GENETIC DIFFERENTIATION OF COTTON CULTIVARS BY POLYACRYLAMIDE GEL ELECTROPHORESIS

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Manuscript received: October 27, 2004; Reviewed: March 2, 2005; Accepted for publication: March 12, 2005

ABSTRACT

Information about genetic diversity and relationships among the breeding materials has a significant impact on crop improvement. Association between parental divergence and progeny performance has not been well documented in cotton. A cotton study was conducted in the department of Plant Breeding and Genetics, University College of Agriculture, Bahauddin Zakaryia University, Multan, (30.2°N, 71.4°E) Pakistan and National Food Research Institute, Tsukuba, Japan. The objective of this study was to estimate genetic diversity among selected cotton genotypes and their reciprocal crosses. Six U.S. and two local (Pakistani) cultivars all belonging to Gossypium hirsutum L were genotyped by polyacrylamide gel electrophoresis and compared with BioRad molecular weight markers. Cluster analysis revealed low genetic diversity among the parents and pooled the crosses with their parents, indicating success of hybridization. The present study, combined PAGE analysis with cluster analysis confirmed the genetic similarities between parents and their crosses while it also confirmed the dissimilarities between the parents as showed by the morphological characters.

KEY WORDS: Cluster analysis, cotton, genetic diversity, Gossypium hirsutum & polyacrylamide gel electrophoresis

INTRODUCTION

Knowledge about the degree and distribution of genetic diversity and relationships among breeding materials has a significant effect on any crop improvement program. Selection of suitable parents is one of the most important criteria used to allocate resources to the most promising crosses and increase the efficiency of breeding programs Gutierrez et al. [6]. Molecular studies increasingly play an important role in crop improvement programs. Genetic variability at the molecular level in plants has been analyzed by various techniques: DNA restriction fragment length polymorphism (RFLPs) Helentjaris et al. [8], isozymes, Glaszman [4], Kochko [11] and random amplified polymorphic DNA (RAPDs), Halward et al. [7]. However, seed protein profiles, Ladizinsky and Hymowitz [12] are still powerful tools for determining genetic homology at the molecular level and for solving problems in systematic methodology, Murtaza et al [14]. Numerous seed protein profile studies have been done with various plant species, such as rice (Gramineae) Aliaga-Morel et al. [1], Capsicum sp. (Solanaceae), Panda et al. [15], Ricinus communis (Euphorbiaceae) Sathaiah and Reddy [17], Manihot sp. (Euphorbiaceae), Grattapaglia et al. [5], and Arachis sp. (Leguminosae) Bianchi-Hall et al. [2], Lanham et al. [13]. However, in this study we used this technique to know the genetic differences and similarities between different cotton G. hirsutum L. cultivars and their crosses.

MATERIAL AND METHODS

A cotton study was conducted on a field at the department of Plant Breeding and Genetics, University College of Agriculture, Bahauddin Zakaryia University, Multan, (30.2^{o}N, 71.4^{o}E) Pakistan. The eight varieties of cotton were chosen based on comparable maturity duration and presence of one or more non-preference traits for insect pests (Table 1). The analysis of cotton seed storage proteins by polyacrylamide gel electrophoresis was conducted at Enzyme Lab, National Food Research Institute, Tsukuba, Japan.

Glasshouse cultivation: Seeds of the parental cultivars were grown in 30 x 30 cm earthen pots containing a mixture of equivalent volumes of sand, soil and farm yard manure from mid November 2000 to mid March 2001 in the greenhouse. Temperature in the glasshouse was maintained at 30°C during the day and 25°C at night by using steam as well as electric heaters. The plants were exposed to natural sunlight and supplemented with artificial lighting, a photoperiod of 16 hours. Seedlings were thinned to one plant per pot after two weeks of planting and after every 14 days 0.25 g of Urea (46% Nitrogen) was added to each pot, plants were watered daily. Crosses were attempted among eight parental cultivars to obtain 56 F_1 (direct and reciprocal) crosses. Parental cultivars were maintained through self pollination.

Field evaluation: The eight varieties of cotton along with their 56 hybrids were planted on a clay loam soil on June 1, 2001. The experimental design was a triplicate randomized complete block design. The growth protocol was identical for all the genotypes. The experimental plot was a 3.3-meter single row with intra and inter row distances of 30 and 75cm, respectively. The F_1 hybrid and parents were self-pollinated to raise F_2 progeny. F_2 progeny was sown in the same field also in a triplicate randomized complete block design (RCBD) on first of June 2002. The plot size for each cross in each replication was 3.3 x 6 meters. Ten plants in F_1 generation in each replication were randomly selected for data recording. Sample size for F_2 generation was sixty competitive plants in each replication. The matured bolls were hand picked after every two weeks as soon as bolls started to open F_1 and F_2 for both the generations 150 days after planting (DAP) for three harvests and seed cotton was collected in Kraft paper bags. Picking was done when the dew had evaporated.

Seed material: The cotton seeds used for protein extraction were collected from eight parent cotton cultivars and their 56 F_2 progenies derived by complete diallel crossing.

Protein extraction: Ten seeds of each accession were dehulled. The kernels were then ground with a mortar and pestle to produce fine flour in liquid nitrogen. Flour (0.4g) was suspended in 6 ml of reagent grade water. The suspension was agitated for 30 minutes in a shaker at room temperature 24^{o}C and the suspension was centrifuged at 10,000 RPM (12,000 g) in a refrigerated centrifuge for 15 minutes at 10°C. The supernatant was then filtered through No. 5A filter paper. The crude protein extract thus obtained was stored in micro centrifuge tubes under refrigeration until its use.

Electrophoresis: The disc electrophoresis system described by Davis [3] including resolving gel and stacking gel was followed with a little modification as reported by Khan [10]. The gels were prepared as follows:

Resolving gel: Tris-HCI buffer stock solution, pH 8.9, acrylamide stock solution and reagent grade water were mixed in an Erlenmeyer flask with a side arm. After adding ammonium persulfate solution to this mixture, it was

swirled gently to avoid the formation of air bubbles, and it was degassed. After this, the solution was immediately pipetted into the prepared gel moulds to a height of 12cm and carefully overlaid with reagent grade water. The gel was left undisturbed for one hour at room temperature in light to polymerize.

Stacking Gel: Tris-HCI stock solution, pH 6.7, was mixed with acrylamide stock solution and reagent grade water in an Erlenmeyer flask with a sidearm. The solution was degassed thoroughly with vacuum. Ammonium persulfate solution was added while swirling gently to ensure proper mixing. A comb was inserted into each gel mould and immediately the gel solution was gently overlaid with reagent grade water and was then left at room temperature exposed to fluorescent light for 1 hr. to polymerize.

Application of samples: 30 ul of each protein sample was loaded into the wells.

Gel Electrophoresis: After loading of the protein samples in the wells, the process of electrophoresis was conducted using an LKB 2001-001 Vertical electrophoresis unit with LKB 2197 constant Power Supply Unit. The chamber accommodated two gels (10 wells each) during each run. Current was kept constant at 40 milliamperes. Each run took from 5-6 hours to complete when dye front was 1 cm from lower end of gels. Temperature of the buffer and gels was kept constant at 10°C, with an LKB 2219 Multi Temp II Thermostatic Circulating Liquid Cooler.

Fixing and Staining: Immediately upon completion of the electrophoresis, the gels were removed and immersed in the staining solution containing 100 ml of 10% acetic acid and 100 ml of stain concentrate (Coomassie Blue R-250). Fixing and staining time was one hour.

De-Staining: Gels were destined in the first destining solution containing 200 ml 95% ethanol and 300 ml 5% acetic acid for 30 minutes. The final destining was done in the second destaining solution containing 150 ml 95% ethanol and 350 ml of 5% acetic acid for 12 hours or overnight.

Photographs: Photographs of the gels were taken after de-staining. Gels were being laid directly on to an illuminator with an opal white screen (avoiding trapped air bubbles) and kept wet during photography by addition of 7 % acetic acid with a Cannon camera.

Analysis of protein bands: The numbers of protein bands revealed by the gels were recorded as present or absent. For analysis, each band was assigned a value of zero (0) when absent, or scored 1 to 4 depending upon their density and sharpness; 1= large band to 4= minor band, when present.

Cluster analysis was performed to provide a statistical basis to establish the number of cluster represented by the 64 genotypes. A clustering procedure (hierarchical cluster analysis) was performed using the unweighted pair group mean with arithmetical averages (UPGMA) method of Sneth and Sokal [18], using computer programme of SPSS/PC+. The output of this analysis was used to derive a dendrogram using PROC TREE, which showed the phylogenetic relationships among all the genotypes.

RESULTS AND DISCUSSION

The results of agglomeration schedule (Table 2) showed the most closely related cultivars/ crosses, which were combined at stage first at coefficients 1.00. This stage had three sub clusters comprised of six genotypes, in which parent 6 (Stoneville-857) and parent 7 (B-557) act as one of the parent. First sub cluster comprised of crosses 49 and 56, while the second consisted of 6 and 34, the third have 36 and 53. The most dissimilar was parents 1 (Laokra 5.5) and parent 4 (Glandless4195-220) which had a coefficient of 46.81.

The dendrogram verified that the bulk of the genotypes were clustered in seven groups (Fig. 5). The first cluster (A) was comprised of thirteen genotypes and made nine sub clusters. In the first sub cluster, the genotypes/ crosses 49 & 56 formed a sister group relationship with a coefficient value of 1.00. Similarly, the second sub cluster contained parent 6 and cross 34 with coefficient value of 1.00. The third sub cluster was constituted of parent 7 and cross 49 with 1.5 coefficients. This cluster (A) include parent 7 (B-557) and parent 6 (Stoneville-857) with their direct and reciprocal crosses along crosses with other parents, i.e. crosses 49, 56, 34, 44, 20,51,27,54,45,48 & 28.

In the second cluster (B), consisted of three crosses i.e. 35, 46 & 55. The cross 35 combine at stage 23 with cross 46 with a coefficient of 5 to make a group, while it make another group with cross 55 with a coefficient of 7.5 at stage 33 respectively. This cluster (B), have crosses of parents, 6, 7, 4 &5, and falls in between clusters A and C. The parent 6 (Stoneville 857) & 7 (B-557) were present in Cluster (A), while parent 4 (Glandless4195-

Table 1: Particular attributes of cotton cultivars

Sr. No.	Cultivar	Distinctive feature
1	Laokra 5.5	Okra type leaf (L^oL^o)
2	DPL -7340-424	Nectariless ($ne_1ne_1ne_2ne_2$)
3	Fregobract	Fregobracts
4	Glandless 4195-220	Glandless
5	SA100	Red leaves (R_1R_1)
6	Stoneville-857	Nectariless ($ne_1ne_1ne_2ne_2$)
7	B-557	Obsolete local cultivar
8	S-14	High ginning outturn local cultivar

Table 2. List of complete set of diallel crosses

Parents/ Self:
P1 = Laokra 5.5
P2 = DPL-7340-424
P3 = Fregobract
P4 = Glandless 4195-220
P5= SA-100
P6= Stoneville 857
P7= B-557
P8 = S-14

Crosses:

9. Laokra 5.5 x DPL-7340-424
10. Laokra 5.5 x Fregobract
11. Laokra 5.5xGlandless-4195-220
12. Laokra 5.5 x SA 100
13. Laokra 5.5 x Stoneville-857
14. Laokra 5.5 x B-557
15. Laokra 5.5 x S-14
16. DPL-7340-424 x Fregobract
17. DPL-7340-424xGlandless-4195-220
18. DPL-7340-424 x Laokra 5.5
19. DPL-7340-424 x SA-100
20. DPL-7340-424 x Stoneville-857
21. DPL-7340-424 x B-557
22. DPL-7340-424 x S-14
23. Fregobract x DPL-7340-424
24. Fregobract x Glandless-4195-220
25. Fregobract x Laokra 5.5
26. Fregobract x SA 100
27. Fregobract x Stoneville - 857
28. Fregobract x B-557
29. Fregobract x S-14
30. Glandless4195- 220 xDPL-7340-424
31. Glandless 4195-220 x Fregobract
32. Glandless 4195-220 x Laokra 5.5
33. Glandless 4195-220 x SA 100
34. Glandless4195-220 x Stoneville-857
35. Glandless 4195-220 x B-557
36. Glandless 4195-220 x S-14
37. SA 100 x DPL-7340- 424
38. SA 100 x Fregobract
39. SA 100 x Glandless 4195-220
40. SA 100 x Laokra 5.5
41. SA 100 x Stoneville-857
42. SA 100 x B-557
43. SA 100 x S-14
44. Stoneville 857 x DPL-7340-424
45. Stoneville 857 x Fregobract
46. Stoneville857 x Glandless 4195-220
47. Stoneville 857 x Laokra 5.5
48. Stoneville 857 x SA 100
49. Stoneville 857 x B-557
50. Stoneville 857 x S-14
51. B-557 x DPL-7340-424
52. B-557 x Fregobract
53, B-557 x Glandless 4195-220
54. B-557 x Laokra 5.5
55. B-557 x SA 100
56. B-557 x Stoneville-857
57. B-557 x S-14
58. S-14 x DPL-7340-424
59. S-14 x Fregobract
60. S-14 x Glandless 4195-220
61. S-14 x Laokra 5.5
62. S-14 x SA-100
63. S-14 x Stoneville-857
64. S-14 x B-557

Table 3. Agglomeration Schedule

	Cluster Combined		Coefficients		Cluster Combined		Coefficients
Stage	Cluster 1	Cluster 2		Stage	Cluster 1	Cluster 2	
1	49	56	1.000	33	35	55	7.500
2	36	53	1.000	34	40	47	8.000
3	6	34	1.000	35	26	31	8.000
4	7	49	1.500	36	5	41	8.200
5	8	62	2.000	37	3	59	8.333
6	5	33	2.000	38	6	51	9.143
7	16	23	2.000	39	6	27	9.500
8	2	18	2.000	40	1	21	9.500
9	1	9	2.000	41	3	52	9.750
10	4	36	2.500	42	13	40	10.000
11	22	58	3.000	43	14	42	10.500
12	5	38	3.000	44	4	5	10.500
13	10	24	3.000	45	8	43	10.750
14	6	7	3.500	46	6	54	10.778
15	8	61	4.000	47	16	25	11.000
16	12	37	4.000	48	8	50	12.167
17	17	32	4.000	49	3	16	13.067
18	15	29	4.000	50	1	13	13.067
19	8	15	4.333	51	14	26	13.333
20	4	39	4.667	52	6	45	13.700
21	6	44	4.800	53	4	11	14.000
22	50	64	5.000	54	6	48	18.091
23	35	46	5.000	55	3	8	18.750
24	4	17	5.000	56	1	14	20.800
25	1	2	5.000	57	4	35	22.476
26	3	10	5.500	58	6	28	24.250
27	5	12	5.667	59	3	63	28.105
28	8	22	5.700	60	1	3	29.862
29	4	30	6.000	61	4	60	30.706
30	14	19	7.000	62	4	6	39.675
31	6	20	7.333	63	1	4	46.806
32	8	57	7.429				

220) & parent 5 (SA100) were reside in the cluster (C) respectively.

The third cluster (C) was composed of fifteen genotypes (17, 32, 36, 53, 39, 30, 33, 38, 12, 37, 41, 11, 60 and parents 4 & 5). This cluster consist of direct and reciprocal crosses of parent 4 (Glandless4195-220) and parent 5 (SA100) with other parents. In this the cross 60 combined with parent 4 at stage 61 with coefficient of 30.706.

The fourth cluster (D) consisted of two parental genotypes: Laokra 5.5 & DPL-7340-424 and their following crosses 18, 9, 21, 40, 47, & 13. The cross 18 combined with its parents at stage 8 with a coefficient of 2.0, while cross 9 combined with parent 1 at stage 9 with a coefficient of 2.0. At stage 34 crosses 40 & 47 combine with a coefficient of 8, while parent 1 combined with crosses 21 & 13 at stage 40 & 50 with a coefficients of 9.5 and 13.067 respectively.

The fifth cluster (E) consisted of five crosses i.e. 26, 31, 14, 19 & 42. The crosses 14 and 19 made a sub cluster at stage 30 with a coefficient of 7.0, while the crosses 26 and 31 combined with each other at stage 35 with a coefficient of 8.0. The last sub cluster was formed between cross 14 and 42 at stage 43 with a coefficient of 10.50.

The cluster (F) comprised of parent 8 (S-14) and its ten crosses with other parents, i.e. 50, 64, 22, 58, 62, 61, 15, 29, 57 & 43. In this cluster parent 8 combined with cross 62 (S-14 x SA100) at stage 5 with a coefficient of 2.0.

The last cluster (G) consisted of parent 3 (Fregobract) along with its seven crosses, i.e. 10, 24, 59, 52, 16, 23 & 25. The cross 63 (S-14 x Stoneville -857) also combined with this cluster at stage 59 with a coefficient of 28.105.

In this study, we wanted to use the molecular and conventional morphological similarities to evaluate the cotton genotypes and their crosses. Poly acrylamide Gel Electrophoresis (PAGE) is used because in this method samples are analyzed in a more direct manner. This method is relatively easy and many samples can be analyzed at the same time. It is also cheaper than other fingerprinting methods. Moreover, the results obtained by PAGE of whole-cell proteins can discriminate at much the same level as DNA fingerprinting, Priest and Austin [16] in some cases.

Cluster analysis of the seed protein data placed the parents and crosses of the Gossypium hirsutum species into seven main groups. The genetic similarity between the genotypes and crosses ranged from 1.0 (reciprocal crosses) to 46.806 % between two parents. The application of UPGMA clustering produced two large clusters within the population, each consisting of several sub clusters.

These results suggested that protein profiles data could clearly separate different parents and their crosses. A high correlation between protein dendrogram and geographic origin of tested genotypes was found. However, as all the genotypes belong to the same species Gossypium hirsutum they formed one group at the end. In the present study, PAGE analysis combined with cluster analysis confirmed the genetic similarities between parents and their crosses while it also confirmed the dissimilarities between the parents as showed by the morphological characters.

In early 1970s, high yielding tetraploid cotton varieties of American origin were introduced into Pakistan, and of these, the varieties that were better adapted were released directly for general cultivation. Those that were less adapted were crossed with local breeding lines. The same gene pool was used repeatedly and resulted in a narrow genetic base, Iqbal et al. [9]. This confirmed the relatedness of the two local parent's similarities with the exotic cultivars of US origin.

CONCLUSION

In summary, the diversity within the species Gossypium hirsutum was determined by using seed storage protein's PAGE analysis could prove useful for the rapid classification of parents and confirmation of hybridization of their crosses.

Figure 1. PAGE of seed storage proteins in G. hirsutum L. genotypes.

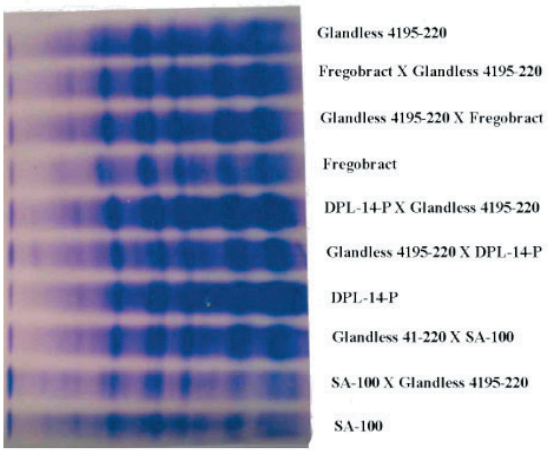


Figure 2. PAGE of seed storage proteins in G hirsutum L. genotypes.

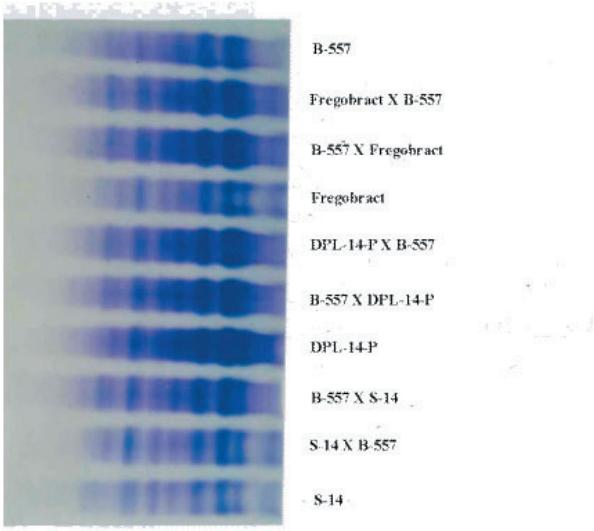


Figure 3. PAGE of seed storage proteins in G hirsutum L. genotypes.

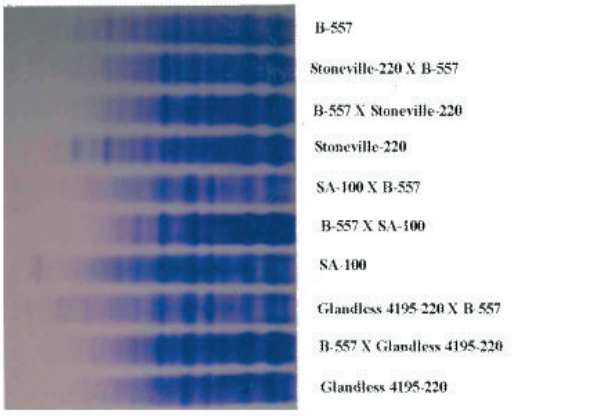


Figure 4. PAGE of seed storage proteins in G hirsutum L. genotypes.

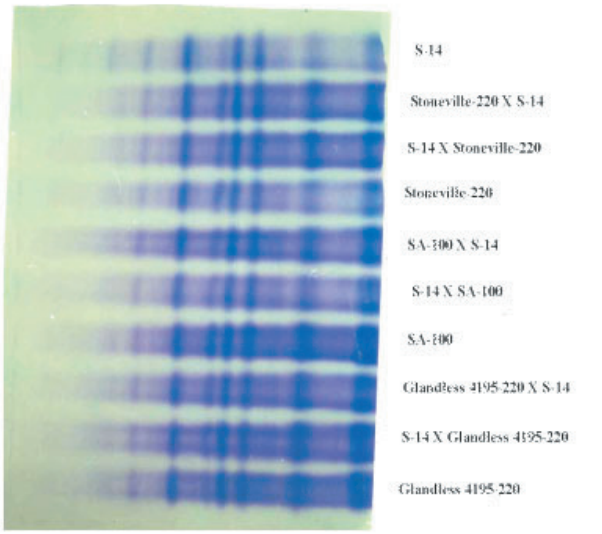
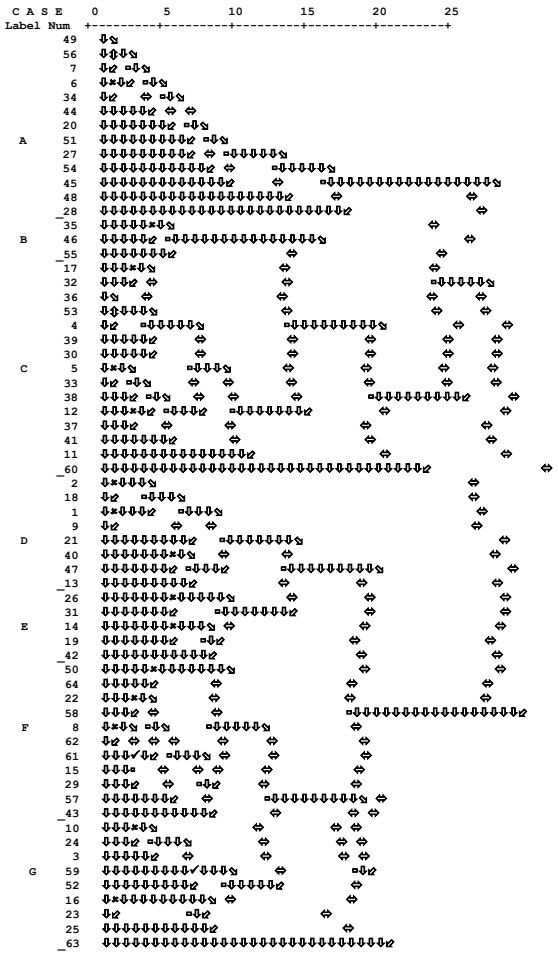


Figure 5. Dendrogram using Hierarchical Cluster analysis Average Linkage (Between Groups).



ACKNOWLEDGMENTS

The special thanks to Dr. Ghulam Mustafa Chaudhary, Vice chancellor, Bahauddin Zakaryia University Multan – Pakistan for providing special facilities. Naveed Murtaza thanks the Japan Society for the Promotion of Science (JSPS) for providing postdoc fellowship award.

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