Dynamics of QoI Sensitivity in *Mycosphaerella fijiensis* in Costa Rica During 2000 to 2003

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ABSTRACT

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From 1997 onward, the strobilurin fungicide azoxystrobin was widely used in the main banana-production zone in Costa Rica against *Mycosphaerella fijiensis* var. *difformis* causing black Sigatoka of banana. By 2000, isolates of *M. fijiensis* with resistance to the quinolene oxidase inhibitor fungicides were common on some farms in the area. The cause was a single point mutation from glycine to alanine in the fungal target protein, cytochrome *b* gene. An amplification refractory mutation system Scorpion quantitative polymerase chain reaction assay was developed and used to determine the frequency of G143A allele in samples of *M. fijiensis*. Two hierarchical surveys of spatial variability, in 2001 and 2002,

found no significant variation in frequency on spatial scales <10 m. This allowed the frequency of G143A alleles on a farm to be estimated efficiently by averaging single samples taken at two fixed locations. The frequency of G143A allele in bulk samples from 11 farms throughout Costa Rica was determined at 2-month intervals. There was no direct relationship between the number of spray applications and the frequency of G143A on individual farms. Instead, the frequency converged toward regional averages, presumably due to the large-scale mixing of ascospores dispersed by wind. Using trap plants in an area remote from the main producing area, immigration of resistant ascospores was detected as far as 6 km away both with and against the prevailing wind.

Additional keywords: black Sigatoka, dispersal, evolution, fungicide resistance, sampling.

Mycosphaerella fijiensis, the causal pathogen of black Sigatoka disease, first was observed as a new pathogen of banana in Fiji in 1963 (23). It is virulent and infects a wider range of banana genotypes (34) than the related and earlier disseminated *M. musicola* and *M. musae*. When a plantation is heavily infected, the foliar disease produced by *M. fijiensis* has a devastating effect through yield loss and premature and uneven fruit ripening (22,35). In commercial production, black Sigatoka disease is managed by regular fungicide sprays.

The fungicide azoxystrobin inhibits ubiquinol cytochrome *c* reductase. It is described as a member of the quinolene oxidase inhibitor (QoI) family of fungicides, originally derived as analogues of naturally occurring fungicidal products (6). It was introduced in the late 1990s and, by 2002, six other members of the family were in commercial use (2). The fungicidal activity of the strobilurins is very specific: it inhibits mitochondrial respiration by binding at the Qo site of the cytochrome *b*. When strobilurin binds at the Qo site, it blocks electron transfer between cytochrome *b* and cytochrome *c*₁ in the cytochrome *bc*₁ complex III. This interferes with production of ATP (2,3,26).

Strobilurin-resistant isolates now have been detected in field populations of a range of important plant pathogens, including *M. fijiensis* (29), *Blumeria (Erysiphe) graminis* f. sp. *tritici* (8,30), *Venturia inaequalis* (7,37,38), *Podosphaera xanthii* (= *Sphaerotheca fuliginea*), *Plasmopara viticola* (9), *Pseudoperonospora cubensis*, *M. graminicola* (16), and *Alternaria alternata* (11). In all cases, resistance was conferred by a point mutation in the cytochrome *bc*₁ gene. The commonest change, and that found in

M. fijiensis, leads to an amino acid change from glycine to alanine at position 143 and is referred to as the G143A mutation.

Strobilurins, in the form of azoxystrobin, were introduced in the banana industry in 1997 and soon were extremely widely used. When first sold in Costa Rica, in 1997, the recommendations were to (i) use a maximum of eight strobilurin spray applications per year, (ii) use no more than two successive spray applications of strobilurin before alternative fungicides from different cross resistance groups were used, (iii) apply the full recommended rates of strobilurin whether alone or in mixtures with other fungicides with different mode of action and, lastly, (iv) spray at intervals of no more than 14 to 16 days under severe infection pressure. These recommendations were known to achieve excellent disease control, and conformed to general industry guidelines to slow the evolution of fungicide resistance issued by the Fungicide Resistance Action Committee (FRAC).

In August 2000, we discovered a shift of sensitivity in samples from several locations across Costa Rica, using a germination bioassay. DNA sequencing showed the resistant isolates to have the G143A mutation. Following an emergency meeting of the FRAC on 8 August 2000, guidelines for the growers in Costa Rica were modified as follows: (i) a maximum of four strobilurin spray applications per year (not eight), (ii) do not spray QoIs in regions where high levels of resistance were detected until there was evidence of substantial increases in the frequency of sensitive population, (iii) spray at intervals of no more than 14 to 16 days under severe infection pressure, (iv) do not make successive sprays of strobilurins, (v) maintain a 3-month strobilurin-free period in each year, and (vi) apply the full recommended rates of the strobilurin whether alone or mixtures. For preference, growers were advised to use mixtures with other fungicides with different modes of action.

Guidelines in 2007 are slightly more stringent, adding three recommendations—(i) to apply QoI fungicides only in mixtures

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with other, non-cross-resistant modes of action; (ii) to alternate with other, non-cross-resistant modes of action; and (iii) to apply at the end of the minimum of the annual disease severity cycle and when disease pressure is low—and reducing the maximum number of sprays to three. In 2000, growers in several farms with high levels of resistance decided to cease spraying strobilurins, while growers in other farms within the same area continued to spray. These decisions provided an opportunity to study the relationship between spray programs and the frequency of G143A allele.

The specific objectives of this study were to (i) determine the spatial scale on which resistance frequency varied, to allow us to sample effectively and interpret other results; (ii) test whether resistance frequency declined on individual farms if QoI sprays were withheld; (iii) determine the relationship between increase of resistance frequency and number of sprays; and (iv) relate changes in frequency on individual farms to regional trends.

MATERIALS AND METHODS

Collection of infected leaves from the field. Infected leaves with necrotic “burnt” tissue were collected after 2 or 3 days without rain. The samples were air dried for 1 to 2 days before shipping to Jealott’s Hill, UK, in paper bags to avoid condensation. Samples that were not used immediately were refrigerated at 4 to 11°C on arrival.

DNA extraction. Infected areas of leaves to be sampled were sprayed with water and incubated in a plastic bag for 48 h, then cut into pieces of ≈ 2 by 2 cm. Five pieces were stapled to a filter paper and fitted into the lid of a petri dish. Ascospores were discharged from the infected banana leaves onto water agar and incubated at 25°C for 48 h. After incubation, plates were viewed under the microscope to check that ascospores had discharged. Once ascospores were located, they were removed from the plates by scraping the surface of the agar with a scalpel blade. Scrapings were put in a falcon tube containing 10 ml of Czapek Dox broth and yeast extract at 1 g/liter, streptomycin sulfate at 100 mg/liter, and oxytetracycline at 100 mg/liter, and the tubes incubated at 28 to 30°C under continuous light. Three days later, the mycelium from each tube was harvested by filtering the broth and stored at -80°C until used. A plant Minikit (21) was used according to the manufacturer’s instructions to isolate DNA from 50 mg fresh weight of mycelium disrupted mechanically using a GenoGrinder (Spex, Metuchen, NJ) for 10 min at 600 rpm in the presence of five glass beads.

Estimation of resistance frequency. The proportion of G143A DNA in a sample is proportional to the frequency of resistant isolates, because single ascospore isolates are homoplasic; that is, they contain only one type of mitochondrion. This was determined by collecting single ascospores from a leaf with a mixed infection and determining the G143A frequency in the culture (1). Bioassays of these cultures on banana confirmed that G143A conferred a very high level of resistance to azoxystrobin. An amplification refractory mutant system Scorpion assay was used in a real-time quantitative polymerase chain reaction (PCR) to estimate the frequency of G143A allele in field samples (36). The cytochrome *b* gene contains a 1,064-bp intron 78 bp downstream from the second base of the G143 codon. The assay was designed on the coding sequence between this intron and the mutation. Amplification refractory mutation system primers (18) of 32 bp were designed with a selective 3’ C residue (binding to the G143A mutant: 5’ GTT TTA CCT TAT GGT CAA ATG TCT TTA TGA CC 3’) or a selective 3’ G (binding to the wild type: 5’ GTT TTA CCT TAT GGT CAA ATG TCT TTA TGA TG 3’). A control primer amplified both G143 and A143 (5’ ATG TTT TAC CTT ATG GTC AAA TGT CTT TAT GA 3’). Underlined bases were deliberately mismatched to the *cyt b* sequence to enhance specificity (18). A common Scorpion primer also was designed, with

sequence 5’ FAM-CC GCG C ATA CCC TGA GTA GGA CAA GCG CGG MR-HEG AAA CC TCC TCA AAT AAA CTC AAC TAT ATC 3’ (FAM = 6-carboxyfluorescein, MR = methyl red, and HEG = hexethylene glycol to block elongation). In the single-stranded form, this forms a loop so that the MR quenches the FAM; in double-stranded form, the FAM fluoresces when excited at 490 nm. Real-time quantitative PCR was done with an ABI PRISM 7700 (PE Biosystems, UK), and the difference in the number of cycles required until fluorescence was above background cycle threshold (ΔCt) noted. Assays consisted of Beacons buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl; 3.5 mM MgCl_2 ; and 0.01% gelatine) (Qiagen, Crawley, UK), bovine serum albumin at 0.25 $\mu\text{g}/\text{reaction}$, 100 μM dNTP (Qiagen), 500 nM primers (Oswel, Southampton, UK), and 1 unit of AmpliTaq Gold enzyme (Qiagen). The final reaction volume was 25 μl (5 μl of template plus 20 μl of the assay mix). An initial PCR step of 94°C for 10 min was followed by 50 cycles of 94°C for 15 s and 60°C for 45 s. The assay was calibrated using dilution series of known types of DNA, and the frequency of G143A allele calculated from a regression of frequency on ΔCt . The assay was almost unaffected by DNA concentration and the frequency estimated by this method was well correlated to bioassay results. All assays were duplicated.

Migration estimate. In March 2001, disease-free banana plants (cv. Grand Naine) were planted 300, 1,000, 3,000, and 6,000 m from the edge of an isolated farm (F) in northwest Costa Rica, in both directions along a northeast-southwest transect. From March to September, when the experiment was done, the prevailing wind is from the northeast. All leaves were removed at planting to eliminate latent infection. Bulk samples of *M. fijiensis* were collected from farm F in March. In September 2001, infected leaves were collected from each plant in the transect and the G143A frequency determined in two pooled samples, each of five leaf pieces as above. There were no commercial farms to the northeast of the farm, and few other farms in close proximity. There were few plantains and no commercial banana production close to farm F and no plantains close to the transect plants. *M. fijiensis* on plantains in the vicinity of farm F had been sampled in November 1999, shortly after the first detection of the G143A allele, and showed no resistance to strobilurin in bioassay.

Population structure. In October 2001 and October 2002, spatially structured samples of *M. fijiensis* were taken across Costa Rica. The sites chosen included farms with and without an aerial spray program with azoxystrobin and with high and low levels of G143A. The design was intended to provide information about variation on all spatial scales, without excessive sampling at small scales. Two or three farms 5 to 10 km apart were selected in three regions across Costa Rica with differing disease pressure, 50 to 70 km apart; on each farm, samples were taken at two locations per farm at 0.5 to 1 km apart; in each location, upper and lower leaves were taken from one tree, and a single leaf from another tree ≈ 10 m away; tissue pieces were taken from the tip and the base of one of these leaves, selected at random. From the other leaves, a single piece was taken. At one of these leaf zones, two samples of 2 cm^2 were taken, a few centimeters apart; at the other zone, a single leaf piece was taken. The full spatial structure was analyzed using samples collected on two dates, October 2001 for the first survey and October 2002 for the second. Data were analyzed by REML using Genstat (v6-v8; VSN International, Oxford, UK).

Evolution of resistance. In August 2000, bulk leaf samples were taken from 16 farms across the country, with varied spray histories. Nine were chosen for detailed study. Spray schedules were provided by the estate managers. Farms A to D in the southern region (Siquirres) had zero or low levels of G143A. Farms E and F in the central region (Guápiles) had a high level of G143A. In the northern region (Puerto Viejo), farm H had a medium G143A level; we had no resistance frequency data for

farm G in August 2000, but the farm was included because its management decided to continue spraying strobilurin throughout the course of the study. Bimonthly from October 2001 to October 2002, five tissue pieces were taken from each leaf collected as above from two trees in two fixed locations on each farm. The discharged ascospores were mixed and the overall frequency in the sample estimated.

RESULTS

Migration. The frequency of the G143A allele at the center of the transect was 9% in March 2001 when the transect was first established. Six months later (September 2001), the first samples from the transect were collected and the percentage of G143A at the center of the transect had increased almost three times, to 27%; the average frequency on the farm was still higher, at 78%. The G143A allele was found on all trap plants, the frequency decreasing the further the plant was from the source. The frequency at the closest trap plants was anomalously low. The decline in log frequency was significant ($P < 0.001$) and approximately linear with log distance, suggesting a power-law dispersal

Fig. 1. Percent frequency of G143A in *Mycosphaerella fijiensis* on trap plants at various distances from a source farm along transects aligned with or opposed to the prevailing wind. Transect to the southwest, downwind, □; and to the northeast, upwind, ■. Frequency in the source is that at the start of the experiment. By the time of sampling in the transects, the frequency had risen to ≈78%.

curve, that is spore density $\propto 1/(1 + \text{distance})^b$. The exponent b of the decline was 0.6, fitting parallel lines to the two directions (Fig. 1). The decline continued to the maximum distance observed. Frequencies were lower at all distances against the prevailing wind ($P < 0.001$ by analysis of variance) (Table 1), but the pattern and rate of decline with distance was the same in both directions.

Population structure. In October 2001, only location and farm contributed significantly to the variance among samples (Table 2). In October 2002, only differences between farms contributed significantly to the variance among samples (Table 2).

Evolution of resistance. There was a very clear positive relation ($P < 0.002$) between the number of strobilurin sprays applied by August 2000 and G143A frequency at that time (Fig. 2A); each additional spray, on average, increased resistance frequency by a factor of $e^{0.62} \approx 2$. However, there was no relationship between the number of sprays applied during August 2000 to October 2002 and the change in G143A frequency (Fig. 2B). Also, the frequency of resistance increased or remained the same in all farms, regardless of the use of strobilurins on those farms. Therefore, there is no evidence of back-selection when sprays are withdrawn.

However, there were clear regional groupings in both the frequency of G143A and the pattern of change. Farms in the southern region (Siquirres), central region (Guápiles), and northern region (Puerto Viejo) converged to a regional mean, regardless of the treatments applied (Figs. 3 to 5). The frequency of resistance rose from negligible to several tens of percent in the period 2000 to 2001, but then stabilized. The isolated northern region farm P remained fully sensitive from 2000 to 2003.

DISCUSSION

Our main conclusions are (i) there is little spatial structure to the *M. fijiensis* population at scales < 10 m; (ii) ascospore dispersal rapidly homogenizes *M. fijiensis* populations on scales of 1 to 10 km; therefore (iii) evolution of resistance to strobilurins is a regional phenomenon: individual farms converge on a regional average frequency regardless of their own spray practices; and (iv) there is no evidence for rapid selection against the resistant form on withdrawal of the fungicide. The implications for practice are discussed below.

Dispersal and population structure. No sprays were applied to the trap plants in the transect, and are unlikely to have been

TABLE 1. Analysis of variance of log frequency of resistant isolates of *Mycosphaerella fijiensis* on trap plants located up- or downwind of a source field at various distances^a

Source of variation	df	Sum of squares	Mean square	Variance ratio	P
Wind direction	1	30.17	30.17	34.35	0.001
Distance	3	32.93	10.98	12.53	0.002
Of which regression on log(distance)	1	20.11	20.11	22.96	0.001
Distance × direction	3	2.94	0.98	1.12	0.4
Replicates	8	7.01	0.88	...	...

^a Source frequency not included.

TABLE 2. Estimated variance components of G143A frequency of *Mycosphaerella fijiensis* at each spatial level sampled in October 2001 and January 2002

Source of variation	df	October 2001				January 2002			
		Variance component	Stratum variance	Variance ratio ^a	P	Variance component	Stratum variance	Variance ratio ^a	P
Farm	7	10.30	312.86	2.09	0.16	18.52	300.88	65.87	0.001
Between location on a farm	8	17.96	149.69	20.28	0.001	0.27	4.57	1.89	0.13
Between trees at one location	16	0.00	7.38	1.00	0.5	0.00	2.41	1.00	0.5
Between leaves on a tree	32	1.70	7.36	1.89	0.04	0.52	2.41	1.75	0.06
Between positions on a leaf	30	0.00	3.90	1.00	0.5	0.00	1.38	1.00	0.5
Between leaf pieces at one position	33	3.90	3.90	...	...	1.38	1.38	...	...

^a Ratio between the variance at this stratum and the one below.

applied to any local sources of ascospores from small-scale plantain production; therefore, the resistance frequency on the trap plants represents the accumulated immigration of resistant spores. Farm F was the closest large source of resistant ascospores, but other farms at greater distances than farm F may have been contributing to the level found. The continuing decline in proportion of resistance as far as 6 km from farm F suggests that farm F was the dominant source. However, the relatively (though not absolutely) large amounts of dispersal to long distances implied by a power-law dispersal curve with an exponent of approximately ≤ 1 means that background levels of ascospores will reflect levels of resistance over a large region (28).

It is surprising that the dispersal gradient was the same both upwind and downwind. Presumably, this is because most of the movement is due to advection in a consistent windstream, and the upwind movement mostly reflects rare occasions when the wind did not blow from the prevailing direction, rather than genuine upwind dispersal by turbulence. Some authors have suggested that long-distance dispersal of *M. fijiensis* ascospores and conidia by wind is limited to a few kilometers because of their susceptibility

to ultraviolet radiation (5,10,14,20,31–33,35). However, Meredith (13) reported that ascospores dispersed over 1 km infected new plants in the field. Similarly, Romero et al. (25) reported that farms with no previous application of benzimidazole contained benomyl-resistant isolates, presumably due to dispersal by wind from neighboring farms (24). Lastly, Burt (4) reported that the entry of *M. fijiensis* in the Caribbean is likely through windborne immigration.

The hierarchical survey of spatial variability in resistance frequency was fully consistent with relatively efficient medium-range dispersal. Locations differing in position by the order of 1 km contributed most of the variance among samples in the first survey whereas, in the second survey, only farms contributed significantly. In other words, in October 2001, populations appeared well mixed on scales of ≤ 10 m; by October 2002, they were well mixed on scales of ≤ 1 km.

In practical terms, this suggests that the frequency of percentage of G143A at a farm can be estimated by averaging over multiple locations. However, there is no advantage to increasing the sample size at a single location. A suitable sampling structure that can represent the whole population would be to collect many single samples in a zigzag design across the whole farm and pool them. For temporal studies, good temporal data can be obtained from any sample that covers the whole farm. There is no need to revisit individual trees.

Variation among locations may be related to variation in spray application pattern within the farm, or to chance factors. For example, one location of a farm may receive a significantly different dosage or droplet volume from another due to operator differences, differences in the topography below an aircraft flight pattern, or slight overlaps or gaps in flight paths.

Muller et al. (17) examined variability in random amplified polymorphic DNA loci of *M. fijiensis* in Nigeria; the results are consistent with ours. They found most variation in Nigerian *M. fijiensis* within locations, implying that the population was well mixed on scales of a few hundred meters, although there was significant extra variation between isolates from different geographical origins.

Relation between spray frequency and changes in resistance frequency. There was no indication that ceasing strobilurin spraying on individual farms caused a rapid decrease in G143A frequency. Spraying strobilurins undoubtedly decreased dramatically after 2000, but the frequency of G143A was essentially stable in each region.

In the period up to 2000, there seemed to be a strong relationship between strobilurin sprays and the frequency of resistance (Fig. 2A), but this was absent in the later period, when spraying was less common and resistance more widespread (Fig. 2B). A consistent picture of what was happening is the following. Initially, G143A was present at the DNA mutation frequency, probably of the order of 10^{-10} on the basis of codon mutation rates in other fungi. This will have made the farms in which it first appeared a matter of chance. The frequency then increased (Fig. 2A), suggesting an approximate doubling of frequency at each spray; this is consistent with other field measurements of selection for resistance in the field (12,15). Once any farms in a region had reached moderate (but still possibly undetectable) levels, dispersal would ensure that G143A individuals were available for selection in all farms. With intensive spraying, homogenization was relatively slower than selection, and so we see the clear relationship of Figure 2A. Once resistance had reached high-enough frequencies to affect control and spraying was withdrawn by many growers and slowed by others, homogenization by mixing was faster than continued selection, and no relationship between spraying on an individual farm and resistance could be seen (Fig. 2B); instead, farms in a region converge on a common frequency. Therefore, an approach to the management of fungicide resistance is a total withdrawal of strobilurin applications across

Fig. 2. Relationship between number of sprays and frequency of resistance in *Mycosphaerella fijiensis* **A**, up to August 2000 and **B**, between August 2000 and October 2002. Dotted line in **B** shows slope from **A**, for comparison.

Costa Rica. Because of the homogenization produced by long-distance dispersal in this windborne pathogen, such a large-scale approach also would be necessary in order to determine how fast (if at all) the G143A allele frequency would decrease in *M. fijiensis* in the absence of selection from sprays.

The same results were found in a study of benomyl resistance in *M. fijiensis*, where persistent high levels of resistance were found in farms where benomyl had not been used for 3 to 5 years; the presence of resistant isolates also was detected in a farm that had no benomyl spray application. This was believed to have been due to wind-blown immigration (24). A similar pattern also was found in *M. graminicola*; whether there was spray application or withdrawal, the isolates with G143A increased (19).

In countries where resistance has not been detected, a further experimental investigation should be conducted to study the population of *M. fijiensis* in the spray overlap zone between boundaries of banana farms owned by different companies to test the common belief that this is the source of the initial resistance build-up. In the situation described here, with good population mixing on scales of tens of meters, this has the effect of slightly increasing the average intensity of selection, but would not have a profound effect on the population dynamics.

Two questions arise from the situation described above. First, were the measures recommended by FRAC effective in slowing the emergence of resistance? Second, were there strategies which might have prevented the emergence of resistance altogether?

Because resistance reached the point where it was a serious problem in 4 years from first use and 1 year from first detection, the early FRAC recommendations can have been, at best, of marginal use. They hinged on reducing frequency of use and alternating with other chemicals. Intermittent use of a chemical only alters the relationship between sprays and selection if there is sub-

stantial selection against resistance in the absence of the chemical (27). This appears not to be the case here; therefore, increases in resistance frequency following a spray will remain indefinitely apart from changes due to drift and immigration. Alternation of active ingredients is more effective than intermittent use only if the alternated fungicide is actually more effective on the resistant form than the susceptible form. With a specific resistance, this will never be true in general, and there is little published evidence that it is true for strobilurins and their likely (but unspecified) alternates.

The extra strategies included in the current FRAC guidelines are the use of mixtures and use of strobilurins at times of low disease pressure. The effectiveness of mixtures—as opposed to using the mixture partner alone—in a situation like that described, in which disease is managed but never eliminated, depends on the alternative component or the specific mixture preferentially acting against resistant isolates. If it does not, there is no change in the extent of selection for resistance. There has been no systematic study of how chemically unrelated mixture partners select resistance alleles; however, it is unlikely to be universally true that they select against resistance. Likewise, use of a fungicide when disease is rare and conditions for spread less favorable is unlikely to reduce selection for resistance by the large factors required to substantially slow the emergence of resistance. This is because conditions will be unfavorable to both resistant and susceptible individuals alike. The *difference* in rate of growth of individuals with and without a resistance allele is not necessarily reduced and is very unlikely to be removed altogether.

The unpalatable conclusion is that, with this group of fungicides, there probably were a certain number of applications (15 to 20) to be made before resistance emerged. This number may be quite variable regionally if the initial appearance of the resistance

Fig. 3. Frequency of G143A allele in *Mycosphaerella fijiensis* (gray) in August 2000 across Costa Rica.

Fig. 4. Frequency of G143A allele (gray) in *Mycosphaerella fijiensis* in October 2001 across Costa Rica.

Fig. 5. Frequency of G143A allele (gray) in *Mycosphaerella fijiensis* in October 2002 across Costa Rica.

allele does not depend on a single class of mutation. However, it remains the case that the only choice was over what period of time to use this number of sprays. It is critical to note that individual growers cannot make this decision singly, because of the strong influence of surrounding farms. If one grower decides to conserve activity but their neighbors do not, they gain nothing by abstaining.

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