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Research Article

Antagonistic Activities of Endophytic Fungi Isolated from *Eleutherine palmifolia* Flower

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Abstract

Background and Objective: Endophytic fungi live in plant tissue and show no symptoms of disease in their host plants. It is known that endophytes as biological agents, can control plant diseases. In this study, isolated endophytic fungi from healthy Dayak Onion flowers were used as biocontrol agents in the control of the pathogenic fungi *Fusarium* spp. that causes molar disease in shallot plants. **Materials and Methods:** This study identifies the type of endophytic fungi molecularly isolated from Dayak onion flowers and determine the antagonistic effect of the endophytic extract against *Fusarium* spp. screening for endophytic fungi as antagonizing agents is carried out using the poisoned food method. **Results:** The results showed that two endophytic fungi isolates were obtained from healthy Dayak onion flowers, namely, EnI which was identified with the primers ITS1 and ITS4 as *Fusarium solani* and EnK as *Neoscytalidium* sp. *Fusarium* wilt caused by pathogenic fungi was identified as *Fusarium oxysporum*. The inhibitory percentage of EnI extract against the pathogenic fungus *Fusarium oxysporum* was 71.09% (high inhibition percentage) and the inhibition percentage of EnK was 38.54% (low inhibition percentage). **Conclusion:** Based on the results of this study, recommend using the endophytic fungus EnI extracts (*Fusarium solani*), extracted from Dayak onion flowers to control the pathogen *Fusarium oxysporum*.

Key words: Antagonism, biological agents, percent inhibition, plant diseases, pathogen

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Endophytic fungi are fungi that live in plant tissue and show no symptoms of disease in their host plants. These microorganisms cooperate with the host plant and thus, the plants somewhat regulate the metabolic cycle of these endophytes to create particles that could show defensive capacities towards the organism and the host.

Endophytic organisms of the variety *Fusarium*, *Colletotrichum aspergillus* and *Alternaria*, confined from plants that are therapeutically significant show different natural exercises like antimicrobial, antitubercular, immunomodulatory, anticancer, antifungal and cell reinforcement exercises with wide application in agrochemical and drug ventures¹⁻⁴. Accordingly, investigating endophytic parasites that dwell in medicinal plant species would give a huge opportunity to find new metabolites that are medicinally significant.

Endophytic fungi are fungi that live in the asymptomatic plant cell and do not damage the host plant⁵. These endophytic fungi have the same potential to produce phytochemical compounds as their hosts⁶ and endophytic fungi can induce a reactive metabolism against plant pathogens known as induced systemic resistance⁷. Endophytic fungi are easy to grow and do not pollute the environment when used. Endophytic fungi can also be extracted to isolate their secondary metabolite, which can also be used as medicinal substances. The secondary metabolite of endophytic fungi has been shown to reduce the infection rate of *Fusarium* spp. up to 47.28%⁸.

Endophytes occupy a unique biotope and have a global estimate of up to one million species, these microorganisms help natural products to assist in solving not only plant diseases but also human and animal health problems. Endophytes are chemical synthesizers inside plants, in other words, the pharmaceuticals active substances are produced by Endophytes as they play selective role system to microbes and from 1981-2006 all list of natural drugs have been presented from which a large number of natural drugs are produced.

Research on the existence of phytochemical compounds in Dayak onion plants (*Eleutherine palmifolia*) has been carried out on a large scale and the phytochemical compounds found in these plants include alkaloids, saponins, tannins, phenolics, flavonoids, triterpenoids and steroids. The number of phytochemical compounds found in this plant makes it one of the most important ingredients in the world of medicine that people are looking for, especially in the Kalimantan region. Besides being beneficial as a medicine for humans, Dayak onion plants have the advantage of preventing the

spread of the onion disease *Fusarium* spp., with their root exudate⁹. This makes people in the wetlands cultivate Dayak onion plants.

Dayak onions (*Eleutherine palmifolia*) contain more phytochemical compounds like phenols and flavonoids compared to tubers¹⁰. However, the small part of the flower is ineffective in producing compared to tubers or leaves. Recently, the isolation and production of bioactive compounds from plants can be replaced by isolating the endophytic fungi present in the plant tissue and using secondary metabolites produced by these endophytes¹¹.

In general, the genetic resources of Dayak onion plants found in Kalimantan are a veritable source of pharmaceuticals and therapeutics with significant information on their phytochemistry. Hence this study was carried out to determine the antagonistic activities of Endophytic Fungi extract from *Eleutherine palmifolia* flower to *Fusarium* spp. causing *Fusarium* wilt on Shallot.

MATERIALS AND METHODS

Study area: The research was carried out from December, 2019 to December, 2020 at the Production Laboratory of the Department of Agroecotechnology, Faculty of Agriculture, Lambung Mangkurat University, Basic Laboratory of Lambung Mangkurat University, Laboratory of Animal Husbandry, Faculty of Agriculture, Lambung Mangkurat University, Laboratory of the Center for Biological Sciences (LSIH) Brawijaya University Malang and PT. Genetics Science Indonesia Banten.

Sample collection: *Fusarium oxysporum* and *F. solani* used in this study were isolated from shallot plants presenting characteristics of disease expressed as *Fusarium* wilt. Pathogen isolate was obtained from the Lambung Mangkurat University, Faculty of Agriculture, Department of Agroecotechnology in Banjarbaru, South Kalimantan Indonesia. In a sealed plastic bag was the plant sample kept and returned to the laboratory on the same day for the isolation of the endophytic fungi.

Isolation of *Fusarium* spp: *Fusarium* spp. was isolated from shallot plants showing *Fusarium* wilt symptoms in Banjarbaru City and then the diseased pieces were placed in PDA (Potatoes Dextrose Agar) culture media, incubated for 2 days and purified.

Endophytic fungi isolation and purification: After collection, the Dayak onion flowers were washed under running water, the purpose of washing is to remove dirt and dust, they were

drained and cut into sizes of 1 × 1 cm. In the plant segments, a serial process of sterilization with sodium hypochlorite and alcohol was used to kill all organisms on the plant tissue surfaces. They were sterilized with 70% alcohol for 30 sec, soaked in 1% NaOCl for 2 min, then rinsed with sterile distilled water 3 times, then dried in sterile tissue. The next step is to insert the pieces into PDA media and incubate and purify them.

Identification of endophytic fungi using PCR: This stage starts with the isolation and amplification of fungal DNA based on the Castillo method, that is, by using polyvinyl pyrrolidone (PVP) and sodium acetate extraction buffer in CTAB. Using ITS1 and ITS4 primer pairs for fungal DNA amplification. The amplified DNA was analyzed by 1% agarose gel electrophoresis in Tris Boric EDTA buffer, visualized with ultraviolet light and DNA tracking analysis using the dideoxynucleotide chain termination method. Also, the Bioedit program was used for DNA preparation and the information provided by Genbank was used for analysis using the BLAST program.

Extracting endophytic fungi: Identified endophytic fungi are then cultured and grown in PDB media for 21 days at 25°C while being shaken at a speed of 120 rpm. The mycelium and the culture filtrate are then filtered with a vacuum filter. The culture filtrate was macerated with two solvents, ethyl acetate 1:1 and n-hexane 1:10, while the mycelium was macerated with ethyl acetate, n-hexane and ethanol solvents at a ratio of 1:5. The maceration and extraction processes were repeated 3 times. The solvent was then evaporated using a rotary evaporator and the resulting extract was then diluted using 10% Dimethyl sulfoxide (DMSO).

Antagonist test of endophytic fungal extract to *Fusarium* spp: The antagonist test was carried out using the poisoned food method¹. The antagonistic fungi extract was poured into the molten agar at a certain concentration and mixed well and then the mycelial pathogen was inoculated into the centre of the plate and incubated at room temperature for 7 days. Fungal endophytes were categorised into three including the control using only the *Fusarium* spp. based on this assessment. The inhibition of mycelial growth was measured by comparing the mycelial growth of *Fusarium* spp. on the treatment plate containing the antagonistic fungi extract (Ds) to the mycelia growth of *Fusarium* spp. which has been placed on the control plate (Dc):

$$\text{Antifungal activity (\%)} = \frac{D_c - D_s}{D_c} \times 100$$

Where, Dc is the mycelial growth of *Fusarium* spp. on the treatment plate and Ds is the mycelial growth of *Fusarium* spp. present in the control plate.

Volatile compounds test: Using the method of the previous study¹² volatile compound products evaluated, dishes of 9 cm that contains PDA was used to incubate isolates of *Fusarium* spp. for 5 days. After 3 days, another set of dishes (5 mm) was used containing *Fusarium* spp. isolates incubated for 48 hrs. The phytopathogenic fungus plates were inverted on top of each isolated fungus using parafilm and adhesive tape to seal the top so that the volatiles will not diffuse. The control was also inverted in a plate containing only PDA. Incubated the dishes at 25°C under a photoperiod of 12 hrs, the treatment was performed in triplicate. The diameter of the colonies pathogen was measured on the 3rd, 5th and 7th day as well as calculating the inhibition percentage.

Non-volatile compounds test: Flasks of 100 mL of Potato Dextrose Broth (PDB) were inoculated using a mycelia disc of 5 mm from margins of actively growing colonies of fungal isolates and incubated for 7 days at 25°C 12 hrs under photoperiod. Then a millipore was used of 0.45 µm pore diameter to filter the solution utilizing the vacuum filter system. Then autoclaved the fungal filtrate at 121°C for 10 min. Three replicate were used for each treatment, *Fusarium* colonies diameter were measured on the 3rd, 5th and 7th day after incubation.

RESULTS

DNA barcode of endophytic fungi and pathogenic

***Fusarium* spp:** The results of the DNA samples of the three fungi with 1.5% agarose gel electrophoresis showed that only two fungal DNAs were amplified, namely EnI endophytic fungal DNA and Pathogenic *Fusarium* spp. which is shown in Fig. 1. It can be seen that the DNA bands of the endophytic fungi EnI and the DNA of the pathogenic fungi *Fusarium* spp. Are both 500 bp in size. Partial sequence of EnI and Pathogenic *Fusarium* spp. using ITS1 and ITS4 primers details given in Table 1.

Antifungal activity of endophytic extract EnI and EnK to

***Fusarium* spp:** As shown in Fig. 2 the growth of mycelia *Fusarium* spp. on the EnI plate was one-third smaller than the *Fusarium* spp. in the control medium, showing 0.92 cm in the EnI plate while the control extract is 2.70 cm. Mycelia *Fusarium* spp. on the EnK treatment medium which was also half times

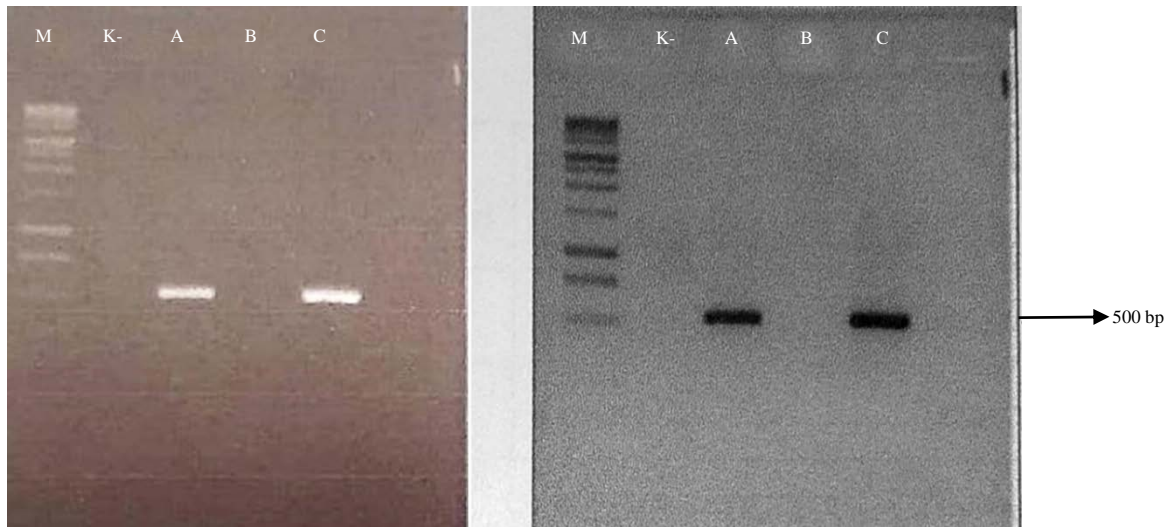


Fig. 1: Results of agarose gel electrophoresis were 1.5% of DNA samples fungi amplified using ITS1 and ITS4 primers
M: Marker 1 kb, K-: Negative control, A-C: Sample

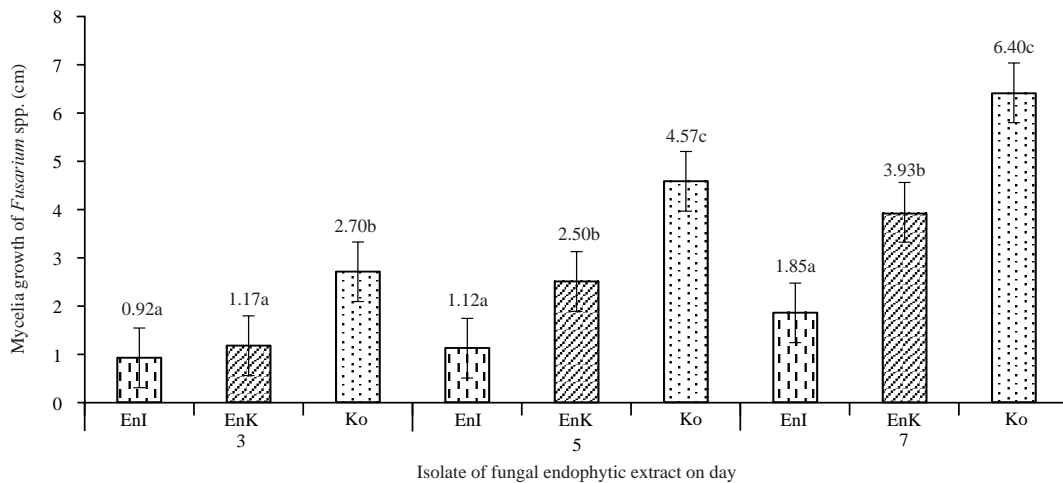


Fig. 2: Mycelia growth of *Fusarium* spp. observed on the 3rd, 5th and 7th day after inoculation of fungi during the volatile compound test

Table 1: Partial sequence of ENI and pathogenic *Fusarium* spp. using ITS1 and ITS4 primers

Isolate	Partial sequence	BLAST analysis
EnI	ACTCCCAAACCCCTGTGAACATACCACTTGTGCCTCGGCGGATCAGCCCCTCCCGGTAAAACGGGACGGCCCGCCAGA GGACCCCTAAACTCTGTTTCTATATGTAACCTCTGAGTAAAACCATAAATAAATCAAAAACCTTCAACAACGGATCTCTTG GTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGA ACGCACATTGCGCCCGCAGTATTCTGGCGGGCATGCCTGTTTCGAGCGTCATTCAACCCTCAAGCACAGCTTGGTGTG GGACTCGCGTTAATTCGCGTTCCTCAAATGATTGGCGGTACGTCGAGCTCCATAGCGTAGTAGTAAACCCTCGTTA CTGGTAATCGTCGCGCCACGCCGTTAAACCCCACTTCTGAATGTTGACCTCGGATCAGGTAGGAATACCCGCTGAAC TAAGCATATCAATAGCCGGAGGAA	<i>Fusarium solani</i>
Pathogenic <i>Fusarium</i> spp.	ACTCCCAAACCCCTGTGAACATACCACTTGTGCCTCGGCGGATCAGCCCCTCCCGGTAAAACGGGACGGCCCGCCAGA GGACCCCTAAACTCTGTTTCTATATGTAACCTCTGAGTAAAACCATAAATAAATCAAAAACCTTCAACAACGGATCTCTTG GTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGA ACGCACATTGCGCCCGCAGTATTCTGGCGGGCATGCCTGTTTCGAGCGTCATTCAACCCTCAAGCACAGCTTGGTGTG GGACTCGCGTTAATTCGCGTTCCTCAAATGATTGGCGGTACGTCGAGCTCCATAGCGTAGTAGTAAACCCTCGTTA CTGGTAATCGTCGCGCCACGCCGTTAAACCCCACTTCTGAATGTTGACCTCGGATCAGGTAGGAATACCCGCTGAAC TAAGCATATCAATAGCCGGAGGAA	<i>Fusarium oxysporum</i>

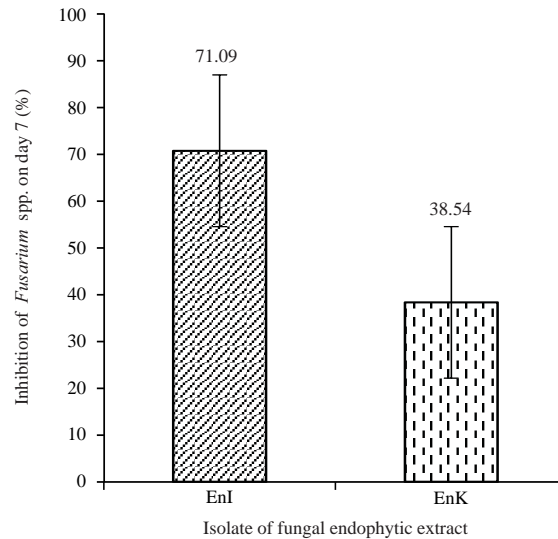


Fig. 3: Percent inhibition of *Fusarium* spp. observed 7 days after inoculation of both fungi during the non-volatile compound test



Fig. 4: Food poisoned technique of endophytic extract of *Eleutherine palmifolia* flower on day 3rd inoculated with pathogenic *Fusarium* spp.

EnI: Endophyte extract En (PDA+1 mL), EnK: Endophyte extract EnK (PDA+1 mL), Ko: Sterile dimethyl (PDA+1 mL)

lower in growth than the mycelia of *Fusarium* spp. on control media on the fifth day, which shows 1.12 cm EnI plate and control, respectively.

Mycelial growth of *Fusarium* spp. on day 7 reached 6.40 cm in the control medium but only 1.85 cm in the medium with EnI extract. This indicates that the presence of EnI extract on PDA inhibits the growth of Mycelia *Fusarium* spp. by about 71% in Fig. 3.

Fusarium spp. have low mycelia growth in both EnI and EnK treatment plates, compared with the control plates, there was a significant difference in the variable values at the 5% level on the 3rd and 5th days (Fig. 2). The growth of *Fusarium* spp. on the EnI extract plate and the EnK extract plate were not significantly different on the 3rd day in Fig. 4 but differed significantly from the growth of *Fusarium* spp.

in the control plate. This indicates that at the beginning of the observation, the endogenous secondary metabolites EnI and EnK can inhibit growth since they both have the same strength level. On the 5th and 6th day of the observation, EnI extract has the greatest inhibitory effect on the pathogen *Fusarium* spp.

DISCUSSION

In this study, endophytic fungi were isolated from the leaves of the sterilized surface of Dayak onions. The DNA bands of the endophytic fungi EnI and the DNA of the pathogenic fungi *Fusarium* spp. are both 500 bp in size. In contrast with the current study the DNA band size ranges from 400-700 bp⁸.

The result from this study indicated that all fungal isolates inhibit the mycelia growth of *Fusarium* spp.

Also, the result indicated that Mycelial growth of *Fusarium* spp. on day 7th reached 6.40 cm in the control medium but only 1.85 cm in the medium with Enl extract. This indicates that the presence of Enl extract on PDA inhibits the growth of Mycelia *Fusarium* spp. by 71%.

In the current study, there is no genetic difference between the endophytic fungi Enl and the pathogenic fungi *Fusarium* spp. while the DNA of the endophytic fungi EnK may not be able to amplify due to lack of purity of the EnK, Enoph-DNA from endophytic fungi, so it affects the primary binding to the DNA of these prints¹³ and this is evidenced by the purity level of EnK DNA, which is higher than other fungal DNA.

The inhibition percentage of EnK extract to *Fusarium* spp. was 38.54%, which was half less than Enl in inhibiting the growth of *Fusarium* spp. mycelia. The difference in inhibitory strength between endophytes is based on the difference in the antagonistic ability of each species. Another study¹³ stated that the difference in the antagonist's strength is based on the difference in its secondary metabolites also¹⁴ found that the metabolites produced by the antagonist cause different fungi responses.

Similarly, current results are supported by a previous study that reported that endophytic *Trichoderma* spp. isolated from the roots of *Coffea arabica* antagonized *Fusarium* spp. and *S. sclerotiorum*¹⁵.

Contrarily, the endogenous Enl was identified as *Fusarium* spp. and *Fusarium* spp. has *mycotoxins* that can inhibit the growth of pathogenic mycelia. *Fusarium* has *mycotoxins*, *fumonisin*, *bleomycin* and *zearalenone*¹⁶. The high concentration of dissolved mycotoxins in Enl extract may cause the growth of *Fusarium* spp. Pathogenicity was inhibited by up to 71% on day 7 but further studies are needed to identify secondary metabolites in Enl extracts.

EnK's was identified as *Neoscytalidium* sp. *Neoscytalidium* sp. is biologically active and can inhibit the growth of *Fusarium in vitro* as stated previously¹⁷. *Neoscytalidium* sp. and *Fusarium solani* has strong mycelial growth characteristics on cassava plants⁷. This characteristic has the effect of inhibiting the pathogenic *Fusarium* spp. by the bioactive compounds produced by its endophytes.

Both fungal isolates tested produced volatiles that caused some inhibition on the growth of *Fusarium* spp. Although differences between isolates were significant, the most effective was Enl. isolate with an inhibition growth of 71.09% and while Enk 38.59%.

All isolates of *Fusarium* spp. produced VOC that inhibited significantly the mycelial growth of *F. solani* and

F. oxysporum from the third day of incubation onwards (Fig. 2). The growth of mycelia *Fusarium* spp. on the Enl plate was one-third smaller than its *Fusarium* spp. the control medium.

Based on the antagonist test results of the extract of the endophytic, it is known that the inhibitory power of the extract of the endophytic fungus *Neoscytalidium* sp. against the pathogen *Fusarium* spp. has a low inhibition, while the inhibitory power of the endophytic fungi extracts of *Fusarium* spp. against the pathogen *Fusarium* spp. has a high inhibition. The criterion for high inhibition is when the percentage of achievement is between 70 and 100%, moderate inhibition when the percentage of inhibition is between 40 and 69% and low inhibition when the percentage of inhibition is between 0 and 39%¹⁸.

The factors influencing the difference in inhibition are the differences in the mechanism of inhibition of each type of endophytic fungus and the origin of these endophytic fungus isolates. In general, the mechanism of inhibiting endophytic fungi against pathogens comprises three mechanisms, namely the production of antibiotics, the competition for food and space and the induction of plant resistance^{19,20}.

CONCLUSION

Two endophytic fungi (Enl and EnK) were obtained from Dayak onion flowers. *Fusarium* spp. isolated from shallot plants showing *Fusarium* wilting symptoms. The results of the DNA samples of the three fungi with 1.5% agarose gel electrophoresis showed that only Enl and *Fusarium* spp. were amplified and the result of the BLAST analysis showed that Enl was *Fusarium solani* while *Fusarium* spp. was *Fusarium oxysporum*. The inhibitory percentage of the extracts Enl (*Fusarium solani*) was 71.09% (high inhibition) and EnK was 38.54% (low inhibition) for the pathogen *Fusarium oxysporum*.

Based on the results of this study recommend using the endophytic fungus Enl extracts (*Fusarium solani*), extracted from Dayak onion flowers to control the pathogen *Fusarium oxysporum*.

SIGNIFICANCE STATEMENTS

This study discovered that the endophytic fungus Enl extracts (*Fusarium solani*), extracted from Dayak onion flowers can be beneficial for the control of pathogen (*Fusarium oxysporum*), this study will help the researchers to

uncover the critical areas of flower endophytic fungi either as a biocontrol agent or an antifungal metabolite that many researchers were not able to explore. Thus a new theory on flower endophytic fungi may be arrived at.

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