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Foliar Application of Extract from an Azalomycin-Producing *Streptomyces malaysiensis* Strain MJM1968 Suppresses Yam Anthracnose Caused by *Colletotrichum gloeosporioides*

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Yam anthracnose caused by *Colletotrichum gloeosporioides (C.g)* is the most devastating disease of yam (*Dioscorea* sp.). In the present study, we evaluated the culture filtrate extract (CFE) of azalomycin-producing *Streptomyces malaysiensis* strain MJM1968 for the control of yam anthracnose. MJM1968 showed strong antagonistic activity against *C.g* in vitro. Furthermore, the MJM1968 CFE was tested for inhibition of spore germination in *C.g*, where it completely inhibited spore germination at a concentration of 50 μg/ml. To assess the in planta efficacy of the CFE and spores of MJM1968 against *C.g*, a detached leaf bioassay was conducted, which showed both the treatments suppressed anthracnose development on detached yam leaves. Furthermore, a greenhouse study was conducted to evaluate the CFE from MJM1968 as a fungicide for the control of yam anthracnose. The CFE non-treated plants showed a disease severity of >92% after 90 days of artificial inoculation with *C.g*, whereas the disease severity of CFE-treated and benomyl-treated yam plants was reduced to 26% and 15%, respectively, after 90 days. Analysis of the yam tubers from the CFE-treated and non-treated groups showed that tubers from the CFE-treated plants were larger than that of non-treated plants, which produced abnormal smaller tubers typical of anthracnose. This study demonstrated the utility of the CFE from *S. malaysiensis* strain MJM1968 as a biofungicide for the control of yam anthracnose.

Keywords: Anthracnose, biofungicide, *Colletotrichum gloeosporioides*, *Streptomyces malaysiensis*, yam

Introduction

Yam is a group of several edible-tuber-producing plants belonging to the genus *Dioscorea*. Yam tubers are consumed as food in tropical and subtropical regions around the world [6]. There are about 600 species of yam, of which *Dioscorea rotundata* and *Dioscorea alata* are widely cultivated around the world [7]. Annual world production of yam is between 37 to 40 metric tonnes, among which Nigeria alone produces 70% [8].

Anthracnose of yam (*Dioscorea* sp.) caused by the fungus *Colletotrichum gloeosporioides* (*C.g*) is the single largest constraint for yam production in the tropical and temperate regions [6]. The disease spreads mainly through air and via raindrop splashes, which require moist weather with higher humidity as favorable conditions for their development [19]. *C.g* infects mainly leaves, although it causes anthracnose symptoms on all plant parts, which appear initially as small brown spots that expand and cause extensive blackening of the leaves as the disease progress [1]. Anthracnose reduces the photosynthetic efficiency of yam plants, which severely limits tuber production and results in heavy yield loss in susceptible genotypes [1, 6]. Anthracnose-affected yam plants produce several smaller tubers instead of the normally larger tuber. Although *C.g* is known to cause foliar disease, the fungus can infect and survive in yam tubers [9] and in decomposing yam residues in soil, which allows them to survive between growing seasons [15].

The genus *Streptomyces* comprises several biocontrol agents that suppress various plant diseases. The mechanisms of

disease suppression by *Streptomyces* strains are diverse and include production of antibiotics and cell-wall-degrading enzymes, hyperparasitism, production of volatiles, competition, and induction of host resistance [14].

In this study, we evaluated the culture filtrate extract (CFE) of the azalomycin-producing *Streptomyces malaysiensis* strain MJM1968 [5], which showed antagonism towards *C.g*, in controlling yam anthracnose under greenhouse condition.

Materials and Methods

Plant Material and Preparation of Pathogen Inoculum

Seed tubers of *Dioscorea batatas* Decne were obtained from the National Academy of Agricultural Science, Rural Development Administration, Suwon, Republic of Korea. Yam anthracnose-causing *C. gloeosporioides* (*C.g*) KACC 40693 was obtained from the Korean Agricultural Culture Collection (KACC), South Korea. The *C.g* inoculum for the bioassay and greenhouse experiments was prepared as described previously [12].

Antagonistic Activity of Strain MJM1968 against *C.g*

The antagonistic activity of *S. malaysiensis* MJM1968 against *C.g* was evaluated by dual-culture in vitro assay on potato dextrose agar as described previously [12].

Preparation of Culture Filtrate Extract from Strain MJM1968

The CFE was prepared as described previously [12] with slight modification. Strain MJM1968 was grown on ISP4 agar (Difco) at 28°C for 10 days. The plates were flooded with sterile distilled water containing 0.02% Tween 80, and the spores were disrupted using a sterile toothpick. The spore suspension was collected in a 50 ml Falcon tube and washed three times with sterile distilled water by repeated centrifugation. The suspension was added to 2 L of Bennett's broth (glucose 10 g/l, yeast extract 1 g/l, peptone 2 g/l, and beef extract 1 g/l) at a concentration of 10^8 CFU per 100 ml of liquid medium. The strain was grown in Bennet medium in 600 ml batches, contained in 2 L baffled flasks, and shaken at 200 rpm at 28°C for 6 days. After 6 days, the culture supernatant was collected by centrifugation, extracted, and stored according to our previous report [12].

Effect of CFE on *C. gloeosporioides* Spore Germination

Ten microliters of *C.g* spore suspension (10^5 spores/ml) was mixed with 20 µl of medium containing 0.4% (w/v) yeast extract and sucrose with different concentrations of CFE (*viz.*, 0, 10, 25, 50, 100, and 150 µg/ml) on a sterile microscope glass slide and were incubated at 28°C in a humidified chamber. Spore germination was observed under a phase contrast microscope (Olympus, Japan) and images were taken using a digital camera (Olympus DP70) after 24 h of incubation. Total number of spores and number of germinated spores were counted using a hemocytometer under a phase contrast microscope and the percentage of germinated spores was calculated.

Evaluation of Anthracnose Suppressive Activity of MJM1968 and the CFE

Detached leaf bioassay. The detached leaf bioassay was performed according to our previous report [12] with modifications. Yam leaves of the same age were collected from healthy plants and washed with running tap water. The detached leaves were surface sterilized by immersing in 70% ethanol for 1 min, and then in 0.05% sodium hypochlorite solution for 5 min with shaking. The leaves were then washed with sterile distilled water for five times, 5 min each. The surface-sterilized leaves were treated with MJM1968 spores (at 10^4, 10^6, and 10^8 CFU/ml) and CFE (1 mg/l) for 20, 40, and 60 min by immersing the leaves in the spore suspension and CFE solution, respectively. The treated leaves were spray-inoculated with a *C.g* spore suspension (10^5 CFU/ml), placed on sterile polystyrene Petri dishes (5 leaves per dish) and incubated at 28°C under long-day (16-h light/8-h dark) conditions in a humidified chamber for 2 weeks. The control (non-treated) was inoculated with the pathogen as mentioned above and received no other treatment. After 2 weeks, the area of leaf tissue affected with typical lesions of anthracnose was recorded as reported previously [16].

Assessment of CFE suppression of yam anthracnose by greenhouse study. The efficacy of CFE in suppressing anthracnose in yam plants was assessed in the greenhouse study following our previous report [12]. Healthy 8-week-old yam seedlings were selected and transplanted in trays of 4 × 1 feet dimension at two seedlings per tray. The trays were maintained at 16-h light/8-h dark photoperiod at 32°C (day) and 25°C (night) in a greenhouse with 80% relative humidity and watered regularly. The trays were arranged in a completely randomized design. The seedlings were divided into different treatment categories, with 20 plants per treatment as follows: (i) treatment with *C.g* spore suspension, (ii) treatment with commercial fungicide benomyl 250 mg/l and *C.g* inoculation, and (iii) treatment with 500 mg/l CFE and *C.g* inoculation. Yam plants were also maintained separately without any treatment and *C.g* inoculation, which served as uninfected control plants. The treatment was done by spraying the CFE using an atomizer until runoff at a rate of 100 ml per plant. The CFE and benomyl were applied 3 days before, at the same time, 3 days after, and then once weekly for up to 30 days in relation to pathogen inoculation. The yam plants were observed for anthracnose at regular intervals, and disease incidence and severity assessments were made every 15 days until 90 days after pathogen inoculation. The disease severity index was calculated according to Simons and Green [18]. The disease severity of each plant was rated using a 6-point scale based on the percentage of anthracnose affected area of the whole plant, where 0 = 0%, 1 = 1%, 2 = 2%, 3 = 5%, 4 = 10%, 5 = 25%, and 6 = ≥50% [18].

Effect of CFE Treatment on Yam Tuber Yield

The yam plants were uprooted after 90 days and the tubers were washed with tap water and photographed after removing surface moisture with blotting paper. The tubers were chopped

and dried in an oven at 70°C for 3 days. The dried tubers from each plant from different treatment categories were weighed and compared.

Statistical Analysis of Data

All the experiments were performed three times. Data of disease severity from detached leaf bioassay, disease severity from greenhouse experiments, and yam tuber weights were subjected to one-way ANOVA.

Results

Antifungal Activity of MJM1968 against *C.g*

Strain MJM1968 showed strong antagonistic activity on *C.g* in dual-culture agar plate assay (Fig. 1). Furthermore, the CFE was tested for inhibitory activity on *C.g* spore germination. Spore germination was greatly affected by increasing concentrations of CFE (Fig. 2), and at higher concentrations (100 and 150 µg/ml), spore lysis was observed (Figs. 2A(v) and 2A(vi)).

MJM1968 Spores and CFE Suppressed Anthracnose Symptoms on Detached Yam Leaves

Yam leaves treated with strain MJM1968 spores and CFE showed low disease severity compared with *C.g*-inoculated, biocontrol-non-treated leaves (Fig. 3). The *C.g*-inoculated leaves showed an average disease severity of 48% (Fig. 3). A significant reduction in disease symptoms ($p < 0.05$) was observed in the MJM1968 spores- and CFE-treated leaves compared with non-treated leaves, and no significant difference was observed among the various treatment categories (Fig. 3).

Effect of CFE Treatment on Yam Anthracnose under Greenhouse Conditions

Disease symptoms on *C.g*-inoculated control plants were visible at 25 days post challenge-inoculation (Fig. 4A) and the disease severity reached about 50% after 60 days. Treatment with the MJM1968 CFE delayed the onset of disease symptoms on yam plants, where the symptoms were not visible at 25 days (Fig. 4B) and were visible only after 45 days of challenge-inoculation and reached about 26% after 90 days (Fig. 4). The disease severity was greatly reduced upon treatment with MJM1968 CFE, which was significantly lower ($p < 0.05$) compared with non-treated *C.g*-inoculated plants (Fig. 4). Benomyl-treated yam plants showed the least disease severity among the treatments, which showed disease symptoms after 75 days of challenge inoculation with *C.g*.

Fresh tuber sizes of CFE- and benomyl-treated yam plants were different from the *C.g*-inoculated control yam plants (Figs. 5A, 5B, and 5C). Tubers from *C.g*-inoculated control plants were smaller in size and multiple smaller tubers were observed in some infected plants instead of a single tuber of normal size (Fig. 5A). Tubers from the CFE- and benomyl-treated plants were of normal size (Figs. 5B and 5C), indicating alleviation of the anthracnose effect. The dry tuber weights of CFE- and benomyl-treated yam

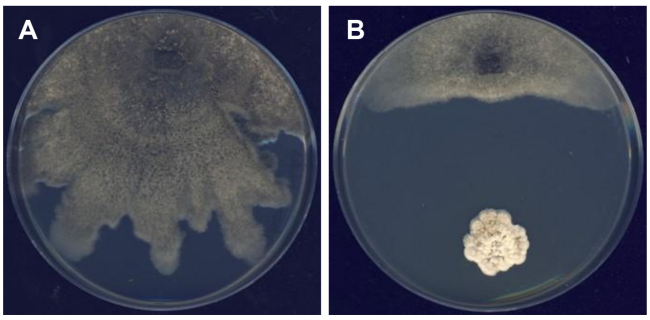

Fig. 1. Antagonistic activity of *S. malaysiensis* MJM1968 against *C.g*.
(**A**) *C.g* growth without antagonist challenge. (**B**) Inhibition of *C.g* growth by MJM1968.

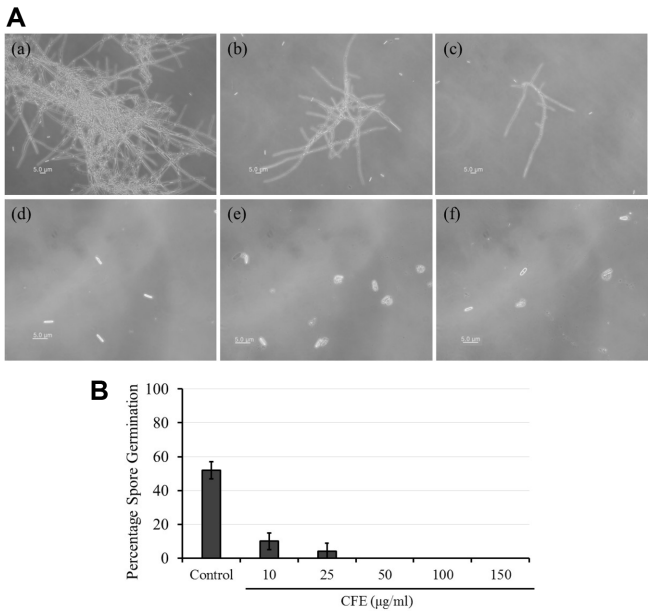

Fig. 2. Inhibition of *C.g* spore germination by culture filtrate extract (CFE) from MJM1968.
(**A**) Photomicrographs showing germination of *C.g* spores incubated with various concentrations of CFE from MJM1968. (a), Control; (b), 10 µg/ml; (c), 25 µg/ml; (d), 50 µg/ml; (e), 100 µg/ml; (f), 150 µg/ml. The scale bar indicates 5 µm. (**B**) Bar diagram showing the percentage of *C.g* spore germination in various concentrations of CFE.

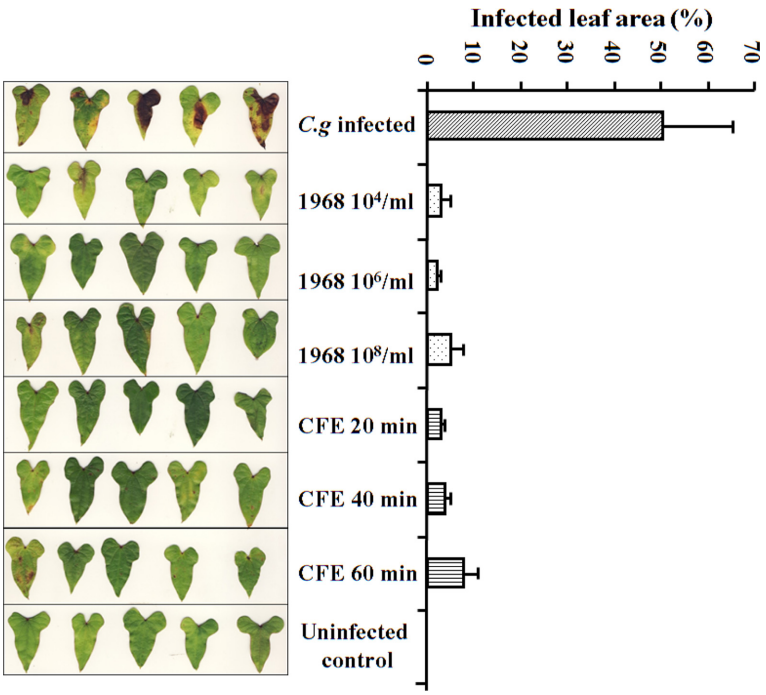

Fig. 3. Effect of *S. malaysiensis* MJM1968 spore treatment and CFE treatment on disease suppression on detached leaves.
C.g infected, leaves inoculated with *C.g* and non-treated with biocontrol preparations, showing disease severity; Treatments, leaves challenge-inoculated with *C.g* were treated with MJM1968 spores 10^4/ml, 10^6/ml, and 10^8/ml or MJM1968 CFE for 20 min, 40 min, and 60 min, respectively; uninfected control, leaves non-inoculated with *C.g*, non-treated with biocontrol preparations and not showing disease symptoms. The total area of lesion on individual leaves was observed after 15 days of challenge-inoculation. Twenty detached leaves were used in each treatment. Error bars indicate the standard deviation of three independent experiments (n = 3).

plants were significantly higher ($p < 0.05$) from that of tubers from *C.g*-inoculated control plants (Fig. 5D), indicating reduction of anthracnose severity on yam tubers by CFE and benomyl treatment.

Discussion

S. malaysiensis strain MJM1968 was originally isolated for the purpose of suppressing phytopathogenic fungi in agricultural soil that cause rot disease in yam tubers [5]. In the present study, we investigated the efficacy of the CFE from MJM1968 on anthracnose of yam. The strain was antagonistic to *C.g*, and hence we hypothesized that it could suppress anthracnose disease development in yam plants. The study of the effect of CFE on spore germination showed that the extract efficiently suppressed spore germination in *C.g*. The inhibition of spore germination by the CFE could be due to the presence of azalomycin. Crude preparation of MJM1968 was observed to inhibit several fungal strains and we confirmed that the active principle was azalomycin F complex in our previous study [5]. Azalomycin was reported to have broad-spectrum antifungal and antibacterial activities [2, 4] and stability over a broad range of pH and temperature [5].

The suppression of anthracnose symptoms on detached yam leaves by MJM1968 spores and CFE are due to the antagonistic activity and the spore germination inhibiting activity of the strain and its CFE, respectively, on *C.g*. Anthracnose development occurs at high humidity after the initial adherence and germination of *C.g* spores on the foliar surface, which are critical in disease development [10, 17]. Inhibition of spore germination will affect disease development [3, 13]. The mechanism of anthracnose suppression on yam leaves by CFE of MJM1968 is due to the inhibition of *C.g* spore germination, which is indicated by the delayed onset of anthracnose in CFE-treated yam plants. Inhibition of spore germination was shown as the mechanism of anthracnose suppression on tomato fruits in our previous study [13], where a protease preparation from *Streptomyces phaeopurpureus* strain ExPro138 was shown to inhibit *C.g* spore germination. In another previous study, we observed that the CFE from *Streptomyces* sp. strain MJM5763 showed inhibition of spore germination, which showed consistent suppression of yam anthracnose in

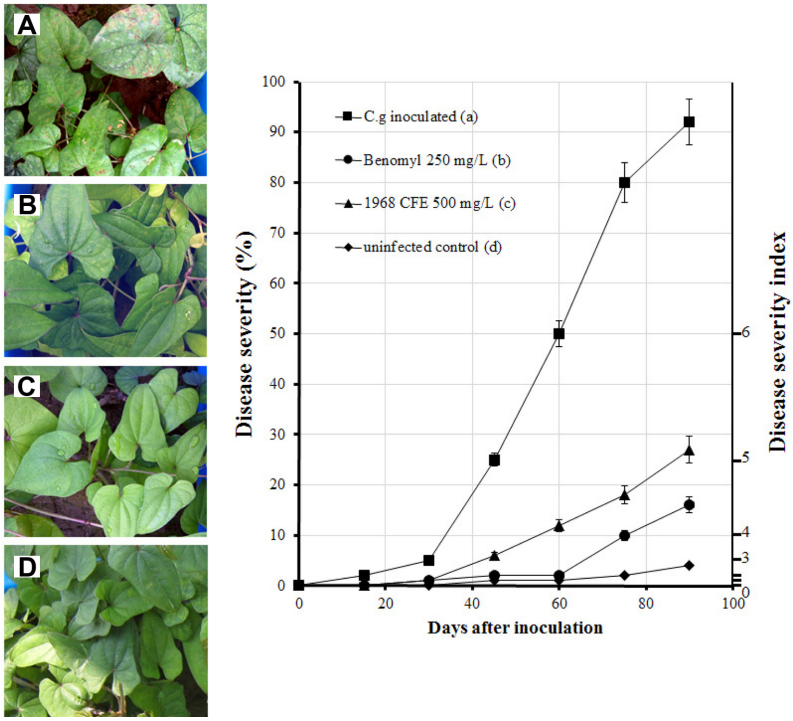

Fig. 4. Effect of *S. malaysiensis* MJM1968 CFE treatment on anthracnose caused by *C.g* under greenhouse conditions.
(**A**) Anthracnose symptoms on *C.g*-inoculated yam plants. (**B**) Leaves from yam plants treated with benomyl 250 mg/l after challenge-inoculation with *C.g*. (**C**) Leaves from yam plants treated with MJM1968 CFE 500 mg/l after challenge-inoculation with *C.g*. (**D**) Leaves from uninfected control yam plants. Error bars indicate the standard deviation of three independent experiments ($n = 3$).

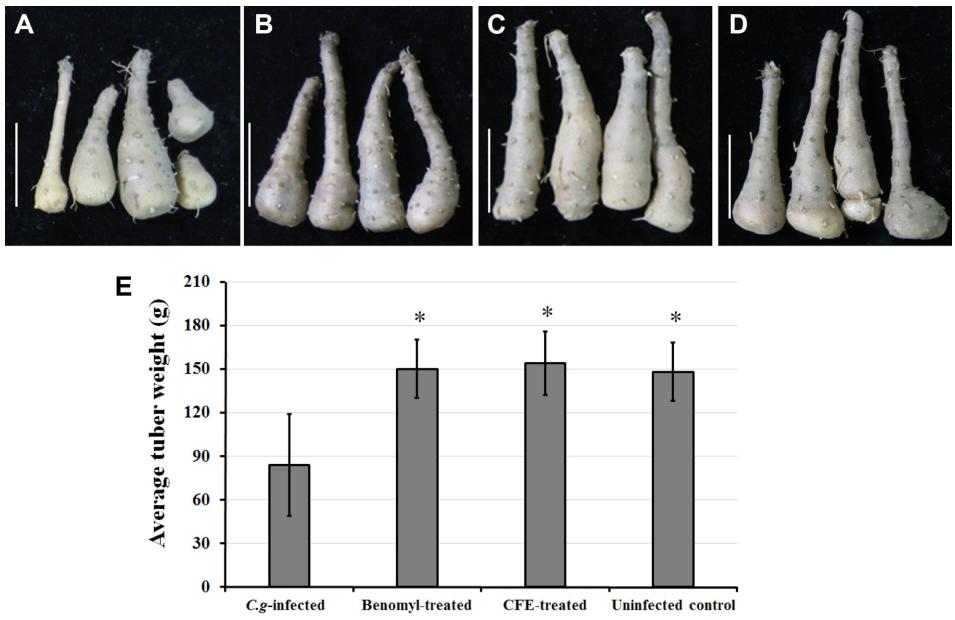

Fig. 5. Effect of MJM1968 CFE treatment on yam tuber yield.
(**A**) Tubers from diseased plants (infected with *C.g*). (**B**) Tubers from benomyl-treated yam plants after challenge-inoculation with *C.g*. (**C**) Tubers from yam plants treated with MJM1968 CFE 500 mg/l after challenge-inoculation with *C.g*. (**D**) Tubers from uninfected yam plants. The scale bar indicates 5 cm. (**E**) Bar diagram showing average dry weight of tubers from each of the treatment categories. Error bars indicate the standard deviation of three independent experiments ($n = 3$). Bars with an asterisk indicate significant difference ($p < 0.05$) from infected plants.

greenhouse and field experiments [12].

CFE treatment of yam plants under greenhouse conditions showed a reduction of disease severity to the level of disease protection provided by benomyl. The reduction of disease severity could be due to the low incidence of anthracnose in the CFE-treated yam plants. The low incidence of anthracnose is due to the low level of active spores on the foliar surface because of inhibition of spore germination by CFE treatment. Young seedlings are more susceptible to the disease than the fully grown yam plants [11]. Infection of young seedlings causes dieback, which results in great yield loss due to less number of surviving plants and also infection of the plants during tuber initiation [1]. Treatment with CFE was started before challenge-inoculation with *C.g* in 2-month-old yam seedlings and continued until 30 days post challenge-inoculation. We observed that this treatment strategy could effectively prevent *C.g* from infecting the yam plants, which resulted in less disease severity compared with non-treated plants. The CFE and benomyl treatments reduced the effect of anthracnose severity on tuber initiation and bulking, as evidenced by the size and weight of the tubers from these treatment categories compared with tubers from *C.g*-inoculated non-treated plants.

In conclusion, the reduction in anthracnose symptoms on yam plants was achieved by treatment with CFE from *S. malaysiensis* MJM1968, which exhibited inhibition of *C.g* spore germination and growth. Therefore, the CFE preparation from MJM1968 could be used as an effective fungicide for the control of yam anthracnose.

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