Inhibition of orange (Citrus reticulata) green mold with antifungal yeast Debaryomyces hansenii and Aureobasidium pullulans

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Abstract. Green mold rot is an important disease that generally affects post-harvest and storage oranges. The attack of this disease quickly causes a decrease in the quality and shelf life of citrus fruits during storage. Post-harvest handling of citrus fruit from green mold attack is generally by coating the fruit using synthetic fungicides so that the fungicide residue often sticks to the orange peel when marketed. Fruit protection with a protective material can be used to increase shelf life and maintain the quality or quality of fruit stored in a room without refrigeration. The use of yeast as a protective material for postharvest fruit is still a little developed. The yeast fructoplan D. hansenii and A. pullulans have anti-fungal properties, so they have the potential to be developed as fruit protective agents. The experiment objective to find out the yeast ability of fructoplan D. hansenii and A. pullulans to protect postharvest citrus fruits with different ripeness levels against pathogens of green mold, storage capacity and quality of citrus fruits. The study was conducted using a two-factor randomized block design with four replications. The first factor, the type of antifungal yeast treatment, consisted of 4 levels; namely treatment without yeast and pathogens as negative control; pathogen inoculation alone, without application of yeast as positive control; A. pullulans application treatment, and D. hansenii application treatment. The second factor was the level of maturity of the citrus fruit, consisting of 3 levels, namely 25% yellow, 75% yellow; and 100% yellow. Measurement variables included disease severity, intensity of green mold rot, fruit storage time, weight loss, fruit hardness, vitamin C content, and total dissolved solids. The results of the experiment proved that the coating treatment of citrus fruits with yeast D. hansenii and A. pullulans was able to maintain the quality of oranges, increase shelf life, and increase the resistance of oranges to post-harvest diseases without reducing the quality of citrus.

1. Introduction
Green mold disease is one of the main post-harvest diseases of citrus. This disease mainly attacks oranges in storage. Green mold disease is caused by the fungus Penicillium digitatum [1]. These pathogens are able to attack various varieties of citrus. In Indonesia, oranges are one of the horticultural commodities that have high economic value. Oranges are widely cultivated in Java and
Bali, the types planted are especially Siamese and tangerines. The fruit quality that is not good enough is one of the problems of citrus fruit agribusiness, which causes the price of oranges to fall. One of the causes of poor quality citrus fruit in storage is the attack of green mold rot disease pathogens.

So far, post-harvest citrus fruit protection from pathogens in storage has been carried out chemically using synthetic fungicides. Before being marketed or stored, the fruit is coated with a fungicide, so the fungicide residue is often still attached to the fruit. Protection of the surface of the fruit with various natural or biological ingredients, in addition to reducing the evaporation of water from inside the cells, reducing oxygen entering the cells, it is also able to protect the fruit from postharvest disease attacks [2]. This can extend the economic life of a product. Fructoplan yeast-based biological materials have been reported to be applied as a coating for fruit both pre-harvest and post-harvest [3]. This method has several advantages. Besides being harmless, yeast on the surface of the fruit is also capable of producing various substances or important metabolite compounds that have a good and beneficial impact on postharvest fruit. Application of yeast on the surface of the fruit generally aims to protect against postharvest pathogens that infect after harvest [4, 5].

2. Methods
The experiment was carried out at the Agrotechnology Laboratory of the Agriculture-Animal Husbandry Faculty, University of Muhammadiyah Malang. The tested citrus fruit used tangerines obtained from citrus orchards in Batu city. The biological material for coating the fruit is *Debaryomyces hansenii* and *Aureobasidium pullulans* with cell concentration of $10^9$ cells / ml. The two yeasts were isolated from the surface of apples from the Batu area. The fungi *D. hansenii* and *A. pullulans* besides morphological characterization, it is also identified molecularly. The experiment was carried out using a two-factor randomized block design, with four replications. The first factor was the type of antifungal yeast, which consisted of 4 treatments, namely without yeast application and without pathogen inoculation, pathogen inoculation without yeast application, citrus coating with *A. pullulans*, and citrus coating with *D. hansenii*. The second factor is the level of maturity of citrus fruit which consists of 3 treatments, namely: 25% yellow, 75% yellow; and 100% yellow. Measurement of observation variables was carried out on citrus fruits in storage for 15 days. Data recording includes 1) The disease severity, measured using the equation: $P = \frac{\text{na}}{\text{N}}$ (P = severity of disease; na = value of area affected; N = number of fruits observed); 2) The intensity of the disease, measured by the equation: (Number of infected fruits / number of fruits observed) x 100%; 3) The shelf life of citrus fruits; 4) Weight loss is measured using the formula: $((a-b) / a) \times 100\%$, (a = initial weight, b = final weight); 5) Fruit hardness is measured by means of a Hardness Tester; 6) Total dissolved solids, measured using a hand refractometer; 7) The content of vitamin C, measured using the iodimetric method; 8) Interaction test of antifungal yeast with green mold rot pathogens. This test was carried out by taking four oranges which were cut 6 mm wide. The injured part of the citrus fruit was given a layer of sterile cellofan film. A total of 20 μl (10⁶ conidia /ml) of green mold spore suspension was dropped onto cellofan. Furthermore, 20 μl of the yeast suspension were dropped onto the same cellofan. Then, incubated at room temperature in a damp plastic box for 24 hours. Two small slices of cellofan from each citrus fruit showing the interaction of yeast and fruit rot pathogens were observed under a microscope. Observations were made on the cellofan slices of the sample before and after washing with distilled water containing 1% (vol / vol) Tween 20.

The data obtained were analyzed using analysis of variance, to determine whether the treatment had an effect or not, it was followed by the F test. If the results of the analysis of variance had a significant effect, the differences between the treatments were analyzed using the 5% Duncan test.

3. Results and Discussion
3.1 The shelf life of citrus fruits
The treatment of yeast barrier application and the level of citrus maturity had a very significant on the citrus fruits shelf life. The difference in shelf life of citrus fruits can be seen in Table 1.
Table 1. The average of citrus fruits shelf life was due to the treatment of yeast type and the level of fruit maturity

| Yeast application | Orange fruit maturity level | Disease severity (%) | Disease intensity (%) | Effectivity (%) |
|-------------------|-----------------------------|----------------------|----------------------|-----------------|
| Negative control (without pathogens and yeast) | 25 % | 11.0 d | 9.5 e | 8.7 bc |
| Positive control (pathogen inoculation only, without yeast) | 75 % | 7.9 b | 8.1 b | 5.4 a |
| Aureobasidium pullulans | 100 % | 14.8 e | 15.0 e | 14.5 e |
| Debaryomyces hansenii | 1,0 d | 7.9 e | 15.0 e | 15.3 e |

Note: The numbers followed by different letters show significant according to the Duncan test of 0.05.

The application of D. hansenii at the maturity level of 25% yellow citrus fruit, can extend the shelf life for 6 to 7 days for negative controls, and 9 days for positive controls. Giving A. pullulans yeast was able to extend the citrus shelf life for 4 to 6 days for negative control, and 7 to 8 days for positive control. The longer shelf life of oranges at room temperature is due to the ability of the two fructoplane yeasts to suppress the attack of green mold that causes fruit rot, as well as the optimal level of ripeness of citrus fruits. Freshly harvested fruit requires adequate post-harvest processing technology to minimize both qualitative and quantitative losses after harvest [6].

3.2 Disease Severity and Intensity

The treatment of antifungal yeast application had a very significant on the variable of disease severity and intensity. The average of disease severity and intensity can be seen in Table 2.

Table 2. The citrus green mold severity and intensity during the shelf life

| Yeast application treatment | Disease severity (%) | Disease intensity (%) | Effectivity (%) |
|----------------------------|----------------------|----------------------|-----------------|
| Negative control (not application pathogens and yeast) | 10.13 b | 18.67 b | - |
| Positive control (inoculation pathogen, not yeast) | 32.77 c | 30.00 c | - |
| Aureobasidium pullulans | 5.40 a | 6.33 a | 78.9 |
| Debaryomyces hansenii | 1.83 a | 1.67 a | 94.4 |

Orange fruit maturity level treatment

| Orange fruit maturity level treatment | Disease severity (%) | Disease intensity (%) | Effectivity (%) |
|--------------------------------------|----------------------|----------------------|-----------------|
| 25% Yellow                           | 10.88 a | 12.75 a | - |
| 75% Yellow                           | 12.63 a | 14.75 a | - |
| 100% Yellow                          | 14.10 a | 15.00 a | - |

Note: The numbers followed by the different letters show significant according to the Duncan test of 0.05.

Shown in Table 2 that coating citrus fruits with A. pullulans and D. hansenii yeasts is able and effectively protects oranges from infection with green mold pathogens. The effectiveness of D. hansenii yeast application reached 94.4%, while A. pullulans yeast reached 78.9%. During storage, the quality of oranges does not decrease with the application of the two yeasts, so that the oranges are still fit for consumption. Spraying fruit with fructoplane yeast can suppress the growth, development and infection of post-harvest disease pathogens, because the yeast acts as a formidable competitor for both space and nutrition [7][8]. The disease affecting post-harvest citrus fruits is mainly green mold disease caused by Penicillium digitatum (Figure 1). The characteristic of infected fruit is the appearance of fungus on the fruit. The fungal hyphae initially turns white, then turns green, which indicates the pathogen has sporulated. The surface of the citrus fruit becomes soft and crumbles easily, then greenish white mold mycelium grows on the surface of the citrus fruit. Pathogens that cause post-harvest disease in fruit are generally from fungi, and some from bacterial groups [9]
3.3 Microscopic Analysis of the Interaction of Green Mold Hyphae versus Antifungal Yeast

Microscopic observations of the fungal hyphae of *P. digitatum* interacting with the yeast *D. hansenii* showed strong adhesion of yeast cells on the surface of the green mold pathogenic hyphae (Figure 2). This strong attachment is a form of yeast parasitization against pathogenic hyphae. *D. hansenii* exhibits a strong parasitic mechanism. The hyphae exposed to the cells of *D. hansenii* appeared lysis. Pathogenic hyphae appear defective, abnormal, and do not produce conidia. Hypha abnormalities are also in the form of many hyphal structures that are circular, not straight and branched. The area where the hyphae is parasitized shows a concave surface. This is thought to be due to the biological activity of antagonistic yeasts in the form of hydrolytic enzyme secretion which hydrolyzes the cell walls of green mold hyphae. Anti-fungal yeast cells remain firmly attached to all parts of the pathogenic hyphae, even though washing has been carried out with running water containing a washing agent in the form of Tween 20.

Khamir fructoplan *D. hansenii* and *A. pullulans* are reported to have the ability to produce volatile organic compounds which are toxic which can suppress the growth and development of postharvest pathogens. Hyphae exposed to VOCs will experience morphological deformation and cause death; or hyphae grow abnormally into circular or swollen structures [10, 11]. Two strains of *Aureobasidium pullulans*, are effective against several postharvest fruit pathogens such as *Botrytis cinerea*, *Colletotrichum acutatum*, *Penicillium expansum*, *Penicillium digitatum*, and *Penicillium italicum*. The inhibition mechanism is mediated through the production of toxic VOCs which are capable of inhibiting the germination of pathogenic conidia [12].

![Figure 1. Symptoms of green mold attack on tangerines (left); healthy citrus fruit (right)](image)

![Figure 2. Strong parasite of green mold hyphae by the *D. hansenii*
Keys : Kh = yeast cells; Hf = hyphae ; Bar = 10 µm](image)
3.4 Content of Vitamin C
The treatment of fruit maturity level was very significant on the vitamin C content of citrus fruits at the end of the observation. The mean vitamin C levels of citrus fruit can be seen in Table 3.

Table 3. Average Vitamin C Levels of Oranges for 15 Days of Storage

| Yeast application treatment | Vit. C levels | Vit. C levels |
|-----------------------------|---------------|---------------|
|                             | early save    | end save      |
| Negative control             | 9.50 a        | 3.59 a        |
| Positive control             | 9.54 a        | 3.79 a        |
| *Aureobasidium pullulans*    | 9.53 a        | 3.76 a        |
| *Debaryomyces hansenii*      | 9.54 a        | 3.72 a        |
| Orange maturity level        | 6.39 a        | 3.88 a        |
| 25% Yellow                  | 8.57 b        | 4.28 a        |
| 75% Yellow                  | 12.04 c       | 3.85 a        |

Note: The numbers followed by the different letters show significant according to the Duncan test of 0.05.

Table 3 shows that before storing citrus fruits (freshly harvested), the 100% yellow fruit ripeness level indicates a higher vitamin C content. After the citrus fruit was stored for 15 days, the vitamin C content in all treatments decreased and had the same content. These data suggest that consumption of fresh citrus fruits immediately after harvest has the highest vitamin C content and is therefore more recommended. Citrus fruits with a 100% yellow color indicate a higher vitamin C content. The content of vitamin C in the early stages of fruit maturity generally increases, then decreases during the storage period of the fruit until it approaches rot [13].

3.5 Weight Loss, Fruit Hardness, Total Dissolved Solids, and Fruit Color Change
The treatment of citrus maturity level had a very significant on the variable of fruit weight loss, fruit hardness, total dissolved solids, and fruit color change. The average of each observation variable can be seen in Table 4.

Table 4. The mean of shrink weight, citrus color change, fruit hardness, and total of dissolved solids during 15 days in storage

| Yeast application treatment | Shrink weight | Change in citrus color | Fruit hardness | Total of Dissolved Solids |
|-----------------------------|---------------|------------------------|----------------|--------------------------|
| Negative control             | 2.36 a        | 4.46 a                 | 3.40 a         | 7.33 b                   |
| Positive control             | 2.50 a        | 4.54 a                 | 3.31 a         | 7.44 b                   |
| *Aureobasidium pullulans*    | 2.06 a        | 4.48 a                 | 3.08 a         | 7.76 b                   |
| *Debaryomyces hansenii*      | 1.86 a        | 4.52 a                 | 3.19 a         | 7.27 b                   |
| Orange maturity level        | 2.10 b        | 3.11 a                 | 5.71 b         | 7.56 a                   |
| 25% Yellow                  | 1.69 a        | 3.97 b                 | 5.23 b         | 7.88 a                   |
| 75% Yellow                  | 1.84 a        | 4.92 c                 | 4.47 a         | 8.84 b                   |

Note: The numbers followed by the different letters show significant according to the Duncan test of 0.05 (in the column and the same treatment factor).

The data in Table 4 shows that giving the two antifungal yeasts as a protector of citrus fruits does not cause a decrease in fruit quality. The presence of *D. hansenii* and A. pullulans on the surface of the citrus can still maintain the freshness of the fruit. The quality of citrus fruit as indicated by change in fruit color, weight loss, total of dissolved solids, and fruit hardness as good as the treatment without
antifungal yeast. Fruit weight loss that occurs is caused by various important factors, including fruit maturity level, temperature, rate of respiration, and attack by pathogens. The factor that affects the rate of water loss is the aging or ripeness of the fruit. The rate of water loss can occur more rapidly in physiologically immature fruit [14].

During in storage, there is a change in the color of the citrus fruit. This color change indicates the fruit is experiencing a citrus ripening process, which is marked by a yellowish green color change to yellow. The citrus color change that occurs during the ripening process is caused by breakdown of the leaf green dye or chlorophyll on the fruit so that the green color fades, and is replaced by a yellow-orange carrier compound. In ripe fruit that is stored there will be an increase in the number of compounds from the carotenoid group. Ethylene gas in oranges can degrade green pigments in the skin of the fruit to form orange pigments (carotenoids)[15] [16]. Citrus fruits with 25% yellow maturity have a higher hardness. This is because the fruit, which is still dominated by green color, is not fully ripe, although physiologically the fruit is ripe. The results of the analysis of the total dissolved solids variable showed that 100% yellow orange had the highest. The reason is because during ripening, there is a process of hydrolysis of starch compounds turning into sugar compounds, which causes the TDS content of fruit to gradually increase during the ripening process, especially after the fruit is harvested [17, 18].

4. Conclusion
Application of D. hansenii and A. pullulans proven to be able to increase the shelf life of citrus fruits up to 9 days longer, maintain the quality of oranges, and protect from postharvest pathogen infections of green mold. The protection effectiveness of D. hansenii reached 94.4%, while A. pullulans was able to provide protection of 78.9%. Khamir D. hansenii showed a strong parasitism mechanism in suppressing green mold pathogens.

5. References
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