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Identification of Groundnut Germplasm Lines for Foliar Disease Resistance and High Oleic Traits using SNP and Gene-based Markers and Their Morphological Characterization

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ABSTRACT

Background: Resistance to foliar fungal diseases along with oleic acid trait, are important objectives of groundnut breeding. Among foliar fungal diseases, rust and late leaf spot (LLS) cause significant economic loss and high oleic trait is preferred in industry that enhances economic values of crop.

Methods: Morphological characterization of the 186-groundnut germplasm lines/genotypes for ten yield attributing traits and their significance of correlation was analyzed using SPSS ver. 19 software at 1% and 5% probability level of significance. Screening for LLS and rust diseases was done employing 10X SNP assay at ICRISAT, Hyderabad, India. Selected superior groundnut germplasm line(s) were screened for presence of FAD2B allele responsible for high oleic acid traits using allele specific marker.

Result: Significant and positive correlation was found between dry weight and hundred pod weight (r=0.0.801) and harvest index (r=0.0.830). Molecular characterization along with morphological characterization identified highly diversified lines of groundnut. This study reports 78 foliar fungal disease resistant groundnut germplasm lines. Selected 11 groundnut germplasm lines represented resistance against LLS and rust diseases along with FAD2B allele for oleic acid trait.

Key words: Foliar fungal diseases, Germplasm, Groundnut, Oleic acid content, Single nucleotide polymorphism.

INTRODUCTION

Groundnut (*Arachis hypogaea* L.) is an important self-pollinated oilseed crop grown in more than 100 countries on about 26.5 million ha with total production of 43.9 million tons. India is second largest producer of groundnut and its oil after China followed by USA and Nigeria. India ranks first with an area of 5.30 Mha and second in production with 9.17 MT of pods (FAOSTAT 2017, Deshmukh *et al*., 2020) Groundnut is valued as a rich source of energy in form of oil (48-50%) and protein (25-28%) in the kernels. Groundnut haulms provide nutritious fodder for livestock. It contains protein (8-15%), lipids (1-3%), minerals (9-17%) and carbohydrate (38-45%) higher than cereal fodder.

Foliar fungal diseases are the major production constraints of groundnut worldwide wherever the crop is grown. These diseases can cause more than 70% loss in yield besides adversely affecting the quality of the produce (pods, seeds and haulms). Among foliar fungal diseases, three major foliar diseases *viz*., early leaf spot [*Cercospora arachidicola* Hori), late leaf spot (*Phaeoisariopsis personata* (Berk. and Curt.) Van. Arx.] and rust (*Puccinia arachidis* Speg.) are the most widely distributed and economically important. Conventional methods of controlling foliar diseases neither ecofriendly nor time saving. For development of foliar resistance varieties, superior germplasm identification is one of the foremost tasks. Several molecular markers for LLS and rust resistance have been validated and used to develop LLS and rust-resistant lines (Yeri and Bhat, 2016; Pandey *et al.,* 2017; Bhawar *et al.,* 2019). Screening of foliar disease resistance in groundnut germplasm using SNP markers are proved very effective, accurate and with exact allele calls.

Consuming oils with high levels of oleic acid is beneficial to human health because it reduces low-density lipoproteins, maintaining high-density lipoprotein, slow down atherosclerosis and reversing the inhibitory effect of insulin production. Unlike oleic acid, higher linoleic acid is vulnerable to oxidation causing off-flavors, rancidity and negatively impacts the oil stability. Oleic acid content in conventional peanut cultivars is 48%-54% and in high-oleic-acid peanut cultivars the oleic acid content may be up to

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80% (Norden *et al.,* 1987). Fatty acid desaturase (FAD) enzyme facilitates the conversion of oleic acid to linoleic acid by adding double bond to oleic acid. This enzyme is coded by two homologous genes (ahFAD2A and ahFAD2B) located on A and B sub genomes. In conventional breeding, selection for fatty acid composition is carried out in advance generations, thus requires huge resources and time to handle. The linked allele-specific (Chen *et al*., 2010) and cleaved amplified polymorphic sequences (CAPS) (Chu *et al*., 2009) markers for both the ahFAD2 genes (ahFAD2A and ahFAD2B) are available for use in molecular breeding programme. Until now, more than 80 high oleic groundnut varieties have been registered globally, which have developed through conventional breeding, marker-assisted backcrossing (MABC), marker-assisted selection (MAS) and mutagenesis (Wang *et al.,* 2015; Bera *et al.,* 2018). In Asian and African countries high oleic groundnut has been recently commercialized, however, combining must-have traits such as late leaf spot (LLS) and rust resistance with high oleic are limited (Shasidhar *et al*., 2020; Deshmukh *et al*., 2020). Screening of groundnut germplasm to get resistance to foliar fungal diseases and high oleic trait, are important objectives of groundnut crop improvement at present. Morphological characterization is important to observe yield performance and their attributing traits (Sahu *et al.,* 2020; Upadhyay *et al.,* 2020; Mishra *et al.,* 2021) So current study was conducted to screen LLS and rust resistant groundnut genotype(s)/ germplasm line (s) with high oleic acid content and higher yield.

MATERIALS AND METHODS

Plant material

The plant materials consisted 166 uncharacterized groundnut germplasm lines, 12 advance breeding lines including 6 check varieties. These germplasms were received from Directorate of Groundnut Research, Junagadh (Gujarat). Foliar disease resistance and high yielding varieties *viz*., GPBD4 and KDG128; high oleic acid containing line Sunoleic95 R and sensitive to foliar diseases varieties *i.e*., TG26, ICGS44 and JGN3 were used as check.

Methodology

Morphological characterization

The field experiment was conducted at Research Farm, Department of Plant Breeding and Genetics, Rajmata Vijayaraje Scindia Krishi Vishwa Vidyalaya Gwalior (M.P.) The material was grown during *kharif* 2018-19 in field with inter and intra row spacing of 30 and 10 cm in augmented design. Seeds were disinfected with combination of Dithane M-45 @ 2 g/kg seed + Bavistin @ 1 g/kg seed. The crop was raised following the recommended cultural practiceswith NPK in ratio 20:60:20 and essential dose of gypsum.

Morphological characterization

Initial plant stand/eow, final plant stand/row, days to 50% flowering, days to maturity, fresh weight/plant, dry weight/ plant, 100 pod weight (gm), kernel yield (gm/plant), 100-kernel weight (gm) and harvest index were documented for five plants and their mean value was considered for further analysis. The coefficient of correlation among all morphological traits at maturity was calculated using SPSS ver19.0 software. The similarity matrices were used to construct a dendrogram for all the germplasm lines and genotypes using NTSYS-pc 2 (Rohlf, 2000).

DNA extraction

The young leaves of 20 days old seedlings from each germplasm lines were sampled from field. The genomic DNA was extracted using CTAB method (Murray and Thompson, 1980) with minor modification (Tiwari *et al.* 2017). The quality of the DNA was checked on 1% agarose gel and the DNA concentrations were estimated with the micro volume spectrophotometer (Helix Biosciences, New Delhi, India).

SNP genotyping for foliar fungal disease resistance

SNP genotyping work was carried out at Molecular Genomics Division, ICRISAT, Hyderabad, Telangana, India. Total 10 plex SNP assay was used having all the SNPs specific for foliar disease resistance including snpAH0002, snpAH0004, snpAH0005, snpAH0010, snpAH0011, snpAH0015, snpAH0017, snpAH0018, snpAH0021 and snpAH0026. Sampling of leaf tissue for all germplasm lines from field was done by Standard operating procedures (SOPs) from young and tender leaves. Total, 4 discs of 2 mm diameter for peanut were taken to get good amount of DNA for Kompetitive allele specific PCR (KASP) platform. After finishing the collection procedure, the plates were sealed properly and transferred to dry ice box for further SNP genotyping.

Genotyping with gene based molecular markers

For screening of oleic acid, gene-based markers were used (Table 1) (Chu *et al*., 2011; Chen *et al*., 2010) while for screening of oleic acid containing germplasm, 3 oleic acid gene-based markers were used *viz*., WT wild type, SUB substitution, INS insertion, SUB + INS substitution plus insertion. The primers were synthesized by Eurofins Genomics India Pvt Ltd. Polymerase chain reaction was performed in 10 µl reaction mixture comprising of 1X PCR buffer, 0.1 U *Taq* DNA polymerase, 1 µl dNTP (1 mM), 0.5 µl of forward and reverse primers each (10 pM) and 20 ng/µl of genomic DNA in a thermocycler (Bio-Rad, USA). The PCR protocol comprised of initial denaturation step of 94°C for 3 min followed by 35 cycles of 94°C for 1 min, annealing at 55°C for 30 sec, elongation at 72°C for 1 min with final extension at 72°C for 10 min. The PCR products were resolved on 3% agarose gel at 120V for 2-3 hrs and documented using Syngene, Gel Documentation System (USA).

RESULTS AND DISCUSSION

Morphological characterization

Significance of correlation of different traits was analyzed using SPSS ver. 19 software at 1% and 5%, respectively (Table 2). Significant and positive correlation was found

between initial plant stand to final plant stand (r=0.963), dry weight (r=0.212) and hundred pod weight (r=0.227) at 1% significant level. Similarly, significant and positive correlation was detected between dry weight and hundred pod weight (r=0.0.801) and harvest index (r=0.0.830). Hundred pod weight is highly significant to harvest index (r=0.0.675) at 1% significant level of significance. Dendrogram representing clustering of 186 genotypes based on mean value of morphological observations (Fig 1). Clustering of groundnut genotypes based on fresh weight and kernel yield divided all the genotypes into four groups in 2D plot (Fig 2). Most of the genotypes are presented in group III and IV.

SNP genotyping

Allele data for SNP genotyping was received in A/G/C/T form and it was converted in A/B alleles for further analysis. A total of 29 alleles were identified with an average of 2.9 alleles per locus. The number of alleles per locus ranged from 2.0 to 3.0 (Table 3). The gene diversity and PIC values varied between 0.02-0.1601 with an average of 0.1461, respectively. The primers that showed highest gene diversity were 9 in number while the lowest gene diversity and PIC values was observed for the primer snpAH0002. The major allele frequency varied between 0.9063 (all the highly polymorphic 9 markers) to 0.9896 (snpAH0002) with a mean value of 0.9146 (Table 3).

Dendrogram for SNP genotyping

Genotyping data of 186 germplasm lines using SNP markers was used for phylogenetic cluster analysis in A/B form. Total three distinct clusters were formed cluster I having 82 germplasm including check varieties KDG124, GPBD4, ICGS44 and TG 26 and foliar disease resistant lines. Cluster II contains Sunoleic95R and 42 groundnut germplasm. Cluster III represented 61 germplasm and included susceptible check variety JGN3 (Fig 3). Out of 82 germplasm present in cluster I, 14 germplasms *i.e.,* ICGV27127, R 7-47-9, RS 1, S 7-1-9, US 64, S-7-24-13, S-7-1-16, S7-2-18, AH7457, AH7218, S7-2-8, MIRLAP1-2-3, AH7999and RCM453-4 were having higher yield as compared to other germplasm used in the study. They were resistant to foliar fungal diseases at field condition also (Fig 4). These germplasm were selected for screening of FAD2B allele responsible for high oleic acid contents.

Screening for oleic acid contents

Screening for high oleic acid contents, three allele specific markers were used. Check variety Sunoleic 95R revealed that total 11 genotypes were showing FAD2B allele for oleic acid content *i.e*., ICGV27127, R 7-47-9, RS 1, S 7-1-9, S-7-24-13, S-7-1-16, AH7218, S7-2-8, MIRLAP1-2-3, AH7999 and RCM453-4 (Fig 5).

Table 1: Details of molecular markers used for screening of high oleic acid traits of groundnut.

Markers name/Type	Annealing temp	Sequence (5′ → 3′)	References
FAD2A (SENSE)/CAPS	48.5	GATTACTGATTATTTGACTT	Chu *et al.,* 2009
FAD2A (ANTISENS)/CAPS	48.5	CCAACCCAAACCTTTCAGAG	Chu *et al.,* 2009
F435-F/AS-PCR	As per R primer mixed	ATCCAAGGCTGCATTCTCAC	Chen *et al.,* 2010
F435IC-R/AS-PCR (CONTROL)	As per R primer mixed	CTCCCTGGTGGATTGTTCATGT	Chen *et al.,* 2010
F435WT-R/AS-PCR	63	ACTTCGTCGCGGTCG	Chen *et al.,* 2010
F435SUB-R/AS-PCR	64	TGGGACAAACACTTCGTT	Chen *et al.,* 2010
F435INS-R/AS-PCR	66	AACACTTCGTCGCGGTCT	Chen *et al.,* 2010
FAD2B-F/CAPS	As per R primer mixed	CAGAACCATTAGCTTTGTAGTAGTG	Chu *et al.,* 2009
FAD2B-A/CAPS	53	AACATTCGTCGCGGTTT	Chu *et al.,* 2009
FAD2-R/CAPS	51	CTCTGACTATGCATCAGAACTTGT	Chu *et al.,* 2009

Table 2: Correlation coefficient between morphological observations of groundnut germplasm.

	Correlations									
	IPS	FPS	DTF	DTM	FW	DW	HPW	KYLD	HKW	HI
IPS	1	0.963**	-0.047	-0.073	0.181*	0.212**	0.227**	-0.160*	0.160*	0.110
FPS		1	-0.039	-0.051	0.176*	0.149*	0.175*	-0.168*	0.175*	0.046
DTF			1	0.034	0.025	-0.123	-0.118	-0.072	0.079	-0.151*
DTM				1	0.087	-0.040	-0.006	-0.077	0.088	-0.101
FW					1	0.079	0.007	-0.050	0.059	-0.399**
DW						1	0.801**	-0.064	0.040	0.830**
HPW							1	0.110	-0.121	0.675**
KYLD								1	-0.996**	-0.049
HKW									1	.012
HI										1

**: Correlation is significant at the 0.01 level (2-tailed). *: Correlation is significant at the 0.05 level (2-tailed).

IPS= Initial plant stand; FPS= Final plant stand; DTF= Days to 50% flowering; DTM= Days to maturity; FW= Fresh weight; DW= Dry weight; HPW= Hundred pod weight; KYLD= Kernel yield; HKW= Hundred kernel weight; HI= Harvest index.

In plant breeding, molecular markers can be used for several purposes like germplasm characterization, diversity analysis, selection of parents for hybridization, testing for genetic purity, gene introgression, gene pyramiding, MAS in segregating populations and marker assisted backcrossing (Tiwari *et al.,* 2017; Pramanik *et al*., 2019; Mishra *et al.,* 2020; Sahu *et al.,* 2020). Marker-assisted selection is an important tool to enhance tolerance/resistance to biotic and abiotic stresses. Present study included use of gene-based markers which are cost effective as they are few in numbers and can be used for screening and identification of resistant germplasm. Allele specific markers can be simply scored on agarose gel electrophoresis are the most cost effective assays to genotype the breeding population in order to select plants with desired allele of foliar disease resistance. Our study utilized 10 plex SNP assay used for selection of LLS and rust resistant genotypes. It is very cost effective, fast and accurate method for selection of foliar disease resistant groundnut genotypes (Adlak *et al.,* 2019).

Table 3: Summary of SNP data analysis of groundnut germplasm.

Marker	Major allele frequency	Allele no	PIC
snpAH0002	0.9896	2.0000	0.0204
snpAH0004	0.9063	3.0000	0.1601
snpAH0005	0.9063	3.0000	0.1601
snpAH0010	0.9063	3.0000	0.1601
snpAH0011	0.9063	3.0000	0.1601
snpAH0015	0.9063	3.0000	0.1601
snpAH0017	0.9063	3.0000	0.1601
snpAH0018	0.9063	3.0000	0.1601
snpAH0021	0.9063	3.0000	0.1601
snpAH0026	0.9063	3.0000	0.1601
Mean	0.9146	2.9000	0.1461

Eight fatty acids can be routinely detected in peanut seeds; however, two major fatty acids, oleic acid (C18:1, D9) and linoleic acid (C18:2, D9, D12), account for approximately 80% of the fatty acid composition (Moore *et al*., 1989; Norden *et al.,* 1987). Major fatty acids in groundnut oil are palmitic acid (8-11%), oleic acid (36-52%) and linoleic acid (24-43%). Fatty acid composition of groundnut oil is an important trait from human nutrition point of view as well as oil stability during the storage. To facilitate marker-assisted selection

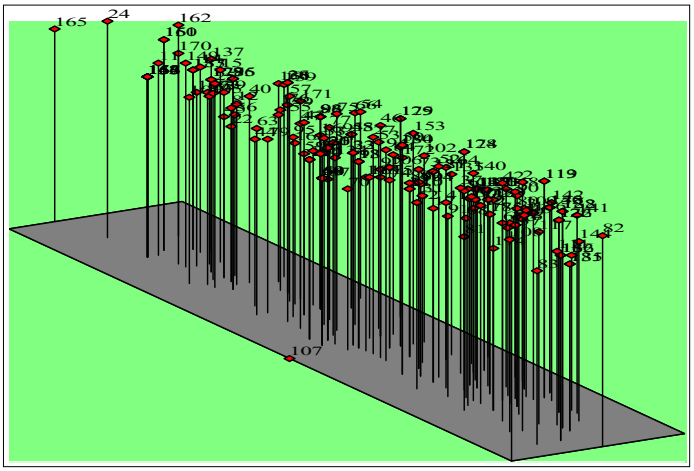

Fig 1: 3D clustering of 186 groundnut germplasm for morphological observations.

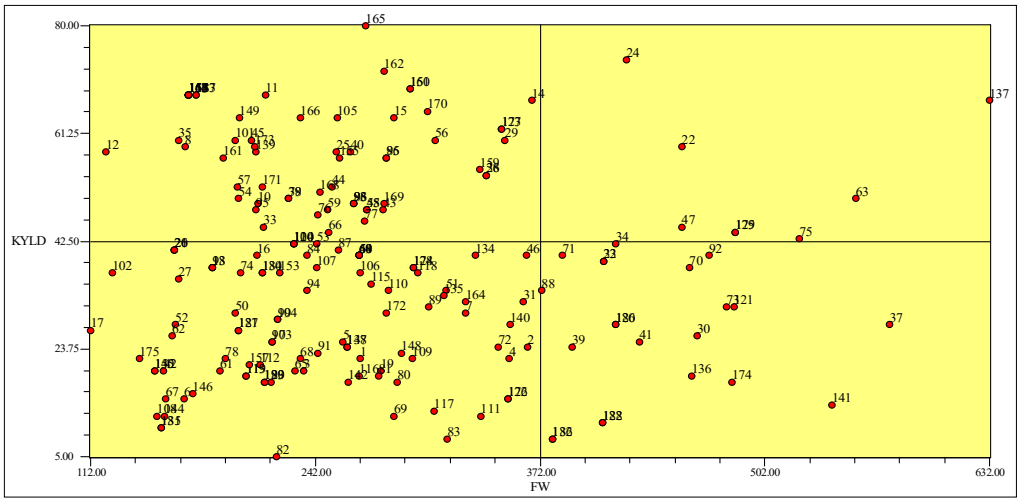

Fig 2: Clustering of groundnut germplasm 2D based on fresh weight and kernel yield.

for the high-oleate trait, different types of DNA markers from these two genes have been developed. The first high oleate peanut line, "SunOleic95R" was developed through conventional breeding methods, (Gorbet and Knauft, 1997) and "Tifguard High O/L" was developed using MAS. Recent advancement of genomic tools accelerated marker assisted breeding (MAB) to enhance efficiency of selection of target traits in groundnut.

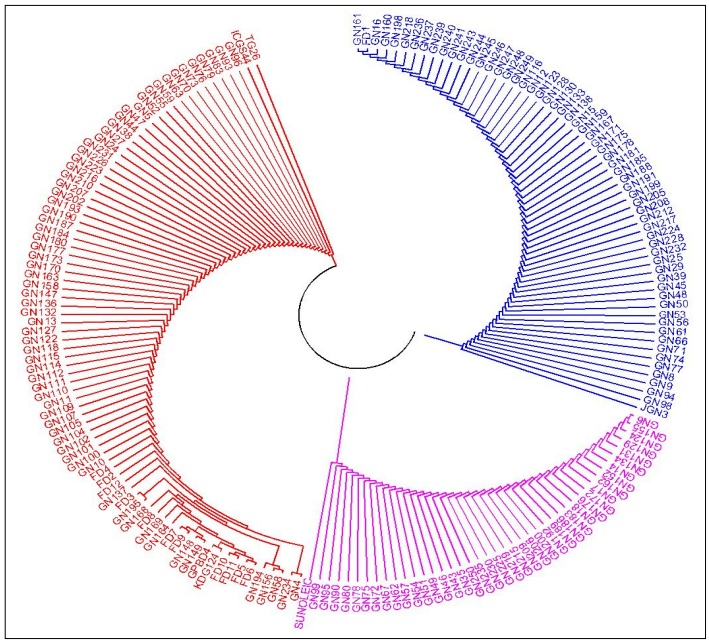

Fig 3: Dendrogram of 186 groundnut germplasm/genotypes showing clusters for 10 plex SNP assay using UPGMA relationship.

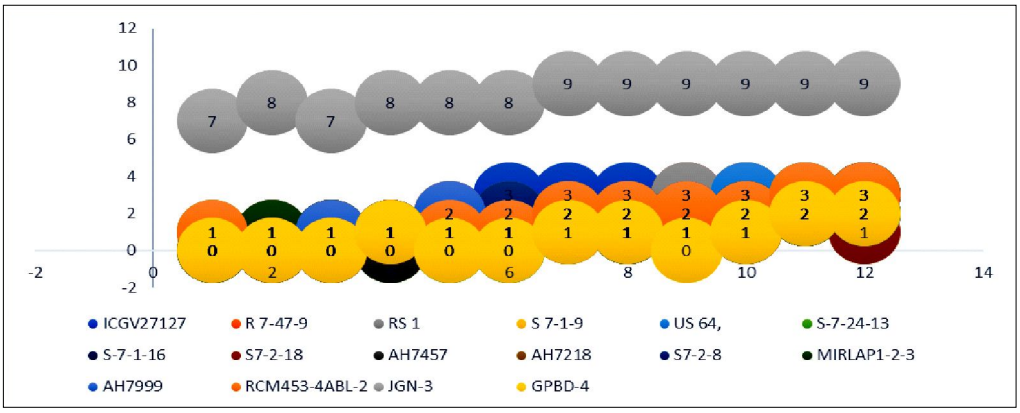

Fig 4: Disease score of selected groundnut germplasm for early and late leaf spot at field condition along with check varieties JGN 3 and GPBD 4.

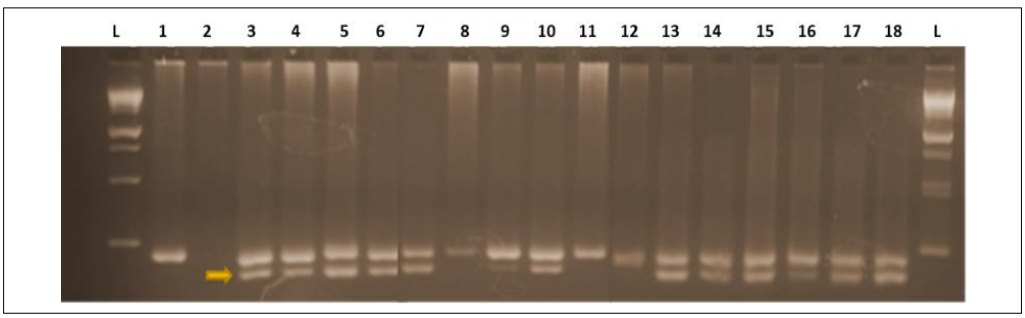

Fig 5: Gel picture representing banding pattern of oleic acid containing allele FAD2B in selected groundnut germplasm. L, 100 bp DNA ladder; 1, GBPD4; 2, KDG128; 3, Sunoleic 95R; 4 to 18 selected germplasm of groundnut.

CONCLUSION

Improvement of groundnut for foliar fungal diseases and high oleic acid traits are major thrust area for groundnut improvement. In our study, we have screened 186 groundnut germplasm for these two traits using marker assisted selection approach. We are reporting ICGV27127, R 7-47-9, RS 1, S 7-1-9, S-7-24-13, S-7-1-16, AH7218, S7-2-8, MIRLAP1-2-3, AH7999 and RCM453-4 groundnut germplasm lines having resistant to LLS and rust diseases and FAD2B allele of high olein traits. These germplasm could be used in crossing programme for new variety development of groundnut with superior agronomic traits.

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