COMBATING THE PURPLE BOTANICAL PLAGUE: EVALUATION OF COLLETOTRICHUM GLOEOSPORIOIDES F. SP. MICONIAE FOR BIOLOGICAL CONTROL OF MICONIA CALVESCENS IN MO'OREA, FRENCH POLYNESIA

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Abstract. Classical biological control aims to actively manage threats that cause immense losses in biological diversity. The introduction of a biological control agent Colletotrichum gloeosporioides f. sp. miconiae (Cgm) in April 2000 to Tahiti, French Polynesia was intended to control the massive spread of an invasive weed Miconia calvescens. However, while Cgm has subsequently spread to Mo'orea, its impact on reducing M. calvescens remains uncertain. The main objectives of this study are: (1) to quantify the amount of the fungal pathogen infecting the *M. calvescens* plants at three elevation ranges on Mo'orea, (2) to understand the impact of moisture on the proliferation of disease development, and (3) to test the influence of endophytic fungal communities on the competitive ability of Cgm. Results from quantifying leaf damage showed that at higher elevations, Cgm disease development is more rampant. In laboratory experiments, varying moisture did not significantly affect the health of the seedling. While Cgm growth rate correlates with competitive ability, endophytic fungal growth rate does not, leading to speculation that other modes such as chemical interactions allow for endophytic competitive ability. Lastly, data supports the hypothesis that Cgm becomes a better competitor against the endophytic fungal species at higher elevations. Results of this study suggest that other microclimatic factors such as temperature and humidity may play a role in disease development. While Cgm may decelerate the growth of M. calvescens, Cgm alone is not likely to obliterate the massive damage M. calvescens has done on the native flora of Mo'orea and its surrounding islands. Conservation biologists must urgently attack this pest, or the fragile ecosystem of the islands will lead to massive losses in biological diversity.

Key words: invasive plants, Miconia calvescens, biological control, Colletotrichum gloeosporioides, elevation, endophyte community, Mo'orea, French Polynesia

INTRODUCTION

With the arrival of the first Polynesians around AD 600, approximately thirty domestic plant species were introduced, including the Tahitian chestnut *Inocarpus fagifer*, breadfruit tree *Artocarpus altilis*, and kava *Piper methysticum*, which benefited their lifestyles as food sources or medicinal sources. When the first European explorers arrived on Mo'orea, native habitat health dramatically decreased with the overuse of natural resources and the introduction, both accidental and planned, of plants and animals (Meyer and Florence 1996). With more than 1500 exotic plant species present today in the Society Islands, several have become unruly pests that endanger endemic vegetation. These biological invasions represent a humaninduced breakdown of the native biological diversity, shaking the dynamics of a once undisturbed ecosystem (Vitousek et al 1997).

The invasion process of epidemic organisms follow three main stages. First, a small number of individuals are initially established and colonized in a non-infected region. Following initial establishment, naturalization occurs as the species is able to reproduce by sexual or vegetative means without any assistance. Finally, the invasive species spreads, damaging native vegetation and destroying the natural ecosystem (Meyer 1997). The most damaging plant pest in the Society Islands has accomplished these three stages of the invasion process.

Miconia calvescens, also called Miconia *magnifica*, deemed the "purple botanical plague," is a melastome native to Central and South America. Introduced to the Papeari Botanical Garden on Tahiti in 1937, M. calvescens served as a garden ornamental. With its striking dark green leaves and purpleviolet underside, the plant may grow to the size of a small tree, reaching 10 to 15 meters in height. Its introduction in the neighboring island of Mo'orea, about 20 km northwest of Tahiti, was not exactly documented, but was noticed in the early 1970s on mountain trails (Meyer 1998). Because this invasive plant thrives in wet forest environments where the mean annual rainfall is above 2000 mm (Meyer 1997), in less than 50 years, M. calvescens now covers over two thirds of the island of Tahiti and has spread to its surrounding islands of Mo'orea, Raiatea, and Taha'a and poses a huge threat to the other five islands in the Society Islands (Bora Bora, Huahine, Maiao, Maupiti, Mehetia (Meyer 1996). It is estimated M. calvescens threatens 60% of endemic plants of the Society Islands (Meyer 1997).

Certain specific ecological and biological characteristics allow *M. calvescens* to flourish in French Polynesia. With its ability to withstand a variety of germination conditions, *M. calvescens* can tolerate low light levels, reach reproductive maturity early, and continuously produce a large number of seeds. *M. calvescens* produces small berries which are effectively dispersed by birds and small rodents (Meyer et al. 1998). A previous study done by Gaubert (1992) showed a significant decrease in the number of native plants as dense monotypic stands of *M*. *calvescens* increased. Alongside its ability to sprout vigorously after being cut, *M. calvescens* suppresses native vegetation growth as it is not able to survive in low light conditions (Meyer 1996).

Declared a noxious species in French Polynesia in 1990, the search for a biological control ensued. Classical biological control exploits the natural enemies of the invasive plants and pests that are lacking in the newly invaded area (Barton 2004). Non-native plants often become invasive and damaging because the natural herbivores and pathogens that limit their abundance are unavailable to do so in the newly invaded area. Ideally, classical biological control introduces the biological control agent to the newly invaded area, resulting in a reduction in abundance of the damaging plant. Scientists carefully screen and select agents that will not attack crops or other non-target plant species (Tu et al. 2001).

Colletotrichum gloeosporioides f. sp. miconiae (Cgm),а fungus from Subdivision Deuteromycotina, class Coelomycetes, Order Melanconiales, was the first successful organism found. Cgm was isolated from leaf lesions on M. calvescens in Brazil and later sent to the University of Hawaii for further testing of host specificity (Killgore et al. 1995). Due to the presence of native species in the family Melastomataceae in French Polynesia, further testing was done. Repeated tests showed that *Cgm* was highly specific to M. calvescens (Killgore et al. 1997). Finally in April 2000, *Cgm* was released in Tahiti (Meyer et al 2008).

Fruiting structures called acervuli produce asexual spores, or conidia, which allow *Cgm* to reproduce. Acervuli appear on the surface of leaf spots under highly humid conditions, and because conidia are easily dispersed by wind and rain, germination occurs with available moisture on the surface of leaves. Once germinated, the conidia produce mycelium which allows the hypha to penetrate into the leaf's epidermis. Once penetrated, a small dark lesion will show and continue to expand (Killgore et al. 1997). The lesions of *Cgm* on *M. calvescens* leaves are readily visible with "chlorotic halos" approximately one week after infection. As the lesions expand circularly around the necrotic center that becomes dry and brittle, extensive leaf damage and leaf abscission occurs about one month later. Although far less common, stem lesions from the pathogen can cause dieback on the branch (Seixas et al. 2007).

In one study, researchers monitored the pathogen's dispersal and its impact on M. *calvescens* over a 6 year period and found that within 3 years of release, the fungus had spread throughout the island of Tahiti – all M. *calvescens* trees, young plants, and seedlings between sea level and 1400 m in elevation had been infected (Meyer et al. 2008). Although *Cgm* was never intentionally introduced to Mo'orea, a previous study done by Watkins (2004) surveyed the spread of *Cgm* on Mo'orea and showed that the pathogen had also spread throughout the island.

The purpose of this study examines the impact of Cgm on stands of M. calvescens in Mo'orea, French Polynesia. The study proposes to answer the following four questions: (1) How much is the fungal pathogen infecting the plant? While it is evident that the biological control pathogen infects M. calvescens' leaves, it is unclear how much damage the pathogen incurs on the leaves of the plant. (2) Does infection of M. calvescens leaves increase at higher elevations? I hypothesize that at higher elevations, there will be more infection on M. calvescens leaves due to a moister environment. (3) What environmental conditions of surrounding moisture favor the expansion of *Cgm*? Due to moisture requirement of the fungi, I expect to find *Cgm* infections more in areas with greater rainfall. As moisture and elevation are related, it is hypothesized that higher elevations will exhibit more Cgm infections. (4) Do the endophytic fungal communities within the leaves of M. calvescens impact the degree of infection by the pathogen? I hypothesize that the interaction between the endophytic fungus and biological control fungus at three elevations is different. The results of this study will help conservation biologists have a more complete understanding of the importance of abiotic and biotic factors that impact the proliferation of *Cgm*. With this knowledge, further deductions can be made to ultimately help control the infestation of *M*. *calvescens* on the Society Islands.

METHODS

Study site

I evaluated the effects of elevation on *M. calvescens'* infection using three elevation ranges. I defined low elevation as a range of 0-200 meters and used Mari Mari Kellum's property (17°30'48.1''S 149°50'55.1''W, 18 m). A mid elevation site, with a range from 200-500 meters, is represented by Three Coconut Pass (17°32'36.0''S 149°49'40.3''W, 210 m). Mount Rotui (17°30'35''S 149°50'19''W, 875 m) and Mount Mouaputa (17°31'35''S



FIG. 1. Sites sampled in this study. (1) Mari Mari Kellum's Property – low elevation, (2) Three Coconut Pass – mid elevation, (3) Mount Rotui – high elevation, and (4) Mount Mouaputa – high elevation.

Data SIO, NOAA, U.S. Navy, NGA, GEBCO Image © 2009 DigitalGlobe, Image © 2009 Terrametrics, ®2009 Google 149°48′12″W, ~800m) represented the high elevation sites, with high elevation defined as >500 meters (Fig. 1).

Experimental design

Leaf damage and elevation

The purpose of this experiment was to understand the impact of elevation on leaf damage. At low elevation (Mari Mari Kellum's property), I constructed a quadrat of 10m X 10m and sampled at five plots. Within each plot, I randomly selected five M. calvescens plants and calculated the percent of leaves infected on each plant and the number of leaf spots on the most infected leaf. I collected the most damaged leaf from the plant and using Image J, I determined the percent damage on the most infected leaf, which elucidates the maximum damage of the Cgm fungus on the given M. calvescens plant. Additionally, I categorized the maximum damage into three categories: minimally damaged (0-10%), moderately damaged (10-25%), and severely damaged (>25%). In total, I sampled a total of 25 M. calvescens plants at low elevation. I repeated this exact method of sampling at Three Coconut Pass, the mid elevation site.

Because of a lack of flat terrain, at the high elevation sites, I constructed a 10 m transect line along the trails of Mount Mouaputa and Mount Rotui. I chose M. calvescens plants in reachable distance along the trails, sampled three plots on Mount Mouaputa, and collected 10 of the most damaged leaves of each plant from each plot. On Mount Rotui, I sampled two plots and collected 15 of the most damaged leaves per plant. In total, I collected 60 leaves from high elevation sites. In order to standardize the number of samples used among the three elevations, at high elevation, I randomly selected 25 leaves (using a random number generator) from the 60 leaves collected to determine its leaf damage. Due to the amount of leaf damage on the most infected leaves, I categorized the leaf damage by minimally damage (0-10%), moderately

damaged (10-25%), and severely damaged (>25%). For the leaves that were minimally or moderately damaged, I used Image J to quantify the actual percent damage.

Moisture variation in laboratory

In order to determine the effects of moisture on *Cgm*'s ability to damage *M. calvescens* health, I inoculated *M. calvescens* seedlings and altered moisture in the laboratory. Within my 10m X 10m plot, I extracted six seedling *M. calvescens* plants with no visible infections. With five plots, I collected a total of 30 seedlings at low elevation and 30 seedlings at mid elevation.

I mixed a spore solution by collecting the *Cgm* spores in a Petri dish (100 mm x 15 mm) by scraping the culture with gloved hands and adding 100 ml of bottled water. This was the concentration of *Cgm* used when released in Tahiti as a biological control agent (Ravahere Taputuarai, personal communication).

From the 30 seedlings collected at each elevation, I randomly selected 20 seedlings to be inoculated with the spore solution. A low moisture treatment included one spray of water per day whereas a high moisture treatment included four sprays of water per day. At low elevation, I inoculated 10 seedlings with the spore solution and grew them in low moisture conditions. Additionally, Ι inoculated another 10 seedlings with the spore solution and grew them in high moisture conditions. As a negative control, I did not inoculate 6 seedlings and grew 3 in low moisture and 3 in high moisture conditions (Table 1). I used this design at mid elevation as well.

I grew all seedlings outside to minimize light and temperature variations. To prevent moisture from transferring between each treatment group, I separated each treatment group approximately 2 meters away. Two weeks after I inoculated the seedlings, I observed whether the seedling had actually been infected by *Cgm* and recorded the plant's overall health (alive, dead).

TABLE 1. *Moisture variation in laboratory* – Ten seedlings at both low and mid elevations were inoculated and either treated with low moisture conditions or high moisture conditions. As a negative control, 6 seedlings in each elevation were not inoculated but still treated with low and high moisture water conditions.

Number of samples	Elevation	Treatment	Moisture
10	Low	Yes	Low
10	Low	Yes	High
10	Mid	Yes	Low
10	Mid	Yes	High
3	Low	No	Low
3	Low	No	High
3	Mid	No	Low
3	Mid	No	High

Field inoculations

In order understand the effects of Cgm at different elevations and moisture, I inoculated leaves in the field and quantified the percent leaf damage after two weeks. Using the same spore solution as above, I sprayed the youngest leaf on each plant in my 10 m X 10 m quadrat and covered the leaf with a Ziploc bag to prevent the rain from washing away the spores. At each of the four plots, I inoculated five leaves. I used plot 5 as a negative control, meaning no leaves were inoculated. I took a digital image of the leaves prior to inoculation. At low and mid elevation, I inoculated 20 leaves each. One week post inoculation, I returned and removed the Ziploc bags from the leaves. After two weeks, I took a digital image of the inoculated leaf and used Image J to determine the percent of infection on the leaf.

Endophytic competitions

In order to determine the ability of endophytic fungi on *M. calvescens* to repel Cgm, I tested competition between the endophytic fungus and the biological control fungus at the three elevations. At each of the five plots within the low elevation site, I randomly selected five leaves within my 10 m

X 10 m quadrat. Thus, at the low elevation site (Mari Mari Kellum's property), I collected 25 leaves. On each leaf, I cut three portions of the leaf, labeled each either A, B, or C, and plated all three of them on 1.5% malt extract agar amended with 100 mg/ml Streptomycin sulfate.

After one week of growth, I tested fungus A against the Cgm fungus on 1.5% malt extract agar amended with 100 mg/ml Streptomycin sulfate. However, if fungus A did not grow or had been contaminated, I competed fungus B with the Cgm fungus. Likewise, if fungus B did not grow or had been contaminated, I used fungus C in competition studies. I plated the endophytic fungus approximately 1.5 cm apart from the Cgm fungus and allowed the competition plates to grow in room temperature in the laboratory (25°C-30°C). I measured the diameter of each colony everyday and measured their respective growth rates prior to contact using linear regression analysis.

Three outcomes could occur upon contact: the endophyte fungus outcompeted the *Cgm*, the *Cgm* outcompeted the endophytic fungus, or the fungus formed an inhibitory zone. I defined "outcompeted" or a "win" as fungus A covering more area than fungus B without forming a line of barrage. A "win" also existed if fungus A enveloped and grew over fungus B. A "draw" occurred if a line of barrage existed (Fig. 2). In order to score these interactions, I used a +1, 0, -1 scoring system to indicate dominance, mutual antagonism, and inhibition, or in other words, win, draw, and lose, respectively (S. Marin et al. 1998). For example, if the endophytic fungus wins, I assigned a score of +1. Consequently, I assigned Cgm a -1 score. If the interaction is a draw, I assigned both fungi with a 0 score. I followed the same procedure for mid elevation and high elevation competition plates, with each elevation gradient having 25 endophyte competition plates. Lastly, I discarded contaminated competition plates.





Statistical analysis

Leaf damage and elevation

All statistical analyses were conducted on JMP statistical software 8.0.1. First, I used a chi-squared analysis to determine if there were statistically significant differences in leaf (minimally damage damaged [0-10%], moderately damaged [10-25%], and severely damaged [>25%]) among the three elevations (low, mid, and high). Because there was continuous data for low and mid elevation sites, I ran a more detailed analysis using a ttest that compared percent damage of the most infected leaf between the low and mid elevation groups. Lastly, using a one-way ANOVA, I determined if the percent of leaves damaged on a given plant differed significantly among low, mid and high elevations.

Moisture variation in Laboratory

I used a chi-squared analysis to examine the relationship between elevation and successfully infected plants. To determine the effect of variation in moisture on variation in the overall health of the plant, I conducted another chi-squared analysis. Lastly, to test the efficacy of my negative controls, I used a chi-squared analysis to compare successfully infected plants and the overall health of the plant. A Bonferoni correction for three comparisons was used to determine statistical significance (p<.016).

Field Inoculations

I used a t-test to determine the effect of elevation on percent damage of the leaf after inoculation.

Endophytic Competitions

I used logistic regression with growth rate as the x-variable and competitive outcome as the y-variable. I used a log likelihood test to assess goodness of fit and assessed significance of test using chi-squared distributed test statistic. This was done for both endophytic fungi and *Cgm*. Lastly, using a chi-squared analysis, I tested the effect of elevation on competitive ability.

RESULTS

Leaf damage and elevation

Increasing elevations exhibits significant increases in damage of the most infected leaf (χ 2 test, χ 2=68.03, p<<0.001) (Fig. 3). This pattern holds true for randomly selected plants as well, as there are significant differences in percent leaf damage on a given plant among the three elevations (ANOVA, F=73.4616, p<<0.0001).

Additionally, the difference between the mean percent damage of the most infected leaf at low and mid elevations is statistically significant (t-test, t=2.62, p=0.01). The average percent infection of the most damaged leaves among the five plots at low elevation was $4.9\pm3.3\%$. At mid-elevation, the average percent infection of the most damaged leaves among the give plots were $8.5\pm1.9\%$. Infection of the most damaged leaf increases with increasing elevation.

Moisture variation in laboratory

A Bonferoni correction for three comparisons suggested a corrected p-value of p<0.016 for statistical significance. There was no significant difference in the number of plants infected between the elevation groups (χ 2 test, χ 2=3.059, p=0.083). There was also no significant difference between the health of the plant when varying moisture (χ 2 test, χ 2=1.359, p=0.244). However, there was a significant effect of successful infection on plant health (χ 2 test, χ 2=12.495, p<0.004).



Effect of elevation on percent damage of most infected leaf

FIG. 3. Effect of elevation on percent damage of most infected leaf. Increasing elevation shows a significant increase in damage of the most infected leaf. Low, mid, and high refers to elevation.

Thus, plants that have not been infected are more likely to stay alive and healthy compared to plants that have been infected.

Field inoculations

A t-test showed that there were no significant differences between elevation groups in terms of the means of percent leaf damage on an infected leaf (t-test, t=1.30, p=0.203).

Endophytic competitions

A logistic regression analysis showed that Cgm's growth rate positively correlates with its competitive ability (Logistic regression, $\chi 2$ =10.06, p=0.007). The faster that *Cgm* grows, the more competitive of a fungus it becomes. There is also a significant correlation between elevation and *Cgm's* competitive ability ($\chi 2$ -squared, $\chi 2$ =17.30, p=0.002). As elevation

increases, *Cgm* becomes a better competitor against the endophytic fungus (Fig. 4). However, the same test showed that there is no statistical difference between the endophytic fungus' growth rate and its competitive ability (Logistic regression, χ 2=3.713, p=0.156).

DISCUSSION

This study shows that as elevation increases, an increase in *Cgm* infection on *M. calvescens* occurs. Observations in the field have shown increase *M. calvescens* damage at higher elevations, but the results of this study quantify what had been expected but previously not evaluated. Climatic conditions directly impact the propagation of *Cgm*, as production of conidia favor high humidity conditions, with wind, rain, and humans easily dispersing the spores over a wide range. Because *M. calvescens* succeeds in a similar



Cgm's competitive ability at three elevations

FIG. 4. Frequency of competitive ability at three elevations. *Cgm* becomes a better competitor against the endophytic fungus in *M. calvescens* with increasing elevations.

humid environment, disease development occurs readily (Killgore et al. 1997). The increased severity of leaf damage caused by *Cgm* at higher elevations may be attributed to the cooler and moister conditions at high elevation (Meyer et al. 2008). Delayed propagation of the fungal pathogen at an infected site in Tahiti as a result of drought supports the idea that moisture is important for disease progression. In one study, optimum temperature for *Cgm* occurs at 28°C and disease development is severely limited at 36°C (TeBest 1991). Thus, temperature and moisture are potentially important factors for disease development.

The results from the moisture variation experiment in the laboratory, however, did not follow the elevation trend as expected. The statistical analysis showed that the negative controls for the experiment were valid uninfected M. calvescens plants were more likely to stay alive and healthy compared to infected plants. However, my field data as well as previous studies suggest a trend of increasing infection from low moisture to high moisture conditions (Meyer et al. 2008). It was also hypothesized that increasing moisture would lead to decline of health of the plants. And with field inoculations, it was expected that at mid elevations, there would be more infection on the leaves over the two week period as compared to leaves at low elevations. However, though the results showed otherwise, several confounding factors may help to explain the lack of statistical significance between elevation and percent infection. A larger sample size and longer time frame may be needed to fully understand the impact of moisture on infection and plant health. Killgore et al. (1997) found that Cgm causes lesions on the leaves of M. calvescens six to eight days post inoculation. Extensive leaf damage resulting in leaf yellowing and premature defoliation and ultimately leaf abscission occurred after three to four weeks (Killgore et al. 1997). In lab experiments done by Meyer et al. (2008), mortality rates for M. calvescens seedlings was

74.1% one month after inoculation. However, in field conditions where larger *M. calvescens* exist, mortality rate was only 30% for seedlings four to six years after release of Cgm (Meyer et al. 2008). Thus, though favorable results were presented in lab conditions, the full impact of Cgm can be assessed only with its success in the field. In addition to moisture, an evaluation of temperature and humidity effects should also be conducted to understand its collective impact on disease development. These three factors cannot be exclusively tested as one factor impacts its effect on another. А more holistic understanding could thus be made from the integration of these factors that may influence disease development.

In addition to the abiotic factors that can affect Cgm disease development in M. calvescens, it is also possible that biotic factors play a significant role. The results from competition experiments showed that as Cgm growth rate increases, it becomes a better competitor against the endophytic fungi tested. However, the growth rate of the endophytic fungi tested does not show increased competitive ability. Instead of using growth rate to increase competition, the endophytic fungal species may rely upon other characteristics such as chemical interactions that could allow for increased competitive ability. At lower elevations, the endophytic fungal species outcompete the *Cgm* more often than at mid and high elevations. Most often, there would be a distinct inhibition line (barrage zone) between the endophytic fungus and *Cgm* at high elevation. This observation may be due to some chemical interaction between the differing fungi. Only in competition experiments with the endophyte obtained from high elevation sites did Cgm ever outcompete the endophytic fungus. Two possible explanations exist for this difference in competition between the endophytic fungi and *Cgm*. The endophytic fungal communities may be different at the three elevations, and the endophytic fungus at low elevations are

better able to combat the Cgm than compared to mid and high elevations. However, another possible explanation is that the endophytic fungal communities are the same at the three elevations, but their ability to grow and compete against the Cgm is limited at higher elevations where the conditions for growth are different.

In all aspects of the study, the power to detect climatic and elevation differences in *Cgm* efficacy would be improved by employing larger sample sizes. Furthermore, the ability to strengthen my conclusions would benefit from increased replication of sites and of individual plants within sites. Lastly, further research on endophytic fungal communities may give a more complete understanding of the biological factors affecting disease development.

Although *Cgm* has successfully invaded *M. calvescens* stands on Mo'orea, the goal of its introduction still has not yet been met - M. calvescens stands are still rampant, endangering native biota. It is clear from these four isolated experiments that several factors must be taken into account when assessing the impact of Cgm on M. calvescens. In addition to abiotic environmental factors of temperature, moisture, humidity, elevation on disease development, other biological characteristics such as endophytic fungal communities of the M. calvescens plant must also be understood. And as it appears that the endophytic fungal communities may actually impact the degree of infection by the pathogen, it would be interesting to understand the potential chemical interactions that occur at the interface between the pathogen and the endophytic fungus.

With any biological control program, the level of control that a biological agent has on the target organism is hard to predict. Though it appears that the plant pathogen *Cgm* may reduce the growth of established *M. calvescens* plants, *Cgm* alone will not be able to control the invasion of *M. calvescens* (Meyer et al. 2007). However, what is known is that reducing stands of *M. calvescens* is critical for

the conservation of the native biota of Mo'orea. This invasion is a pressing issue that must be dealt with immediate urgency. The introduction of other native enemies of *M. calvescens* may be a viable option. Perhaps with these concerted efforts of multiple controls, conservationists can fully control the invasion by *M. calvescens* in the Society and Hawaiian islands to prevent the green cancer from obliterating the native vegetation of the island ecosystem.

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