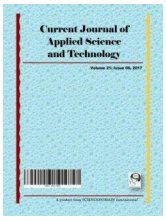

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Molecular Detection of Antimicrobial Peptide Genes and Identification of Antifungal Compounds of *Bacillus* sp. against *Fusarium oxysporum* f. sp *cubense* Causing Wilt in Banana

Rajesh Kumar Ponnusamy[1*], Paranitharan Vaikuntavasan[1], Rajesh Subramanian[2], Kavino Mathiyazhagan[3], Muthulakshmi Palanisamy[1] and Kamalakannan Ayyanar[1]

[1]Department of Plant Pathology, TNAU, Coimbatore, India.
[2]Department of Plant Biotechnology, CPMB, TNAU, Coimbatore, India.
[3]Department of Fruits Crops, HC&RI, TNAU, Coimbatore, India.

Authors' contributions

This work was carried out in collaboration between all authors. Authors KA, PV, RS, MP and KM designed the study, supervised and facilitated the research and wrote the first draft of the manuscript. Author RKP performed the experiments and analyzed the results obtained in the study. All authors read and approved the final manuscript.

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ABSTRACT

Fusarium oxysporum f. sp *cubense* (*Foc*) is a serious infectious agent that causes *Fusarium* wilt of banana within tropical and semi-tropical regions of India. It is both necessary and critical to find an efficient biocontrol strategy to ensure banana production around the world. Twenty strains of *Bacillus* species from various banana rhizosphere regions were analysed using 16s rRNA and tested against *Foc in vitro*. Among 20 strains, B18, B4 and B7 were observed to inhibit the mycelial growth of the pathogen. The *Bacillus* sp strains were analysed through PCR with specific primers for

**Corresponding author: E-mail: rajeshkumarponnusamy89@gmail.com;*

the presence of antimicrobial biosynthetic genes *viz.,* Iturin A, Iturin C, Bacillomycin A, Surfactin and Bacillomycin D. The effective strain B18 was identified as *Bacillus cereus* based on biochemical and 16s rRNA region. In addition to antimicrobial peptide gens, the crude antibiotic extract from strain B18 was highly effective against *Foc* possibly owing to the presence of diverse antifungal compounds namely, 1- Dodecene, heptadecane, Pyrrolidine, Tetradecane, 1- Hexadecanol and Nonadecane, detected and identified using Gas chromatography and Mass spectrometry (GC/MS).

Keywords: Bacillus; Fusarium oxysporum f. sp cubense; antifungal compounds; antimicrobial genes; GCMS.

1. INTRODUCTION

Banana is commercially cultivated in many tropical and subtropical areas and remains to be one among the world's most important fruit crops [1]. India has become the largest producer of bananas in the world with an annual production of 29.5 million tonnes from an area of 8.48 lakh ha (Indiastat, 2016). Among the diseases, *Fusarium* wilt or Panama disease of banana (*Musa* spp.), caused by *Fusarium oxysporum* f. sp *cubense* (E.F. Smith) "Snyder and Hansen", is the major restraint to banana cultivation and production worldwide [2]. Application of fungicides and resistant cultivars are the only management techniques available for controlling *Fusarium* wilt in banana [3,4]. The loss of non-targeted valuable soil microorganisms due to chemicals, have alerted researchers to look for alternative methods of management. *Bacillus* species carries 24 varied Anti-Microbial Peptide (AMP) genes that allow the biosynthesis of antibiotics like iturin, bacilysin, bacillomycin, fengycin, surfactin, mersacidin, ericin, subtilin, subtilosin, and mycosubtilin [5,6,7]. The generation of metabolites through bacterial cell culture with antimicrobial action is a proven way to control plant pathogens [8]. The detection of diverse antifungal compounds of *Bacillus* species by GC-MS and their strong inhibitory effects against pathogenic fungi [9,10]. Therefore, *Bacillus* species were considered effective in the production of antifungal compounds and antimicrobial peptide genes against *Fusarium* wilt of banana. The objectives of the present study include (i) Isolation and characterization of *Bacillus* spp from banana rhizosphere, (ii) Detection of diverse antibiotic genes and (iii) Identification of antifungal compounds.

2. MATERIALS AND METHODS

2.1 Isolation of Pathogen, *Fusarium oxysporum* f. sp cubense (*Foc*)

Infected banana plants showing the typical wilt symptom were collected from different varieties of banana in Tamil Nadu. Corm portion was excised from the infected plant. The infected corm was cut into 1cm bits and surface sterilized with 0.5% sodium hypochlorite solution for 30 seconds and washed thrice in the series of sterile distilled water to remove the traces of sodium hypochlorite, and transferred to sterilized Petri plates containing potato dextrose agar (PDA) medium amended with 1000 ppm of streptomycin sulphate to avoid bacterial contamination [11]. Then, the Petri plates were incubated at room temperature (27±2°C) for 7 days. The pathogen was identified through morphological and molecular characterization.

2.2 Isolation of *Bacillus* spp and Screening against *Foc*.

Twenty strains of *Bacillus* spp., were isolated from the rhizhosphere of banana. Antifungal activity of the *Bacillus* spp., was evaluated through a dual culture technique [12]. Plates were incubated at 27 ± 2◦C. After 7 days of incubation, per cent inhibition (PI) of mycelial growth was calculated with the formula proposed by Dennis and Webster [13].

2.3 PCR Amplification of Antibiotic Genes

Bacterial genomic DNA was extracted [14] and 16S rRNA interveining sequence specific BCF1 (5′CGGGAGGCAGCAGTAGGGAAT-3′) and BCR2 (5′- CTCCCCAGGCGGAGTGCTTT-3′) primer pair were used to confirm bacterial strain as *Bacillus* species described by Cano et al. [15]. These *Bacillus* species were also analysed for the presence of AMP genes with their respective primer pairs following procedures of Vinod Kumar *et al.,* 2017 and were described below for six genes.

2.3.1 Iturin A

The forward primer ITUD-1F (5'GATGCGATCTCCTTGGATGT3') and reverse

primer ITUD-1R (5'ATCGTCATGTGCTGCTTGAG3') were used for amplification of iturin A gene (647 bp) [16]. PCR conditions: 94°C for 3 min, 35 cycles consisting of 94°C for 1 min, 60°C for 30 s, 72°C for 1 min 45 s and 72°C for 6 min.

2.3.2 Iturin C

The forward primer ITUC-F (5' TTCACTTTTGATCTGGCGAT3') and reverse primer ITUC-R (5' CGTCCGGTACATTTTCAC 3') were used for amplification of Iturin C gene (506 bp) [16]. PCR conditions: 94°C for 3 min, 40 cycles consisting of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min and 72°C for 10 min.

2.3.3 Surfactin

The forward primer SUR-3F (5'ACAGTATGGAGGCATGGTC3') and reverse primer SUR-3R (5'TTCCGCCACTTTTTCAGTTT3') were used for amplification of surfactin gene (441 bp) [10]. PCR conditions: 94°C for 3 min, 40 cycles consisting of 94°C for 1 min, 57°C for 1 min, 72°C for 1 min and 72°C for 10 min.

2.3.4 Bacillomycin A

The forward primer BACA-F (5'TGAAACAAAGGCATATGCTC3') and reverse primer BACA-R (5'AAAAATGCATCTGCCGTTCC3') were used for amplification of Bacillomycin A gene (344 bp) [16]. PCR conditions: 94°C for 3 min, 40 cycles consisting of 94°C for 1 min, 57°C for 1 min, 72°C for 1 min and 72°C for 10 min.

2.3.5 Bacillomycin D

The forward primer BACC-1F (5'GAAGGACACGGCAGAGAGTC3') and reverse primer BACC-1R (5'CGCTGATGACTGTTCATGCT3') were used for amplification of Bacillomycin D gene (875 bp) [16]. PCR conditions: 94°C for 3 min, 35 cycles consisting of 94°C for 1 min, 62°C for 30 s, 72°C for 1 min 45 secs and 72°C for 6 min.

2.3.6 Extraction and bioassay of crude metabolites against *Foc*

The isolates of three antagonistic bacteria (B18, B4, B7) were incubated at 28±2° C for 7 days. After incubation they were centrifuged at 10000 rpm for 10-15 min and the supernatant was adjusted to pH 2.0 with concentrated HCl. The mixture was stirred at 100 rpm in an orbital shaker for 8 hrs. After shaking, the precipitate was collected by centrifugation, re-suspended in 1mL of l M NaOH to adjust the pH 7.0. The resultant suspension was extracted twice with ethyl acetate. The ethyl acetate phase was transferred into the vacuum flash evaporator maintained at 60°C, at 80 rpm, till this fraction gets evaporated. The crude antibiotics was re-suspended in 1mL of methanol-chloroform mixture (1:1) and used for further bioassay and GC/MS analysis [9]. The bioassay was performed by Agar diffusion method [17]. The area of inhibition was also recorded.

2.3.7 Characterization of crude metabolites by GC-MS

Volatile antifungal and anti-bacterial compounds were identified by GC-MS using a column Elite-5MS (100% Dimethyl poly siloxane), 30 x 0.25 mm x 0.25 µm of equipped with GC Clarus 500 Perkin Elmer. The turbo Mass-Gold-Perkin-Elmer detector was used. The carrier gas flow rate was 1 mL/min, split 10:1, and injected volumes were 3 µL. The column temperature was maintained initially at 110°C at the rate of 10°C/min-No hold followed by increases up to 280°C at the rate of 5°C /min-9 min (hold). The injector temperature was 250°C and this temperature was held constant for 36 min. The electron impact energy was 70 eV, Julet line temperature was set at 2000°C and the source temperature was set at 200°C. Electron impact (EI) mass scan (m/z) was recorded in the 45-450 aMU range. Using computer searches on the NIST Ver.2006 MS data library and comparing the spectrum obtained through GC/MS the compounds present in the crude sample were identified. Crude metabolites of the most effective strain of *B. cereus* B18 were analysed by GC-MS for the detection of active antifungal compounds responsible for the inhibition of *F. oxysporum* f. sp *cubense* (*Foc*) as proposed by Vinod kumar et al. [10].

2.4 Statistical Analysis

Data was analysed using IRRITSAT v. /93 software programme developed by International Rice Research Institute (IRRI), Philippines. Means differences of the treatment were evaluated with ANOVA and compared by Duncan's Multiple Range-Test (DMRT) [18].

3. RESULTS AND DISCUSSION

The *Fusarium* wilt of banana pathogen produced white cottony mycelia growth with microconidia, macroconidia and chlamydospores 7 days after incubation at 27 ± 2ºC. Based on the morphological characters, the pathogen was confirmed as *Fusarium oxysporum* f. sp *cubense*. In Addition, the *F. oxysporum* genus specific primers amplified a fragment of approximately 550 bp corresponding to the region of the 18S-23S rRNA intervening sequence for *F. oxysporum*. The amplified genomic product was partially sequenced at priority life sciences Pvt. Ltd., Bangalore, India. The rRNA homology searches were performed using the BLAST program through the internet server at the National Centre for Biotechnology Information, USA. The sequences analysis for isolates revealed that they had a nucleotide sequence identity of 98 - 100% with the *F. oxysporum*. Since, the pathogen was isolated from banana, the identified pathogen was referred as *F. oxysporum* f. sp. *cubense*. The partial sequence of *F. oxysporum* f. sp. *cubense* isolates were submitted to the NCBI, Genbank, New York, USA. (MF576349). The molecular approaches are rapidly used for species level identification. Various techniques, including rDNA PCR (rDNA-intergenic spacer length polymorphisms) and 16S–23S intergenic transcribed spacer region have been used for identification and differentiation of bacterial species. The 16S rRNA gene sequence analysis has been described to be necessary mainly to detect some misidentification of *Bacillus* and related strains [19]. In present study, *Bacillus* spp., amplified at 546 bp corresponding region confirmed through 16s rRNA and the effective isolate B18 was sequenced and shows 100% similarity in NCBI database to be identified as *Bacillus cereus* (MH046059) (Fig. 1). Among twenty isolates of *Bacillus* spp., screened against *Foc* only three isolates were identified as effective. *B. cereus* strain B18 highly inhibited the growth of mycelium up to 58.89 % over control (Table 1). Further, 75 µl of crude extracts inhibited the mycelial growth of 407.00 mm^2 area of Foc under *in vitro. Bacillus* spp. produce a broad range of AMPs such as cyclic lipopeptides such as iturins, surfactins, fengycins, bacilysin, bacillomycin, mersacidin, and subtilin [3,7,6] reported that antimicrobial peptide (AMP) biosynthetic genes surfactin, bacylisin, fengycin, bacyllomicin, subtilin and iturin was examined in

Table 1. Efficacy of *Bacillus* spp against *Fusarium oxysporum* f. sp *cubense* under *in vitro*

S. No	Isolates	Radial growth of pathogen (mm)*	Percentage of inhibition
1.	B1	51.00^{gh}	43.33 $(41.16)^{ef}$
2.	B2	64.00^{b}	28.89 $(32.51)^{k}$
3.	B3	60.00^{c}	33.33 $(35.26)^{j}$
4.	B4	40.00^{k}	55.56 $(48.19)^{b}$
5.	B5	50.00^{h}	44.44 $(41.81)^{e}$
6.	B6	58.00^{de}	35.56 $(36.60)^{hi}$
7.	B7	42.00^{j}	53.33 $(46.90)^{c}$
8.	B8	57.00^{e}	36.67 $(37.26)^{h}$
9.	B9	47.00^{i}	47.78 $(43.72)^{d}$
10.	B10	53.00^{f}	41.11 $(39.87)^{g}$
11.	B11	48.00^{i}	46.67 $(43.08)^{d}$
12.	B12	59.00^{cd}	34.44 $(35.93)^{ij}$
13.	B13	51.00^{gh}	43.33 $(41.16)^{ef}$
14.	B14	52.00^{fg}	42.22 $(40.52)^{fg}$
15.	B15	47.00^{i}	47.78 $(43.72)^{d}$
16.	B16	59.00^{cd}	34.44 $(35.93)^{ij}$
17.	B17	69.00^{a}	23.33 $(28.88)^{l}$
18.	B18	37.00^{l}	58.89 $(50.12)^{a}$
19.	B19	63.00^{b}	30.00 $(33.20)^{k}$
20.	B20	64.00^{b}	28.89 $(32.51)^{k}$
21.	Control	90.00	0.00

Values are mean of three replications
Data in parentheses are arcsine transformed values
Means followed by a common letter are not significantly different at 5% level by DMRT

Table 2. Detection of antimicrobial biosynthetic genes among of *Bacillus* spp. through PCR

S. No	Isolates	Bacillomycin D	Bacillomycin A	Iturin A	Iturin C	Surfactin
1	B1	+	+	+	+	-
2	B2	+	+	+	+	+
3	B3	-	-	+	-	+
4	B4	+	+	+	+	+
5	B5	+	+	+	+	+
6	B6	+	+	+	-	+
7	B7	+	+	+	+	+
8	B8	+	+	+	+	+
9	B9	+	-	+	-	+
10	B10	-	-	+	-	+
11	B11	+	-	-	-	+
12	B12	-	-	+	-	+
13	B13	+	+	+	-	+
14	B14	+	-	-	-	-
15	B15	-	-	-	-	+
16	B16	+	-	+	-	+
17	B17	+	+	+	+	+
18	B18	+	+	+	+	+
19	B19	+	+	+	+	+
20	B20	+	-	+	+	+

(+) = Positive *(-) = Negative*

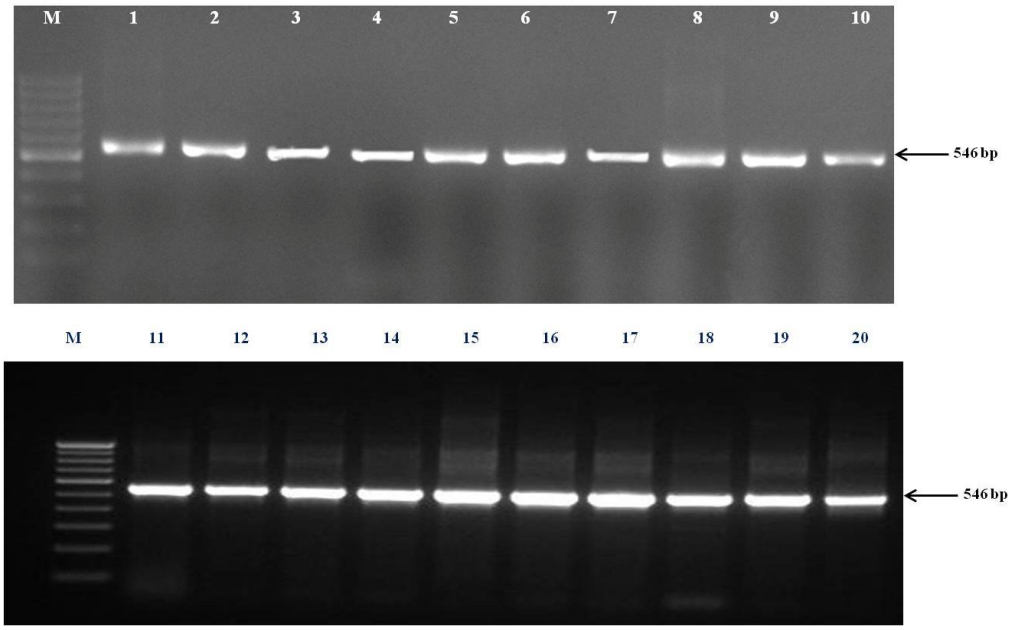

Fig. 1. PCR amplification of 16S-rRNA region of *Bacillus* isolates

Table 3. Antifungal compounds produced by *Bacillus cereus* strain B18 and identified by GC-MS.

S. No	Retention time (min)	Chemical name	Molecular formula
1.	2.55	1-Dodecene	$C_{12}H_{24}$
2.	13.97	Heptadecane	$C_{17}H_{36}$
3.	18.75	Pyrrolidine	$C_{22}H_{41}NO_2$
4.	22.49	1-Tetradecene	$C_{14}H_{28}$
5.	32.48	Tetradecane	$C_{17}H_{36}$

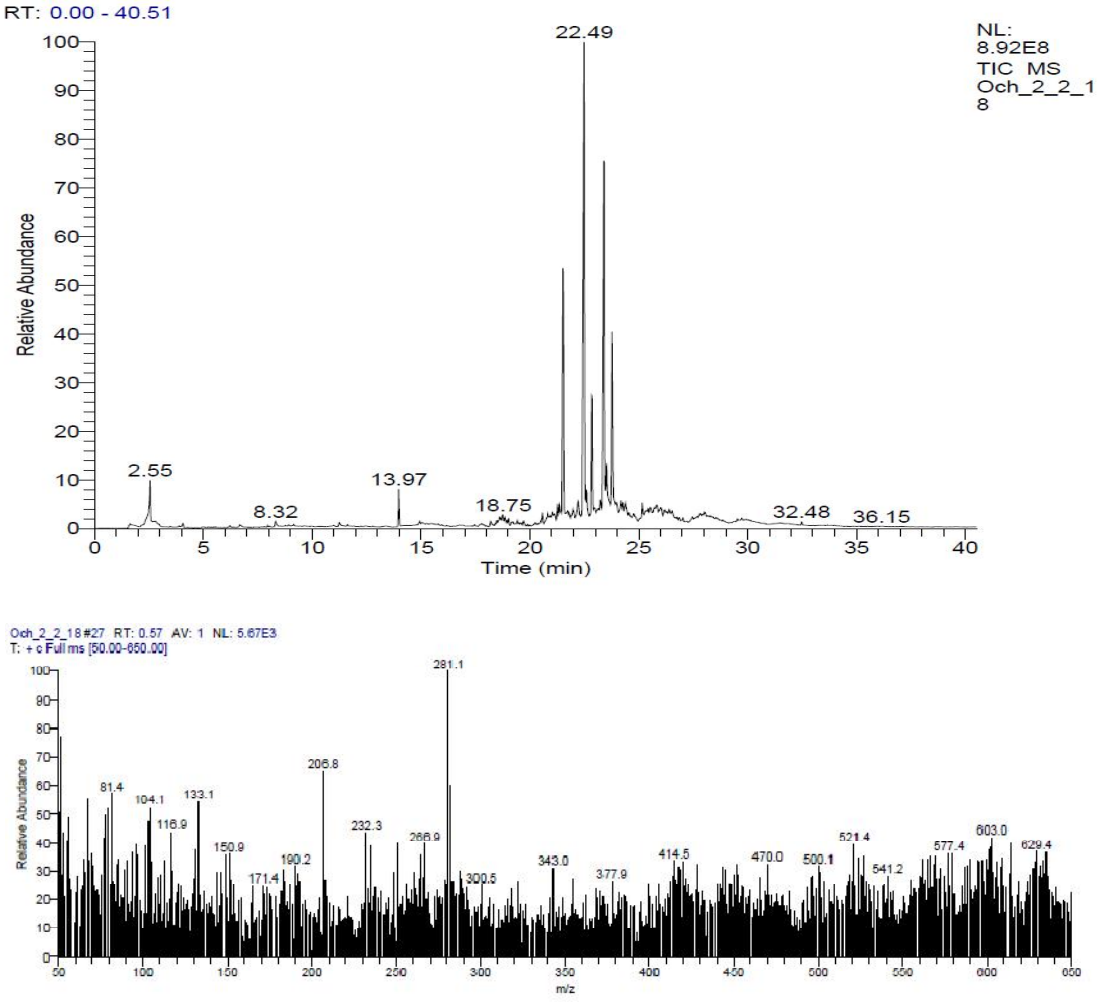

Fig. 2. Total Ion Chromatogram (TIC) and Mass Spectra (MS) of antifungal compounds identified from *B. cereus* strain B18 using GC/MS.

184 isolates of *Bacillus* spp., from rhizosphere, and non-rhizosphere regions of Mediterranean farm land area in Spain. Most strains had between two and four AMP genes. Out of ten AMP genes were found from 55 *Bacillus* spp., only which *B. amyloliquefaciens* (VB7) highly inhibited the growth of *S. sclerotiorum* [10] that supported the finding of present investigation in Banana. The AMP biosynthetic gene of iturin penetrates cell membranes and it leads to cellular leakage [20]. Zhang et al. [21] reported that bacillomycin produced by *B. cereus* disturbed the plasma membrane of *R. solani* abnormality in conidia and mycelia were also observed. In our study, the 20 isolates of *Bacillus* spp, five AMP genes associated to the antibiotics iturin A, iturin C, bacillomycin A, bacillomycin D, and surfactin were prevalent. The effective *Bacillus* spp., with potential strains of *B. cereus* strain B18 and B4, had five AMP genes responsible for the biosynthesis of antibiotic genes (Table 2). *Bacillus* spp., are capable of producing an ample variety of secondary antifungal metabolites that are varied in structure and function. Production of antifungal metabolites determines the ability of *Bacillus* species to control plant pathogens. The crude metabolites of *B. cereus* strains B18 were analysed by GC/MS to identify their production of antifungal compounds (Fig. 2). Mainly vital antifungal compounds detected were, 1-dodecane, heptadecane, pyrrolidine, 1-tetradecene and tetradecane (Table 3). Similarly, the results are also supported by findings of Zhang et al. [22], which insist the crude lipopeptides extracted from cell-free

culture broth of *B. amyloliquefaciens* strain TF28 exhibited a wide-spectrum of antifungal activity, but it strongly inhibited the growth and spore germination of *F. moniliforme.* Crude extracts of *B. subtilis* have been reported to inhibit mycelial growth in *F. oxysporum* f. sp *gerberae* [23]. Dodecanoic acid belonging to fatty acid with antibacterial and antifungal activity [24]. Too, Wang et al. *[25]* have identified 18 antifungal compounds produced by *B. amyloliquefaciens* W19 strain with high significant antagonistic effect on *F. oxysporum* f. sp *cubense*. Possibly, the biocontrol by bacteria can be owing to the production of AMP genes, antifungal and antibacterial inhibitory compounds.

4. CONCLUSION

Management of *Fusarium* wilt of banana usually relies on several control methods. Hence, a study was conducted to understand the role and efficacy of antimicrobial genes and antifungal compounds from *Bacillus* spp., by extracting crude metabolites. GC/MS analysis of crude metabolite extract of the effective *Bacillus cereus* strain B18 revealed active antifungal compounds responsible for inhibition of *Foc*. Results showed five amplified AMP genes from all *Bacillus* spp*.,* Therefore, the antifungal and antibacterial compounds identified through present investigation would help to control the *Foc* pathogen causing wilt in banana.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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