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CHRYSANTHEMUM VIRUS B, ITS SEROLOGICAL DIAGNOSIS IN CHRYSANTHEMUM, AND ITS RELATIONSHIP TO THE POTATO VIRUSES S AND M AND TO CARNATION LATENT VIRUS[1]

Met een samenvatting: De serologische diagnostiek van virus B in chrysant en de verwantschap van dit virus met S-virus en M-virus van aardappel en met latent anjervirus

BY

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1. INTRODUCTION

Chrysanthemum virus B was first described by NOORDAM (1952) in The Netherlands. The virus is common in the United States of America (BRIERLEY & SMITH, 1953) and HOLLINGS (1957) found it widespread in English chrysanthemum stocks. The virus can be detected by means of sap inoculation of test plants. Provided NOORDAM's *Cucumis* virus 1 st. Chr. is absent, *Petunia hybrida* Vilm. var. 'Celestial' reacts with yellowish spots about two to five weeks after inoculation. To avoid this long incubation period, however, a search was made for a more rapid method of detection.

The first positive results with an antiserum were published by NOORDAM (1952) but sometimes his reactions were obscured by spontaneous flocculations or no precipitation ocurred at all. HOLLINGS (1957) also attempted to prepare an antiserum but this serum did not react with the antigen.

In the present paper a serological method is described giving satisfactory results with chrysanthemum material by using a homogenizing medium which prevents spontaneous flocculations. The results of this method were in good agreement with those obtained in infection tests. Moreover, the production of an antiserum of sufficient titre made it possible to investigate the serological relationship of *Chrysanthemum* virus B to other viruses.

2. PREPARATION OF AN ANTISERUM TO *CRYSANTHEMUM* VIRUS B

2.1. *Purification of antigenic material*

By means of a Waring blendor, 80 g of leaves from infected plants of the variety 'Evelyn Bush' were homogenized in 160 ml of a 0.25 percent solution of Na_2SO_3 and the homogenate was pressed through cheesecloth. To 210 ml of this juice was added 21 ml of n-butanol. The mixture was then shaken for 10 minutes after which 210 ml of chloroform were added under continued shaking. The aqueous phase, separated by centrifugation (STEERE, 1959), was supplemented with 100 ml of distilled water to a total of 280 ml and centrifuged at 60000 $\times$ *g* for 1 hour. The sediment was resuspended in 3 ml of saline and mixed with 3 ml of Freund's Bacto Adjuvant Complete (Difco Laboratories).

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2.2. *Immunization of rabbits*

Two rabbits were injected intramuscularly, receiving 1 ml of the emulsion into each hindleg. The injection was repeated 19 days later with the same amount of emulsion. Blood was taken from the ear 25 days after the second injection.

For absorption of the antisera an extract from virus-free chrysanthemum plants was prepared by homogenizing 8 g of leaves in a 0.25 percent solution of Na_2SO_3. The green liquid obtained by squeezing the homogenate through cheesecloth was centrifuged for 15 minutes at 60000 $\times$ *g*, the sediment being resuspended in 8 ml of saline. One volume of antiserum mixed with 9 volumes of this suspension was incubated for 2 hours at 37°C and then left overnight at a temperature just above zero, whereupon the precipitate was centrifuged off. The clear supernatant was then divided in small portions and freeze-dried in glass ampoules.

3. Methods for detecting the virus

3.1. *Method of inoculating* Petunia *plants*

Three or four chrysanthemum leaves were crushed in a mortar in 1–2 ml of a freshly prepared 0.5 percent solution of Na_2SO_3. The extract was rubbed with the finger on the leaves of *Petunia* plants, previously dusted with carborundum powder, after which the plants were rinsed with water and were placed in an aphid-proof greenhouse.

3.2. *Serological method*

For titration of the antisera as well as for the routine serological tests, the microprecipitation test described by van Slogteren (1955) was employed.

In preparing extracts from chrysanthemum leaves, Na_2SO_3 was included in the extraction medium to prevent the sap from turning brown by enzymatic action (Oertel, 1959). The extraction giving satisfactory results was as follows: 15 g of fresh leaves were homogenized with 45 ml of a phosphate buffer solution consisting of 6 parts 1/30 molar Na_2HPO_4 and 4 parts 1/30 molar KH_2PO_4. In this phosphate buffer Na_2SO_3 was dissolved to make a 0.5 percent solution. The supernatant obtained by centrifuging the extract at 3000 r.p.m. for 10 minutes was used for the reactions.

From the absorbed antisera a series of twofold dilutions was made with saline in glass tubes, the initial serum dilution by absorption being 1:10. Droplets taken from these various antiserum dilutions were mixed with droplets of clarified extracts from virus B-infected and virus-free plants respectively.

When tested against extracts from plants with a sufficient virus concentration the antisera showed precipitin end-point titres varying between 1:320 and 1:640, best results being obtained during the winter months.

4. Comparison between the results of serological reactions and infection tests

The results obtained from serological reactions and infection tests with extracts from the same chrysanthemum plants are shown in table 1. In 153 out of a total of 154 plants belonging to 22 varieties the results obtained by

TABLE 1. The results of parallel serological and infection tests with extracts from 154 chrysanthemum plants belonging to 22 varieties.
De resultaten van serologische reacties en infectieproeven, uitgevoerd met extracten van 154 chrysanteplanten, behorende tot 22 cultivars.

Variety *Cultivar*	Number of plants tested *Aantal getoetste planten*	Serological reaction *Serologische reactie*	Infection test on *Petunia* *Infectieproef op* Petunia
'Autumn Tints'	11	−	−
'Copperhead'	3	+	+
do.	9	−	−
'Delamere'	1	+	+
'Delmar'	5	−	−
'Dr. Wasscher'	1	+	+
'Espoir de René'	4	+	+
'Evelyn Bush'	11	+	+
'Golden Chord'	12	−	−
'Goldene Sonne'	4	+	+
do.	8	−	−
'Illini Formal'	12	−	−
'Illini Golddrift'	1	+	+
do.	11	−	−
'Long Island Beauty Bronze'	1	+	+
'Matterhorn'	10	−	−
'Pinkette'	12	−	−
'Pink Fred Shoesmith'	7	−	−
'René Molineaux'	2	+	+
'Stylish'	12	−	−
'Super Form'	1	+	+
'White Popcorn'	6	−	−
'Woking Perfection'	1	+	+
'Yellow Beauregard'	8	−	−
'No. 100'	1	−	+

both methods in parallel tests were in complete agreement. In only one case did yellow lesions appear on an inoculated *Petunia* plant whereas the serological reaction was negative.

The tests mentioned above were carried out in the autumn and winter months. Serological tests done in summer gave less satisfactory results. This is in agreement with the seasonal variation in the reaction of virus B on *Petunia* already described by HOLLINGS (1957). In his experience the summer months were unfavourable for reliable infection tests.

5. SEROLOGICAL RELATIONSHIPS OF *CHRYSANTHEMUM* VIRUS B WITH OTHER VIRUSES

5.1. *Serological reactions with antisera to other viruses*

Antisera to 23 plant viruses known to have either rod- or thread-shaped particles, including antiserum to *Chrysanthemum* virus B itself, and also normal serum were absorbed with an extract from virus-free chrysanthemum plants as described above. The results of the precipitin tests between these absorbed sera and extracts from respectively virus B-infected and virus-free plants of the chrysanthemum variety 'Evelyn Bush' are shown in table 2.

TABLE 2. Serological reactions of extracts from virus B-infected and virus-free chrysanthemums respectively with antisera to 23 different plant viruses and with normal serum.
Serologische reacties van extracten van met virus B geïnfecteerde, resp. virusvrije chrysanteplanten met antisera tegen 23 verschillende plantevirussen en met normaalserum.

Antiserum to *Antiserum tegen*	Reaction with chrysanthemum *Reactie met chrysant*		Antiserum to *Antiserum tegen*	Reaction with chrysanthemum *Reactie met chrysant*	
	Virus B-infected *Met virus B besmet*	Virus free *Virusvrij*		Virus B-infected *Met virus B besmet*	Virus free *Virusvrij*
Potato virus F	−	−	*Narcissus* "grey" virus	−	−
Potato virus M	+	−	*Ornithogalum* mosaic virus	−	−
Potato virus S	+	−	*Odontoglossum* ringspot virus	−	−
Potato virus X	−	−	*Cymbidium* mosaic virus	−	−
Potato virus Y	−	−	*Scilla* mosaic virus	−	−
Alfalfa mosaic virus	−	−	Sugarbeet yellows virus	−	−
Carnation latent virus	+	−	Tobacco mosaic virus	−	−
Phaseolus virus 2	−	−	*Tulipa* virus 1	−	−
Freesia mosaic virus	−	−	*Allium* virus 1	−	−
Hyacinth mosaic virus	−	−	*Chrysanthemum* virus B	+	−
Iris mosaic virus	−	−	Normal serum	−	−
Iris "grey" virus	−	−			
Narcissus mosaic virus	−	−			

The positive reactions between *Chrysanthemum* virus B and antisera to the Potato viruses S and M and to Carnation latent virus were interesting, the more so as a serological relationship between these three viruses has already been established by previous authors, reference being made here to BAGNALL, LARSON & WALKER (1956), KASSANIS (1956), ROZENDAAL & VAN SLOGTEREN (1958) and BAGNALL, WETTER & LARSON (1959).

5.2. *Reciprocal reactions and cross-absorption tests*

5.2.1. Materials and methods

For titration of the respective antisera as well as for purposes of absorption and cross-absorption, partially purified preparations were made as follows from infected as well as from virus-free host plants: 20 g of leaves were homogenized in 60 ml of 1/30 molar phosphate buffer pH 7, including 0.5 percent Na_2SO_3 and the homogenate was pressed through cheesecloth. The extract was mixed and shaken with diethylether, whereupon the aqueous layer, separated by centrifugation, was mixed and shaken with CCl_4 (WETTER, 1960). The clear liquid separated from CCl_4 by centrifugation was stirred with two thirds of its volume of saturated $(NH_4)_2SO_4$ and the precipitate, sedimented by centrifugation, was dissolved in 6 ml of water and dialysed against tapwater. NaCl was then added to give a 0.85 percent solution. Such preparations represented approximately a threefold concentration of the original extract and were used for absorbing the sera, initial serum dilutions thus being kept at a low level. Virus preparations for titration were diluted 1:2 with saline.

Carnation latent virus, *Chrysanthemum* virus B, Potato virus M and Potato virus S will henceforth be indicated as CLV, CVB, PVM and PVS respectively,

partially purified virus preparations as pCLV, pCVB, pPVM and pPVS, and preparations from the virus-free host plants, viz. *Dianthus barbatus*, chrysanthemum and potato, as pD, pC and pP. In order to prevent spontaneous flocculations arising when preparations from different host plants were involved in a reaction, it was found desirable to absorb the antisera with mixtures of pD, pC and pP. In the case of cross-absorption a virus preparation was added to these mixtures.

For cross-absorptions with either CLV or CVB 0.8 ml of either pCLV or pCVB + 0.2 ml of each preparation pD, pC and pP was added to 0.1 ml of antiserum. Similarly for cross-absorptions with either PVM or PVS, 0.1 ml of antiserum received 0.7 ml of either PVM or PVS + 0.1 ml each of pD and pC. For comparison, 0.4 ml of antiserum was absorbed with a mixture of 0.4 ml pD + 0.4 ml pC + 0.4 ml pP.

5.2.2. Interpretations of the results

The titres of the respective cross-absorbed sera and, for comparison, the same sera absorbed with virus-free mixtures, to all homologous and heterologous virus preparations are shown in table 3. The cross-absorptions were carried out in two sets, one with pCLV and pCVB along with a mixture p(D + C + P)1 for comparison, and the other with pPVM and pPVS with a mixture p(D + C + P)2. In evaluating the data the relative strengths of the antisera as well as the relative amounts of virus in respective preparations must be taken into account. The homologous titres of the four antisera estimated in preliminary tests averaged 8192 for CLV-antiserum, 2048 for CVB-antiserum, 2048 for PVM-antiserum and 4096 for PVS-antiserum. For practical reasons the titrations shown in table 3 were made up to serum dilution 1:1024, the reciprocal value 1024 being attained or surpassed only by homologous titres.

1024^{++} in table 3 stands for a strong flocculation with the homologous virus at serum dilution 1:1024, indicating a serum titre of at least 2048. The precipitin end-points of the respective virus preparations in a preliminary test against their homologous antisera were 1:256 for pCLV, 1:64 for pCVB, 1:64 for pPVM and 1:32 for pPVS.

The homologous titres of the four antisera are high as compared to those against heterologous viruses, pointing to a major antigenic fraction distinguishing each virus from the three others. With respect to the homologous titre of PVS-antiserum, its value of 1024, which is at least one step lower than the other three homologous titres, is apparently due to the low antigenic strength of pPVS. PVS-antiserum was also the only one for which the homologous titre was not fully eliminated by homologous absorption.

In attempting to get more information about relationships among the four viruses by comparison of heterologous titres it may be helpful to present the data from table 3 in the form shown in table 4 so that the three heterologous viruses to each antiserum are arranged in the decreasing order of their corresponding serum titres.

Taking into account the fact that the heterologous antiserum titres, apart from being proportional to the degree of relationship, are proportional also to the relative strength of the particular antiserum and, for this type of elon-

TABLE 3. Homologous and heterologous titres of the antisera to CLV, CVB, PVM and PVS absorbed and cross-absorbed respectively with homologous and heterologous virus preparations and, for comparison, with a mixture of preparations from virus-free host plants.
Homologe en heterologe titers van de antisera tegen CLV, CVB, PVM en PVS na verzadiging, respectievelijk kruisverzadiging, met homologe en heterologe virusprepa-raten en ter vergelijking met een mengsel van extracten uit virusvrije waardplanten.

Antiserum	Absorbing preparation *Preparaat voor verzadiging*	Test virus preparation *Viruspreparaat voor toetsing*			
		pCLV	pCVB	pPVM	pPVS
CLV-antiserum	p(D + C + P)1	1024^{++}	32	128	32
	pCLV	0	0	0	0
	pCVB	1024	0	64	(6)0
	p (D + C + P)2	1024^{++}	32	128	16
	pPVM	1024	16	0	8
	pPVS	(1) 1024^{++}	16	64	0
CVB-antiserum	p(D + C + P)1	256	1024^{++}	32	64
	pCLV	0	1024	(7)0	32
	pCVB	0	0	0	0
	p(D + C + P)2	128	1024^{++}	32	64
	pPVM	128	1024	0	32
	pPVS	128	1024	(8)0	0
PVM-antiserum	p(D + C + P)1	16	16	1024^{++}	16
	pCLV	0	(9)0	(3) 512	(10)0
	pCVB	(11)0	0	(4) 512	(12)0
	p(D + C + P)2	16	32	1024^{++}	8
	pPVM	0	0	0	0
	pPVS	8	16	1024	0
PVS-antiserum	p(D + C + P)1	64	128	32	1024
	pCLV	0	64	(13) 0	512
	pCVB	32	0	16	(5) 256
	p(D + C + P)2	32	128	32	1024
	pPVM	16	64	0	(2) 1024
	pPVS	0	0	0	32

gated virus, to the virus concentration of the test-suspension, some evidence for lower and higher degrees of relationships among the four viruses can be obtained from these data. Virus S is apparently more closely related to CVB than to CLV and is least related to virus M. CLV-antiserum being the serum with the largest relative strength displays the largest titre against virus M. M-antiserum seems to be the poorest of the four sera in antibodies to heterologous viruses. In analysing further the data shown in table 3 special attention is given to either elimination or at least two-step reductions in titre by homologous and heterologous absorption respectively and to any absence of reduction.

Some cases of particular interest are indicated by numbers between brackets in table 3 as well as in the text. In all cases homologous absorption eliminated

TABLE 4. Heterologous viruses to the different antisera arranged in the decreasing order of their corresponding heterologous antiserum titres taken from table 3, and, for comparison, the average homologous titres.
De heterologe virussen, voor de verschillende antisera gerangschikt volgens afnemende grootte der aan tabel 3 ontleende heterologe antiserumtiters met ter vergelijking de gemiddelde homologe titers.

Antiserum	Heterologous antiserum titres *Heterologe antiserumtiters*	Homologous titre *Homologe titer*
CLV-antiserum	PVM (128–128); CVB (32–32); PVS (32–16)	8192
CVB-antiserum	CLV (256–128); PVS (64–64); PVM (32–32)	2048
PVM-antiserum	CVB (16–32); CLV (16–16); PVS (16–8)	2048
PVS-antiserum	CVB (128–128); CLV (64–32); PVM (32–32)	4096

heterologous titres and heterologous absorption eliminated titres against the absorbing virus itself. In 2 out of 12 cases, viz. CLV-antiserum absorbed with pPVS (1) and PVS-antiserum absorbed with pPVM (2), heterologous absorption caused no reduction in homologous titres. On the other hand, in three cases, viz. PVM-antiserum absorbed with pCLV (3) and pCVB (4) and PVS-antiserum absorbed with pCVB (5), the homologous titre was reduced by two steps.

In 8 out of 24 cases heterologous absorption of an antiserum eliminated its titre against one or two of the remaining heterologous viruses. The 8 instances where this occurred were the following: CLV-antiserum against pPVS by absorption with pCVB (6), CVB-antiserum against pPVM by absorption with pCLV (7) as well as with pPVS (8), PVM-antiserum against both pCVB (9) and pPVS (10) by absorption with pCLV and against both pCLV (11) and pPVS (12) by absorption with pCVB and, finally, PVS-antiserum against pPVM by absorption with pCLV (13).

A more distant relationship between CLV and PVS as indicated by (1) is uncertain as the relatively high relative strength of CLV-antiserum is disproportional to the low antigenic strength of pPVS. On the other hand (2) supports the assumption of a more distant relationship between PVS and PVM.

That CLV probably has a distinct antigenic fraction in common with PVM on the one hand and with both CVB and PVS on the other is supported by the relatively high titre of CLV-antiserum against pPVM which is little affected by absorption with either pCVB or pPVS whereas its titre against pPVS is eliminated by absorption with pCVB (6). As far as CLV, PVM and PVS are concerned, the analogous interpretation given by BAGNALL, WETTER & LARSON (1959) is thus endorsed. Moreover (6) supports the assumption of a relatively close relationship between CVB and PVS which assumption is strengthened also by (5). The assumption of a relatively close relationship between PVM and CLV is supported by (13).

The low titres (16–16) of PVM-antiserum against CLV in comparison to the high reciprocal titres (128–128) of CLV-antiserum against PVM, the fact that all heterologous titres of PVM-antiserum are eliminated by absorption with both pCLV and pCVB (9), (10), (11), (12), and the marked reduction of its homologous titre by absorption with both pCLV and pCVB (3), (4), suggest that PVM-antiserum has a poorer assortment of antibodies to distinct antigenic fractions than the relatively stronger CLV-antiserum. It seems that, in

addition to "PVM-specific" antibodies, PVM-antiserum has only just sufficient antibodies to a fraction which is common to all viruses of the group to make it detectable. It is conceivable that for antisera to viruses of this group the number of antibodies of different specificities increases with the relative strength of the antiserum concerned. If this is true the strongest antisera, viz. CLV- and PVS-antisera are the most suitable ones for differentiating distinct antigenic properties among heterologous viruses. Consequently, the arrangements of the heterologous viruses as shown in table 4 for CLV- and PVS-antisera would reflect the sequence of relationships better than those found for CVB- and PVM-antisera.

CVB appears to have an antigenic fraction common to CLV, PVM and PVS, besides sharing a distinct fraction with PVS. This is suggested by the fact that the titre of CVB-antiserum against pPVM is eliminated by absorption with both pCLV (7) and pPVS (8), whereas its titre against pPVS is affected to a lesser extent by absorption with either pCLV or pPVM. On the other hand, the relatively high heterologous titres (256–128) of CVB-antiserum against pCLV, although perhaps somewhat out of proportion owing to the high virus concentration of pCLV, suggests an antigenic fraction common to CLV and CVB, which view is strengthened by the fact that absorption with either pPVM or pPVS caused no perceptible reduction of this titre.

The overall results of heterologous reactions and cross-absorption tests present enough evidence to range *Chrysanthemum* virus B as a distinct entity among the group of viruses hitherto represented by Potato virus S, Potato virus M and Carnation latent virus. If an attempt is made to arrange these viruses according to their mutual degrees of serological relationships, the results obtained under imperfect conditions make it reasonable to propose a provisinal arrangement with the viruses CVB and CLV in the centre and PVS and PVM on either side, thus: PVS–CVB–CLV–PVM.

BRANDES (1961), in arranging elongated plant viruses according to their "normal length", placed *Chrysanthemum* virus B in a group including among others, CLV, PVM, and PVS, the normal length of CVB being slightly longer than that of CLV, PVM and PVS. Our serological results indicate that further attempts to obtain highly accurate electronmicroscopical measurements of the four viruses are warranted, as also are further serological studies with other viruses of the same morphological group.

SUMMARY

An antiserum prepared against *Chrysanthemum* virus B reacted positively with extracts from 39 chrysanthemum plants out of a total of 154, belonging to 22 varieties. In all 39 cases, *Petunia* plants developed symptoms of virus B after inoculation with extracts from the same plants. Of the *Petunia* plants inoculated with the remaining 115 chrysanthemum extracts which did not react with the antiserum, only one developed symptoms. Reliable results were obtained only with an extraction medium consisting of 1/30 molar phosphate buffer solution, pH 7, including 0.5 percent sodium sulfite.

The serological relationship of *Chrysanthemum* virus B to Carnation latent virus, Potato virus M and Potato virus S was established during the testing of 23 available antisera against extracts from infected chrysanthemum, and

was confirmed by reciprocal tests. By comparing homologous and heterologous titres of the respective antisera against the four viruses and also in cross-absorption tests, some evidence was obtained for different degrees of serological relationship among the four viruses.

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Samenvatting

Uit bladextracten van met *Chrysanthemum*-virus B (CVB) besmette chrysanteplanten kon het virus met behulp van de butanol-chloroform-methode worden gezuiverd. Door intramusculair inspuiten van konijnen met geconcentreerde virussuspensies, gemengd met Freund's adjuvant olie, werd een specifiek antiserum tegen dit virus verkregen. Met dit antiserum werden 154 planten, behorende tot 22 cultivars, getoetst op de aanwezigheid van CVB, terwijl tevens met een extract van deze planten *Petunia*'s werden geïnoculeerd (tabel 1). Het extract van de 39 planten die serologisch positief reageerden, gaf na inoculatie op *Petunia*'s karakteristieke gele vlekken op deze toetsplanten. Bij de 115 planten die serologisch niet reageerden was er slechts één geval, waarbij op *Petunia* toch een ziektebeeld ontstond.

Voor een betrouwbaar resultaat was het van essentieel belang om als extractie-vloeistof 1/30 molair fosfaatbuffer met pH 7 te gebruiken, waaraan natriumsulfiet tot een concentratie van 0,5% was toegevoegd. De beste resultaten werden verkregen in de herfst- en wintermaanden.

Van 23 antisera, bereid tegen verschillende plantevirussen, reageerden antiserum tegen latent anjervirus (CLV) en de antisera tegen M-virus (PVM) en S-virus (PVS) van aardappel positief met extracten uit chrysanteplanten besmet met CVB. Tabel 2 geeft een overzicht van deze reacties.

In een proef, waarbij antisera tegen respectievelijk CLV, CVB, PVM en PVS werden getitreerd met gezuiverde preparaten der vier virussen in alle homologe en heterologe combinaties, werd de bovengenoemde verwantschap van *Chrysanthemum*-virus B met de virussen CLV, PVM en PVS reciprook bevestigd. Door onderlinge vergelijking van de heterologe titers en door bovendien de invloed na te gaan op homologe en heterologe titers teweeggebracht indien de antisera met telkens één van de vier virussen werden verzadigd („kruisverzadiging"), werd een voorlopige indruk verkregen van de mate der onderlinge serologische verwantschap tussen de vier virussen. Deze zou dan kunnen worden aangegeven door de volgorde PVS–CVB–CLV–PVM.

De homologe en heterologe titers en de titers der vier antisera na kruisverzadiging met elk der vier virussuspensies worden weergegeven in de tabellen 3 en 4. Uit de verhouding tussen homologe en heterologe titers kwam duidelijk naar voren dat de antigene fractie, specifiek voor elke virus afzonderlijk, groter is dan de fracties welke twee of meer virussen onderling gemeen hebben.

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