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Antagonist Test of Streptomyces spp. from Shallot Fields in Bojonegoro Against Fusarium sp. Which Causes Moler Disease In vitro

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Abstract

Background: Background: Shallots are a horticultural product widely consumed in Indonesia. In 2021, shallot production will decline. This is due to pest and disease attacks on plants. *Fusarium* sp. is a pathogen that causes the primary disease in shallots, namely moler disease, which can cause losses of up to 50%, so control activities must be carried out. This research aims to determine the potential of *Streptomyces* sp. bacteria from exploring shallot planting areas in Bojonegoro against *Fusarium* sp. fungi *in vitro*. **Methods:** This study used 15 isolates of *Streptomyces* sp. from exploration results tested with Fusarium sp. using the dual culture method, and negative control (only Fusarium sp.) repeated 3 times for each treatment. Observations were carried out every day for 7 days by observing the growth of *Fusarium* sp. hyphae and their inhibitory power. **Result:** The research showed that isolate S8 had the highest inhibitory power, 49.5%. Meanwhile, the one with the lowest results was isolated S1, 34.5%. From the chitinase test results, the chitinocytic index of isolate S8 was 5.2, which is in the high category. Meanwhile, the isolate S8 cellulolytic index was 4.8 and was classified as high. **Conclusions:** All *Streptomyces* sp.p isolates inhibited the growth of *Fusarium* sp., which was characterized by an inhibition zone compared to the control treatment.

Keywords: Bojonegoro; *Fusarium sp.*; Moler; Shallots; *Streptomyces* sp.

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Shallots (*Allium ascalonicum* L.) are horticultural crops consumed by many people daily and have high economic value. The increase in shallot production continued from 2017 to 2021, reaching 2.004.590 tonnes, but a decline occurred in the following year to 1,982,360 tonnes (BPS, 2023). One of the reasons for this decrease in production is the attack of moler disease due to *Fusarium* sp. Symptoms include yellowing, curling, and twisting of the leaves. Then, if the attack is severe, the plants grow stunted and wither because the water and nutrient transportation process is disrupted (Deden & Umiyati, 2017). Shallot plants with a high attack level are easily uprooted because root growth is disrupted, the bulbs rot, and the plants die (Budiarti et al., 2022).

Streptomyces sp. is a bacterium from the Actinomycetes class whose population is the most dominant in the soil, so its existence is easy to find (Kawuri, 2016). Exploration of *Streptomyces* in recent years has been widely carried out because of its ability to produce secondary metabolites, and its benefits in agriculture, health, and animal husbandry have been proven (Kurnijasanti, 2013). *Streptomyces* sp. is known to produce antibiotics and extracellular hydrolytic enzymes such as chitinase and β 1,3-glucanase, which can suppress pathogen growth (Miranda et al., 2022).

In the agricultural sector, *Streptomyces* sp. bacteria have been proven to be able to produce secondary metabolites as antimicrobials and produce Plant Grow Promoting Rhizobacteria (PGPR) in extreme environments so they can increase plant growth and protect plants from infection by disease-causing pathogens (Newitt et al., 2019). Research by Rahmiyati et al. (2021) states that *Streptomyces* sp. with a concentration of 15% can suppress *Fusarium* sp. by 52.2%, increase the height of shallot plants by 41.5 cm, the number of tillers by 8.89, and the final plant weight by 42 .84 grams.

Streptomyces sp. tested with *Fusarium oxysphorum* f.sp. *lycopersici* based on research by Sari et al. (2012), can produce an inhibitory power of 75%. Nellawati et al. (2016) stated that *Streptomyces roseoflavus* can inhibit the growth of *Xanthomonas* sp., which causes blight in rice plants *in vitro* by producing an inhibition zone of 17.8%. In addition, *Streptomyces* sp. suspension and Fobio biopesticide, when applied, can be correlated with increasing the resistance of shallot plants and preventing *Fusarium* sp. fungal infections as evidenced by the disease intensity from the start of the attack to the end of observation remaining 0.17% (Hasyidan et al., 2021). This research aims to determine the potential of *Streptomyces* sp. bacteria from exploring shallot fields in Bojonegoro against the *Fusarium* sp. fungus, which causes moler disease *in vitro*. The *Streptomyces* sp. isolate was isolated from exploration and never tested.

Methods

Exploration and Isolation of *Streptomyces* sp.

Streptomyces exploration was carried out in the shallot fields of Krondonan Village, Gondang District, Bojonegoro Regency, East Java, Indonesia. Samples were taken by uprooting the shallot plants, and then the soil attached to the plant roots was collected and transferred to a plastic bag. The soil samples were air-dried for 1 – 2 days before being isolated. Isolation of *Streptomyces* sp using the 10-5 serial dilution method. Samples from dilutions 10^{-4} and 10^{-5} were taken at 200 microns using a micropipette. They inoculated on Glucose Nitrate Agar (GNA) media that consists of glucose (1 gram), $NaNO_3$ (0.85 grams), KH_2PO4 (1.75 grams), KCl (0.75 grams), $MgSO_4.7H_2O$ (2.5 grams), agar (20 grams), and sterile distilled water (1000 ml) to isolate *Streptomyces* by the pouring technique and then spread it thoroughly with an L rod on the media. The plates were incubated for 14 days. *Streptomyces* colonies were selected and purified on slanted GNA media using the streak plate technique.

Isolation and Pathogenicity Test of *Fusarium* sp.

Fusarium isolation was taken from shallot bulb samples, which showed symptoms of moler disease. The bulbs are cut into small pieces using a scalpel and sterilized by dipping them in 70% alcohol sterile distilled water and dried on a tissue. Bulb cuttings were transferred in Potato Dextrose Agar (PDA) media consisting of PDA MERCK and 1000 ml of sterile distilled water and incubated for 2 x 24 hours. *Fusarium* fungi that grow are selected and purified on other PDA media. The pathogenicity test used 10 shallot plants with one replication. *Fusarium* fungus is inoculated on plants, and visible symptoms are observed.

Antagonist Test of *Streptomyces* sp. and *Fusarium* sp.

Fifteen isolates of *Streptomyces* spp from the isolation results were used for this antagonist test. The negative control was only inoculated with *Fusarium* sp without *Streptomyces*. Each treatment was repeated 3 times. Antagonist test using the dual culture method. The *Fusarium* sp isolate was cut using a cork borer with a diameter of 0.5 mm and placed in the middle of the PDA media (Hanif, 2018). Then, there is a *Streptomyces* streak on the right and left of *Fusarium*. The dish was incubated and

observed every day for 7 days. The observation parameter is Streptomyces sp's inhibitory power in disrupting Fusarium's growth. The inhibitory power calculation formula from Mayadanti et al. (2020):

$$\text{Inhibitory Power} = \frac{\text{Control} - \text{Treatment}}{\text{Control}} \text{ x } 100$$

Chitinase Test and Cellulose Test

The *Streptomyces* sp isolate was cut using a *cork borer* with a diameter of 0.5 mm and transferred to the middle of Colloidal Chitin Agar (CCA) media consisting of 3 gram KH_2PO4, 6 gram NH_2HPO_4, 0,5 gram NaCl, agar 15 gram, 0,05-gram yeast extract, 1000 ml distilled water, and colloidal chitin 0,5% (Nurul et al., 2015). The dish was incubated for 14 days, then dripped with an iodine solution to see the inhibition zone formed. The cellulose test is the same as the chitinase test. Still, *Streptomyces* was transferred to Carboxymethyl Cellulose (CCM) media consisting of 1 gram CMC, 0,02 gram $MgSO_4.7H_2O$, 0,0075 gram KNO_3, 0,0002 gram KH_2PO_4, 0,0004 gram $CaCl_2.2H_2O$, 1,5 gram agar and 100ml distilled water (Suyanto et al., 2022). The dish was incubated for 3 days and then dripped with an iodine solution.

Data analysis

Data analysis uses Analysis of Variance (ANOVA). If there is a significantly different effect from the treatment, then a further test will be carried out using DMRT at a level of 5%.

Results and Discussion

Isolation of *Streptomyces* sp.

Isolation uses GNA (Glucose nitrate agar) media because it is a selective medium for Streptomyces spp., which can minimize the growth of other organisms—a total of 15 *Streptomyces* spp. Isolates were found in exploration samples in Bojonegoro. The morphology of these isolates is shown in Fig. 1. The *Streptomyces* spp. The obtained isolates differed based on their color because all isolates had round colony shapes, uneven surfaces, and powdery. Color differences between isolates include isolates with red, light cream, dark grey, light gray, grayish white, pink, brownish cream, dark red, reddish gray, brownish white, white, and gray. Based on research by Rahmiyati et al. (2021), the color of *Streptomyces* colonies consists of white, gray, brownish gray, greenish gray, and pink. Different pigment contents between isolates caused bacterial colonies to have different colors (Ferdiyanti et al., 2021).

Based on Table 1, it is known that from the isolation results, the *Streptomyces* sp. isolates obtained had different characteristics between the isolates. Macroscopically, all *Streptomyces* sp. isolates found had the same colony shape: round, uneven, and powdery. However, the colonies' color differed between several isolates, as in Table 1. Based on research by Rahmiyati et al. (2021), the color of *Streptomyces* colonies consists of white, gray, brownish gray, greenish gray, and pink. The difference in colony color is due to the different pigment content in the spore chain, making bacterial colonies appear to have different colors (Fardiyanti et al., 2021).

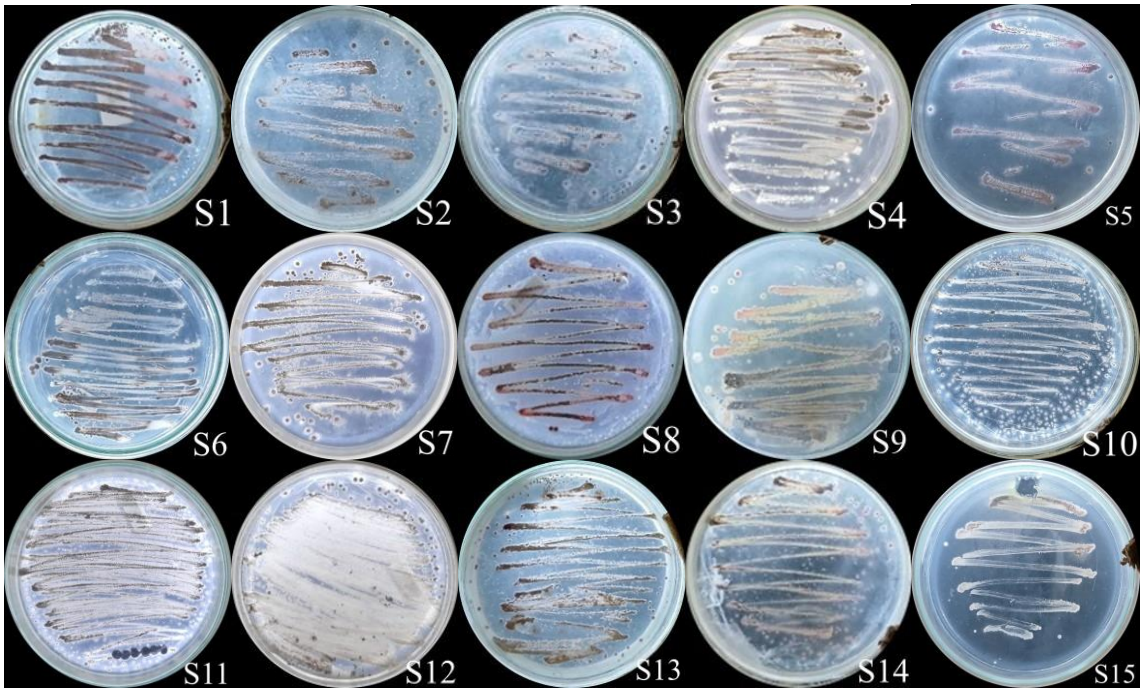

Figure 1. *Streptomyces Isolates from Isolation Results with Color Differences Between Isolates*

Table 1. Characteristics of *Streptomyces* spp. Isolates from Isolation Result

Isolat *Streptomyces* spp.	Characteristics
S1	Round colonies, red color, powdery surface, and earthy odor
S2	Round colonies, light cream color, powdery surface, and earthy odor
S3	Round colonies, dark gray color, powdery surface, and earthy odor
S4	Round colonies, light gray color, powdery surface, and earthy odor
S5	Round colonies, pink color, powdery surface, and earthy odor
S6	Round colonies, grayish-white color, powdery surface, and earthy odor
S7	Round colonies, brownish cream color, powdery surface, and earthy odor
S8	Round colonies, dark red color, powdery surface, and earthy odor
S9	Round colonies, reddish-gray color, powdery surface, and earthy odor
S10	Round colonies, white color, powdery surface, and earthy odor
S11	Colonies are round, light gray, powdery surface, and earthy.
S12	Colonies are round, brownish cream color, powdery surface, and earthy odor.
S13	Colonies are round, dark gray color, powdery surface, and earthy odor.
S14	Colonies are round, reddish-gray color, powdery surface, and earthy odor.
S15	Colonies are round, brownish white color, powdery surface, and earthy odor.

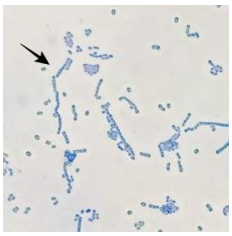

Figure 2. Microscopic Observation of *Streptomyces* sp.

Based on microscopic observations, all the *Streptomyces* spp. The obtained isolates have the same microscopic characteristics: round spores and spore chains (Fig 2). According to Rahmiyati et al. (2021), *Streptomyces* sp. spores are round, and the conidia are chains that cluster at the ends of the aerial hyphae. *Streptomyces* sp. is a Gram-positive bacterium of the Actinomycetes class, which has a small colony size of around 1 – 10 nm, grows optimally at a temperature of 25 – 35°C, pH 6.5 – 8.0, and has an earthy aroma. This is due to the content of acetic acid, acetaldehyde, ethanol, isobutanol, and

isobutyl acetate produced by *Streptomyces* sp. (Raharini et al., 2014). Microscopically, the bacteria *Streptomyces* sp. looks like a fungus because it has hyphae and conidia that form chains and cluster at the tips of the aerial hyphae (Raharini et al., 2014).

Isolation of *Fusarium* sp.

Fusarium fungi have two types of asexual sp. ores: macroconidia and microconidia. The characteristics of macroconidia are that they are sharp and curved at the ends like a crescent moon, measuring 10 - 45 x 3.2 - 8 µm, and 3 - 5 insulated. Meanwhile, microconidia are round to straight, measuring 1 - 5 x 2.3 – 3.5 µm, non-separated, single cells, and thin cell walls (Hikmawati et al., 2020).

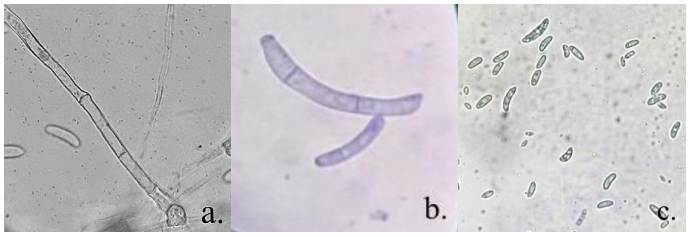

Figure 3. Microscopic Observation of *Fusarium* sp.; a.) Hyphae of *Fusarium* sp.; b.) Macroconidia of *Fusarium* sp.; c.) Microconidia of *Fusarium* sp.

Fig. 3. shows microscopic observations of the *Fusarium* sp. fungus, namely that it has macroconidia that are insulated, curved, and sharp at the ends like crescent moons, as well as microconidia that are round to straight and not insulated. *Fusarium* sp. hyphae are slender and insulated.

Pathogenicity Test of *Fusarium* sp.

The pathogenicity test aims to prove whether an isolate is pathogenic and can cause disease symptoms. From the results of the pathogenicity test, moler symptoms began to appear on the 14th day and died on the 21st day with a 100% pathogenicity rate. All shallot plants from the pathogenicity test results died. Symptoms of the disease include wilting, paleness, yellowing, twisting, and eventually death. This shows that the *Streptomyces* sp obtained is the pathogen that causes the disease.

Figure 4. Pathogenicity Test Results A.) Healthy Plants; B.) Wilted and Pale Plants; C.) Crooked Plants; D.) Dead Plants

Antagonist Test of *Streptomyces* sp. and *Fusarium* sp.

The antagonist test aims to determine the effect of *Streptomyces* sp. on the *Fusarium* sp. fungus *in vitro*. The antagonist test parameter is the inhibitory power known by looking at the clear zone that forms. The clear zone indicates the activity of the chitinase enzyme, which means that *Streptomyces* can inhibit the growth of other microorganisms by producing extracellular metabolites in the form of antibiotics and other compounds to inhibit other microorganisms from obtaining nutrients. The percentage category of inhibitory power adheres to the statement (Prastya et al., 2014), namely the strong

category for > 40%, weak for < 30%, and no ability for 0%. The following table shows *Streptomyces* sp. and *Fusarium* sp. antagonist test results.

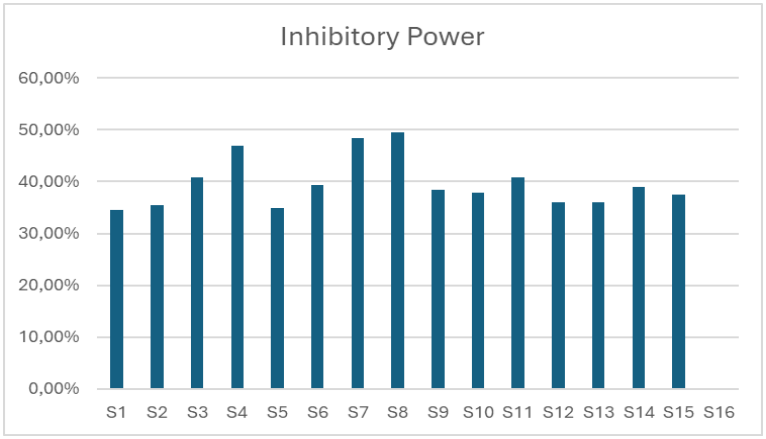

Figure 5. Inhibitory Power Calculation of *Streptomyces* spp. with *Fusarium* sp.

The results of the inhibitory power calculation are shown in Figure 5. Isolate S8 is the isolate with the highest inhibitory power, namely 49.5%, while the isolate with the lowest inhibitory power is isolate S1 at 34.5%. The difference in the percentage of inhibition results is thought to be due to the influence of the type, quantity, and quality of antibiotics produced by *Streptomyces* sp. Differences in the inhibitory power formed by *Streptomyces* sp can be caused by differences in the antibiotic compounds produced, the origin of the isolate, and environmental conditions (Raharini et al., 2014).

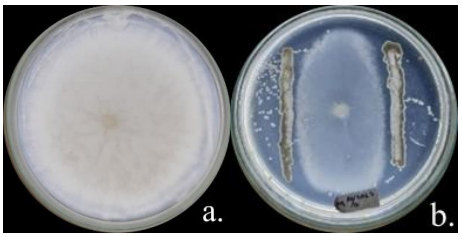

Figure 6. *In Vitro* Test Results a) Control (Only *Fusarium* sp.); b) Antagonist Test Isolates S8 with *Fusarium* sp.

The growth of *Fusarium* sp, which Streptomyces inhibits, is shown in Fig. 6b, as can be seen from clear zones between isolates. On the 7th day after isolation, the growth of *Fusarium* sp should have filled the entire petri dish (Fig. 6a); however, if tested with *Streptomyces* sp, the growth of *Fusarium* sp was hampered and could not pass through *Streptomyces* sp so that the hyphae grew upwards (Fig. 6b). It is thought that the growth of *Fusarium* sp is inhibited because there is an antagonistic mechanism in the form of antibiosis. Antibiosis is an antagonistic mechanism that results in lysis-causing metabolites, enzymes, volatile and non-volatile compounds, or toxins produced by microorganisms (Berlian et al., 2013). The antagonistic mechanism used by Actinomycetes is to produce antibiotic and antifungal compounds in the form of chitinase enzymes, which can hydrolyze chitin and glucans in fungal cell walls. *Streptomyces* produces antibiotics, biosurfactants, volatile compounds, and toxins, which can be biocontrol agents against plant pathogens (Rahmiyati et al., 2021). The existence of an antagonistic mechanism causes *Fusarium* sp not to be able to grow past *Streptomyces* sp.

Fusarium sp hyphae abnormalities are another impact of the antagonistic activity carried out by *Streptomyces* sp. Abnormal *Fusarium* sp hyphae that grow to swell, lysis, and form chlamydospores due to *Streptomyces* sp are shown in Fig. 7. *Fusarium* sp. hyphae that are affected by antagonistic activity grow abnormally, such as lysis, curling, swelling, twisting, coiling, and forming chlamydospores as a form of defensive structure

Agustin et al., (2023). Hyphal abnormalities are a form of pathogenic effort to defend themselves.

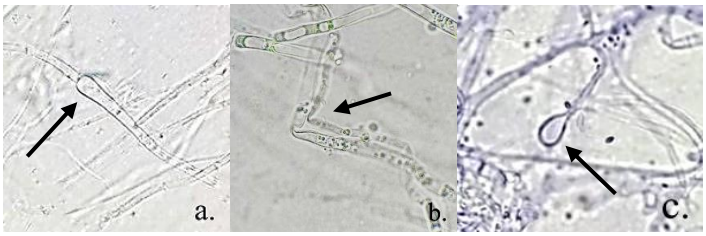

Figure 7. Abnormal Hypha Form of *Fusarium* sp. After Antagonist Test a) Hyphae Swell; b) Hyphae Lysis; c) Chlamydiospores

Ctitinase Test of *Streptomyces* sp.

The chitinase test was carried out to determine the chitinase enzyme content in *Streptomyces* sp. isolates. The activity of the chitinase enzyme is generally optimal at temperatures of 30 – 40°C and pH 5 – 7. In the agricultural sector, chitinase produces chitooligosaccharides, which play a role in plant defense. The research results showed that the *Streptomyces* sp. isolate contained the chitinase enzyme, which was characterized by the formation of a clear zone around the colony (Fig. 8). This shows that the chitinase enzyme degrades the chitin contained in the media.

The breaking of the β-1,4-homopolymer N-acetylglucosamine bond in chitin by chitinase to become N-acetylglucosamine monomer forms a clear zone in the media. The isolate tested with the chitinase test was only isolated S8 because this isolate had the highest inhibitory power compared to other isolates. Isolate S8 has a chitinolytic index of 5.2 and is classified as high. The chitinolytic index is classified as high if >2 and low if <2 (Nurul et al., 2015). This shows that isolate S8 can produce the chitinase enzyme, which can degrade chitin in CCA media. The difference in the diameter of the clear zone formed indicates the difference in the activity of each secreted chitinolytic enzyme (Nurul et al., 2015). Dropped iodine reacts and changes the color of the media containing chitin to brown.

Figure 8. Citinase Test Results From Isolate S8

Cellulose Test

The cellulose test aims to determine the hydrolytic activity of the cellulase enzyme in the bacteria *Streptomyces* sp. The isolate tested for cellulose was only isolated S8 because this isolate produced the highest inhibitory power compared to other isolates. Isolate S8 contains the cellulase enzyme characterized by a clear zone formed around the colony, as shown in Fig 9. The cellulolytic index of isolate S8 was 4.8 and is classified as a high category. According to Dewi et al. (2020), the cellulocytic index value is classified as low if <2, medium between 1 – 2, and high if >2. Differences influence the differences in the clear zones formed in the ability of each isolate to degrade cellulose (Siruwahni & Rasyidah, 2023). This shows that isolate S8 can produce the cellulase enzyme, which can degrade cellulose in CMC media. The cellulase enzyme degrades

cellulose by breaking the β-1,4 glycoside bonds capable of producing oligosaccharides derived from cellulose and glucose (Siruwahni & Rasyidah, 2023).

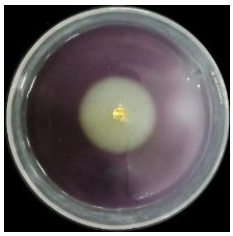

Figure 9. Cellulose Test Results

Conclusions

The results of the research showed that 15 isolates of *Streptomyces* sp. Isolate S8 was the best isolate from the *in vitro* test results, with the highest inhibitory power at 49.5%. Meanwhile, the one with the lowest results was isolated S1, 34.5%.

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Declaration statement

The authors reported no potential conflict of interest.

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