The Antibacterial Effect of the Extract of Eupatorium indica Leaves on Two Main Postharvest Diseases of Pineapple

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Abstract. Phytophthora nicotianae var. Parasitica and Thielaviopsis paradoxa, often occur in pineapple production. In order to explore the bacteriostatic effect of the extract on the pathogen of pineapple disease, and to provide theoretical basis for the application of the extract on the prevention and control of pineapple fungal disease, the antibacterial activity of three extracts of Eupatorium adenophorum against two pathogenic bacteria of pineapple was studied. Two components with antibacterial activity extracted from the leaves of Eupatorium indica leaves were used as "fungicide". The antibacterial effects of the three extracts on the pathogens of pineapple heart rot and black rot were studied. The results showed that the crude extracts of H, B, and C components at a concentration of 2 mg / ml against pineapple heart rot and black rot pathogens reached 76.14%, 77.94%, 80.40% and 40.15%, 60.50%. The EC90 values of the crude extracts H, B, and C for pineapple heart rot pathogens were 4.48, 2.67, and 4.45 mg / ml, respectively. Respectively, the EC90 values for pineapple black rot pathogens were 10.60, 7.17, and 3.27 mg / ml. Relatively speaking, the three kinds of extracts have better antibacterial effects on pineapple heart rot pathogens than pineapple black rot pathogens; B and C two component substances have great potential to prevent and cure pineapple heart rot and black rot.

1. Introduction
Pineapple (Ananas comosus (L.) Merr), also known as pineapple, king pear, yellow pear, is a perennial herb of the Bromeliaceae family. It is the third largest tropical fruit in the world and the seventh largest fruit in the world. During the growth, storage and transportation of pineapples, pineapples will suffer from diseases such as dead plants, leaf spots and fruit rot. Pathogenic fungi are the main cause of disease. About 50 species of pineapple fungal diseases have been found in the world, and 22 species of pineapple diseases have been recorded in Taiwan [1]. There are more than 10 kinds of pineapple fungal diseases in South China. Pineapple heart rot and black rot are the most serious infectious diseases in pineapple producing areas in China [2]. The prevention and control of these two diseases in production depend on mainly the use of chemical fungicides. Chemical fungicides have certain
disadvantages in environmental protection. Plant-based fungicides can use certain antibacterial substances contained in plants themselves or induced plant defensins to kill or effectively inhibit the growth and development of certain pathogens. Studying the bacteriostatic effect of plant-derived fungicides on the pathogens of pineapple diseases is of great significance to the production and trading activities of non-polluting and pollution-free safe pineapples.

Pineapple heart rot is a soil-borne disease. It harms not only seedlings, but also adult plants and near-fruiting plants, causing the pineapple roots to rot. It had occurred in pineapple producing areas such as Guangdong, Guangxi, Fujian and Taiwan, with serious damage in some areas [3]. Pineapple black rot is an important disease in the process of fruit ripening and storage. When the disease was serious, the disease rate could reach 50-60%, and the pathogens can also attack pineapple seedlings, causing seedling rot [4]. Tang et al. reported that carbendazim, sec-butylamine or belenide and other fungicides mixed with GA3 could significantly control pineapple black rot [5]. Gu et al. have found that prochloraz and difenoconazole have a good inhibitory effect on the spore germination of black rot pathogen [6]. At present, pineapple disease prevention and control mainly rely on chemical fungicides. The use of chemical sterilization is prone to drug resistance of pathogens, but also pollutes the environment and fruits and endangers public health. The use of green pollution-free biological prevention and control technology for prevention and control of pineapple diseases and preservation has increasingly attracted the attention of producers and researchers. Reyes et al. reported that antagonistic bacteria isolated from epiphytic microorganisms on pineapple fruit could inhibit the spore germination, germ tube length and dry weight of black rot pathogens, and can effectively inhibit black rot pathogens [7]. Wijesinghe et al. reported that the use of antagonistic antibacterial mixed fungicides could completely control black rot [8]. Qin et al. used Bacillus subtilis and Streptomyces to control pineapple heart rot disease, the mycelial growth inhibition rate of heart rot disease mycelia was 71.6% and 53.3%, and the inhibition rate of sporangia of the pathogen was 83.3% and 61.7% [9].

Eupatorium adenophorum (Eupatorium adenophorum) is a perennial, tufted evergreen semi-shrub plant and a worldwide vicious weed. Eupatorium adenophorum plant is rich in natural active substances, and its extract has a strong allelopathy effect on various plants, pathogenic microorganisms and pests, significantly inhibits the growth and development of plants, and has a certain control effect on plant fungal diseases and insect pests [10-11], it is expected to be developed as a plant fungicide, which is applied to the green prevention and control of plant fungal diseases and fruit postharvest diseases.

At present, there is no research report on the antibacterial activity of Eupatorium adenophorum against pineapple disease pathogens. In this test, the crude ethanol extract of Eupatorium adenophorum and the petroleum ether and ethyl acetate extract components of the crude extract were used to target the bacterium pathogenic bacteria that are susceptible to pineapple heart rot and black rot. The antibacterial activity of three extracts of Eupatorium adenophorum against two pathogenic bacteria of pineapple was studied. The purpose is to provide a theoretical basis for the application of Eupatorium adenophorum extract in the green prevention and control of pineapple fungal diseases.

2. Materials and methods

2.1 Materials
Pineapple heart rot fungus (Phytophthora nicotianae var. Parasitica (Dast.) Waterh) and pineapple black rot fungus (Thielaviopsis paradoxa) were transferred to PDA medium plate (28°C) culture 2-5 d, for testing after meeting the inoculation requirements. The mature leaves of Eupatorium adenophorum plants (collected from Pu’er City, Yunnan Province); Carbendazim (50% wettable powder, produced by Jiangsu Yanfeng Biochemical Co., Ltd.); Prochloraz water emulsion (active ingredient 450g / L, produced by Shanghai Yuelian Chemical Co., Ltd.).
2.2 Measurement items
The preparation method of the components of different Eupatorium adenophorum extracts. After the leaves of Eupatorium adenophorum are shade dried, crushed to a size of 2-5 mm, add 95% ethanol according to the material-liquid ratio of 1: 8-1: 12, extract under reflux at 80-85℃ for 2 h, extract twice, and combine extracts Concentrate to obtain the crude extract (code: H); load the crude extract into the chromatography column and elute with petroleum ether (60-90℃) and ethyl acetate solvent successively. Concentrate the eluent to obtain petroleum ether extracts And ethyl acetate extract. Load the petroleum ether extract into the chromatography column, elute with a mixture of petroleum ether and ethyl acetate and concentrate the eluent to obtain the component B substance; pack the ethyl acetate extract into the column, use petroleum ether and ethyl acetate The ester mixed eluent is eluted, and the eluent is concentrated to obtain the component C substance. Place the crude extract (H) and the components of B and C in a low-temperature vacuum drying oven to evaporate the eluent to a balanced weight. Store at 4℃ in the dark at a low temperature and save for use.

Antibacterial activity of different Eupatorium adenophorum extracts against two pathogens. Using the growth rate method, according to the preliminary experiment on the sterilization activity of Eupatorium adenophorum extract, three kinds of Eupatorium adenophorum extract were dissolved with a small amount of ethyl acetate (1 g / 10ml) to prepare a mother liquor with a concentration of 100 mg / ml. And then formulate a drug-containing PDA plate with an active ingredient concentration gradient of 0.1, 0.5, 1.0, 1.5, and 2.0 mg / ml, respectively, and prepare a bacterial dish with a hole punch (Φ = 0.50 cm) for two pathogens, and then inoculate On medicine-containing tablet. Taking 2.0 mg / ml ethyl acetate PDA plate as a control (CK), it was incubated at 28 ℃ under constant temperature. After 5 days of cultivation, the diameter of the colony was measured to calculate the growth inhibition rate of the colony at different concentrations. Find the probability value of bacteriostatic rate (y), and calculate the logarithmic value of each concentration (x), and use the logarithmic value of concentration (x) and the probability of bacteriostatic rate (y) to find the virulence regression equation y = a + bx, and calculate the half-maximal effect concentration (EC50) and correlation coefficient (r) for inhibiting colony growth [12]. The colony growth inhibition rate was repeated 3 times for every 3 dishes. Colony growth inhibition rate /% = (control colony diameter - treated colony diameter) / control colony diameter x 100.

Comparison of antibacterial activity of different Eupatorium adenophorum against two pathogens. In the same way as above, three kinds of Eupatorium adenophorum extracts were formulated into 0.1, 0.5, 1.0, 1.5, 2.0 mg / ml concentration gradient drug-containing PDA plates, and two pathogens were inoculated on the drug-containing plates. The culture was carried out at a constant temperature at 28℃. After 5 days of cultivation, the diameter of the colony was measured to calculate the growth inhibition rate of the colony at different concentrations. Set carbendazim (code D) and prochloraz (code M) (concentration recommended for production) at a concentration of 1 mg / ml as reference, and compare the crude extract and two component substances with carbendazim and prochloraz Antibacterial effect on 2 kinds of pathogenic bacteria.

2.3 Statistical analysis
The statistics of experimental data was conducted using Excel 2007 and was analyzed using Duncan’s new complex range method by software DPS v9.50.

3. Results and analysis
3.1 Antibacterial activity of three Eupatorium adenophorum extracts against two pathogens
It can be seen from Figures 1 and 2 that the three kinds of Eupatorium adenophorum extract gradually increase the antibacterial activity of the two pathogens with increasing concentration, and the antibacterial rate increases. When the concentration of crude extract H and components B and C was 0.1 mg / ml, the bacteriostatic rates of pineapple heart rot (Figure 2A) and black rot pathogen (Figure 2B) were 27.08%, 25.68%, and 25.47, and 2.78%, 5.02%, 2.60%, respectively; at 2 mg / ml, the
bacteriostatic rates of pineapple heart rot and black rot pathogens were 76.14%, 77.94%, 80.40% and 40.15%, 60.50%, 72.30%; Relatively speaking, the bacteriostatic effect of H crude extract and B and C components on pineapple heart rot pathogen was better than pineapple black rot pathogen. At a relatively low concentration (0.5mg / ml), the inhibitory rates of crude extract H and component B and C on pineapple heart rot pathogens were 46.59%, 44.22%, and 33.33% (inhibition rates were all greater than 30%); And the bacteriostatic rate of pineapple black rot pathogens was 12.08%, 19.33% and 24.53% (less than 30%); the pineapple heart rot pathogen was examined separately, at high concentration (2.0 mg / ml), the inhibition of the three extracts Bacterial effect, C component substance B component substance H crude extract. Examine the pineapple black rot pathogen separately. At 0.5, 1, 1.5 and 2.0 mg / ml concentration, the antibacterial effect was C component substance > B component > substance H crude extract.

Fig.1 Bacteriostatic effect of three extracts on pineapple heart rot (a) and black rot (b) pathogens

Fig.2 Bacteriostasis rates of three extracts on pineapple heart rot (a) and black rot (b) pathogens

3.2 Toxicity of 3 extracts to 2 pathogens
It can be seen from Table 1 that the EC50 values of the crude extract of H and the components B and C against pineapple heart rot pathogens were 1.00, 0.59 and 0.44 mg / ml, and the EC90 values were 4.48, 2.67 and 4.45 mg / ml, respectively; H The EC50 values of the crude extract and the B and C component substances on the pineapple black rot pathogen were 3.10, 1.16 and 0.94 mg / ml, and the EC90 values were 10.60, 7.17 and 3.27 mg / ml, respectively. The results indicated that the two components B and C were more toxic to the two pathogens than the crude H extract. H crude extract, B and C components were more toxic to pineapple heart rot pathogens than black rot pathogens, EC90 value (all less than 5.0 mg / ml). The two component substances B and C can be used as good fungicides for the two pathogens.

| Extract component | Pathogen     | Y= aX+b     | R       | EC50 (mg/ml) | EC90 (mg/ml) |
|-------------------|--------------|-------------|---------|--------------|--------------|
| H                 | P. nicotianae| y=1.9729x+4.9961| 0.9554**| 1.00         | 4.48         |
| T. paradoxa       | y=2.3998x+3.8209| 0.9795**| 3.10        | 10.60        |
| B                 | P. nicotianae| y=1.9529x+5.4472| 0.9975**| 0.59         | 2.67         |
3.3 Comparison of antibacterial effects of three kinds of extracts with carbendazim and prochloraz on two kinds of pathogenic bacteria

From Figure 3 and Figure 4, the recommended concentration of 1 mg / mL prochloraz (M) has good bacteriostatic effect on two pathogens, and the bacteriostatic rate on heart rot pathogens is 78.60% (4A). The inhibitory rate against black rot pathogens reached 91.63% (Figure 4B). Carbendazim has good bacteriostasis against black rot pathogens, and the bacteriostasis rate was 54.19%, but it has poor bacteriostasis against heart rot pathogens, and the bacteriostasis rate was only 21.23%; 1 mg / mL H extract, B and C on the two pathogens were not as good as prochloraz, and the antibacterial rates on the heart rot pathogens were 48.77%, 58.61% and 53.12%. Bacteriostatic effect was shown as component B> C Substance> H extract, and the difference between the three extracts was significant. The antibacterial rates of black rot pathogens were 14.50%, 40.70% and 46.29% respectively. The antibacterial effect of component C> component B> H extract, the difference between the three extracts was also significant; the antibacterial effect of three extracts against heart rot pathogens was better than carbendazim. The antibacterial effect against black rot pathogens is not as good as carbendazim. It can also be seen from Figure 3 that the H extract, B and C component substances have a better bacteriostatic effect on heart rot pathogens than black rot pathogens.

**Table:**

|            | Formula          | R²      | Bacteriostatic Rate (%) |
|------------|------------------|---------|------------------------|
| T. paradoxa| $y=1.6214x+4.8946$ | 0.9891** | 1.16                   |
|            |                  |         | 7.17                   |
| P. nicotianae| $y=1.2771x+5.4542$ | 0.8992  | 0.44                   |
|            |                  |         | 4.45                   |
| T. paradoxa| $y=2.3727x+5.0595$ | 0.9994**| 0.94                   |
|            |                  |         | 3.27                   |

**Fig. 3** Bacteriostatic effects of three extracts, carbendazim, and prochloraz on pineapple heart rot (a) and black rot (b) pathogens

**Fig. 4** Bacteriostatic rate of three extracts with carbendazim and prochloraz against pineapple heart rot (a) and black rot (b) pathogens

4. Discussion and conclusion

According to reports, there are more than 6,300 higher plants with pest control in the world, including more than 2,000 sterilized plants. China is very rich in plant resources. At present, there are more than 1,400 kinds of plants with fungicidal activity. Some medicinal plants have a good inhibitory effect on plant pathogens [13]. Studies by Yang et al. had found that Eupatorium adenophorum extract has significant inhibitory effects on banana anthracnose, anthracnose citrus, Phytophthora, Penicillium citrinum, etc. [14]. Research by Ding et al. found that the petroleum ether extraction of the ethanol extract of Eupatorium adenophorum has a very strong bacteriostatic effect on Phytophthora infestans [15]. Studies by Li et al found that 70% ethanol extract of Eupatorium adenophorum has a significant
inhibitory effect on Staphylococcus aureus, Bacillus subtilis, Bacillus thuringiensis, Escherichia coli, and Ralstonia solanacearum [16]. Zhao et al. reported that 100 mg / mL ethanol, acetone, and ether extracts had a good inhibitory effect on Fusarium graminearum and soybean anthracnose [11]. Ma et al. studies have shown that the ethanol extracts and petroleum ether extracts of Eupatorium adenophorum can inhibit melon wilt, powdery mildew, tomato early blight, late blight, wheat powdery mildew, pear black spot and other pathogenic bacteria. The bacterial rate can reach more than 80%, and the EC50 value of Eupatorium adenophorum extract and petroleum ether extract against pathogenic bacteria were both below 2mg / mL [16].

Utilizing the natural bactericidal active substance in Eupatorium adenophorum, it will be developed into a plant-derived fungicide for pineapple disease green prevention and control and post-harvest fruit preservation, which has broad application prospects. However, the chemical composition of Eupatorium adenophorum is complex, and the main reported ones are monoterpenes, sesquiterpenes, triterpenes, steroids, flavonoids, phenylpropanoids and related derivatives [17]. Its antibacterial active substance may be a single component, or it may be a combination of several components. According to the results of this study, the B component and C component of the crude ethanol extract (H) and crude extract of Eupatorium adenophorum leaves were further isolated, and the antibacterial effect of crude extract H under in vitro conditions is obviously inferior to that of B and C components. It is speculated that the bactericidal active substance in Eupatorium adenophorum leaves may be one or several single-component substances, and it is necessary to further group B and C. Separation and identification of substances. The active substance's antibacterial mode and mechanism also need further study.

According to the results of this experiment, although the B and C components at a concentration of 1 mg / mL had less bacteriostatic effect on the two pathogens than prochloraz, the bacteriostatic rate on the two pathogens is above 40%. The EC50 values of the two pathogenic bacteria were relatively small (<2mg / mL). Considering the environmental friendliness of plant-derived bactericidal active substances, the bactericidal effect on pineapple organisms needs to be further studied in order to compare the two groups Substances developed into botanical fungicides are used to provide theoretical basis for green prevention and control of pineapple diseases.

The three Eupatorium adenophorum extracts tested have certain inhibitory effects on pineapple heart rot and black rot pathogens. The 2mg / mL concentration of crude extracts H, B, and C components inhibited the pineapple heart rot and black rot pathogens at 76.14%, 77.94%, 80.40%, and 40.15%, 60.50%, and 72.30%, respectively; Relatively speaking, the three kinds of extracts have better bacteriostatic effect on pineapple heart rot pathogen than pineapple black rot pathogen. The EC90 values of crude extracts H, B, and C components for pineapple heart rot were 4.48, 2.67, and 4.45 mg / mL, respectively. The EC90 values of pineapple black rot pathogens were 10.60, 7.17 and 3.27 mg / mL, respectively. The two components of B and C have great potential for the prevention and treatment of pineapple heart rot and black rot. It is worth further separation, purification and structural identification, and they are developed into plant-derived fungicides for green prevention and control of pineapple diseases Keep the fruit fresh.

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Recommended reason

1. The work described has not been submitted elsewhere for publication, in whole or in part, and all the authors listed have approved the manuscript.
2. Pineapple heart rot and black rot are main postharvest diseases. Crude extracts of Eupatorium adenophorum have great potential against two pathogenic bacteria of pineapple

References
[1] M. M. Xiang, Journal of Zhongkai College of Agri Technol, 07, 69-75(1994)
[2] N. Zhang, Z. T. Chen, X. D. Zhang, M. Y. Ren, South China Fruits, 38, 52-55(2009)
[3] J. J. Luo, Q. G. Liu, X. B. He, Z. B. Li, C. D. Wei, Guangdong Agri Sci, 12,90-92, (2012)
[4] B. X., Gong, Plant Protection Technol and Promotion, 21,26(2001)
[5] Y. L. Tang, Y. C. Zhou, Y. C. Zhou, X. J. Tan, Plant Protection, 04,13-15(1997)
[6] H. Gu, S. J. Zhu, R. L Zhan., D. Q. Gong, L. B. Zhang, Journal of Fruit Sci, 31,448-453+340(2014)
[7] M. E. Q. Reyes, K. G Rohrbach., R. E. Paull, Postharvest Biol Tech, 33,193-203(2004)
[8] C. J. Wijesinghe, R. S. W. Wijeratnam, J. K. R. R. Samarasekara., Crop Prot, 30,300-306(2011)
[9] H. L. Qin, Hainan University, 2015.
[10] J. H Ma, G. F. Xing, W. X. Yang, Acta Ecologica Sinica, 32,50-56(2012)
[11] C. F. Zhao, Y. Y. Wang, D. H. Liu, X. M. Xie, Hubei Agri Sci, 51,1133-1135(2012)
[12] F. Liu, R. L. Zhan, J. G. Wei, J. M. Chang ,J of Fruit Trees, 29,428-433. (2012)
[13] Z. Yan, X. L. Mo, Y. S. Wang, Chinese Journal of Traditional Chinese Medicine, 30, 1714-1717(2005)
[14] F. B. Yang, Y. G. Zhou, Z. M. Xia, H. M. Ou, T. Y. He, W. X. Gu, Guangdong Agri Sci, 38,83-84+97(2011)
[15] J. H Ding, Y. L. Yang, F. Y. Zhu, R. Z. Wang, L. T. Xiao, J of Hunan Agri U (natural science edition), 06,751-753(2007)
[16] L. P. Li, X. M. Xie, H. Y. Song, B. Q. Niu, Biotechnology Bulletin, 07, 46-152(2010)
[17] M. Zhang, Z. Y. Zhou, H. Ren, J. W. Tan, F. H. Wan, Acta Tropical and Subtropical Flora, 21, 63-68(2013)