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International Union for the Protection of New Varieties of Plants

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Technical Working Party for Vegetables

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PARTIAL REVISION OF THE TEST GUIDELINES FOR TOMATO

Document prepared by an expert from the Netherlands (Kingdom of)

Disclaimer: this document does not represent UPOV policies or guidance

1. The purpose of this document is to present a proposal for a partial revision of the Test Guidelines for Tomato (document TG/44/12).

2. The Technical Working Party for Vegetables (TWV), at its fifty-eighth session[1], agreed that the Test Guidelines for Tomato (*Solanum lycopersicum* L.) be partially revised (see document TWV/58/11 "Report", Annex II).

3. The following changes are proposed:

(a) Addition of "Resistance to *Passalora fulva* (Pf) - Race H"
(b) Revision of explanation "Resistance to *Passalora fulva* (Pf)"
(c) Addition of an alternative molecular marker method (using makers on I2) for characteristic 48 "Resistance to *Fusarium oxysporum f. sp. lycopersici* (Fol) - Race 1EU/2US" next to the bioassay.

4. The proposed changes are presented below in highlight and underline (insertion) and ~~strikethrough~~ (deletion).

[1] held via electronic means, from April 22 to 25, 2024.

Proposed addition of "Resistance to *Passalora fulva* (Pf) - Race H"

Current wording

57.	**QL**	**VG**	**(+)**				
	Resistance to *Passalora fulva* (Pf) - Race F		**Résistance à *Passalora fulva* (Pf) - Race F**	**Resistenz gegen *Passalora fulva* (Pf) - Pathotyp F**	**Resistencia a *Passalora fulva* (Pf) - Raza F**		
	absent		absente	fehlend	ausente	Monalbo, Moneymaker	1
	present		présente	vorhanden	presente	Chelino, Completo	9
58.	**QL**	**VG**	**(+)**				
	Resistance to *Passalora fulva* (Pf) - Race J		**Résistance à *Passalora fulva* (Pf) - Race J**	**Resistenz gegen *Passalora fulva* (Pf) - Pathotyp J**	**Resistencia a *Passalora fulva* (Pf) - Raza J**		
	absent		absente	fehlend	ausente	Chelino, Completo	1
	present		présente	vorhanden	presente	Mogami	9

Proposed new wording

57.	**QL**	**VG**	**(+)**				
	Resistance to *Passalora fulva* (Pf) - Race F		**Résistance à *Passalora fulva* (Pf) - Race F**	**Resistenz gegen *Passalora fulva* (Pf) - Pathotyp F**	**Resistencia a *Passalora fulva* (Pf) - Raza F**		
	absent		absente	fehlend	ausente	Monalbo, Moneymaker	1
	present		présente	vorhanden	presente	Chelino, Completo	9
58.	**QL**	**VG**	**(+)**				
	Resistance to *Passalora fulva* (Pf) - Race H		**Résistance à *Passalora fulva* (Pf) - Race H**	**Resistenz gegen *Passalora fulva* (Pf) - Pathotyp H**	**Resistencia a *Passalora fulva* (Pf) - Raza H**		
	absent		absente	fehlend	ausente	Sprigel	1
	present		présente	vorhanden	presente	Chelino, Completo	9
~~**58.**~~ **59**	**QL**	**VG**	**(+)**				
	Resistance to *Passalora fulva* (Pf) - Race J		**Résistance à *Passalora fulva* (Pf) - Race J**	**Resistenz gegen *Passalora fulva* (Pf) - Pathotyp J**	**Resistencia a *Passalora fulva* (Pf) - Raza J**		
	absent		absente	fehlend	ausente	Chelino, Completo	1
	present		présente	vorhanden	presente	Mogami	9

Proposed revision of explanation “Resistance to *Passalora fulva* (Pf)”

Ad. 51: Resistance to *Passalora fulva* (Pf) - Race 0

1.	Pathogen	*Passalora fulva*
2.	Quarantine status	-
3.	Host species	*Solanum lycopersicum*
4.	Source of inoculum	Naktuinbouw[2] (NL) or GEVES[3] (FR)
5.	Isolate	Races 0, A, B, C, D, E, F, H and J
6.	Establishment isolate identity	with genetically defined differentials A breaks *Cf-2*, B *Cf-4*, C *Cf-2* and *Cf-4*, D *Cf-5*, E *Cf-2*, *Cf-4* and *Cf-5*, F *Cf-2* and *Cf-9*, H *Cf-4* and *Cf-9*, J *Cf-2, Cf-6* and *Cf-9* https://www.worldseed.org
7.	Establishment pathogenicity	symptoms on susceptible tomato
8.	Multiplication inoculum	
8.1	Multiplication medium	Potato Dextrose Agar or Malt Agar or a synthetic medium
8.8	Shelflife/viability inoculum	4 hours, keep cool
9.	Format of the test	
9.1	Number of plants per genotype	at least 20 plants
9.3	Control varieties	
	Susceptible	Monalbo, Moneymaker
	Resistant for Race A:	Purdue, IVT1154, IVT1149, Antique, Pink Treat, Sprigel, Triatlon
	Resistant for Race B:	Vétomold, IVT1154, IVT1149, Antique, Retinto, Sprigel, Triatlon
	Resistant for Race C:	IVT1154, IVT1149, Antique, Sprigel, Triatlon
	Resistant for Race D:	Vétomold, IVT1154, Antique, Pink Treat, Retinto, Sprigel
	Resistant for Race E:	IVT 1154, Antique, Sprigel
	Resistant for Race F:	Purdue 135, IVT1149, Ontario 7818, Chelino, Completo
	Resistant for Race H:	Vétomold, IVT1149, Ontario 7818, Chelino, Completo
	Resistant for Race J:	Purdue 135, IVT1149
9.5	Test facility	glasshouse or climate room
9.6	Temperature	day: 22° C, night: 20°or day: 25°C, night 20°C
9.7	Light	12 hours or longer
9.8	Season	
9.9	Special measures	depending on facility and weather, there may be a need to raise the humidity, e.g. humidity tent fully closed 3-4 days after inoculation and after that partly closed (66% to 80%, 24 h per day), until end
10.	Inoculation	
10.1	Preparation inoculum	prepare evenly colonized plates, e.g. 1 for 36 plants; remove spores from plate by scraping with water with Tween20; filter through double muslin cloth
10.2	Quantification inoculum	count spores; adjust to 10^5 spores per ml or more
10.3	Plant stage at inoculation	19-20 d (incl. 12 d at 24°), 2-3 leaves
10.4	Inoculation method	spray on dry leaves
10.7	Final observations	14 days after inoculation; when susceptible control does not show clear symptoms the test may be prolonged until for example 18 days after inoculation
11.	Observations	
11.1	Method	visual inspection of abaxial side of inoculated leaves
11.2	Observation scale	Symptom: velvety, white spots
11.3	Validation of test	evaluation of variety resistance should be calibrated with results of resistant and susceptible controls
12.	Interpretation of data in terms of UPOV characteristic states	absent [1] symptoms present [9] no symptoms

[2] Naktuinbouw; resistentie@naktuinbouw.nl

[3] GEVES; matref@geves.fr

13.	Critical control points	Pf spores have a variable size and morphology. Small spores are also viable. Fungal plates will gradually become sterile after 6-10 weeks and repeated subculturing. Do not subculture more often than strictly necessary for multiplication. Excessively high humidity may cause rugged brown spots on all leaves.

Ad. 52: Resistance to *Passalora fulva* (Pf) - Race A

See Ad. 51

Ad. 53: Resistance to *Passalora fulva* (Pf) - Race B

See Ad. 51

Ad. 54: Resistance to *Passalora fulva* (Pf) - Race C

See Ad. 51

Ad. 55: Resistance to *Passalora fulva* (Pf) - Race D

See Ad. 51

Ad. 56: Resistance to *Passalora fulva* (Pf) - Race E

See Ad. 51

Ad. 57: Resistance to *Passalora fulva* (Pf) - Race F

See Ad. 51

Ad. 58: Resistance to *Passalora fulva* (Pf) - Race H

See Ad. 51

Ad. ~~58.~~59 Resistance to *Passalora fulva* (Pf) - Race J

See Ad. 51

Proposed addition of an alternative molecular marker method (using makers on I2) for characteristic 48 "Resistance to *Fusarium oxysporum f. sp. lycopersici* (Fol) - Race 1EU/2US" next to the bioassay.

Current wording

Ad. 47: Resistance to *Fusarium oxysporum* f. sp. *lycopersici* - Race 0EU/1US (Fol: 0EU/1US)

1.	Pathogen	*Fusarium oxysporum* f. sp. *lycopersici*
3.	Host species	*Solanum lycopersicum* L.
4.	Source of inoculum	GEVES[4] (FR), INIA - CSIC[5] (ES) or Naktuinbouw[6] (NL)
5.	Isolate	e.g. Reference strain validated in an interlaboratory test[7]. Race 0EU/1US (e.g. isolate Orange 71 or PRI 20698 or Fol 071), race 1EU/2US (e.g. isolate 4152, PRI40698 or RAF 70) and race 2EU/3US
6.	Establishment isolate identity	use differential varieties, see ISF website: https://www.worldseed.org
7.	Establishment pathogenicity	on susceptible tomato varieties
8.	Multiplication inoculum	
8.1	Multiplication medium	Potato Dextrose Agar or Medium "S" of Messiaen or Czapek-Dox
8.4	Inoculation medium	water for scraping agar plates or Czapek-Dox culture medium (7 d-old aerated culture)
8.6	Harvest of inoculum	filter through double muslin cloth
8.7	Check of harvested inoculum	see 10.2
8.8	Shelflife/viability inoculum	4-8 h, keep cool to prevent spore germination
9.	Format of the test	
9.1	Number of plants per genotype	at least 20 plants plus at least 5 non-inoculated plants
9.2	Number of replicates	plants have to be divided into at least 2 replicates
9.3	Control varieties	
9.3.1	Control varieties for the test with race 0EU/1US	Susceptible: Marmande, Marmande verte, Resal, Moneymaker Resistant: Marporum, Larissa, "Marporum x Marmande verte", Motelle, Gourmet; and Riesling as additional resistant control for medium level
9.3.2	Control varieties for the test with race 1EU/2US	Susceptible: Marmande verte, Cherry Belle, Roma, Marporum, Ranco, Moneymaker Resistant: Tradiro, Motelle, "Motelle x Marmande verte"; and Agostino as additional resistant control for medium level
9.3.3	Control varieties for the test with race 2EU/3US	Susceptible: Marmande verte, Motelle, Marporum Resistant: Alliance, Florida, Murdoch, "Marmande verte x Florida"
9.5	Test facility	glasshouse or climate room
9.6	Temperature	24-28°C (severe test, with mild isolate), 20-24°C (mild test, with severe isolate)
9.7	Light	12 hours per day or longer
9.8	Season	all seasons
10.	Inoculation	
10.1	Preparation inoculum	3-5 days in aerated liquid cultures like PDB, Czapek Dox or S of Messiaen or scraping of plates of 10 days cultures on agar medium.

[4] GEVES, matref@geves.fr

[5] INIA – CSIC, resistencias@inia.es

[6] Naktuinbouw, resistentie@naktuinbouw.nl

[7] Harmores 3 CPVO project: https://cpvo.europa.eu/sites/default/files/documents/report_harmores_3_final_meeting_v0_0.pdf

10.2	Quantification inoculum	spore count, adjust to 10^6 spores per ml, in case of very aggressive isolate inoculum concentration can be decreased
10.3	Plant stage at inoculation	10-18 d, cotyledon to first leaf
10.4	Inoculation method	plants at the inoculation stage are harvested carefully, roots and hypocotyls are immersed in spore suspension for 5-15 min; trimming of roots is an option, and transplanted in trays
10.7	Final observations	14-21 days after inoculation
11.	Observations	
11.1	Method	visual
11.2	Observation scale	

Class 0	Class 1	Class 2	Class 3
Healthy compared to the non-inoculated control.	Healthy compared to the non-inoculated control with brown vessel above the cotyledon (observed when plants are cut in case of variety with different levels of symptoms)	Higher than 50% of growth reduction and/or yellowing and/or wilting on cotyledons and/or leaves.	Nearly dead: strong reduction with plants look dwarf (there can be necrosis but not always) or dead
			
If all plants in class 0 or if all plants in classes 2 and 3, it is not necessary to cut the plants.			
In case of variety or control with different levels of symptoms, cut the plants to check presence or not of strong brown vessel above cotyledons. In case of no brown vessels or below cotyledons, the plant is note 0. In case of brown vessels above cotyledons, the plant is note 1.			

11.3	Validation of test	Validation on controls. Expected response of controls: Susceptible control: most plants in class 2 and 3, max.10% of plants class 0 and 1 Resistant control: most plants in class 0 and 1, max. 10% of plants class 2 and 3. Controls with medium level of resistance can show a higher number of plants in class 2 and 3.
12.	Interpretation of data in terms of UPOV characteristic states	[1] absent: Average symptom level higher than in the medium-resistant control [9] present: Average symptom level not different from the medium-resistant control or the high-resistant control

Ad. 48: Resistance to *Fusarium oxysporum* f. sp. *lycopersici* - Race 1EU/2US (Fol: 1EU/2US)

See Ad. 47

Ad. 49: Resistance to *Fusarium oxysporum* f. sp. *lycopersici* - Race 2EU/3US (Fol: 2EU/3US)

See Ad. 47

Proposed new wording

Ad. 47 and Ad 48: Resistance to *Fusarium oxysporum* f. sp. *lycopersici* - Race 0EU/1US (Fol: 0EU/1US) and Race 1EU/2US (Fol: 1EU/2US)

Resistance to *Fusarium oxysporum* f. sp. *lycopersici* (Fol) - Race 0EU/1US to be tested in a bio-assay (method i).

Resistance to *Fusarium oxysporum* f. sp. *lycopersici* (Fol) - Race 1EU/2US to be tested in a bio-assay (method i) and/or in a DNA marker test on gene *I-2* (method ii).

In case of a bio-assay, type of observation is MS/VS/VG. In case of a DNA marker test, type of observation is MS.

(i) Bio-assay

1.	Pathogen	*Fusarium oxysporum* f. sp. *lycopersici*
3.	Host species	*Solanum lycopersicum* L.
4.	Source of inoculum	GEVES[8] (FR), INIA - CSIC[9] (ES) or Naktuinbouw[10] (NL)
5.	Isolate	e.g. Reference strain validated in an interlaboratory test[11]. Race 0EU/1US (e.g. isolate Orange 71 or PRI 20698 or Fol 071), race 1EU/2US (e.g. isolate 4152, PRI40698 or RAF 70) and race 2EU/3US
6.	Establishment isolate identity	use differential varieties, see ISF website: https://www.worldseed.org
7.	Establishment pathogenicity	on susceptible tomato varieties
8.	Multiplication inoculum	
8.1	Multiplication medium	Potato Dextrose Agar or Medium “S” of Messiaen or Czapek-Dox
8.4	Inoculation medium	water for scraping agar plates or Czapek-Dox culture medium (7 d-old aerated culture)
8.6	Harvest of inoculum	filter through double muslin cloth
8.7	Check of harvested inoculum	see 10.2
8.8	Shelflife/viability inoculum	4-8 h, keep cool to prevent spore germination
9.	Format of the test	
9.1	Number of plants per genotype	at least 20 plants plus at least 5 non-inoculated plants
9.2	Number of replicates	plants have to be divided into at least 2 replicates
9.3	Control varieties	
9.3.1	Control varieties for the test with race 0EU/1US	Susceptible: Marmande, Marmande verte, Resal, Moneymaker Resistant: Marporum, Larissa, “Marporum x Marmande verte”, Motelle, Gourmet; and Riesling as additional resistant control for medium level
9.3.2	Control varieties for the test with race 1EU/2US	Susceptible: Marmande verte, Cherry Belle, Roma, Marporum, Ranco, Moneymaker Resistant: Tradiro, Motelle, “Motelle x Marmande verte”; and Agostino as additional resistant control for medium level
9.3.3	Control varieties for the test with race 2EU/3US	Susceptible: Marmande verte, Motelle, Marporum Resistant: Alliance, Florida, Murdoch, “Marmande verte x Florida”
9.5	Test facility	glasshouse or climate room

[8] GEVES, matref@geves.fr

[9] INIA – CSIC, resistencias@inia.es

[10] Naktuinbouw, resistentie@naktuinbouw.nl

[11] Harmores 3 CPVO project: https://cpvo.europa.eu/sites/default/files/documents/report_harmores_3_final_meeting_v0_0.pdf

9.6	Temperature	24-28°C (severe test, with mild isolate), 20-24°C (mild test, with severe isolate)
9.7	Light	12 hours per day or longer
9.8	Season	all seasons
10.	Inoculation	
10.1	Preparation inoculum	3-5 days in aerated liquid cultures like PDB, Czapek Dox or S of Messiaen or scraping of plates of 10 days cultures on agar medium.
10.2	Quantification inoculum	spore count, adjust to 10^6 spores per ml, in case of very aggressive isolate inoculum concentration can be decreased
10.3	Plant stage at inoculation	10-18 d, cotyledon to first leaf
10.4	Inoculation method	plants at the inoculation stage are harvested carefully, roots and hypocotyls are immersed in spore suspension for 5-15 min; trimming of roots is an option, and transplanted in trays
10.7	Final observations	14-21 days after inoculation
11.	Observations	
11.1	Method	visual
11.2	Observation scale	

Class 0	Class 1	Class 2	Class 3
Healthy compared to the non-inoculated control.	Healthy compared to the non-inoculated control with brown vessel above the cotyledon (observed when plants are cut in case of variety with different levels of symptoms)	Higher than 50% of growth reduction and/or yellowing and/or wilting on cotyledons and/or leaves.	Nearly dead: strong reduction with plants look dwarf (there can be necrosis but not always) or dead
			
If all plants in class 0 or if all plants in classes 2 and 3, it is not necessary to cut the plants.			
In case of variety or control with different levels of symptoms, cut the plants to check presence or not of strong brown vessel above cotyledons. In case of no brown vessels or below cotyledons, the plant is note 0. In case of brown vessels above cotyledons, the plant is note 1.			

11.3	Validation of test	Validation on controls. Expected response of controls: Susceptible control: most plants in class 2 and 3, max.10% of plants class 0 and 1 Resistant control: most plants in class 0 and 1, max. 10% of plants class 2 and 3. Controls with medium level of resistance can show a higher number of plants in class 2 and 3.
12.	Interpretation of data in terms of UPOV characteristic states	[1] absent: Average symptom level higher than in the medium-resistant control [9] present: Average symptom level not different from the medium-resistant control or the high-resistant control

(ii) DNA marker test on gene *I-2*

The resistance gene I-2 confers resistance to both *Fusarium oxysporum* f. sp. *lycopersici* Fol:1(EU)/2(US) and Fol:0(EU)/1(US). The presence of the resistant allele and/or the susceptible allele can be detected by the co-dominant TaqMan marker based on the dominant marker described in Arens et al., (2010) and El Mohtar, et al., (2007).

Specific aspects: *Fusarium oxysporum* f.sp. *lycopersici* Fol: 1(EU)/2(US)

1	Characteristic	*Fusarium oxysporum* f.sp. l*ycopersici* Fol: 1(EU)/2(US)
2	Genes and alleles	*I-2*
2.1	Targeted gene(s)	Resistance Gene *I-2* Accession no. AF118127 Susceptible gene/ homologs *i-2* I-2C1 (accession no. AF004878), I-2C2 accession no. AF004879), I-2C3 (accession no. AF004880) Arens et al., (2009). Susceptible gene/ homologs i-2 I-2C1 (accession no. AF004878), I-2C2 accession no. AF004879), I-2C3 (accession no. AF004880)
2.3	Allele corresponding to expression state 9	Resistance Gene I-2 Accession no. AF118127 Arens et al., (2009)
3	Primers (and probes)	
3.1	Primers to detect both alleles	Forward Primer: 5'-AATGATGAGAGRGTGAAGAAWCA-3' Reverse Primer: 5'-TCTTTCCCTTCAAACTTTCCTTCA-3'
3.2	Probes to detect both alleles	Recommended probes are MGB probes (Applied biosystems) or XS probes (Biolegio) the Tm of the XS probes must be ordered at 68°C. Susceptible i2 probe: 5'-6FAM*-TTGACAGCTTGGTTTTGT-BHQ1-3' Resistance I2 probe: 5'-TEXASRED*-TTTGAAAGCGTGGTATTGC-BHQ2-3' *Fluorophores and quenchers can be modified according to compatibility with the filters on the real-time PCR machine.
4	Format of the test	
4.1	Number of plants per genotype	20 plants (individual DNA extraction and PCR for each plant)
4.2	Control varieties	
4.3	Process controls	Negative control (H2O), positive controle (sample containing the expected alleles)
5	Preparations	
5.1	Preparation DNA	Harvest per individual plant a part of a young leaf. Isolate total DNA with a standard DNA isolation protocol (for example commercial kit for plant DNA extraction, or lab prepared reagents)

5.2	Preparation PCR	Pipette each DNA sample and a commercial real-time PCR mastermix into individual wells. Analyze the samples in a real-time PCR machine capable of reading the fluorophores of all the probes, with reaction conditions suitable for the mastermix used. For this test the Quanta PerfeCta Multiplex qPCR Toughmix is commonly used.
5.3	Example PCR mastermix	

	Initial concentration	**Volume/ reaction (µL)**	**Final concentration**
PerfeCta Multiplex qPCR Toughmix	5x	4	1X
Forward Primer	10µm	0.75	375nM
Reverse Primer	10µm	0.75	375nM
Probe-Fus-i2-sus	10µm	0.3	150nM
Probe-Fus-I2-res	10µm	1.3	650nM
H_2O	-	9.9	-
subtotal		*17*	-
DNA		3	-
Total		**20**	-

6	Technique of the method	
6.1	Particular conditions	PCR conditions: 1. Initial denaturation step at 94°C for 2-10 minutes (mastermix dependent) 2. 40 cycles at 94°C for 15 sec, 60°C 1 min. Every cycle ends with plate reading 3. Analysis of Ct values for each probe is done to identify positive (+) reactions at Ct<35, or negative reactions (no Ct value). Reactions with Ct values 35-40 should be repeated. Analysis can also be done with a genotyping end point fluorescence reading.
7	Observations	
7.1	Validity of the results	•Check for typical exponential amplification curves for each sample, as expected for normal specific amplification. •Non-specific amplification is possible in a PCR reaction. Check the results for the presence of non-exponential curves and/or curves just above the threshold. These curves should be assessed as negative. •Check if the control samples are as expected (negative control: no signal; positive controls: shows expected signals for the fluorophores).
8	Interpretation of the test results	•Ct values are determined using a set threshold (single threshold) of 200 RFU for each of the fluorescence labels,. this value may need to be adapted to each machine. •For low or high Ct values the DNA concentration should be checked. If the DNA concentration is low, high Ct values are expected. For samples with a high DNA concentration, low Ct values are expected. If two fluorophores are present, both fluorophores will show the high or the low Ct value.

8.1	Decision Matrix:	

Signal specific Fluorophore*				
Fam Susceptible i-2**	Texas Red Resistance I-2 **	**Molecular Interpretation**	**Conclusion regarding resistance to Fol: 1(EU)/2(US)**	**Control variety**
+	-	i-2/i-2	Absent***	Marmande Verte
+	+****	I-2/i-2	Present	Motelle x Marmande Verte
-	+	I-2/I-2	Present	Tradiro
-	-	Invalid result. Repeat assay or bio assay should be performed.		

* + signal is above the threshold and curves are as expected; - signal is not above the threshold or curves are non-exponential.
**Fluorophores can be modified according to compatibility with the filters on the real-time PCR machine.
*** Susceptible, or possibly resistant on another mechanism like gene I3
****Ct value should not be more than +3Ct after the Ct value of the susceptible i-2 fluorophore otherwise the marker is considered as absent.

9	Validation of the method	A conclusion of presence/absence of resistance should be made for each variety based on the results of the 20 individual plant genotypes. A tolerance of 1 individual out of type plant can be made, otherwise the variety should be identified as heterogenous if contradictory results are obtained for a variety. This protocol was validated by a ring-test with three different laboratories (Interlaboratory Comparative Test Report, INVITE 2023). If a different protocol is used, the laboratory must validate its method in comparison to the reference method to show that the alternative protocol gives the same results.
	Contact Examination Office	Naktuinbouw

Ad. 49: Resistance to *Fusarium oxysporum* f. sp. *lycopersici* - Race 2EU/3US (Fol: 2EU/3US)

See Ad. 47

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