



RESEARCH ARTICLE

Evaluation of two eco-friendly botanical extracts on fruit rot pathogens of orange (*Citrus sinensis* (L.) Osbeck)

Hiệu quả của hai loại dịch trích thực vật đối với các tác nhân gây thối trái cam (Citrus sinensis (L.) Osbeck)

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Fruit rot caused by *Aspergillus niger* and *Colletotrichum* sp. could cause rapid and severe damage on orange fruits. Current control method of orange fruits is mainly applied by usage of harmful pesticides, leading to chemical residues on fruits, environmental pollution and human poisoning. One of alternative methods of reducing pesticides is to use botanical extracts. This study was conducted to evaluate the *in vivo* antifungal efficacy of aqueous extracts from the leaves of neem and basket plants against *A. niger* and *Colletotrichum* sp. Orange fruits artificially inoculated by fruit rot pathogens were immersed into leaf extracts of 6% (w/v) neem or basket plants for 30 s, and kept for 11 days to record lesion length at room temperature. Orange fruits immersed into sterile distilled water were used as the control treatment. The results showed that at 11 days after inoculation, extracts of neem and basket plants significantly reduced the *Aspergillus* rot lesions by 109.08 and 124.00 mm, respectively. In addition, anthracnose lesions on orange fruits were statistically inhibited by treatments of neem and basket plants, with the average lesion diameters approximately 160.00 and 154.75 mm, respectively, at day 11 of the conducting experiment. The results of this study showed that leaf extracts of neem and basket plant at the concentration of 6% could be used as a natural alternative to control the *in vivo* growth of rot pathogens of orange fruits. These extracts have a bright future in modern plant protection to replace conventional synthetic pesticides in agro-ecosystem.

Thối trái bởi Aspergillus niger và Colletotrichum sp. gây ra các thiệt hại nghiêm trọng trên cam. Biện pháp phòng trừ bệnh trên trái cam hiện nay chủ yếu dựa vào thuốc hóa học, dẫn đến tồn dư thuốc trên trái cây, ô nhiễm môi trường và gây độc cho con người. Một trong các phương pháp thay thế giúp giảm sử dụng thuốc hóa học là sử dụng dịch trích thực vật. Nghiên cứu này đã được thực hiện để đánh giá hiệu quả in vivo của dịch trích ở nồng độ 6% của neem hoặc lược vàng đối với A. niger và Colletotrichum sp. Các trái cam đã lây nhiễm nhân tạo tác nhân gây thối trái thì được nhúng vào dịch trích ở nồng độ 6% của neem hoặc lược vàng trong 30 giây, và giữ đến 11 ngày để ghi nhận chiều dài vết bệnh ở nhiệt độ phòng. Các trái cam được nhúng vào nước cất thì dùng như nghiệm thức đối chứng. Kết quả cho thấy ở 11 ngày sau khi chủng bệnh, dịch trích neem và lược vàng làm giảm đáng kể vết thối Aspergillus lần lượt là 109,08 và 124,00 mm. Bên cạnh đó, vết bệnh thân thư trên trái cam đã bị ức chế có ý nghĩa thống kê bởi các dịch trích neem và lược vàng, với đường kính trung bình các vết bệnh lần lượt là 160,00 và 154,75 mm, ở ngày 11 của thí nghiệm. Kết quả của nghiên cứu này đã chỉ ra rằng dịch trích neem và lược vàng ở nồng độ 6% có thể sử dụng như một biện pháp thay thế tự nhiên trong việc phòng trừ sự phát triển của tác nhân gây thối trái cam. Các loại dịch trích này có tương lai trong bảo vệ thực vật hiện đại, thay thế các loại thuốc hóa học tổng hợp truyền thống trong hệ sinh thái nông nghiệp.

Keywords: *Aspergillus niger*, *Colletotrichum* sp., leaf extract, orange fruit

1. Introduction

Nowadays, control of post-harvest disease of fruits is mainly implemented by usage of chemicals. In a research of Brown and Chambers (1999), the authors showed that application of aqueous polyhexamethylene biguanide solution to mandarins and oranges effectively managed green mold caused by *Penicillium digitatum*, sour rot caused by *Geotrichum citri aurantii* and stem-end-rot caused by *Botryodiplodia theobromae*. Stenzel et al. (1998) indicated that a fungicide of amide carbamates group had

antifungal activity against *Plasmopara viticola* causing downy mildew in grapevine. Immersing oranges fruits into solutions of sodium molybdate at a concentration of 24.2 mM for 150 s, or 1 mM ammonium molybdate at 48 or 53°C provided effective control of green and blue molds caused by *Penicillium* spp. (Palou et al., 2002). An application of sorbitol liquid retarded the softening of flesh tissue, leading to inhibit an infection by *Aspergillus niger* (Kumpoun et al., 2003). Oliveira et al. (2000) showed that orange fruits which immersed for 1 min in aqueous suspensions of

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imazalil (100 mg l⁻¹) or thibendazole (1000 mg l⁻¹) and surfactant (0/002%) could inhibit the growth of fungal pathogens. The persistence of fungicides might depend on the nature of fruits, concentrations of fungicides applied and storage conditions. Remained residues of harmful chemicals in fruits always affect consumers' health (Mohammadi-far et al., 2012; Hue and Nghiem, 2014).

Based on this context, alternative safe treatments are investigated to manage fruit diseases. Among them, an application of plant extracts is promising due to their antifungal compounds including alkaloids, polyphenols, phenolic acid, flavonoids, quinones, flavones, flavonols, tannins, coumarins, polypeptides and terpenoids (Talibi et al., 2011; Gurjar et al., 2012; Chanh and Jin, 2014). Numerous plant species containing compounds with antimicrobial properties have been screened to manage diseases in many kinds of fruits. Most of these studies provide results of *in vitro* experiments, aimed at assessing the effect on conidial germination and mycelial growth of fungal pathogens causing fruit diseases (Islam et al., 2003). Only a few reports indicate the effectiveness of natural plant extracts in controlling the fruit diseases *in vitro*. Application of *Mentha arvensis* and *Eugenia caryophyllata* extracts reduced blue mold rot caused by *P. italicum* in orange and lime fruits, and enhanced their shelf life by 6 to 8 days (Tripathi et al., 2004; Sukorini et al., 2013). Aqueous extracts of *Vitex negundo* var. *purpurescens* at a concentration of 10% (w/v) offered the protection to carrots against *Cladosporium*, *Fusarium* and *Geotrichum* rot (Prakasam et al., 2001). Extracts of *Solanum torvum* were reported to be more effective in controlling the banana anthracnose disease caused by *Colletotrichum musae*. Furthermore, an increase in shelf-life by 16 to 20 days over control was the additional benefit of treatment with an extract of *S. torvum* (Thangavelu et al., 2004). Aman and Rai (2015) reported that at *in vitro* studies, plant extracts of *Garcinia cambogia* Gaertn, *Orthosiphon diffuses* Benth and *Radermachera xylocarpa* Roxb had significantly antifungal activity against fungal growth and spore germination of *Mycosphaerella musicola* on banana plants. Falade (2017) showed that *Datura stramonium* was the most effective extract, followed by *Ricinus communis* and *Jatropha gossypifolia*, on reducing hyphal growth rates of *Colletotrichum lindemuthianum*. However, these extracts had no significant inhibitory efficacy on conidial germination and sporulation *in vitro*.

In a research of Hidangmayum and Singh (2017), these authors showed that the extracts of *Eupatorium birmanicum* and *Vitex negundo*, both at a concentration of 20% (w/v) had high inhibition rate of the fungus *A. niger* of pineapples *in vitro*. Fungicidal activity was also highly exhibited by *Azadirachta indica* extract at 100% (w/v) against *A. niger* Van Tiegh, *Fusarium oxysporum* Schlecht, *Geotrichum candidum* Link and *Rhizopus stolonifer* Ehrenb ex. Fr. which isolated from tomato (Ngegba et al., 2018).

Management of *Alternaria alternata* causing fruit rot of strawberry was conducted by Patil and Suryawanshi

(2018). The results showed that the extracts of *Zingiber officinale* Rosc, *Mimusops elengi* L., *Aloe vera* L., *Lantana camara* L., *Mentha arvensis* Benth, *Catharanthus roseus* L., *Eucalyptus globulus* Labill, *Allium sativum* L., *Calotropis gigantea* (L.) R. Br. Ex Schult and *Cymbopogon citrate* DC Stapf at concentrations of 25, 50, 75 and 100% (w/v) gave good control efficacy of rot lesions on strawberry fruits. On mango fruits, the aqueous extract of garlic (*A. sativum*) at a concentration of 100% (w/v) had inhibitory effect against *Lasioidiplodia theobromae* *in vitro*. This garlic extract could prevent *Lasioidiplodia* lesion development on mango fruits after soaking the fruits into the extracts for 4 hours (Nur Fatimma et al., 2018).

Citrus (*Citrus* spp.) is a valuable fruit widely cultivated in Viet Nam. A research of Xuyen (2012) showed that there were six kinds of post-harvest fungi of orange fruits in Viet Nam. Among them, *A. niger* and *Colletotrichum* sp. could cause rapid and severe damage on orange fruits (Xuyen, 2012). Moreover, *Aspergillus* fungi could secrete mycotoxins that severely affect human health (Ploetz, 2003). Farmers require an eco-friendly approach for managing *Aspergillus* rot and anthracnose on orange fruits. Utilization of eco-friendly botanical extracts has recently started to get characterized. Many kinds of botanical extracts were reported as biocontrol agents to control fruit rot pathogens *in vitro* conditions. However, the botanical extract potential at *in vivo* conditions is still insufficiently exploited on orange fruits due to deficiency of studies on this subject. Therefore, this study was carried out to evaluate the efficacy of extracts from neem and basket plants on inhibiting lesions of *A. niger* and *Colletotrichum* sp. on orange fruits.

2. Materials and methods

2.1 Materials

Fungal pathogens including *A. niger* and *Colletotrichum* sp. were obtained from Department of Plant Protection, College of Agriculture and Applied Biology, Can Tho University. These fungi were cultured on petri dishes of Potato Dextrose Agar medium at approximately 10-12 days before conducting experiments. Medium of Potato Dextrose Agar was prepared followed a procedure of Atlas (2004).

Fresh mature leaves of neem (*A. indica*) and basket plant (*Callisia fragrans*) were collected at a garden of Department of Plant Protection, Can Tho University. These two kinds of plant were chosen due to their high ability to manage foliar diseases including blast, brown spot and bacterial leaf blight in rice plants (Thuy and Phuoc, 2015; other unpublished data). The leaves were washed thoroughly two times in tap water and then with sterile distilled water for one time, air-dried at room temperature, weighed at 6 g and ground with 100 mL sterile distilled water in a set of sterile mortar and pestle. The macerate was filtered with three folds of cheese cloth.

2.2. Assessment of leaf extract efficacy on orange fruits

The experiment was carried out in completely randomized design, with three treatments including neem leaf extract, basket plant extract and a control treatment; with 12 replications, one orange fruit per one replication, four inoculated points per one fruit. Different aqueous concentrations of neem and basket plant extracts were screened *in vitro* conditions (data not shown). Based on these results, concentrations of neem extract at 6% (w/v) and of basket plant extract at 6% (w/v) were chosen to conduct experiments on orange fruits.

In the experiment, orange fruits were similar on size and color. The fruits were washed on tap water for two times, air-dried at room temperature approximately 30 min, sprayed by ethanol at a concentration of 70% (v/v). A bunch of 4 sterile needles was used to create tiny holes with a depth of 4 mm on fruit epidermis. An orange fruit was inoculated at 4 positions. After 30 minutes, 1 mL of the spore suspension at a density of 10^6 spore mL⁻¹ was dropped on tiny holes on fruit surface. The inoculated orange fruits were put in an incubation chamber at 25°C, with relative air humidity approximately 98% for 24 h. After that, the inoculated orange fruits were immersed in the leaf extracts approximately 30 s. On the untreated control, the fruits were handled identically, but sterile distilled water was used instead of the leaf extract. Finally, each inoculated fruit was kept in a transparent nilong bag, amenmend with wet cotton, at room temperature.

Lesion diameter on orange fruits were recorded at 3, 7 and 11 days after inoculation (DAI). The experiment was performed twice on each kind of fungal rot pathogen.

2.3 Statistical analysis

The data was analyzed using the Excel software (Microsoft Office Excel, 2010). Interpretations using an analysis of variance and Duncan's multiple range test at p=0.05 were performed with SPSS 16.0 software (IBM, USA).

3. Results and discussion

3.1 Leaf extract efficacy on orange fruits inoculated with *Aspergillus niger*

The leaf extract was treated one day after pathogen inoculation. The results showed that treatments of leaf extract had the ability to inhibit the lesion development caused by *A. niger* on orange fruit epidermis. However, their ability varied and depended on the kind of leaf extracts.

Disease incidence on orange fruits was 100% (data not shown). At 3 DAI, lesion diameters of treatments of neem extract at a concentration of 6% and basket plant extract at a concentration of 6% were 51.33 and 55.50 mm, respectively, significantly lower than the control one at approximately 69.08 mm. On the next observations at 7 and 11 DAI, two treatments of leaf extract continued to inhibit lesion development on orange fruit surface, and significantly different to those of the control treatment. Efficacy of neem extract was better than one of basket plant extract (Table 1 and Figure 1).

Table 1. Lesion diameter (mm) of rot lesions caused by *A. niger* on orange fruits

Treatment	Days after inoculation		
	3 ^{1/}	7 ^{1/}	11 ^{1/}
Neem leaf extract of 6%	51.33 ± 6.4 ^b	80.25 ± 8.2 ^{bc}	109.08 ± 9.3 ^{bc}
Basket plant extract of 6%	55.50 ± 5.9 ^b	92.67 ± 8.5 ^b	124.00 ± 9.3 ^b
Control with distilled water	69.08 ± 6.7 ^a	120.58 ± 9.8 ^a	170.92 ± 8.3 ^a
Significance	*	*	*
CV (%)	18.21	22.13	17.64

^{1/} Mean ± SE (standard error) followed by the same letter do not differ significantly according to Duncan's multiple range test at p ≤ 0.05. *: significant at p ≤ 0.05

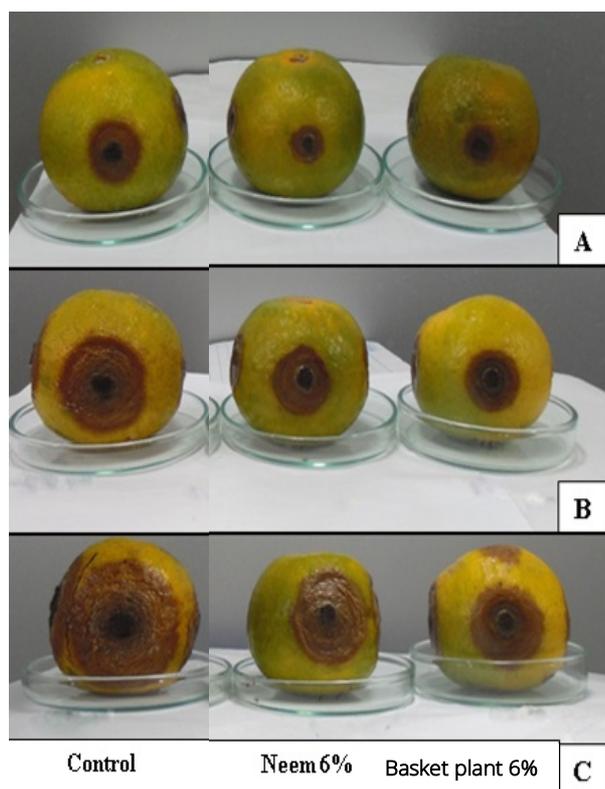


Figure 1. Efficacy of leaf extracts on lesions' development caused by *A. niger* on orange fruits at 3 days (A), 7 days (B) and 11 days (C).

The orange fruits were wounded on epidermis at four various positions by sterile needles and inoculated with *Aspergillus* spore suspension at a density of 10^6 spore mL^{-1} . After that, these fruits were treated with solutions of leaf extracts. Control: orange fruit was treated with distilled water, Neem 6%: the orange fruit was treated with neem leaf extract at a concentration of 6%; Basket plant 6%: the orange fruit was treated with basket plant extract at a concentration of 6%.

3.2 Leaf extract efficacy on orange fruits inoculated with *Colletotrichum* sp.

Both extract treatments inhibited *Colletotrichum* lesions on orange fruits. Therefore, they had high ability on controlling the anthracnose disease in oranges.

Disease incidence on orange fruits was 100% (data not shown). During three observation times at 3, 7 and 11 DAI, lesion diameter of neem and basket plant extract treatments varied approximately 29-38, 72-79, and 154-160 mm, respectively, significantly lower than those of the control treatment (Table 2 and Figure 2).

Table 2. Anthracnose lesion diameter (mm) caused by *Colletotrichum* sp. on orange fruits

Treatment	Days after inoculation		
	3 ^{1/}	7 ^{1/}	11 ^{1/}
Neem leaf extract of 6%	29.08 ± 4.2 ^b	79.00 ± 6.3 ^b	160.00 ± 10.5 ^b
Basket plant extract of 6%	38.00 ± 3.7 ^b	72.17 ± 7.2 ^b	154.75 ± 11.4 ^b
Control with distilled water	58.33 ± 4.1 ^a	119.00 ± 8.0 ^a	209.67 ± 12.6 ^a
Significance	*	*	*
CV (%)	30.32	26.76	14.83

^{1/} Mean ± SE (standard error) followed by the same letter do not differ significantly according to Duncan's multiple range test at $p \leq 0.05$. *: significant at $p \leq 0.05$

The orange fruits were wounded on epidermis at four various positions by sterile needles and inoculated with *Colletotrichum* spore suspension at a density of 10^6 spore mL^{-1} . After that, these fruits were treated with solutions of leaf extracts. Control: orange fruit was treated with distilled water, Neem 6%: the orange fruit was treated with neem leaf extract at a concentration of 6%; Basket plant 6%: the orange fruit was treated with Basket plant extract at a concentration of 6%.

Many plant extracts have been reported as having antimicrobial activities against fruit rot pathogens. The aim of the presented study was to elucidate the effect of two botanical extracts as an alternative to synthetic fungicides in controlling lesion development of *A. niger* and *Colletotrichum* sp. on orange fruits. Our results indicated that both tested leaf extracts of neem and basket plant caused a significant reduction in the length of rot lesions.

Similar efficacy of neem extracts effectively against fungal fruit pathogens was reported by several authors. Efficacy of neem extract at concentrations of 5, 10, 15 and 20% against *A. niger* were 78.12, 72.00, 65.36 and 54.00%, respectively, on pineapple fruits (Hidangmayum and Singh, 2017). The percentage of treatment efficacy on orange fruits against *A. niger* in our study was approximately 23-36%, lower than the efficacy on pineapple. The extract of neem could inhibit the development of hyphal growth of *Penicillium digitatum* *in vitro* (Al-Samarrai et al., 2012) as well as reduce fruit rot *in vivo* (Nath et al., 2013). Efficacy of the neem extract on inhibiting fungal growth was also reported on tomato (Nashwa and Abo-Elyousr, 2012; Ngegba et al., 2018). In this study, extract of basket plant had efficacy on inhibiting the development of rot lesions of both *A. niger* and *Colletotrichum* sp., but lower than those of the neem extract. Studies of application of the extract of basket plant on fruit rot pathogens have not been carried out.

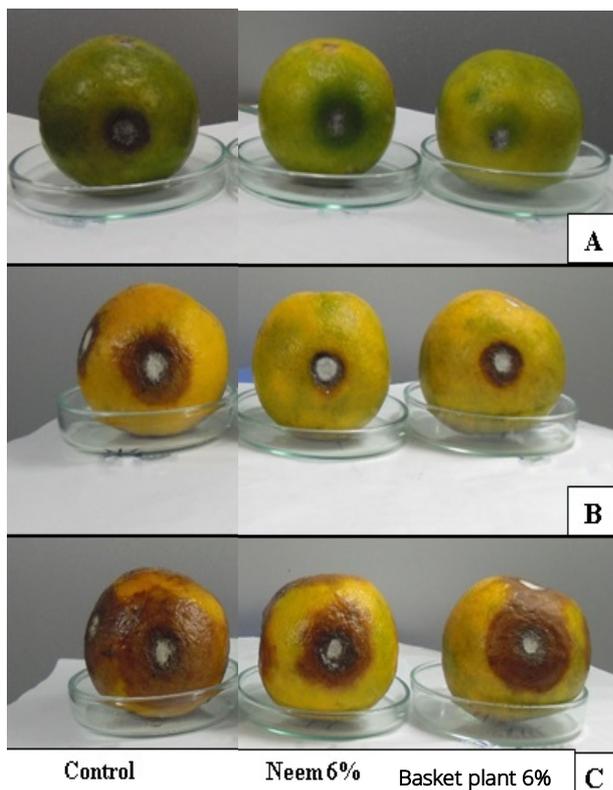


Figure 2. Efficacy of leaf extracts on anthracnose lesions' development caused by *Colletotrichum* sp. on orange fruits at 3 days (A), 7 days (B) and 11 days (C).

Plant extracts including neem and basket plant are an important source of natural agrochemicals which are environmentally friendly and do not have any toxic effects on fruits. They possess fungicidal properties. A research of Cowan (1999) indicated that natural extracts from plants contain many kinds of phytochemicals, most of which are polyphenols or oxygen-substituted derivatives. Other important compounds are phenolic acids, quinones, flavones, flavonols, tannins, coumarins and polypeptides (Cowan, 1999; Gurjar et al., 2012). Water is a universal solvent which is used to do plant extracts easily and this solvent is safe to fruit peels. Water is found to be the best solvent for the extraction of phenolic compounds, tannins, saponins and terpenoids (Gurjar et al., 2012). Phenolic compound, phenolic acids and terpenoids could degrade fungal cell walls, leading to membrane disruption. Tannins bind to proteins, leading to enzyme inhibition inside fungal cells. The bio-components of extracts from neem and basket plant have not fully revealed.

4. Conclusion

This study had shown that the neem and basket plant extracts, both at a concentration of 6%, have the potentials in the protection of orange fruit rots caused by *A. niger* and *Colletotrichum* sp. Therefore, due to the fact that chemical control of fruit rot is hazardous to human health and environmental, this biodegradable, non-toxic extract could be used as an alternative and green approach on reducing

and controlling fruit rot of oranges. Formulation of these extracts will be studied on the further researches.

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