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# Identification of Fusarium oxysporum f.sp cepae race 4 isolated from shallots in East Java Indonesia

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**KEYWORDS**

*Fusarium oxysporum* f.sp *cepae*

Foce

Race 4

Volatile odor test

Vegetative compatibility group

**ABSTRACT**

*Fusarium oxysporum f.sp cepae* (Foce) which causes moler disease, has been mutated into four races. The fourth race is the riskiest due to its vulnerability to being attacked by various cultivars. This research aims to identify the existence of Foce Race 4 in the shallot production center districts. The plant samples attacked by moler disease were taken in three districts (i.e., Magetan, Nganjuk, and Probolinggo) by Purposive Random Sampling method with 5% samples, obtaining 9 Foce isolates. The results were then identified using Volatile Odor Test (VOT), Biochemistry, and Vegetative Compatibility Group (VCG); four were identified as Race 4. Those four isolates were then examined for their virulences towards three cultivars, particularly Bauji, Tajuk, and Biru Lancor. The findings show that all four isolates were noxious and able to plague all the varieties.

## Introduction

*Fusarium oxysporum f.sp cepae* (Foce) is currently a fractious pathogen. It is difficult to control once it plagues the varieties. There have been more than 50% reports about shallots' crop failure due to this fungal attack which is known as Moler disease (Widono, et al., 2023). Moler disease plagues shallots where its pathogen is transmitted through the soil and infects the host plant roots only to grow and outspread the plant's vascular tissue. This fungus delays the distribution of water and nutrients to all plant organs, affecting the growth process so that shallots cannot produce bulbs well. Several symptoms occurred, such as wilted plants, chlorosis leaves, stunted, and rots in the base of the plant stem (Sudantha and Suwardji, 2021).

To manage this pathogen, shallots production needs to be increased in certain ways. Increasing shallots production can be achieved through genetically modified technology by creating superior disease-resistant varieties, especially for Moler disease (Hadiwiyono, et al., 2020). At first, this method was considered an effective way to manage the disease. However, some research has shown that the pathogen has been mutated. This was first reported by Pegg et al. (2019), who discovered that *Fusarium* attacks banana commodities with higher virulence levels than before. This case was re-analyzed by Li et al. (2013), who stated that *Fusarium* had mutated and made a new race. In addition to supporting this discovery, a study by Hokken et al. (2019) reported that the organism will always be able to adapt to the dynamic environment by experiencing mutation and evolution as an adaptation mechanism.

Hence due to recent reports, the most noxious fungus is Race 4, with its specialty that could plague all host plant varieties. The race was first found in the 1970s when the variety itself plagued other varieties that have never been attacked before, such as Cavendish. This caused the spreading fear among farmers after 50 years when no Panama wilt disease reports had been submitted during that era (Bastidas, 2022). The expeditious technological development finally triggered more effective and efficient identification methods, such as *Volatile Odor Test* (VOT), which can identify *Fusarium* Race 4 with identical analysis results or equal to VCG-test, RAPD-PCR, and DNA fingerprint. The result of VOT can be validated by identifying the presence of aldehydes using biochemistry, namely strong acids and strong oxidizing agents (Yudha, 2022; Tian et al. 2023).

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Though it is an urgent matter regarding the shallot production rate, there is little information regarding Foce Race 4 in Indonesia, particularly in shallot's production center areas. This deficiency eventually attracts researchers' attention to validate its existence, considering shallots as a superior commodity with a great number of demands that always increase annually. Magetan, Nganjuk, and Probolinggo districts are chosen as locations of the observation since they are the top production center in East Java (Central of Statistics and Directorate General of Horticulture, 2022). Thus, the result of the identification of race 4 using the *Volatile Odor Test* (VOT), *Vegetative Compatibility Group* (VCG), biochemical test, and virulence test could be used later as the main data to create a distribution map of Foce Race 4 as well as part of Moler disease management efforts, especially in East Java.

## Research Methods

The observation of shallots plagued by Moler disease was carried out in three locations: Magetan with Bauji cultivar, Nganjuk with Tajuk cultivar, and Probolinggo with Biru Lancor cultivar. This research used a purposive random sampling method with a 5% level (Wiyatiningsih, et al., 2016). The identification of the 4 Race was held at UPN "Veteran" East Java Growing Health Laboratory. Meanwhile, the Virulence test was held at UPN "Veteran" Agroclimatology Laboratory, East Java. The research was conducted from early October 2021 until December 2022.

### *The identification of Fusarium oxysporum f.sp. cepae*

The first step before identifying Race 4 was to ensure that the *Fusarium* collection from the results of the exploration of some plants' samples with Moler symptoms is Foce isolates. The identification was carried out on 7-day-aged isolates grown on Potato Dextrose Agar (PDA) medium. Foce identification was conducted in two ways, macroscopically and microscopically. Macroscopic observations were made visually by observing the growth of the growing colonies. The Foce in macroscopic observation has the characteristics of mycelium that grows vertically like cotton-clumping white, with the base color of the colony being yellowish white or purplish. While we observed the growing colonies in macroscopic observation, microscopic observations were carried out under a microscope. Its Foce has oval-shaped microconidia spores like eggs, crescent-shaped macroconidia with 3-5 partitions, and chlamydospores like tadpoles which have a partition between the head and tail (Kalman et al., 2020).

### *The identification of race 4 using the volatile odor test (VOT) method*

The first stage of Race 4 identification used the *Volatile Odor Test* (VOT) method. This method was performed by aseptically inoculating the Foce isolate into a rice medium contained in Erlenmeyer with a Laminar Air Flow (LAF) and then covering it with cotton and foil. After that, the plant culture was stored at room temperature for 7 days. The race 4 identification process was carried out by smelling fermentation aroma after the culture was 7 days old planting. The plant cultures that produce an aroma like fermented *tape* are Race 4 and vice versa. Cultures that do not produce an aroma are not Race 4. The test is failed if the culture smells bad like rotten rice. Nasir et al. (2003) stated that Foce Race 4 can produce aldehydes which can be identified from the aroma of the culture resembling fermented *tape* after 7 days of planting; other races do not share this. Isolates that produce an aroma resembling fermented *tape* are called *odoratum*, while those that do not emit an aroma are called *inodoratum.*

The rice medium was made by inserting 15 g of rice and 45 mL of distilled water into an Erlenmeyer with a capacity of 250 mL. Then the Erlenmeyer was covered with cotton first and layered with foil. The making of rice medium is the same as cooking rice in general. However, the difference here is that the process was done in an autoclave with the same working procedure as the sterilization of the growing medium. The process itself was done by inserting the Erlenmeyer into the autoclave, which was ready for use. The activity lasted for 20 minutes at 1.5 atm pressure, and the medium was ready to use when it turned into rice perfectly (Yudha, 2022).

### *The identification of aldehyde content with biochemical compounds*

Isolates classified as *odoratum* were then tested again to prove the presence of aldehydes from the gas which produced by the Foce culture. This process is carried out by converting gas into liquid through a distillation process using a distillator. The liquid resulting from the distillation process was then combined with 1 mL of potassium permanganate and strong acid. The Aldehyde can be controlled using formalin. If it contains aldehydes, the solvent will change color from purple to red, and incline to brown like the color of

bricks (Yudha, 2022). This happened because the aldehyde compounds reacted with Fehling's reagent and formed a brick-red precipitate. One of Fehling's reagents is potassium permanganate and sulfuric acid (Li et al., 2022).

***The identification of race 4 isolate strains using the vegetative compatibility group (VCG)***

The virulence test using Race 4 isolates from different strains was conducted to obtain more diverse virulence results. Regarding that matter, we used the *Vegetative Compatibility Group* (VCG) as one of our methods. It is a method of grouping fungi based on a genetic exchange between isolates on the reproductive compatibility of different strains (Davis, 2004). The first step is culturing Nitrate Nonutilizing (nit) mutant before carrying out the complementation test to determine the similarity of the strains between each other. This method was carried out by inoculating Foce Race 4 ( 7 days old) from PDA medium to minimal medium added with 1.5% chlorate. The culture was then incubated for 15 days at room temperature. The result showed that colonies with mycelium growing very thin and faster than other mycelia are mutants. The researcher needs to pay attention to the medium to get the result. The minimum medium consists of 30 g sucrose, 1 g monopotassium phosphate, 0.5 g magnesium sulfate heptahydrate, 0.5 g potassium chloride, 10 mg sulfate heptahydrate, 20 g agar, 0.2 ml Trace Element Solution, 1000 mL distilled water. (Hartanti et al., 2016)

After the nit mutant had been gained, the complementation test was continued by inoculating them, which were obtained from several isolates into one petri dish, and then incubated at room temperature for 7 days. The medium used was nitrate media. Then, the observations were made visually by observing the growth of the hyphae. Isolates that are vegetatively compatible with each other are isolates that come from the same strain. It was indicated by the growth of thick hyphae like cotton and coalescing with one another. Vice versa, incompatible vegetative isolates may not experience this condition and are said to come from different strains or are called *Single Self-Compatibility* (SSC) (Correl et al., 1987; Hartanti et al., 2016).

***Virulence test***

The cultivars used as test materials were the sampling locations for the diseased plants: Bauji from Magetan, Tajuk from Nganjuk, and Biru Lancor from Probolinggo. The Foce isolates used were isolates that have passed the test up to the *Vegetative Compatibility Group* (VCG). Foce isolates classified as *Single Self-Compatibility* (SSC) were used as virulence test materials. The isolates used to infect were 4 days old on PDA media. Inoculation was carried out by soaking the shallot seed bulbs to be planted in Foce microconidium suspension for 30 minutes. Please note that the microconidium concentration used was $10^6$/mL. Before the inoculation, the tubers were disinfected by immersing them in 1% Clorox solution for 1.5 minutes, then washed with sterile distilled water and drained on filter paper for at least 12 hours. The inoculated tubers were then planted in 25 x 25 cm polybags containing compost and garden soil with a ratio of 1:2. The research design used was a Completely Randomized Factorial Design (CRD) with three replications. (Prakoso, et al., 2016). In addition, the observation of virulence tests was performed through daily observation of the attack rate of Moler disease.

**Results and Discussion**

***The identification results of Fusarium oxysporum f.sp. cepae***

The results of the 9 isolates from exploring plant samples with Moler symptoms showed that they are all identified as Foce. To specify the information of the results, both the macroscopic and microscopic observations can be seen in Figure 1. The macroscopic side shows that all pure isolates had fine-textured mycelium with long white threads clumping like cotton. If it was being viewed from the bottom of the petri dish, the colonies of all isolates had a purplish-white to yellowish color-brass. The results of these observations were reported by Labanska et al. (2022) and Zhou (2023), who found that Foce isolates with a fine colony texture (by forming long and dense air mycelium) resemble the fine threads of cotton. The color of the colonies also varied from purplish-white to yellowish. Figure 1 was added not only to specify the macroscopic observation but also the microscopic. The microscopic observations shows that all isolates had oval-shaped microconidia, crescent-shaped macroconidia with 3 to 5 partitions, and chlamydospores resembling tadpoles. Johnson et al. (2020) also stated that Foce's spores for microconidia are oval, while macroconidia are curvy with 3 to 5 partitions, thicker cell walls, and colorless.

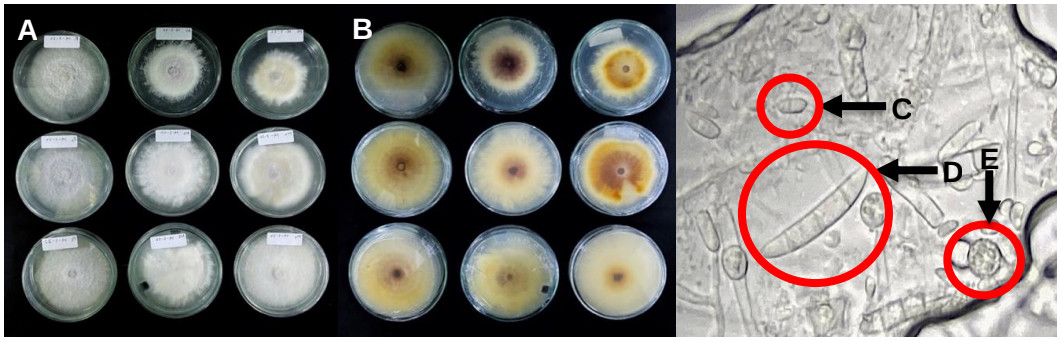


**Figure 1.** Morphology of Foce isolates, (A) Appearance of the upper surface of the colony, (B) Appearance of the lower surface of the colony, (C) Microconidia, (D) Macroconidia, and (E) Chlamidiospores.

**Figure 2.** *Volatile Odour Test* (VOT) culture of 9 collections of Foce isolates as a result of previous exploration

**Table 1.** The result of VOT and Biochemical test from 9 collections of Foce isolates as a result of previous exploration

| No. | Isolates Name | VOT Result | Biochemistry Result | Identify |
|---|---|---|---|---|
| 1 | Magetan 1 | *Odouratum* | Aldehyde containt | Race 4 |
| 2 | Magetan 2 | *Odouratum* | Aldehyde containt | Race 4 |
| 3 | Magetan 3 | *Inodouratum* | Non-Aldehyde | Non-Race 4 |
| 4 | Nganjuk 1 | *Odouratum* | Aldehyde containt | Race 4 |
| 5 | Nganjuk 2 | *Odouratum* | Aldehyde containt | Race 4 |
| 6 | Nganjuk 3 | *Odouratum* | Aldehyde containt | Race 4 |
| 7 | Probolinggo 1 | *Odouratum* | Aldehyde containt | Race 4 |
| 8 | Probolinggo 2 | *Inodouratum* | Non-Aldehyde | Non-Race 4 |
| 9 | Probolinggo 3 | *Inodouratum* | Non-Aldehyde | Non-Race 4 |

***The race 4 identification results using the volatile odor test (VOT)***

Foce is the result of an identified 9 isolates from the previous identification process. Those isolates were then identified again as Race 4 using the VOT method. Based on the plant culture of the rice medium, all 7-day-old cultures were found to grow with the same colony colors as the previous pure cultures, which were yellowish-white to purplish-white, which means the cultures grew well because they did not undergo a decaying process. The color of the cultures can be seen in Figure 2. In addition, from the VOT results of the 9 test samples, it was found that 6 cultures produced a fermented aroma-like *tape* while the other 3 did not emit any aroma. This means that 6 isolates were grouped as *odoratum*, while 3 of them were grouped as *inodoratum* (Table 1).

***Results of identification of aldehyde content with biochemical compounds***

The isolates belonging to the *odoratum* group were then tested again using the biochemical method to prove the presence of aldehydes. The test results show that 6 isolates turned a brownish color, meaning that the isolates contained aldehyde groups (Figure 3). From this, it can be concluded that 6 isolates belong to the Foce Race 4 group. This result is also supported by a report from Yudha (2022), who identified Foce isolates using the same method. He found that 6 out of 9 isolates identified as Race 4. This goes the same with a report from Nasir et al. (2003), who identified Foce isolates using the same method. They found out that 16 out of 18 isolates were identified as Race 4.

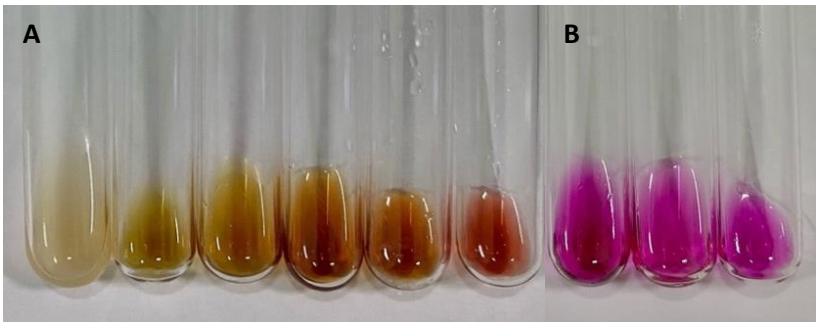


**Figure 3.** Biochemical test sample, (A) The test sample contains aldehydes, (B) The test sample did notcontains aldehydes

***Results identification of race 4 isolate strains using the vegetative compatibility group (VCG)***

The VCG test results on 6 isolates through VOT and Biochemistry test selection can be seen in Figure 4. Figure 4 shows that only 4 isolates formed nit-mutants, characterized by thin mycelium growth, while the other 2 isolates were considered to form heterokaryons or thick mycelium formation. It was concluded that the 4 isolates - Magetan 1, Nganjuk 1, Nganjuk 2, and Probolinggo 1 - could be considered genetically different from each other. They are quite distinct from the other two isolates - Magetan 2 and Nganjuk 3 - of the same genetic origin. This finding is similar to a report by Wiyatiningsih et al. (2016), who also obtained 4 out of 8 test isolates that formed nit-mutants which were considered to have different species from one another and were then characterized into Race 1, 2, 3, and 4.

***Virulence test results***

Based on the virulence test conducted on 4 Foce Race 4 isolates, it was found that all isolates could attack all test varieties: Bauji, Tajuk, and Biru Lancor. The Bauji was the most susceptible because symptoms of Moler disease were found on the 17th day after planting. In contrast, the Tajuk type was seen on the 23$^{rd}$ day after planting. Unlike the previous two, Biru Lancor has the strongest genetic resistance on the 24$^{th}$ day after the planting process occurred. While all the plants gradually withered and died. The condition of the plants and tubers on the 40$^{th}$ day after the planting process occurred can be overlooked in Figure 5. One of the differences in the incubation period is influenced by the resistance of each cultivar itself. Ita et al. (2020) stated that the results of these observations by the average incubation period of the cultivar, Bauji experienced the fastest incubation period of the 18 other isolates, around 5.3 days. In contrast, Tajuk had the longest incubation period in 3$^{rd}$ place, which was 17.0 days, followed by Biru lancer with an average incubation period of 13 days.

The observations results during the incubation period showed that the shorter the incubation period of moler disease, the younger the plants were subjected to fungal attack, and the faster the damage and death of the plants. The slower the incubation period for moler disease, the slower the plant damage, and the plants can still form tubers even though in a small size. The length of the incubation period of a plant disease varies with the specific combination of host-pathogen, the growth stage of the host, and environmental conditions (Mulyana et al., 2021).

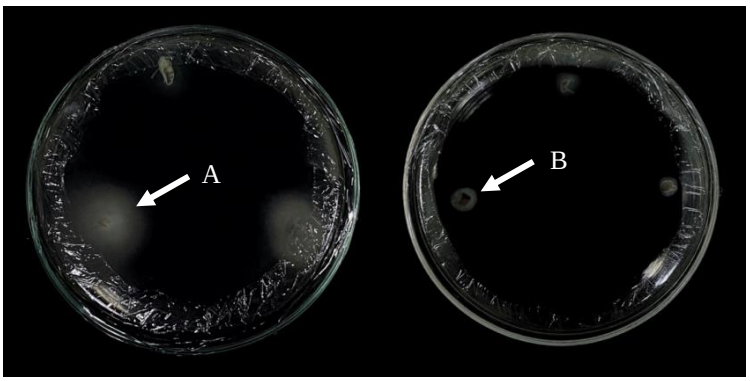


**Figure 4.** VCG test results on 6 isolates, (A) Isolates forming heterokaryons, (B) Isolates forming Nit-Mutants

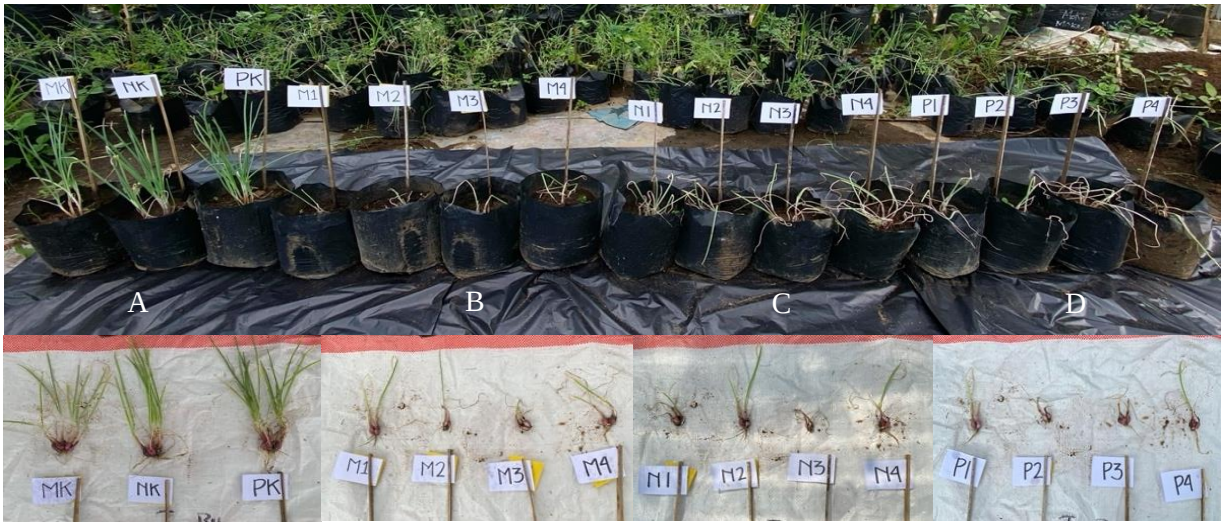


**Figure 5.** Virulence test results of isolates 1, 2, 3, and 4 on the 40th days after planting, (A) Control, (B) Inoculation of isolates to cultivar Bauji, (C) Inoculation of isolates to cultivar Tajuk, (D) Inoculation of isolates to cultivar Biru Lancor

## Conclusion

Race 4 was found in Magetan, Nganjuk, and Probolinggo, with 9 Foce isolates were obtained. The identification results using VOT and biochemistry showed six isolates as Race 4. The identification results using VCG demonstrated four isolates as Race 4 came from different strains. Meanwhile, the virulence test shows that these four isolates from the three tested cultivars could plague all types of tested cultivars. In addition, based on the incubation and the intensity, the Bauji cultivar was the most susceptible, and the Biru Lancor cultivar was the strongest.

## Declarations

**Conflict of interests** The authors declare no competing interests.



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