05$ ) effectiveness of concentration inhibited the growth of *F. oxysporum* on the *C. annuum* seedlings, respectively. It would also be interesting to study the effects of organic extracts of *C. roseus* against other important fungal for developing new antifungal agents to control serious fungal diseases of plant, animal and human beings. The use of organic extracts of *C. roseus* from stems part could be an alternative to replace synthetic fungicides which are commercially used in agro industries sector. Thus, further investigations on screening and developing of novelty selective bio-fungicide compounds should be highly focused in the treatment of many microbial phytopathogens that causes destruction of crop plant, such as vegetable, ornamental plants and also

forest plantation which finally will decreased their production. Besides that, this bio-product is also safe to apply to the environment.

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