Antifungal activity screening for 32 essential oils against *Rhizopus stolonifer*

Yongdong Xie¹,a, Linhong Jiang¹,b, Juan Li²,c, Jiawen Zhu²,d, Caihong Tu¹,e, Qiliang Guo¹,f, Ji Liu¹,g*

¹ Research Institute of Agricultural Product, Chengdu Academy of Agricultural and Forestry Science, Chengdu, Sichuan, China
² Institute of Raising Livestock, Chengdu Academy of Agricultural and Forestry Science, Chengdu, Sichuan, China

*e-mail: xydcom@sina.cn
*Corresponding author: e-mail: xyd@stu.sicau.edu.cn

Abstract: In order to screen out essential oils with better inhibitory effects on *Rhizopus stolonifer* and their appropriate concentrations, an in vitro antibacterial and drug susceptibility tests was conducted to determine the effects of 32 essential oils on the growth of grape *R. stolonifer*. The results showed that 1000 μL/L of clove, geranium, agilawood and lovage essential oils could completely inhibit the growth of *R. stolonifer* within 48 hours. Through follow-up verification, it was found that 1000 μL/L of clove, geranium andLovage essential oils had a continuous inhibitory effect on the growth of *R. stolonifer*, and the inhibition rate was continuing to 100% from 2 to 8 days. When the concentration was 250 μL/L, only Lovage essential oil had high inhibition rate. Therefore, Lovage essential oil can effectively inhibit the growth of *R. Stolonifer* on grape, and the appropriate concentration should not be less than 250 μL/L.

1. Introduction
Postharvest rot is a key issue that restricts the healthy development of the global fruit and vegetable industry. There are many factors that affect the postharvest storage of fruits and vegetables, among which fungal diseases are one of the most important factors leading to the deterioration of postharvest quality [1]. *Rhizopus stolonifer* is a strong parasitic pathogen, which can cause postharvest rot of peach, strawberry, grape and other fruits and vegetables [2-4]. The fungus mainly penetrates the host body through external scratches during the period of harvesting, transportation and sale. Then the mycelial mass grows on the fruit surface and produces long mycelial stolons, the growth, reproduction and infection rate are very fast, and the spores can spread with airflow, once it invades the host, it will spread rapidly, and cause decay and softness within 1-2 days, which seriously affects the transportation and sales of fruits and vegetables [5].

At present, chemical agents are often used in production to prevent and control postharvest diseases of fruits and vegetables, which not only causes environmental pollution, but also increases the amount of pesticide residues in agricultural products, which is potentially harmful to human health. At the same time, it is easy to induce the generation of resistance of pathogenic bacteria and the disease cannot controlled [6]. Essential oil is a general term for oily liquids with a certain volatility extracted from plants, they are widely distributed in plants and can be extracted from flowers, leaves, roots and barks [7]. Plant essential oils are divided into terpenes, aromatic compounds, aliphatic compounds, nitrogen-
containing and sulfur-containing compounds according to their chemical composition, and the roles and functions of plant essential oils are different with different compositions [8]. Many studies have shown that plant essential oils have good antibacterial activity and are considered natural antibacterial agents. Recent study showed that oils from Origanum vulgare and Thymus vulgaris exhibited fungistatic activity against Botrytis cinerea, Phytophthora citrophthora, and Rhizopus stolonifera [9]. 200 and 300ppm clove essential oil exerted antifungal activities with total inhibition of mycelia growth of Rhizopus stolonifer and Fusarium solani of yam tuber [10]. At the same time, plant essential oils also have the advantages of natural green, safe and no residue, unique fragrance, and have broad development prospects in the prevention and control of pathogenic microorganisms. Therefore, this study preliminarily screened the inhibitory effects of 32 plant essential oils at 1000 μL/L on grape R. stolonifer through in vitro antibacterial methods, in order to provide a theoretical basis for the prevention and control of pathogenic bacteria in grapes after harvest.

2. Materials and Methods

2.1. Materials

All 32 essential oils used in the experiment were purchased from Ji’an Shengda Spice Oil Co., Ltd. (Ji’an, Jiangxi, China). 46.0 g powdery potato dextrose agar (PDA) medium was weighed, and added to 1000 mL distilled water, autoclaved at 115℃ for 20 mins, and then stored at 4 ℃. Rhizopus stolonifer was isolated from the mature grapes.

2.2. Methods

2.2.1. Preparation of fungus-containing plates. R. stolonifer was inoculated on PDA medium and cultured in a biochemical tank at 26°C for 48h. The hyphae were picked from the plate with an inoculating loop and placed in a conical flask containing 50 ml of potato dextrose broth (PDB) medium, and placed in a constant temperature shaker with temperature was 26 °C for 48h.

2.2.2. Preparation of essential oil plate and determination of inhibition rate. The 32 essential oils crude oil were respectively dissolved in 5% tween-80 and fully emulsified to a final concentration of 20,000 μL/L. And then 1.5 mL of 20,000 μL/L essential oil was sucked into a centrifuge tube under sterile condition, and poured into a petri dish after shake well, each essential oil was repeated three times. An 8.0 mm stipe was punched out with a sterile perforator from fungal medium that has been grown for 2 days, and placed in the center of different essential oil plates. Then, all plates were placed in biochemical incubator at 26 °C, and the plaque diameter was determined when the fungal was cultivated for 48h.

2.3. Statistical analysis

Statistical analyses were conducted using SPSS 20.0 statistical software (IBM Corporation, Armonk, New York, USA). The data was analyzed using one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test applied at 5% significance interval. The inhibition rate was calculated as follow:

\[ \text{Inhibition rate} = \left( \frac{C - T}{C} \right) \times 100\% \]

C: plaque diameter of control (mm); T: plaque diameter of different plant essential oils (mm).

3. Results & Discussion

3.1. Inhibitory effect of 32 essential oils on R. stolonifer of grape

When R. stolonifer was cultured in medium for 2d, most of the essential oils with concentration of 1000 μL/L could inhibit the growth of R. stolonifer, but the inhibition rates varied with essential oils. The inhibitory effect of clove, geranium, agilawood and lovage were the best, and the inhibition rate were
100%, which were significantly higher than other essential oils except for ilex. The inhibition rate of ilex essential oil on the growth of *R. stolonifer* was 96.39%, ranked only second to the above four essential oils, but the difference was not significant. There were 16 essential oils that inhibit the growth of *R. stolonifer* more than 50%. Ginger essential oil had almost no effect on the growth of *R. stolonifer*. Tea tree, chamomile, mustard essential oils had a promoting effect on the growth of *R. stolonifer*, and their inhibition rates were -3.61%, -5.73% and -5.69%, respectively, which were significantly lower than that of other essential oils. Therefore, through preliminary screening, it was determined that the five essential oils of clove, geranium, agilawood, lovage and ilex had the highest inhibition rate and the best antibacterial effect on *R. stolonifer*, which can be used in subsequent tests.

| Essential oil   | Inhibition rate | Essential oil   | Inhibition rate |
|-----------------|-----------------|-----------------|-----------------|
| Clove           | 100.00±0.00a    | Notopterygium    | 46.69±2.61g     |
| Geranium        | 100.00±0.00a    | Magnolia officinalis | 31.63±2.61h |
| Agilawood       | 100.00±0.00a    | Garlic          | 26.20±2.47h     |
| Lovage          | 100.00±0.00a    | Blumea          | 17.47±5.49i     |
| Ilex            | 96.39±4.24ab    | Mastix          | 16.57±6.56i     |
| Verbena         | 91.27±1.00bc    | Lavender        | 14.16±7.29i     |
| Sandalwood      | 90.36±1.48c     | Sweet wormwood  | 13.25±1.20i     |
| Catmint         | 84.34±2.25d     | Bergamot        | 12.65±1.04i     |
| Patchouli       | 83.43±1.98d     | Myristica fragrans | 6.93±3.11j |
| Angelica dahurica| 83.13±1.90d    | Grapefruit      | 5.13±1.24j      |
| Cedar           | 63.86±2.56e     | Lemon           | 3.07±0.84k      |
| Osmanthus       | 63.25±3.01e     | Pepper          | 1.76±1.34k      |
| Curcuma         | 61.45±4.82e     | Ginger          | 0.26±1.01l      |
| Citronella      | 60.24±4.26e     | Tea tree        | -3.61±1.39m     |
| Cardamom        | 58.73±4.11ef    | Chamomile       | -5.73±2.83m     |
| Cedarwood       | 51.81±3.71fg    | Mustard         | -5.69±1.16m     |

Note: The inhibition rate was determined at 48h. Data represents means (±SE) of three independent replicates. Different lowercase letters indicate significant differences within a column (LSD *P*<0.05), the same as below.

### 3.2. 1000μL/L of 6 essential oils for continuous inhibition on *R. stolonifera* of grape

Through continuous observation of the antibacterial effects of the 6 essential oils initially screened, it was found that 1000 μL/L of clove, geranium and lovage had a continuous inhibitory effect on the growth of *R. stolonifer*, until the 8th day, the inhibition rate was still 100%, indicating that 1000 μL/L of essential oil may cause the fungus to lose its activity. The fungal inhibition rate of agilawood essential oil was 100% in the first four days, and on the 8th day, the inhibition rate was still 59.94%. However, ilex and verbena essential oils had optimal antibacterial effects only on 2d, then continue to decrease, and lost their antibacterial effects on 8th day (Table 2).

| Essential oil | Days of exposure |
|---------------|------------------|
|               | 2d   | 4d   | 6d   | 8d   |
| Clove         | 100.00±0.00a    | 100.00±0.00a | 100.00±0.00a | 100.00±0.00a |
| Geranium      | 100.00±0.00a    | 100.00±0.00a | 100.00±0.00a | 100.00±0.00a |
| Lovage        | 100.00±0.00a    | 100.00±0.00a | 100.00±0.00a | 100.00±0.00a |
| Agilawood     | 100.00±0.00a    | 100.00±0.00a | 84.94±2.09b   | 59.94±4.33b   |
3.3. 250μL/L of 6 essential oils for continuous inhibition on *R. stolonifera* of grape

Low concentration (250 μL/L) of lovage essential oil still had a strong inhibitory effect on the growth of *R. stolonifer*. The inhibition rate was 100% on 2th and 4th day, and the inhibition rate was reduced to 78.92% on the 6th day, which was significantly higher than that of other five essential oils at the same time. 250 μL/L of clove, ilex and verbena essential oils had no inhibitory effect on the growth of *R. stolonifer*. On the 2nd day, the inhibition rates of geranium and agilawood essential oils on the growth of *R. stolonifer* were 53.91% and 35.67%, respectively, and then the inhibitory rate decreased to 0 (Table 3). It suggested that when the concentration of essential oil was reduced to 250 μL/L, only lovage essential oil had better inhibition effect on the growth of *R. stolonifera*.

| Essential oil | Day 2       | Day 4       | Day 6       | Day 8       |
|--------------|------------|------------|------------|------------|
| Clove        | 0.00±0.00d | 0.00±0.00b | 0.00±0.00b | 0.00±0.00a |
| Geranium     | 53.91±7.25b| 0.00±0.00b | 0.00±0.00b | 0.00±0.00a |
| Lovage       | 100.00±0.00a | 100±0.00a | 78.92±8.97a | 0.00±0.00a |
| Agilawood    | 35.67±5.29c | 0.00±0.00b | 0.00±0.00b | 0.00±0.00a |
| Ilex         | 0.00±0.00d | 0.00±0.00b | 0.00±0.00b | 0.00±0.00a |
| Verbena      | 0.00±0.00d | 0.00±0.00b | 0.00±0.00b | 0.00±0.00a |

4. Conclusions

Among the 32 essential oils in this study, lovage essential oil has the best inhibition effect on *R. stolonifer* of grape, geranium and agilawood essential oils also have good antibacterial effect, but the antibacterial concentration should not be lower than 250 μL/L. Therefore, lovage, geranium and agilawood essential oils more than 250 μL/L can effectively inhibit the growth of *R. Stolonifer* on grape, which can be used for the control of pathogenic bacteria during grape storage.

Acknowledgments

This study was supported by Sichuan Science and Technology Program “2021JDRC0134”.

Reference

[1] Chand-Goyal, T., Spotts, R. A. (1996) Postharvest biological control of blue mold of apple and brown rot of sweet cherry by natural saprophytic yeasts alone or in combination with low doses of fungicides. Biol. Control, 6:253-259.

[2] Oliveira, J., Parisi, M. C. M., Baggio, J. S., Silva, P. P. M., Paviani, B., Spoto, M. H. F., Gloria, E. M. (2019) Control of *Rhizopus stolonifer* in strawberries by the combination of essential oil with carboxymethylcellulose. Int. J. Food Microbiol, 292: 150-158.

[3] Zhou, D., Wang, Z., Li, M., Xing, M., Xian, T., Tu, K. (2018) Carvacrol and eugenol effectively inhibit *Rhizopus stolonifer* and control postharvest soft rot decay in peaches. J. Appl. Microbiol., 124: 166-178.

[4] Lisker, N, Keren-Shacham, Z., Sarig, P., Zutkhi, Y. (2010) The biology and pathology of the fungus *Rhizopus stolonifer*, cause of black mould disease of table grapes in Israel. Plant Pathol., 45: 1099-1109.

[5] Yan, J., Wu, H., Shi, F., Wang, H., Chen, K., Feng, J., Jia, W. (2020) Antifungal activity screening for mint and thyme essential oils against *Rhizopus stolonifer* and their application in postharvest preservation of strawberry and peach fruits. J. Appl. Microbiol., 1-15.
[6] Kinay, P., Mansour, M. F., Gabler, F. M., Margosan, D. A., Smilanick, J. L. (2007) Characterization of fungicide-resistant isolates of Penicillium digitatum collected in California. Crop Prot., 26: 647-656.

[7] Lovkova, M. I. A., Buzuk, G. N., Sokolova, S. M., Klimenteva, N. I. (2001) Chemical features of medicinal plants. Appl. Biochem. Microbiol., 37: 229-237.

[8] Nazzaro, F., Fratianni, F., Coppola, R., De Feo, V. (2017) Essential oils and antifungal activity. Pharmaceuticals, 10: 86.

[9] Camele, I., De Feo, V., Altieri, L., Mancini, E., De Martino, L., Luigi Rana, G. (2010) An attempt of postharvest orange fruit rot control using essential oils from Mediterranean plants. J. Med. Food, 13: 1515-1523.

[10] Sameza, M. L., Mabou, L. C. N., Tchameni, S. N., Bedine, M. A. B., Tchoumbougnang, F., Dongmo, P. M. J., Fekam, F. B. (2016) Evaluation of clove essential oil as a mycobiocide against Rhizopus stolonifer and Fusarium solani, tuber rot causing fungi in yam (Dioscorea rotundata Poir.). J. Phytopathol., 164: 1-8.