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Genetic diversity and pathogenic variability among different isolates of *Fusarium udum* causing wilt disease in pigeonpea (*Cajanus cajan* (L.) Millsp.)

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Abstract
Genetic diversity and pathogenic variability among fourteen *Fusarium udum* isolates collected from different climatic conditions of India were studied. All the isolates exhibited variable levels of virulence against a susceptible pigeonpea cultivar (T-21). The genetic diversity allelic variations among these isolates were estimated using RAPD molecular markers. All the ten RAPD primers were found to be highly reproducible and produced a total of 114 bands of which 113 were polymorphic bands and showed 99.17% polymorphism. Primer OPG 3 amplified highest number of polymorphic bands (17). All the primers except OPG 4 showed 100% polymorphism. Genetic similarity was calculated using Jaccard's similarity coefficient and cluster analysis was used to generate a dendrogram showing relationships between them. The dendrogram grouped the isolates in two major groups A (12 isolates) and B (2 isolates) and the group (A) sub divided into three groups based on molecular analysis. The similarity coefficient values for fourteen isolates of *F. udum* ranged between 0.25 (DHO and BRC) to 0.61 (GHED and KNW). The average genetic similarity coefficient observed was 0.39±. The higher value of similarity coefficient close to one was indicative of close resemblance between the different isolates of *F. udum.* Result reflecting correlation of similarity with pathogenicity to some extent. However, there may not be a clear correlation of genetic molecular markers (RAPD) with the virulence, physiological characters and geographical region, however, studies indicate some correlation under specific instances of general nature as observed in the present study

Keywords: *Fusarium udum*, *Fusarium* wilt, pathogenicity, pigeonpea, RAPD

1. Introduction
Pigeonpea (*Cajanus cajan* (L) Millsp.) is one of the most extensively grown legume crops in India, accounting for almost 90% of world's area and production [1]. Vascular wilt of pigeonpea caused by *Fusarium udum* is the most important disease, causing more economic damage to the crop. The fungus can survive on infected plant debris in the soil for about three years and causes serious yield losses, some times upto 100% in susceptible cultivars [2]. The total production loss due to this disease in India alone was estimated to be approximately 97,000 tones per year [3]. Use of resistant cultivars is the most practical and economical method for any disease management practices. However, in case of vascular wilt caused by *F. udum*, deployment of resistant varieties may become extensive because of the high level of genetic variability in the pathogenic population [4]. Moreover, *F. udum* isolates from the same site/host have been shown to exhibit high variability in cultural characteristics [2, 5-7]. Pathogenicity tests are the only means of determining the pathological effect of fungal strains present in diseased plants or in soil samples. However, in some pathosystems, race identification by pathogenicity assays provides very little information about genetic diversity within, or relatedness among races of the pathogen. These assays are cumbersome, time-consuming, require extensive facilities and are often influenced by the conditions of the experimental system. Therefore, characterization of genetic variation among pathogenic isolates of *F. udum* may be a primary step to understand and correlate strain variation with regard to population structure.
Several researchers have grouped *Fusarium* sp population from different plant host by using randomly amplified polymorphic DNA (RAPD) analysis and suggested that RAPD markers can be a quick and reliable alternative for differentiating isolates of *Fusarium* sp. into their respective pathogenic groups [8]. RAPD markers have been used for analysis of genetic diversity among different *F. oxysporum* formae specials [9, 10]. However, no comprehensive

effort has been made to investigate genetic and pathogenic variability among *F. udum* isolates obtained from various agro-climatic zones of India. The present study was aimed to determine the degree of cultural and pathogenic variability among the *F. udum* isolates collected from different pigeonpea growing districts of Rajasthan and other state of India and to estimate the genetic relatedness by using RAPD markers.

2. Material and Method

2.1 Collection of isolates

Out of fourteen isolates of *Fusarium udum* Butler, four isolates obtained from pigeonpea plants of Rajasthan state [Dholpur, Jaipur, Kota and Navgaon (Alwar)], one from M.P. (Khandwa), two from M.S. (Akola, Wardha) and seven from Indian Type Culture Collection (ITCC), New Delhi. Pure culture of these isolates obtained through single spore isolation technique, were maintained on potato dextrose agar slants for further investigation.

2.2 Pathogenicity Test

Pathogenicity of these isolates was tested both in sterilized and unsterilized soil on the susceptible pigeonpea variety T-21. Inoculum of each isolate was multiplied on sterilized sand maize flour medium (partially broken maize grains 10g, sand 10g, and 20ml distilled water in each 250 ml Erlenmeyer's flask). The flask containing the sterilized media was inoculated with pure culture mycelial disc of *F. udum* (5 mm diameter) and inoculated at 28 ± 1^0 C for 15 days. These inocula were used for soil inoculation in ratio of 1: 200 w/w basis and was added in disinfected burnt earthen pots (60 cm diameter) maintained in triplicates. Seed samples were surface sterilized with 0.1 per cent mercuric chloride for 30 second. Five seeds of susceptible variety T-21 were sown in each pot. Observations were recorded after 40 days of sowing.

2.3 RAPD analysis

Genomic DNA was isolated from mycelial mats of isolates raised on Czapek's dox broth medium. The mycelial mats collected through filtration were dried between layers of tissue paper and frozen in liquid nitrogen in foil packets. The tissue was gently broken into fine powder in mortar with a pestle. Five gram of fungal mycelium was homogenized in liquid nitrogen and processed for DNA isolation as protocol described by Doyle and Doyle (1990) [11]. DNA was purified giving RNase treatment (50mg/ml of DNA solutions for 30 min at 37^0 C). The DNA was re-extracted once with chloroform: isoamyl alcohol, and precipitated with two volumes of absolute ethanol. The genomic DNA pellets were washed once with 70% ethanol and dried under vacuum and dissolved in $T_{10}E_1$ (pH 8.0) buffer. Re-suspension of the DNA gave clear, colorless, viscous solution.

Quantification of DNA was performed by running 5.0 µl DNA solutions on 0.8 per cent agrose gel with known concentration of uncut lamda DNA. Concentration was adjusted to 25 ng /µl for use in RAPD-PCR.

RAPD Primers were obtained from Operon Technologies Inc, USA. In present experiment, 10 primers of B and G series were screened with all isolates of *Fusarium udum*. PCR was performed in 25 µl reaction volume containing 1.5 unit Taq DNA polymerase, dNTPs, 20mM Tris HCl (pH8.0); 50mM KCl; 1.5 mM MgCl2, 0.5 ng primer and 25-50 ng of genomic DNA. The reaction mixture was vortexed and centrifuged briefly. DNA amplification was performed in a "Biometra Thermo cycler" using the cycling parameters : Cycle 1: Denaturation 94°C for 5 Minutes, Primer annealing 37°C for 1 Minute, Primer extension 72°C for 2 Minutes; Cycle 2- 43: Denaturation 94°C for 1 Minute, Primer annealing 37°C for 1 Minute, Primer extension 72°C for 2 Minutes; Cycle 44: Denaturation 94°C for 1 Minute, Primer annealing 37°C for 1 Minute, Primer extension 72°C for 7 Minutes.

DNA amplification was performed with the temperature profiles: 94° C for 5 min to denature genomic DNA, 37° C for 01 min for primer annealing and 72° C for 2 min for primer extension. The PCR products (10µl) were resolved by gel electrophoresis by using 1.2 per cent agarose gel in 0.5x Tris Borate EDTA (TBE) buffer, at 50 V for 4-5 h. Gel was observed in UV Transilluminator and photographed by digital camera. In the control treatment, PCR reaction mixture was supplemented with sterile distilled water instead of genomic DNA. One kb DNA ladder was used as DNA markers.

All fingerprint data were converted into a binary matrix based on the presence or absence of individual amplified bands for each isolate. An RAPD band of particular molecular weight present in an isolate was marked as 1 and absence was recorded as 0. The cluster analysis was performed by using un-weighted pair group method and arithmetic average (UPGMA) to distinguish *F. udum* isolates.

The computer software NTSYS-PC Version 2.02c [12] was used for data analysis. Pairwise association coefficients were calculated from qualitative data matrix Jaccard similarity coefficient [13].

3. Results and Discussion

3.1 Pathogenicity Test

All the fourteen isolates tested for their pathogenic nature, using susceptible T-21 variety of pigeonpea were found virulent in sterilized as well as in unsterilized soil. The isolate DHO was highly virulent followed by BHU, AK, NGN, WR, JND, KOTA, KNP, KNW, DLI-1, JP, GHED, BRC and DLI-2. The wilt incidence (%) in sterilized soil and unsterilized soil varied from 20.00 to 100 and 13.33 to 73.33, respectively. In unsterilized soil the reaction of isolates was less and delayed as compared to sterilized one. In general the mortality of plants in unsterilized soil was reduced by 26.67 to 50.00 per cent over sterilized soil. There was practically no direct correlation between the cultural characters and virulence in any of the isolates. Inoculation with *F. udum* was produce more wilt of pigeonpea in sterilized than in unsterilized soils at the same pH [14]. Sukumar and his associates used susceptible T-21 variety of pigeonpea for testing the pathogenic nature of fourteen isolates of *F. udum* as being done in present studies [15]. Rangaswaray and his associates studied pathogenic variations in the isolates of *F. udum* collected from Warangal, Khammam and Ranga Reddy districts of Andhra Pradesh and categorized as highly pathogenic and weak pathogenic thereby confirming the present findings [16].

3.2 Genetical studies

3.2.1 DNA isolation

Intact high molecular weight Fusarium nuclear DNA is essential for successful RAPD analysis. Earilier methods of DNA isolation involving sodium dodecyl sulphate and various enzymes, were not only costlier but also cumbersome, time consuming and yielded less plant and fungal DNA. With the advent of molecular technologies one can overcome these limitations and generate useful information for fungal characterization [17]. The ditergent CTAB (hexa decyltrimethyl ammonium bromide) used to eliminate

polysaccharides generally known to effect the quality and thus digestibility of DNA [18].

The DNA isolated using the CTAB method from all the isolates of *Fusarium udum* occupied a position in the gel higher to 200 bp lambda indicating high molecular weighty DNA. (Table 1 & Plate 1) The DNA bands were observed to be sharp, no streaking or very less streaking is suggestive of negligible DNA shearing [19, 20].

3.2.2. RAPD analysis

The operon random decamer primers procured from OPERON TECHNOLOGY are most commonly used arbitrary primer. They generated a significant information. Most of the primers produced fragments below 200 bp. The pattern of amplification has been found to be isolates and primer dependent. Not only the size of fragments were variable but number of bands generated by different primers were also much variable. A total of 114 bands generated, out of which 113 were polymorphic bands and showed 99.17% polymorphism. All the primers except OPG 4 produced all polymorphic bands (Table 2, Plate 2 & 3). Variation in size and number of amplicons generated by various arbitrary primers in *Fusarium* has also been reported [21, 22].

3.2.3. Genetic relationship among the isolates and cluster analysis

A considerable level of diversity (61%) revealed among the isolates collected from diverse climatic conditions. Higher level of diversity is important for adaptability to the pathogen over wider range of environment. The lower Jaccard's similarity coefficient of DHO – NGN (0.28) and DHO – KNW (0.27) showed that they are more diverse from each other and the isolates from DLI-2 – GHED (0.61), DLI-1 – GHED (0.57), KNP – WR (0.56) and KNW – KOTA (0.55) with higher similarity index were found more closer to each other. (Table 3).

The dendrogram constructed on the basis of RAPD patterns showed that isolates from Dholpur (DHO) and BHU were different from all other isolates and recorded highest genetic distance (66.5%) from other isolates. (Fig. 1) The dendrogram grouped the isolates in two major groups. One major group (A) consisting of 12 isolates and another (B) consisting of only two isolates DHO and BHU. The group (A) can be sub divided into three groups. A^1 consisting of seven isolates A^2 two isolates and A^3 three isolates. Five of the group A^1 isolates *viz*, DLI-I, GHED, KNW, DLI-2, KOTA and BRC are more closely related with 50% similarity are happened to less virulent. At the same time the two isolates of group (B) DHO and BHU that are most divers from rest of the isolates (66.5%) are most virulent (100% DHO and 86% BHU). Rest of the isolates fall in the moderate virulence. (Fig. 1) This indicates grouping on the basis of molecular markers correlate with virulence level.

RAPD analysis of *Fusarium udum* isolates were studied and correlation between pathogenicity and polymorphism was established [21, 23]. High level of DNA polymorphism also recorded using molecular markers [24]. They also suggested the rapid evolutions of new recombinants of the pathogen in pigeonpea growing regions. Four isolates each of *F. oxysporum* f. sp. *ciceri*, *F. oxysporum* f. sp. *lentis* and *F. udum* were analyze using RAPD primes of OPI, OPA and OPX series from Operon Technologies. The isolates grouped into two major cluster, *F. oxysporum* f. sp. *ciceri* and *F. oxysporum* f. sp. *lentis* forming one cluster, further divided in two sub-clusters according to their formae speciales, while *F. udum* isolates grouped into a separate cluster and showed maximum homogeneity among isolates [25]. The discriminatory power of different primers calculated according to Hunter and Gaston [26] has been presented in Table 2. Primers OPB 2, OPB 6, OPG 1, OPG 3, OPG 4 and OPG 7 have discriminatory power (D) as 1. This indicates that these six primers generated unique banding pattern for all the isolates and the isolates can be identified by a single primer. Such primers discriminating all the isolates have been reported by Monga and his associates [27]. These primers can add to identification of isolates prevailing the area and could prove important in formulating disease control strategies. This also indicates higher level of polymorphism available in the genome.

4. Conclusions

However, there may not be a clear correlation of genetic molecular markers (RAPD) with the virulence, physiological characters and geographical region, however, studies indicate some correlation under specific instances of general nature as observed in the present study.

Table 1: DNA yield based on absorbance (260 nm) obtained from various isolates of *Fusarium udum*

Isolate	OD at 260 nm	OD at 280 nm	Ratio of A_{260}/A_{280}	Conc. of DNA µg/ µl
AK	0.289	0.206	1.40	1.45
BHU	0.392	0.228	1.72	1.96
BRC	0.125	0.064	1.95	0.63
DHO	0.375	0.231	1.62	1.88
DLI-1	0.157	0.067	2.34	0.79
DLI-2	0.124	0.045	2.76	0.62
GHED	0.236	0.140	1.69	1.18
JND	0.182	0.066	2.76	0.91
JP	0.180	0.085	2.12	0.90
KNP	0.263	0.124	2.12	1.32
KNW	0.134	0.076	1.76	0.67
KOTA	0.253	0.126	2.01	1.27
NGN	0.376	0.227	1.66	1.88
WR	0.089	0.032	2.78	0.45

Table 2: Analysis of the polymorphism obtained with random primers among various isolates of *Fusarium udum*

Primers	Sequence (5'-3')	Total No. of bands (a)	No. of polymorphic bands (b)	Polymorphism b/a x 100 (%)	Discriminativ power (D)
OPB-1	GTTTCGCTCC	10	10	100	0.99
OPB-2	TGATCCCTGG	12	12	100	1.00
OPB-5	TGCGCCCTTC	10	10	100	0.98
OPB-6	TGCTCTGCCC	11	11	100	1.00
OPB-11	GTAGACCCGT	10	10	100	0.99
OPG-1	CTACGGAGGA	12	12	100	1.00
OPG-3	GAGCCCTCCA	17	17	100	1.00
OPG-4	AGCGTGTCTG	12	11	91.67	1.00
OPG-7	GAACCTGCGG	10	10	100	1.00

OPG-15	ACTGGGACTC	10	10	100	0.97
	Total	114	113	991.67	
	Avg.	11.40	11.30	99.17	

Table 3: Similarity matrix generated by Jaccard's similarity coefficient for fourteen isolates of *Fusarium udum* Butler

Isolates	DLI-1	DLI-2	DHO	GHED	JP	JND	KNP	KNW	KOTA	NGN	BRC	WR	AK	BHU
DLI-1	1.0000													
DLI-2	0.4872	1.0000												
DHO	0.3250	0.3415	1.0000											
GHED	0.5753	0.6081	0.3133	1.0000										
JP	0.4474	0.4615	0.3867	0.4868	1.0000									
JND	0.3867	0.3671	0.3243	0.4459	0.3973	1.0000								
KNP	0.3837	0.4643	0.3780	0.4186	0.4268	0.3704	1.0000							
KNW	0.5479	0.4810	0.2738	0.6111	0.4051	0.4000	0.3793	1.0000						
KOTA	0.4167	0.4819	0.2989	0.4699	0.3929	0.4231	0.4824	0.5584	1.0000					
NGN	0.3690	0.3523	0.2824	0.3563	0.3614	0.3544	0.4186	0.3488	0.4524	1.0000				
BRC	0.3875	0.4024	0.2500	0.4615	0.3625	0.3733	0.3721	0.5135	0.5128	0.4074	1.0000			
WR	0.3529	0.3523	0.2529	0.3258	0.3780	0.3210	0.5641	0.3647	0.4186	0.3882	0.2955	1.0000		
AK	0.4217	0.3556	0.2874	0.3908	0.3810	0.4103	0.3889	0.3678	0.4205	0.3596	0.3605	0.4070	1.0000	
BHU	0.3095	0.4250	0.3867	0.2989	0.3671	0.3421	0.3605	0.3537	0.3765	0.3452	0.3133	0.3294	0.3182	1.0000

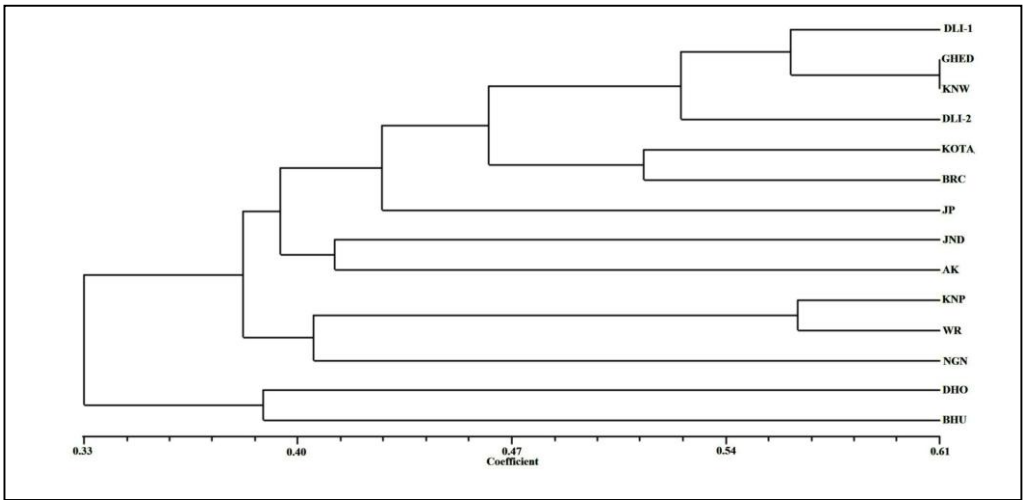

Fig 1: Dendrogram showing relationships among *Fusarium udum* isolates generated by UPGMA analysis bases on RAPD data

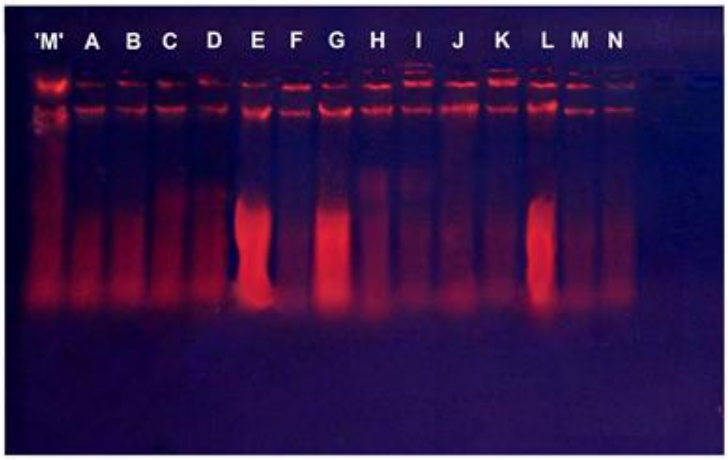

Plate 1. RNase treated high molecular weight *Fusarium udum* genomic DNA

'M'- Lambda DNA marker

A - AK (Akola) B - BHU (BHU-5241) C - BRC (Vadodara)
D - DHO (Dholpur) E - DLI-1 (Delhi-781) F - DLI-2 (Delhi-1050)
G - GHED (GHED-4874) H - JND (Junagarh-4873) I - JP (Jaipur)
J - KNP (Kanpur-4968) K - KNW (Khandwa) L - KOTA
M - NGN (Navgaon, Alwar) N - WR (Wardha)

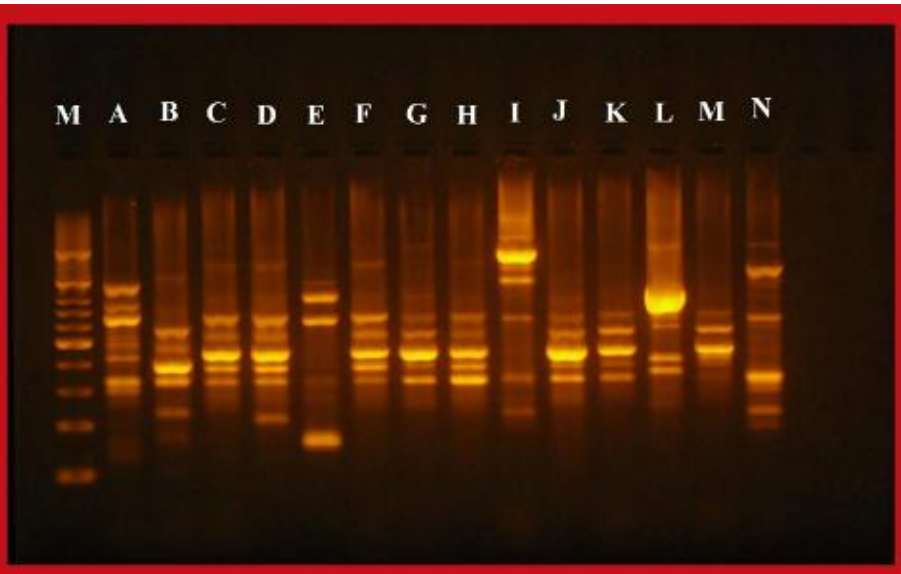

Plate 2. RAPD patterns generated by OPB-11 in isolates of *Fusarium udum*

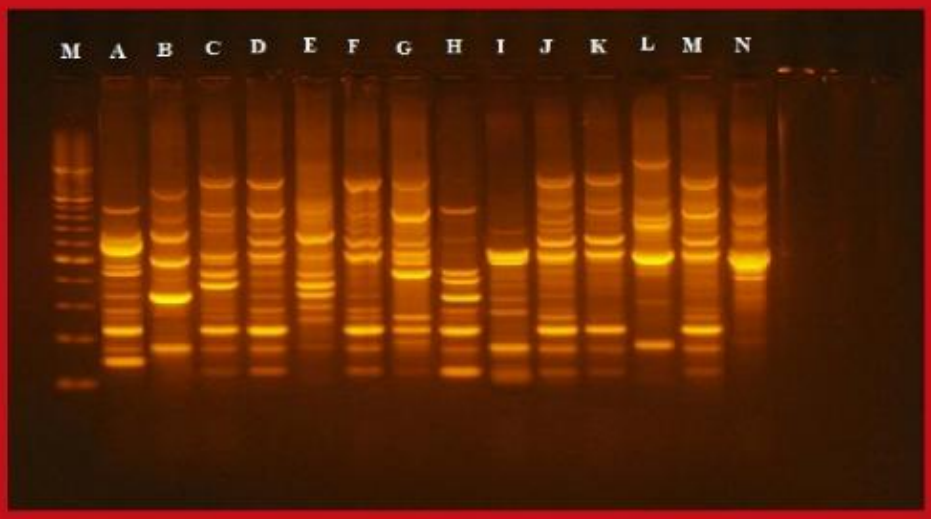

Plate 3. RAPD patterns generated by OPG-3 in isolates of *Fusarium udum*

M - Lambda uncut DNA marker

A - AK (Akola)	B - BIIU (BIIU-5241)	C - BRC (Vadodara)
D - DHO (Dholpur)	E - DLI-1 (Delhi-781)	F - DLI-2 (Delhi-1050)
G - GHED (GHED-4874)	H - JND (Junagarh-4873)	I - JP (Jaipur)
J - KNP (Kanpur-4968)	K - KNW (Khandwa)	L - KOTA
M - NGN (Navgaon, Alwar)	N - WR (Wardha)	

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