2004-021: Draft Annex to ISPM 27 – *Citrus tristeza virus*

Comm. no.	Para. no.	Comment type	Comment	Explanation	Country	SC Responses
1.	*G*	Substantive	I support the document as it is and I have no comments		Guyana, Congo, Australia, Singapore, Mexico	**INCORPORATED** Thank you for your confidence in our work.
2.	*G*	Substantive	Footnotes related to the use of commercial brands should be included in this draft DP. Moreover there are commercial brands associated to a footnote, but text of the footnote is missing (footnote Nº 11 in paragraph 102; footnote 12 in paragraph 108 and footnote 7 in paragraph 137)	The following paragraphs mention commercial brands: 50, 53, 102, 108 and 137. Footnote to be included should read as follows: "The use of the brands ....., in this diagnostic protocol implies no approval of them to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute an endorsment by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results"	Uruguay, Argentina, Chile, Paraguay	**MODIFIED** We agree in including the commercial brands as suggested. Please, note than in paragraph 50 the two mentioned brands are the only ones internationally distributing Tissue print-ELISA kits. In 53 all the brands related to the product are mentioned.
3.	*G*	Technical		General comments about the terminology used in this protocol When preparing the EPPO Standard on interlaboratory comparison Quality Assurance experts (from outside plant pest diagnostics) commented that the term ring test should not be used any longer but test performance study should be used instead. This terminology has now been adopted in EPPO Standards. We suggest replacing ring test by test performance study. Molecular amplification test: this terminology is not used in other IPPC protocols. We propose to replace 'molecular amplification test' by 'molecular tests' as in other protocols. In this protocol 'method', 'procedure' 'test' seem to be used with the meaning of tests in other IPPC protocols. The use of	European Union	**INCORPORATED** We fully agree with the terminology of "performance study". Nevertheless, "molecular amplification test" refers better to PCR based techniques than molecular tests that is too general and includes molecular

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				terms should be checked and be consistent we recommend to continue using test to designate the combination of a method (e.g. molecular/serological to detect/identify a pest in a matrix).		hybridization (see e.g. 20 and 38). So we suggest maintaining the specific term of "molecular amplification techniques" that could be used in future in all protocols. We agree with the suggested use of test instead of method.
4.	*7*	Substantive	Add CTV infestation and distribution information in Asian countries.**1. Pest Information**	It is useful for control and technical cooperation.	China	**CONSIDERED, BUT NOT INCORPORATED** A phrase was added to better specify the putative origin of CTV and citrus ("in South East Asia and Malaysia, putative areas of origin of citrus). CTV (mainly aggressive isolates) is present in all Asian citrus industries with very high prevalence (endemic), We consider it is not necessary to identify country by country, but we can do if the IPPC panel agrees. In fact, CABI is finishing a publication in which a complete list of the

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						situation of CTV by countries is included.
5.	*8*	Editorial	*Replace "Citrus limon (lemon) " to "C. limon (lemon) "*Citrus tristeza virus* (CTV) causes one of the most damaging diseases of citrus resulting in devastating epidemics that have changed the course of the citrus industry (Moreno *et al*., 2008). The term tristeza, in Portuguese "sadness" or "melancholy", refers to the decline seen in many citrus species when grafted on *Citrus aurantium* (sour orange) or *Citrus limon* (lemon) rootstocks. Although tristeza disease is predominantly a bud union disease (Román *et al*., 2004), some CTV strains induce other disorders, including stem pitting, stunting, reduced productivity and impaired fruit quality of many commercial cultivars, even when they are grafted on tristeza-tolerant rootstocks.	The generic name should be abbreviated when it presents for the second time.	China	**MODIFIED** We agree. The text of the manuscript was accordingly modified.
6.	*10*	Editorial	d isease should be disease. Tree losses on sour orange rootstock were first reported in South Africa in the early twentieth century, and in Argentina and Brazil in the 1930s following the introduction of CTV-infected plants and the aphid vector most efficient for transmitting the virus, *Toxoptera citricida* Kirkaldy. CTV-induced tree decline has killed or rendered unproductive trees grafted on *C. aurantium* (sour orange) rootstock (Bar-Joseph *et al*., 1989; Cambra *et al*., 2000a). CTV outbreaks have been observed in the United States of America, some Caribbean countries and some Mediterranean countries (especially Italy and Morocco). CTV has affected an estimated 38 million trees in the Americas (mainly Argentina, Brazil, Venezuela and California (United States of America)), 60 million trees in the Mediterranean Basin (especially Spain, with about 50 million trees affected) and an estimate of 5 million trees elsewhere, making a total of more than 100 million trees. Tristeza di sease can be managed by using citrus rootstock species that induce tolerance to the tristeza syndrome. Some aggressive strains of CTV cause stem pitting in certain citrus cultivars regardless	One more space should be deleted.	China	**MODIFIED** The word has been corrected.

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			of the rootstock used. This has a significant impact on fruit quality and yield in several million trees infected with these aggressive strains in most citrus industries worldwide, with the exception of those in the Mediterranean Basin. To effectively manage this stem pitting syndrome, some citrus industries have adopted a strategy of prophylactically inoculating trees with mild strains of CTV, otherwise known as cross-protection (Broadbent *et al*., 1991; Da Graça and van Vuuren, 2010).			
7.	*10*	Translation	Tree losses on sour orange rootstock were first reported in South Africa in the early twentieth century, and in Argentina and Brazil in the 1930s following the introduction of CTV-infected plants and the aphid vector most efficient for transmitting the virus, *Toxoptera citricida* ((Kirkaldy)). CTV-induced tree decline has killed or rendered unproductive trees grafted on *C. aurantium* (sour orange) rootstock (Bar-Joseph *et al*., 1989; Cambra *et al*., 2000a). CTV outbreaks have been observed in the United States of America, some Caribbean countries and some Mediterranean countries (especially Italy and Morocco). CTV has affected an estimated 38 million trees in the Americas (mainly Argentina, Brazil, Venezuela and California (United States of America)), 60 million trees in the Mediterranean Basin (especially Spain, with about 50 million trees affected) and an estimate of 5 million trees elsewhere, making a total of more than 100 million trees. Tristeza di sease can be managed by using citrus rootstock species that induce tolerance to the tristeza syndrome. Some aggressive strains of CTV cause stem pitting in certain citrus cultivars regardless of the rootstock used. This has a significant impact on fruit quality and yield in several million trees infected with these aggressive strains in most citrus industries worldwide, with the exception of those in the Mediterranean Basin. To effectively manage this stem pitting syndrome, some citrus industries have adopted a strategy of prophylactically inoculating trees with mild strains of CTV, otherwise known as cross-protection (Broadbent *et al*., 1991; Da Graça and van Vuuren,	Clarification needed wheather the CTV was introduced in Brazil and Agentina through either CTV infected plants the Aphid (Toxoptera citricida) or both at the same time.	Kenya	**MODIFIED** Very probably CTV was introduced in Brazil and Argentina through CTV infected plants infested with *T. citricida.* The text was slightly modified in this sense.

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			2010).			
8.	*11*	Editorial	CTV is the largest and most complex member of the genus *Closterovirus* (Moreno *et al*., 2008). The virions are flexuous filamentous, 2 000 ~~nn~~nm long and 11 nm in diameter, and contain a non-segmented, positive-sense, single-stranded RNA genome. The CTV genome contains 12 open reading frames (ORFs), potentially encoding at least 17 proteins, and two untranslated regions (UTRs). ORFs 7 and 8 encode proteins with estimated molecular weights of 27.4 kDa (P27) and 24.9 kDa that have been identified as the capsid proteins. CTV diversity is greater than previously thought; new genotypes have diverged from the ancestral population or have arisen through recombination with previously described strains (Harper *et al*., 2008). CTV populations in citrus trees are complex mixtures of viral genotypes and defective RNAs developed during the long-term vegetative propagation of virus isolates through grafting and the mixing of such isolates with aphid-vectored isolates. This results in CTV isolates containing a population of sequence variants, with one usually being predominant (Moreno *et al*., 2008).	it should be 'nm' not 'nn'	European Union	**MODIFIED** We agree. Must be nm. The text was corrected.
9.	*11*	Technical	"2000 nn" should be changed into " 2000 nm".CTV is the largest and most complex member of the genus *Closterovirus* (Moreno *et al*., 2008). The virions are flexuous filamentous, 2 000 nn long and 11 nm in diameter, and contain a non-segmented, positive-sense, single-stranded RNA genome. The CTV genome contains 12 open reading frames (ORFs), potentially encoding at least 17 proteins, and two untranslated regions (UTRs). ORFs 7 and 8 encode proteins with estimated molecular weights of 27.4 kDa (P27) and 24.9 kDa that have been identified as the capsid proteins. CTV diversity is greater than previously thought; new genotypes have diverged from the ancestral population or have arisen through recombination with previously described strains (Harper *et al*., 2008). CTV populations in citrus trees are complex mixtures of viral genotypes and defective	Mistake of noting.	China	**MODIFIED** nm was introduced in the text in order to correct the mistake.

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			RNAs developed during the long-term vegetative propagation of virus isolates through grafting and the mixing of such isolates with aphid-vectored isolates. This results in CTV isolates containing a population of sequence variants, with one usually being predominant (Moreno *et al*., 2008).			
10.	*12*	Editorial	Replace "Aphis spiraecola Patch, Toxoptera aurantii (Boyer de Fonsicolombe), Myzus persicae (Sulzer), Aphis craccivora Koch and Uroleucon jaceae (Linnaeus)" to "A. spiraecola Patch, T. aurantii (Boyer de Fonsicolombe), Myzus persicae (Sulzer), A. craccivora Koch and Uroleucon jaceae (Linnaeus)"CTV is readily transmitted experimentally by grafting healthy citrus with virus-infected plant material. It is naturally transmitted by certain aphid species in a semi-persistent manner. The most efficient vector of CTV worldwide is *T. citricida*. *T. citricida* is well established in Asia, Australia, sub-Saharan Africa, Central and South America, the Caribbean, Florida (United States of America) and northern mainland Spain and Portugal as well as the Madeira Islands (Ilharco *et al*., 2005; Moreno *et al*., 2008). However, *Aphis gossypii* Glover is the main vector in Spain, Israel, some citrus growing areas in California (United States of America) and in all locations where *T. citricida* is absent (Yokomi *et al*., 1989; Cambra *et al*., 2000a; Marroquín *et al*., 2004). The comparative effects of aphid vector species on the spread of CTV have been reported (Gottwald *et al*., 1997). Other aphid species have also been described as CTV vectors (Moreno *et al*., 2008) including *Aphis spiraecola* Patch, *Toxoptera aurantii* (Boyer de Fonsicolombe), *Myzus persicae* (Sulzer), *Aphis craccivora* Koch and *Uroleucon jaceae* (Linnaeus). Although these listed aphid species were shown to be less efficient vectors of CTV than *T. citricida* and *A. gossypii* in experimental transmission studies, they are the predominant aphid species in some areas and are therefore likely to play a role in CTV spread, compensating for their poor transmission efficiency by their abundance (Marroquín *et al*., 2004).	The generic name should be abbreviated when it presents for the second time.	China	**MODIFIED** We agree. The change was introduced in the text of the manuscript.

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11.	*20*	Technical	Detection and identification of CTV can be achieved using biological, serological or molecular amplification tests. The use of any one of these tests is the minimum requirement to detect and identify CTV (i.e. during routine diagnosis of the pest when it is widely established in a country). In instances where the national plant protection organization (NPPO) requires additional confidence in the identification of CTV (i.e. detection in an area where the virus is not known to occur or detection in a consignment originating from a country where the pest is declared to be absent), further tests may be done. Where the initial identification was done using a molecular ~~amplification~~ test, subsequent tests should be serological, and vice versa. Further tests may also be done to identify the strain of CTV present, in which case sequencing of the PCR amplicon may be needed. In all cases, for the tests to be considered valid positive and negative controls must be included. The recommended techniques for the biological, serological and molecular amplification tests are described in the following sections. A flow chart for strain identification of CTV is presented in Figure 3.	Regarding the following sentence : "Where the initial identification was done using a molecular amplification test, subsequent tests should be serological, and vice versa" : Analytical sensitivity of molecular tests is usually lower than the analytical sensitivity of a serological test. Confirmation of a positive molecular result may consequently not always be possible with a serological test. This comment has been made in the EPPO framework at different occasions.	European Union	**CONSIDERED, BUT NOT INCORPORATED** We would like to maintain molecular amplification because it is more accurate to refer to PCR based molecular tests without including hybridization tests (see 20 and 38). Concerning the general question: Diagnostic and analytical parameters for CTV detection methods were analysed by Vidal et al. (2012). As a summary the most sensitive method (higher analytical sensitivity) for CTV detection is real time RT-PCR and the most specific test (higher analytical specificity) is ELISA (3DF1+3CA5 based). Consequently, in the specific case of CTV, negative results by the most sensitive method are very confident and positive results

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						by the most specific method are very confident by ELISA (3DF1+3CA5 based). In a Bayesian approach at lower prevalence the most appropriate test is ELISA and in higher prevalence real-time RT-PCR is the tests of choice. The combination of both tests, when they agree in the results of a sample, the accuracy of the diagnostic or detection is almost 100%. So taking in account this fact, especially at low prevalence of CTV, the positive result by molecular amplification tests need to be confirmed by specific serological tests. The negative results obtained by the most sensitive test require the confirmation by serological tests. Usually there is a very good agreement in CTV case except when the amplification was inhibited and

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						serology confirms the infection. In summary, for CTV detection there is a very good agreement between both molecular and serological based tests (because both tests use excellent reagents and the tests are both of high sensitivity and specificity). Consequently, we are in favor of the current text of the manuscript because the particular case of CTV was studied and evaluated (Vidal, E., Yokomi, R.K., Moreno, A., Bertolini, E. & Cambra, M. 2012. Calculation of diagnostic parameters of advanced serological and molecular tissue-print methods for detection of *Citrus tristeza virus*. A model for other plant pathogens. Phytopathology, doi.org/10.1094/PHYTO-05-11-0139) related to the

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						prevalence of CTV.
12.	*21*	Technical	In this diagnostic protocol, methods (including reference to brand names) are described as published, as these defined the original level of sensitivity, specificity and/or reproducibility achieved. ~~Use of names of reagents chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable~~. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.	Text deleted as per general comment	Uruguay, Argentina, Chile	**MODIFIED** We agree. The text of the phrase was deleted as suggested.
13.	*23*	Editorial	Replace "Citrus grandis (pomelo)" to "C. grandis (pomelo) " Replace "Citrus reticulata (mandarin), Citrus sinensis (sweet orange) and Citrus latifolia (lime) are among the cultivars most susceptible to natural CTV infection, followed by Citrus paradisi (grapefruit), Citrus unshiu (Satsuma mandarin) and C. limon (lemon) cultivars. Among the species used as rootstock, Citrus macrophylla (alemow), Citrus volkameriana, Cleopatra mandarin and Citrus limonia (Rangpur lime or lemandarin) are highly susceptible to natural CTV infection, whereas Carrizo and Troyer citranges and C. aurantium are rarely infected. Poncirus trifoliata and C. paradisi × P. trifoliata (citrumelo) rootstocks are resistant to most CTV strains. Passiflora gracilis and Passiflora coerulea are experimental non-citrus hosts." to "C. reticulata (mandarin), C. sinensis (sweet orange) and C. latifolia (lime) are among the cultivars most susceptible to natural CTV infection, followed by C. paradisi (grapefruit), C. unshiu (Satsuma mandarin) and C. limon (lemon) cultivars. Among the species used as rootstock, C. macrophylla (alemow), C. volkameriana, Cleopatra mandarin and C. limonia (Rangpur lime or lemandarin) are highly susceptible to natural CTV infection, whereas Carrizo and Troyer citranges and C. aurantium are rarely infected. P. trifoliata and C. paradisi × P. trifoliata (citrumelo) rootstocks are resistant to most CTV strains. Passiflora gracilis and	The generic name should be abbreviated when it presents for the second time.	China	**MODIFIED** We agree and the text was corrected.

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			Passiflora coerulea are experimental non-citrus hosts." Under natural conditions, CTV readily infects most species of *Citrus* and *Fortunella* and some species in genera known as citrus-relatives of the family Rutaceae that are also susceptible hosts of CTV; namely, *Aegle*, *Aeglopsis*, *Afraegle*, *Atalantia*, *Citropsis*, *Clausena*, *Eremocitrus*, *Hespertusa*, *Merrillia*, *Microcitrus,Pamburus, Pleiospermium* and *Swinglea* (Duran-Vila and Moreno, 2000; Timmer *et al*., 2000). Most *Poncirus trifoliata* (trifoliate orange) clones and many of their hybrids as well as *Fortunella crassifolia* (Meiwa kumquat) and some *Citrus grandis* (pomelo) are resistant to most CTV strains (Moreno *et al*., 2008). Consequently, CTV is absent or in very low concentration in these species. *Citrus reticulata* (mandarin), *Citrus sinensis* (sweet orange) and *Citrus latifolia* (lime) are among the cultivars most susceptible to natural CTV infection, followed by *Citrus paradisi* (grapefruit), *Citrus unshiu* (Satsuma mandarin) and *C. limon* (lemon) cultivars. Among the species used as rootstock, *Citrus macrophylla* (alemow), *Citrus volkameriana*, Cleopatra mandarin and *Citrus limonia* (Rangpur lime or lemandarin) are highly susceptible to natural CTV infection, whereas Carrizo and Troyer citranges and *C. aurantium* are rarely infected. *Poncirus trifoliata* and *C. paradisi* × *P. trifoliata* (citrumelo) rootstocks are resistant to most CTV strains. *Passiflora gracilis* and *Passiflora coerulea* are experimental non-citrus hosts.			
14.	*23*	Technical	Citrus reticulata (mandarin) should be removed.Under natural conditions, CTV readily infects most species of *Citrus* and *Fortunella* and some species in genera known as citrus-relatives of the family Rutaceae that are also susceptible hosts of CTV; namely, *Aegle*, *Aeglopsis*, *Afraegle*, *Atalantia*, *Citropsis*, *Clausena*, *Eremocitrus*, *Hespertusa*, *Merrillia*, *Microcitrus,Pamburus, Pleiospermium* and *Swinglea* (Duran-Vila and Moreno, 2000; Timmer *et al*., 2000). Most *Poncirus trifoliata* (trifoliate orange) clones and many of their hybrids as well as *Fortunella crassifolia* (Meiwa kumquat) and some *Citrus grandis* (pomelo) are	Citrus reticulata (mandarin) is tolerant to CTV.	China	**CONSIDERED, BUT NOT INCORPORATED** We do not agree. We agree that mandarin is tolerant but in the phrase mandarin is cited among the cultivars most susceptible to natural CTV infection that is

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			resistant to most CTV strains (Moreno *et al*., 2008). Consequently, CTV is absent or in very low concentration in these species. *Citrus reticulata* (mandarin), *Citrus sinensis* (sweet orange) and *Citrus latifolia* (lime) are among the cultivars most susceptible to natural CTV infection, followed by *Citrus paradisi* (grapefruit), *Citrus unshiu* (Satsuma mandarin) and *C. limon* (lemon) cultivars. Among the species used as rootstock, *Citrus macrophylla* (alemow), *Citrus volkameriana*, Cleopatra mandarin and *Citrus limonia* (Rangpur lime or lemandarin) are highly susceptible to natural CTV infection, whereas Carrizo and Troyer citranges and *C. aurantium* are rarely infected. *Poncirus trifoliata* and *C. paradisi* × *P. trifoliata* (citrumelo) rootstocks are resistant to most CTV strains. *Passiflora gracilis* and *Passiflora coerulea* are experimental non-citrus hosts.			absolutely correct.
15.	*25*	Editorial	Replace “Citrus jambhiri (rough lemon) ” to “C. jambhiri (rough lemon)”.Symptom expression in CTV-infected citrus hosts is highly variable and is affected by environmental conditions, host species and the aggressiveness of the CTV strain. In addition, the virus may remain latent for several years. Some CTV strains are mild and produce no noticeable effects on most commercial citrus species, including citrus grafted on *C. aurantium*. In general, mandarins are especially tolerant to CTV infection. *C. sinensis*, *C. aurantium* as a seedling and not as grafted rootstock, *Citrus jambhiri* (rough lemon) and *C. limonia* are usually symptomless when infected but may react to some aggressive strains. Citrus hosts that manifest symptoms are likely to include lime, grapefruit, some cultivars of pomelo, alemow and sweet orange, some citrus hybrids and some citrus-relatives of the family Rutaceaementioned above.	The generic name should be abbreviated when it presents for the second time.	China	**MODIFIED** We agree and the text was corrected.
16.	*25*	Technical	Symptom expression in CTV-infected citrus hosts is highly variable and is affected by environmental conditions, host species and the aggressiveness of the CTV strain. In addition, the virus may remain latent for several years. Some CTV strains are mild and produce	openin bracket need to be added before "as" closing bracket need to be added after "rootstock". abbreviate to C. jambhiri	Kenya	We agree and we introduced the suggestion in the text.

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			no noticeable effects on most commercial citrus species, including citrus grafted on *C. aurantium*. In general, mandarins are especially tolerant to CTV infection. *C. sinensis*, *C. aurantium* (as a seedling and not as grafted rootstock), *Citrus jambhiri* *C. jambhiri* (rough lemon) and *C. limonia* are usually symptomless when infected but may react to some aggressive strains. Citrus hosts that manifest symptoms are likely to include lime, grapefruit, some cultivars of pomelo, alemow and sweet orange, some citrus hybrids and some citrus-relatives of the family Rutaceaementioned above.			
17.	*26*	Technical	Depending on the CTV strain and citrus species or scion–rootstock combination, the virus may cause three four different syndromes: (1) no symptoms; (2) tristeza decline; (3) stem pitting; or (4) seedling yellows, which is mainly seen under greenhouse conditions. These four outcomes are described in the paragraphs below. Figure 1 shows the main symptoms caused by CTV.	proposed ammendment from four to three. delete the syndrome "no syndromes" as all syndromes must have visible symptoms	Kenya	**MODIFIED** We agree, the text was slightly modified accordingly.
18.	*27*	Editorial	One of the most economically significant outcomes of CTV infection is tristeza disease (bud union disease), which is characterized by the decline of trees grafted on sour orange or lemon rootstocks. Sweet orange, mandarin and grapefruit scions on these rootstocks become stunted, chlorotic and often die after a period of several months or years (i.e. they experience a slow decline), while other scions experience a rapid decline or collapse some days after the first symptom is observed. The decline results from the physiological effects of the virus on the phloem of the susceptible rootstock just below the bud union. Trees that decline slowly generally have a bulge above the bud union, a brown line just at the point of bud union, and inverse pinhole pitting (honeycombing) on the inner face of the sour orange rootstock bark. Stunting, leaf cupping, vein clearing, chlorotic leaves, stem pitting and reduced fruit size are common symptoms observed on susceptible hosts. However ~~S~~some isolates of the virus~~, however~~, particularly in the Mediterranean area, do not induce	redrafted for clarity.	European Union	**MODIFIED** We agree with the suggested text and we modified in the manuscript.

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			decline symptoms until many years after infection, even in trees grafted on sour orange~~, until many years after infection~~.			
19.	*29*	Technical	The seedling yellows syndrome is characterized by stunting, production of chlorotic or pale leaves, development of a reduced root system, and cessation of growth on sour orange, grapefruit and lemon seedlings cultivated under greenhouse conditions ((20–26 ºC)).add more information	more informatin needed to describe the outdoor symptoms of seedlings	Kenya	**CONSIDERED, BUT NOT INCORPORATED** We consider that enough information concerning symptoms is included for this syndrome that usually only appears when indexing and in susceptible seedlings grown in the field in some citrus industries when 20-26ºC is the usual range of temperature.
20.	*31*	Editorial	Replace “Citrus aurantifolia (Mexican, key or Omani lime)” to “C. aurantifolia (Mexican, key or Omani lime)”The objective of biological indexing is to detect the presence of CTV in plant accessions or selections or in samples whose sanitary status is being assessed, and to estimate the aggressiveness of the isolate on *Citrus aurantifolia* (Mexican, key or Omani lime), *C. macrophylla* or Duncan grapefruit seedlings. The indicator is a graft inoculated according to conventional methods and held under standard conditions (Roistacher, 1991), with four to six replicates. Vein clearing in young leaves, leaf cupping or leaf distortion, short internodes, stem pitting or seedling yellows symptoms on these sensitive indicator plants is evidence of CTV infection after graft inoculation. Symptom onset is compared against that on positive and negative control plants. Illustrations of symptoms caused by CTV on indicator plants can be found in Roistacher (1991) and Moreno *et al*. (2008).	The generic name should be abbreviated when it presents for the second time.	China	**MODIFIED** We modified in the text of the manuscript.

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21.	*31*	Technical	The objective of biological indexing is to detect the presence of CTV in plant accessions or selections or in samples whose sanitary status is being assessed, and to estimate the aggressiveness of the isolate on *Citrus aurantifolia* (Mexican, key or Omani lime), *C. macrophylla* or Duncan grapefruit seedlings. The indicator is a graft inoculated according to conventional methods and held under standard conditions (Roistacher, 1991), with four to six replicates (or with two to three replications if sufficient samples cannot be taken). Vein clearing in young leaves, leaf cupping or leaf distortion, short internodes, stem pitting or seedling yellows symptoms on these sensitive indicator plants is evidence of CTV infection after graft inoculation. Symptom onset is compared against that on positive and negative control plants. Illustrations of symptoms caused by CTV on indicator plants can be found in Roistacher (1991) and Moreno *et al*. (2008).	Two to three replicates should be possible in graft inoculation if sufficient samples cannot be taken (e.g., potted seedlings) in order to improve the feasibility of this diagnostic protocol.	Japan	**INCORPORATED** We introduced the suggestion in spite of the current recommendation (according to Chester Roistacher, 1991) is the use of 4 to 6 replications.
22.	*32*	Technical	Biological indexing is used widely in certification schemes, as it is considered a sensitive and reliable method for the detection of a new or unusual strain of the virus. However, it has some disadvantages: it is not a rapid test (symptom development requires three to six months post-inoculation); it can only be used to test budwood; it requires dedicated facilities such as temperature-controlled insect-proof greenhouse space; and it requires dedicated staff who can grow healthy and vigorous indicator host plants that will show appropriate symptoms as well as experienced staff who can accurately interpret observed disease symptoms that can be confused with symptoms of other graft-transmissible agents. Moreover, asymptomatic CTV strains that do not induce symptoms (latent strains) are not detectable on indicator plants (~~i.e.~~ e.g the CTV strain K described by Albertini *et al*. (1988)).	there are more	Kenya	**INCORPORATED** We agree with the suggestion that we incorporated in the current text.
23.	*34*	Technical	**3.4 Sampling and sample preparation for serological and molecular testing add an " biological indexing" or heading**	change sampling for the assays	Kenya	**CONSIDERED, BUT NOT INCORPORATED** We followed the same “chapter’s

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						denomination" than in other IPPC standards. The sampling for indexing is not described here, so we consider that is not pertinent.
24.	*35*	Substantive	1.Add virus detection in aphid. 2.Can the RNA of Citrus tristeza virus be drawn from fruit of Citrus? Which detection method is better for CTV in fruit? **3.4.1 Sampling**	1.The aphid is efficient vector to transmit CTV. It is useful to detect virus in aphid. 2.Add literature to clarify.	China	**MODIFIED** A paragraph was added concerning CTV detection by molecular amplification tests in squashed aphid species (see current text after (38) in (43) and in **3.4.2.2. section.** Literature, already in references, was added. The squash of aphid species is the most convenient method for testing aphids without conventional RNA purification (according to Bertolini et al., 2008) and other authors. RNA can be purified from fruit peduncles or from fruit albedo. The area of the columela in the fruit is the best because is rich in vascular connections. A phrase related to

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						fruits is already in the text.
25.	*35*	Technical	**3.4.1 Sampling**sampling paragraphs and headings need to be moved just below paragraph 29	sampling to be done just before you move to individual assays	Kenya	**CONSIDERED, BUT NOT INCORPORATED** We think that is OK in the current form.
26.	*36*	Editorial	General guidance on sampling methodologies is described in ISPM 31:2008 (*Methodologies for sampling of consignments*). Appropriate sampling is crucial for CTV detection and identification by ~~biological,~~ serological or molecular ~~amplification~~ methods. Changes to an accepted sampling scheme could result in an effective diagnostic protocol generating false positive or false negative results. The standard sample for adult trees is five young shoots or fruit peduncles, ten fully expanded leaves, or five flowers or fruits collected around the canopy of each individual tree from each scaffold branch. Samples (shoots or fully expanded leaves and peduncles) can be taken at any time of the year from sweet orange, mandarin, lemon and grapefruit in temperate Mediterranean areas, but spring and autumn are the optimal sampling periods in tropical and subtropical climates for achieving high CTV titres. In these climates, a reduced CTV titre is observed in Satsuma mandarin during summer; consequently, the recommended period for sampling includes all vegetative seasons, with the exception of hot days (35–40 ºC) in summer. Roots, however, can be sampled during hot periods if required. Flowers or fruits (when available) are also suitable materials for sampling (Cambra *et al*., 2002). Tissue from the fruit peduncle in the region of the albedo, where the peduncle is joined to the fruit, or from the columnella are the most suitable fruit samples. Standard requirements for sampling nursery plants include the collection of two young shoots or four leaves per plant.	This paragraph is for serological and molecular testing only.	European Union	**CONSIDERED, BUT NOT INCORPORATED** A phrase related to indexing was included in the text. For molecular amplification, see comments (e.g. 20 and 38).

Comm. no.	Para. no.	Comment type	Comment	Explanation	Country	SC Responses
27.	*37*	Technical	Shoots, leaf petioles, fruit peduncles and flowers can be stored at approximately 4 °C for up to seven days before processing. Fruits can be stored for one month at approximately 4 °C. Use beyond these time frames may result in lower titres and the potential for false negative results in diagnostic methods.	Affix 'approximately' to temperatures to avoid metrology problem during accreditation.	European Union	**INCORPORATED** We agree and consequently the suggested word was incorporated in the text.
28.	*38*	Editorial	Composite samples, to be tested as a single sample, can be collected together (one to ten nursery plants or adult trees) for serological or molecular ~~amplification~~ tests (see specific recommendations in sections 3.5 and 3.6). In some circumstances (e.g. routine screening for CTV widely established in a country or an area), multiple plants may be tested simultaneously using a bulked sample derived from a number of plants. The decision to test individual plant or bulked plant samples by serological or molecular amplification methods depends on the virus concentration in the plants, the expected prevalence of CTV in the area (Vidal *et al*., 2012), the limit of detection of the test method to be used, and the level of confidence required by the NPPO.	see general comment	European Union	**CONSIDERED, BUT NOT INCORPORATED** We do not agree because we consider more accurate the term "molecular amplification" to refer to PCR based tests and to differentiate with hybridization tests (that are also molecular methods). See also (20 and 38). In addition molecular tests, that is commonly used, do not refer to antibodies that are also molecules.
29.	*38*	Technical	Composite samples, to be tested as a single sample, can be collected together (one to ten nursery plants or adult trees) for serological or molecular amplification tests (see specific recommendations in sections 3.5 and 3.6). In some circumstances (e.g. routine screening for CTV widely established in a country or an area), multiple plants may be tested simultaneously using a bulked sample derived from a number of plants. The decision to test individual plant or composite~~bulked~~ plant samples by serological or molecular amplification methods depends on the virus concentration in the plants, the expected prevalence of CTV in the area	replace bulk with composite	Kenya	**INCORPORATED** We agree and we incorporated to the current text of the manuscript.

Comm. no.	Para. no.	Comment type	Comment	Explanation	Country	SC Responses
			(Vidal *et al*., 2012), the limit of detection of the test method to be used, and the level of confidence required by the NPPO.			
30.	*41*	Technical	Tender shoots, leaf petioles, fruit peduncles or flower ovaries are cut cleanly. The freshly cut sections are carefully pressed against a nitrocellulose or ester of cellulose membrane (0.45 mm) and the trace or print is allowed to dry for 2–5 min. For routine serological testing, at least two prints should be made per selected shoot (one from each end of the shoot) or peduncle and one per leaf petiole or flower. Printed membranes can be kept for several months in a dry and dark place.	Regarding "ester of cellulose membrane" : Consider replacing ester of cellulose membranes. In the EPPO Panel on diagnostics in Virology and Phytoplasmology this denomination was discussed in the framework of the horizontal ELISA protocol and it was agreed to replace it by commercial nitrocellulose membrane'	European Union	**CONSIDERED, BUT NOT INCORPORATED** We have no serious objection to incorporate the suggestion, but today only "ester of cellulose membranes" is currently used for laboratory purposes instead of the classical nitrocellulose that is not recommended specially for aerial transportation (hazardous risk). So we prefer to maintain the paragraph in its current version that accurately refers to the current composition of the commercially available membranes.
31.	*42*	Editorial	*3.4.2.2 Preparation of tissue prints for molecular ~~amplification~~ testing*	see general comment	European Union	**CONSIDERED, BUT NOT INCORPORATED** See previous comments (e.g: 36 and 38).
32.	*43*	Editorial	Collection of plant material by hand is recommended to avoid contamination of samples by scissors. Tender	see general comment	European Union	**CONSIDERED, BUT NOT**

Comm. no.	Para. no.	Comment type	Comment	Explanation	Country	SC Responses
			shoots with fully expanded leaves or mature leaves are collected around the canopy of the tree. The leaf petiole of two leaves or shoots are pressed directly on Whatman[1] 3MM paper (0.45 mm) or positively charged nylon membrane. Several partially overlapping imprints from different leaves are made on approximately 0.5 cm^2 of the membrane, according to Bertolini *et al.* (2008). The trace or print is allowed to dry for 2–5 min. For routine molecular ~~amplification~~ testing one print should be made per selected leaf pedicel (section 3.4.1). Printed membranes can be kept for several months in a dry and dark place.			**INCORPORATED** See previous comments (e. g:36 and 38).
33.	*45*	Editorial	**3.4.3 Preparation of plant extracts for serological and molecular ~~amplification~~ testing**	see general comment	European Union	**CONSIDERED, BUT NOT INCORPORATED** See previous comments (e.g. 20 and 38). .
34.	*46*	Editorial	Fresh plant material, 0.2–0.5 g ~~,~~is cut in small pieces with disposable razor blades or bleach-treated scissors to avoid sample to sample contamination and placed in a suitable tube or plastic bag. Extracts for serological testing can be prepared in tubes or in plastic bags. Samples for molecular amplification testing should only be prepared in individual plastic bags to avoid contamination among samples. The sample is homogenized thoroughly in 4–10 ml (1:20 w/v) extraction buffer using an electrical tissue homogenizer, manual roller, hammer or similar tool. The extraction buffer is phosphate-buffered saline (PBS), pH 7.2–7.4 ($NaCl_2$, 8 g; KCl, 0.2 g; $Na_2HPO_4 \cdot 12H_2O$, 2.9 g; KH_2PO_4, 0.2 g; distilled water, 1 litre) supplemented with 0.2% sodium diethyl dithiocarbamate (DIECA) or 0.2% mercaptoethanol, or an alternative suitably validated buffer.	delete coma.	European Union	**INCORPORATED** Done.
35.	*46*	Technical	Fresh plant material, 0.2–0.5 g ,is cut in small pieces with disposable razor blades or bleach-treated scissors to avoid sample to sample contamination and placed in a suitable tube or plastic bag. Extracts for serological	Some manufacturers mention different ratios for grinding (e.g. 1/10). It is recommended to add . “unless otherwise stated by the manufacturer”.	European Union	**INCORPORATED** We agree. The suggested text was incorporated in the

Comm. no.	Para. no.	Comment type	Comment	Explanation	Country	SC Responses
			testing can be prepared in tubes or in plastic bags. Samples for molecular amplification testing should only be prepared in individual plastic bags to avoid contamination among samples. The sample is homogenized thoroughly in 4–10 ml (1:20 w/v unless otherwise stated by the manufacturer) extraction buffer using an electrical tissue homogenizer, manual roller, hammer or similar tool. The extraction buffer is phosphate-buffered saline (PBS), pH 7.2–7.4 ($NaCl_2$, 8 g; KCl, 0.2 g; $Na_2HPO_4 \cdot 12H_2O$, 2.9 g; KH_2PO_4, 0.2 g; distilled water, 1 litre) supplemented with 0.2% sodium diethyl dithiocarbamate (DIECA) or 0.2% mercaptoethanol, or an alternative suitably validated buffer.			manuscript.
36.	*48*	Technical	Enzyme-linked immunosorbent assay (ELISA) using validated monoclonal antibodies or polyclonal antibodies is highly recommended for screening large numbers of samples for CTV detection and identification. The production of monoclonal antibodies specific to CTV (Vela *et al*., 1986; Permar *et al*., 1990) and others reported by Nikolaeva *et al*. (1996) solved the problem of the diagnostic specificity presented by polyclonal antibodies (Cambra *et al*., 2011) and thus increased the diagnostic sensitivity of serological tests. A mixture of the two monoclonal antibodies 3DF1 and 3CA5, or their recombinant versions (Terrada *et al*., 2000), recognizes all CTV isolates tested from different international collections (Cambra *et al*., 1990). A detailed description, characterization and validation of these monoclonal antibodies is summarized in Cambra *et al*. (2000a). A mixture of the monoclonal antibodies 4C1 and 1D12 produced in Morocco is reported to react against a broad spectrum of CTV strains (Zebzami *et al*., 1999) but there are no validation data available.	Add diagnostic sensitivity/specificity (important specification).	European Union	**INCORPORATED** We agree and the terms were included in the text.
37.	*50*	Technical	Direct tissue print-ELISA, also referred to as immunoprinting ELISA or direct tissue blot immunoassay (DTBIA), is performed according to Garnsey *et al.* (1993) and Cambra *et al.* (2000b) using the method described below. A complete kit (validated in ring tests and in several published studies) based on	remove Agdia as a source of reagents	Kenya	**INCORPORATED** We did in the current version of the manuscript.

Comm. no.	Para. no.	Comment type	Comment	Explanation	Country	SC Responses
			3DF1 + 3CA5[2] CTV-specific monoclonal antibodies (Vela *et al*., 1986), including preprinted membranes with positive and negative controls and all reagents, buffers and substrate, is available from Plant Print Diagnòstics SL. A similar but non-validated kit based on another source of antibodies is available from ~~Agdia.~~			
38.	*51*	Technical	Membranes that have been tissue printed (recommended size: approximately 7 × 13 cm) are placed in an appropriate container (tray, hermetic container or plastic bag), covered with a 1% solution of bovine serum albumin (BSA) in distilled water and incubated for 1 h at room temperature or overnight (about 16 h) at 4 °C (the latter is recommended). Slight agitation is beneficial during this step. The albumin solution is discarded but the membranes are kept in the same container. A conjugate solution is prepared that consists of equal concentrations of CTV-specific 3DF1 + 3CA5 monoclonal antibody linked to alkaline phosphatase (approximately 0.1 µg/ml of each monoclonal antibody in PBS) or of 3DF1 scFv-AP/S + 3CA5 scFv-AP/S fusion proteins[6] expressed in *Escherichia coli* (an appropriate dilution in PBS) (Terrada *et al*., 2000). The conjugate solution is poured onto the membranes, covering them, and the membranes are incubated for 3 h at room temperature, with slight agitation. The conjugate solution is then discarded. The membranes and the container are rinsed with washing buffer (PBS, pH 7.2–7.4, with 0.05% Tween 20), and washed by shaking (manually or mechanically) for 5 min. The washing buffer is discarded and the washing process is repeated twice. The substrate for alkaline phosphatase (Sigma Fast 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) tablets) is then poured over the membranes and the membranes are incubated until a purple-violet colour appears in the positive controls (about 10–15 min). The reaction is stopped by washing the membranes with tap water. The membranes are spread on absorbent paper and allowed to dry. The prints are examined using a low-power magnification	Regarding "The substrate for alkaline phosphatase" : Concentration should be indicated.	European Union	**INCORPORATED** We agree and the concentration was introduced in the text: (“that gives a final concentration of 0.33 mg/ml of NBT and of 0.175 mg/ml of BCIP”).

Comm. no.	Para. no.	Comment type	Comment	Explanation	Country	SC Responses
			(×10 to ×20). The presence of purple-violet precipitates in the vascular region of plant material reveals the presence of CTV.			
39.	*54*	Substantive	Two wells of a microtiter plate are used for each sample and at least two wells for positive and negative controls. An appropriate dilution is prepared of the polyclonal or monoclonal (3DF1 + 3CA5) antibodies (usually 1–2 µg/ml total immunoglobulins) in carbonate buffer, pH 9.6 (Na_2CO_3, 1.59 g; $NaHCO_3$, 2.93 g; distilled water, 1 litre), and 200 µl is added to each well. The plate is incubated for 4 h at 37 °C or overnight (about 16 h) at 4 °C. The wells are washed three times with washing buffer (PBS, pH 7.2–7.4, with 0.05% Tween 20). The plant extract (section 3.4.3) is then added, 200 µl to each well. After incubation for 16 h at 4 °C, the plates are washed three times as described above. Specific polyclonal or monoclonal $(3DF1 + 3CA5)^2$ antibody mixtures linked with alkaline phosphatase are prepared at appropriate dilutions (about 0.1 µg/ml in PBS with 0.5% BSA) then 200 µl is added to each well. Incubation is carried out for 3 h at 37 °C. The plates are again washed as described above. A solution of 1 mg/ml alkaline phosphatase (p-nitrophenyl phosphate) in substrate buffer (97 ml diethanolamine in 800 ml distilled water, pH adjusted to 9.8 with concentrated HCl, and the total volume then made up to 1 000 ml with distilled water) is prepared and 200 µl is added to each well. The plates are incubated at room temperature and read at 405 nm after 30, 60 and 90 min. The ELISA is negative if the absorbance of the sample is less than twice the absorbance of the healthy control, and positive if the absorbance of the sample is equal to or greater than twice that value.	Regarding "positive if the absorbance of the sample is equal to or greater than twice that value" : Setting a threshold too strict, often leads to a lot of retesting. When the healthy control material gives very low values, this results in all samples considered positive, which is often not the case when checked with another method. Therefore, we propose that other options of interpretation should be included in the text. An EPPO Standard on ELISA is under development that proposes options however the standard is under consultation so no specific proposal can be made at this stage. As soon as the Standard is adopted, the EPPO Secretariat will communicate elements for the TPDP to consider	European Union	**INCORPORATED** We agree and the option of the EPPO ELISA standard was introduced in the text. Also the “reading time” phrase was modified accordingly EPPO ELISA standard (“… read at 405 nm at regular intervals within 120 min, or following the instructions of the supplier”.
40.	*54*	Substantive	Add buffer control.Two wells of a microtiter plate are used for each sample and at least two wells for positive and negative controls. An appropriate dilution is prepared of the polyclonal or monoclonal (3DF1 + 3CA5) antibodies (usually 1–2 µg/ml total immunoglobulins) in carbonate buffer, pH 9.6 (Na_2CO_3, 1.59 g; $NaHCO_3$, 2.93 g; distilled water, 1 litre), and	Buffer control is also important when make DAS-ELISA besides positive and negetive control.	China	**CONSIDERED, BUT NOT INCORPORATED** We do not agree. The inclusion of buffer control is not necessary/important

Comm. no.	Para. no.	Comment type	Comment	Explanation	Country	SC Responses
			200 µl is added to each well. The plate is incubated for 4 h at 37 °C or overnight (about 16 h) at 4 °C. The wells are washed three times with washing buffer (PBS, pH 7.2–7.4, with 0.05% Tween 20). The plant extract (section 3.4.3) is then added, 200 µl to each well. After incubation for 16 h at 4 °C, the plates are washed three times as described above. Specific polyclonal or monoclonal (3DF1 + 3CA5)[2] antibody mixtures linked with alkaline phosphatase are prepared at appropriate dilutions (about 0.1 µg/ml in PBS with 0.5% BSA) then 200 µl is added to each well. Incubation is carried out for 3 h at 37 °C. The plates are again washed as described above. A solution of 1 mg/ml alkaline phosphatase (p-nitrophenyl phosphate) in substrate buffer (97 ml diethanolamine in 800 ml distilled water, pH adjusted to 9.8 with concentrated HCl, and the total volume then made up to 1 000 ml with distilled water) is prepared and 200 µl is added to each well. The plates are incubated at room temperature and read at 405 nm after 30, 60 and 90 min. The ELISA is negative if the absorbance of the sample is less than twice the absorbance of the healthy control, and positive if the absorbance of the sample is equal to or greater than twice that value.			because already healthy control prepared in buffer control is recommended. Buffer control alone usually gives yellow color that not appears in healthy control prepared with the same buffer (that is crucial to include, especially if polyclonal antibodies are used).
41.	*54*	Technical	Two wells of a microtiter plate are used for each sample and at least two wells for positive and negative controls. An appropriate dilution is prepared of the polyclonal or monoclonal (3DF1 + 3CA5) antibodies (usually 1–2 µg/ml total immunoglobulins) in carbonate buffer, pH 9.6 (Na_2CO_3, 1.59 g; $NaHCO_3$, 2.93 g; distilled water, 1 litre), and 200 µl is added to each well. The plate is incubated for 4 h at 37 °C or overnight (about 16 h) at 4 °C. The wells are washed three times with washing buffer (PBS, pH 7.2–7.4, with 0.05% Tween 20). The plant extract (section 3.4.3) is then added, 200 µl to each well. After incubation for 16 h at 4 °C, the plates are washed three times as described above. Specific polyclonal or monoclonal (3DF1 + 3CA5)[2] antibody mixtures linked with alkaline phosphatase are prepared at appropriate dilutions (about 0.1 µg/ml in	Regarding "after 30, 60 and 90 min" : In most laboratories 120 min is the standard for incubation, so this may be added.	European Union	**INCORPORATED** We agree. Please see the current text (see 54) of the manuscript.

Comm. no.	Para. no.	Comment type	Comment	Explanation	Country	SC Responses
			PBS with 0.5% BSA) then 200 µl is added to each well. Incubation is carried out for 3 h at 37 °C. The plates are again washed as described above. A solution of 1 mg/ml alkaline phosphatase (p-nitrophenyl phosphate) in substrate buffer (97 ml diethanolamine in 800 ml distilled water, pH adjusted to 9.8 with concentrated HCl, and the total volume then made up to 1 000 ml with distilled water) is prepared and 200 µl is added to each well. The plates are incubated at room temperature and read at 405 nm after 30, 60 and 90 min. The ELISA is negative if the absorbance of the sample is less than twice the absorbance of the healthy control, and positive if the absorbance of the sample is equal to or greater than twice that value.			
42.	*54*	Technical	Two wells of a microtiter plate are used for each sample and at least two wells for positive and negative controls. An appropriate dilution is prepared of the polyclonal or monoclonal (3DF1 + 3CA5)* antibodies (usually 1–2 µg/ml total immunoglobulins) in carbonate buffer, pH 9.6 (Na_2CO_3, 1.59 g; $NaHCO_3$, 2.93 g; distilled water, 1 litre), and 200 µl is added to each well. The plate is incubated for 4 h at 37 °C or overnight (about 16 h) at 4 °C. The wells are washed three times with washing buffer (PBS, pH 7.2–7.4, with 0.05% Tween 20). The plant extract (section 3.4.3) is then added, 200 µl to each well. After incubation for 16 h at 4 °C, the plates are washed three times as described above. Specific polyclonal or monoclonal (3DF1 + 3CA5)[2] antibody mixtures linked with alkaline phosphatase are prepared at appropriate dilutions (about 0.1 µg/ml in PBS with 0.5% BSA) then 200 µl is added to each well. Incubation is carried out for 3 h at 37 °C. The plates are again washed as described above. A solution of 1 mg/ml alkaline phosphatase (p-nitrophenyl phosphate) in substrate buffer (97 ml diethanolamine in 800 ml distilled water, pH adjusted to 9.8 with concentrated HCl, and the total volume then made up to 1 000 ml with distilled water) is prepared and 200 µl is added to each well. The plates are incubated at room temperature and read at 405 nm after 30, 60 and 90 min. The ELISA is negative if the absorbance of the	(3DF1+ 3CA5) should be a footnote unless it applies ton the whole document	Kenya	**CONSIDERED, BUT NOT INCORPORATED** The IPPC panel decision will be welcome. Nevertheless, we prefer to maintain the current text because both CTV specific monoclonal antibodies are a well-established, and internationally recognized as reference for CTV detection and diagnosis. Both antibodies are well validated, commercially available and extensively used.

Comm. no.	Para. no.	Comment type	Comment	Explanation	Country	SC Responses
			sample is less than twice the absorbance of the healthy control, and positive if the absorbance of the sample is equal to or greater than twice that value.			
43.	*57*	Editorial	**3.6 Molecular ~~amplification~~ tests**	see general comment	European Union	**CONSIDERED, BUT NOT INCORPORATED** See previous comments (e.g.: 20 and 38).
44.	*60*	Editorial	**3.6.1 Controls for molecular ~~amplification~~ tests**	see general comment	European Union	**CONSIDERED, BUT NOT INCORPORATED** See previous comments (e.g: 20 and 38).
45.	*68*	Editorial	modify "3.6.1" to "3.6.2".**3.6.1 RNA purification, immunocapture and cDNA synthesis**	Repeated with Para. 60.	China	**MODIFIED** We agree. The modification was introduced in the text. The numbers related to 3.6.1 were revised and corrected.
46.	*70*	Technical	RNA purification should be done using appropriately validated protocols or using RNA purification kits according to the manufacturer's instructions. The extracted RNA should be stored at –70 ºC (preferably) or at –20 ºC until its use as a template and for less than one year. Storage should be in small quantities to avoid degradation of RNA due to repeated freeze–thaw cycles.	Important specification.	European Union	**INCORPORATED** We agree. The suggestion was introduced in the current text.
47.	*70*	Technical	RNA purification should be done using appropriately validated protocols or using RNA purification kits according to the manufacturer's instructions. The extracted RNA should be stored at –70 ºC (preferably) or at –20 ºC clarify until its use as a template. Storage should be in small quantities to avoid degradation of RNA due to repeated freeze–thaw cycles.	propose to either delete the temperature of 20 degrees or state the length of duration of storage at the above temerature	Kenya	**CONSIDERED, BUT NOT INCORPORATED** We do not agree. We prefer to maintain the current text completed after introduction of (Comm nº46, 70)

Comm. no.	Para. no.	Comment type	Comment	Explanation	Country	SC Responses
						technical comment.
48.	*72*	Substantive	Whether 2 h is long enough for the tube incubated on ice.Immunocapture is an alternative option to RNA purification. For this procedure, a diluted antibody mixture is prepared, consisting of 1 µg/ml CTV-specific polyclonal antibodies or a dilution of monoclonal antibodies (3DF1 + 3CA5, 0.5 µg/ml + 0.5 µg/ml) in carbonate buffer, pH 9.6 (Na_2CO_3, 1.59 g; $NaHCO_3$, 2.93 g; distilled water, 1 litre). The antibody mixture is then dispensed into microtubes (100 µl per tube) and the tubes are incubated for 3 h at 37 ºC. The coated tubes are washed twice with 150 µl sterile washing buffer (PBS, pH 7.2–7.4, with 0.05% Tween 20; see section 3.4.3 for the composition of PBS). Plant extract (100 µl) is clarified by centrifugation (section 3.4) and aliquots of the supernatant are dispensed into the antibody-coated microtubes. The tubes are incubated for 2 h on ice or alternatively for 2 h at 37 ºC. After this immunocapture phase, the microtubes are washed three times with 150 µl sterile washing buffer. It is in these washed tubes that cDNA synthesis and PCR amplification are performed.	Add literature to clarify	China	**INCORPORATED** Literature was added in the first version of this standard but the IPPC panel recommended a significant reduction of the references that we applied. Historically, the two different options were proposed. i) The immunocapture phase for 2 h on ice (Rosner et al., 1998) or ii) alternatively at 37 ºC (Wetzel et al., 1992). The specific reference for incubation on ice is: Rosner A, Shilboleth Y, Spiegel S, Krisbai L & Kölber M (1998) Evaluating the use of immunocapture and sap-dilution PCR for the detection of *Prunus necrotic ringspot virus*. Acta Horticulturae 472, 227–233.
49.	*72*	Technical	Immunocapture is an alternative option to RNA purification. For this procedure, a diluted antibody	Regarding the sentence "The tubes are incubated for a minimum of 2 h on ice or alternatively for 2 h at 37	European Union	**MODIFIED** We partially agree

Comm. no.	Para. no.	Comment type	Comment	Explanation	Country	SC Responses
			mixture is prepared, consisting of 1 µg/ml CTV-specific polyclonal antibodies or a dilution of monoclonal antibodies (3DF1 + 3CA5, 0.5 µg/ml + 0.5 µg/ml) in carbonate buffer, pH 9.6 (Na_2CO_3, 1.59 g; $NaHCO_3$, 2.93 g; distilled water, 1 litre). The antibody mixture is then dispensed into microtubes (100 µl per tube) and the tubes are incubated for 3 h at 37 ºC. The coated tubes are washed twice with 150 µl sterile washing buffer (PBS, pH 7.2–7.4, with 0.05% Tween 20; see section 3.4.3 for the composition of PBS). Plant extract (100 µl) is clarified by centrifugation (section 3.4) and aliquots of the supernatant are dispensed into the antibody-coated microtubes. The tubes are incubated for a minimum of 2 h on ice or alternatively ~~for 2 h~~ at 37 ºC preferably overnight. After this immunocapture phase, the microtubes are washed three times with 150 µl sterile washing buffer. It is in these washed tubes that cDNA synthesis and PCR amplification are performed.	ºC preferably overnight.": This instruction does not seem logic, as this would mean that any temperature between on ice and 37 would be possible.		and the suggestion of "minimum" was incorporated in the text. Nevertheless, we did not incorporate "preferably overnight" for the alternative incubation at 37ºC. This specific incubation can be performed at any time without preferences (overnight or "overday").
50.	*72*	Technical	Immunocapture is an alternative option to RNA purification. For this procedure, a diluted antibody mixture is prepared, consisting of 1 µg/ml CTV-specific polyclonal antibodies or a dilution of monoclonal antibodies (3DF1 + 3CA5, 0.5 µg/ml + 0.5 µg/ml) in carbonate buffer, pH 9.6 (Na_2CO_3, 1.59 g; $NaHCO_3$, 2.93 g; distilled water, 1 litre). The antibody mixture is then dispensed into microtubes (100 µl per tube) and the tubes are incubated for 3 h at 37 ºC. The coated tubes are washed twice with 150 µl sterile washing buffer (PBS, pH 7.2–7.4, with 0.05% Tween 20; see section 3.4.3 for the composition of PBS). Plant extract (100 µl) is clarified by centrifugation (section 3.4) and aliquots of the supernatant are dispensed into the antibody-coated microtubes. The tubes are incubated for 2 h on ice or alternatively for 2 h at 37 ºC. Clarify After this immunocapture phase, the microtubes are washed three times with 150 µl sterile washing buffer. It is in these washed tubes that cDNA synthesis and PCR amplification are performed. lack references	2hours on ice cannot substitute the 2hour at 37 degrees . is one step before the other There are no eerences to paragraph 41, 72	Kenya	**CONSIDERED, BUT NOT INCORPORATED** We do not agree. The method was assayed and validated and is included in some EPPO standards. See previous comment (72) related to references. The references could be included if the panel agrees.
51.	*74*	Technical	Because the preservation of RNA during storage is	Regarding "the preservation of RNA during storage is	European Union	**MODIFIED**

Comm. no.	Para. no.	Comment type	Comment	Explanation	Country	SC Responses
			difficult, it is recommended to synthesize complementary DNA (cDNA), which can be preserved for long periods with minimal temperature requirements compared with RNA. Several commercial kits are available for cDNA synthesis.	difficult": What is meant here? Storage prior to testing or storage of RNA for e.g. positive control material?.		The RNA is degraded during storage especially at room temp. even at -20ºC (see previous comments). May be "difficult "could be replaced by "problematic". We introduced "problematic" in the text.
52.	*75*	Technical	IC-RT-PCR should be taken twice**3.6.2 IC-RT-PCR**	As the expected product size is 131 base pairs (bp), it is very difficult to detect, and positive results are easily encountered as well.	China	**Noted.** Nevertheless it is not very difficult to detect a product of 131 bp.
53.	*81*	Technical	Nested IC RT-PCR in a single closed tube should be think twice.**3.6.3 Nested IC RT-PCR in a single closed tube**	As the expected product size is 131 base pairs (bp), it is very difficult to detect, and positive results are easily encountered as well.	China	**See response above**
54.	*87*	Technical	0.5 ml should be changed into 0.2 ml.The device for compartmentalization of a 0.5 ml microtube for nested RT-PCR in a single closed tube is according to Olmos *et al*. (1999). The RT-PCR master mix consists of two reaction mixtures:	The usual volume of PCR tube is 0.2ml.	China	**INCORPORATED** We agree that the conventional volume for PCR tubes currently is 0.2 ml. Nevertheless, time ago 0.5 was very popular, in fact many thermal cycler machines still have blocks for tubes of 0.2 and of 0.5 ml. For nested RT-PCR in a single closed tube 0.5 ml tubes are required to contain the device (plastic tip) necessary to guarantee the

Comm. no.	Para. no.	Comment type	Comment	Explanation	Country	SC Responses
						physical separation of both cocktails. Please, see the paper of Olmos et al. (1999) where the device is described. We fully agree that nowadays, nested RT-PCR is overcome by real-time RT-PCR, but still in some laboratories without real-time thermal cycler machine, the "nested" is a valid and simple option to increase drastically the sensitivity.
55.	*95*	Substantive	Add virus sequence on determination of the positive results.The test on a sample is negative if the CTV-specific amplicon of the expected size is not detected in the sample in question but is detected for all positive controls. The test on a sample is positive if the CTV-specific amplicon of the expected size is detected in the sample in question, providing that there is no amplification from any of the negative controls.	To eliminate the possibility of false positive results in PCR tests.	China	**CONSIDERED, BUT NOT INCORPORATED** We consider that is not necessary. In other protocols the viral sequence is not included. In any case, several CTV sequences are available in the literature.
56.	*103*	Technical	For the tissue print real-time RT-PCR, a diagnostic sensitivity of 0.98, a specificity of 0.85, and a positive and negative likelihood ratio of 6.63 and 0.021, respectively, were estimated (Vidal *et al*., 2012). These diagnostic parameters show that tissue print real-time RT-PCR was the most sensitive technique, validating its use for routine CTV detection and diagnosis, and highly recommending it for assessing the CTV-free status of any plant material. The high sensitivity of this	Important : Specify diagnostic sensitivity/specificity.	European Union	**MODIFIED** We agree and the text was consequently modified.

Comm. no.	Para. no.	Comment type	Comment	Explanation	Country	SC Responses
			technique allows the accurate analysis of composite samples (up to ten batched trees or nursery plants) as one diagnostic sample when tested in any season of the year, and it also allows analysis of aphid species to detect low concentrations of CTV. For additional diagnostic parameters of validation of tissue print real-time RT-PCR, see section 3.7.			
57.	*103*	Technical	For the tissue print real-time RT-PCR, a sensitivity of 0.98, a specificity of 0.85, and a positive and negative likelihood ratio of 6.63 and 0.021, respectively, were estimated (Vidal *et al*., 2012). These diagnostic parameters show that tissue print real-time RT-PCR was the most sensitive technique, validating its use for routine CTV detection and diagnosis, and highly recommending it for assessing the CTV-free status of any plant material. The high sensitivity of this technique allows the accurate analysis of composite samples (up to ten batched trees or nursery plants) as one diagnostic sample when tested in any season of the year, and it also allows analysis of aphid species to detect low concentrations of CTV. For additional diagnostic parameters of validation of tissue print real-time RT-PCR, see section 3.7. clarify	testing or diagnoses of aphids has no procedure on sample preparation of aphids. clarify	Kenya	**INCORPORATED** Testing of aphid species was introduced in the text. Please, see e.g: 35 and the current text of the manuscript.
58.	*111*	Technical	**Conventional RT-PCR and IC-RT-PCR**	The use of internal controls is not described in this part. If internal controls are included in duplex format, the test has to be validated in this format.	European Union	**Noted.** In general we are not in favor of internal controls that could compete with the target reducing sensitivity. If appropriate positive controls are included, the internal control is not necessary.
59.	*115*	Substantive	Add the primer sequence of nad5 internal control.If the mRNA mitochondrial gene *NADH dehydrogenase* 5 (*nad5*) internal control primers are also used, then the negative control (healthy plant tissue) (if used), positive control and each of the test samples must produce a	Helpful for practice.	China	**INCORPORATED** The coamplification of plant mRNA as internal control using *nad5* is based

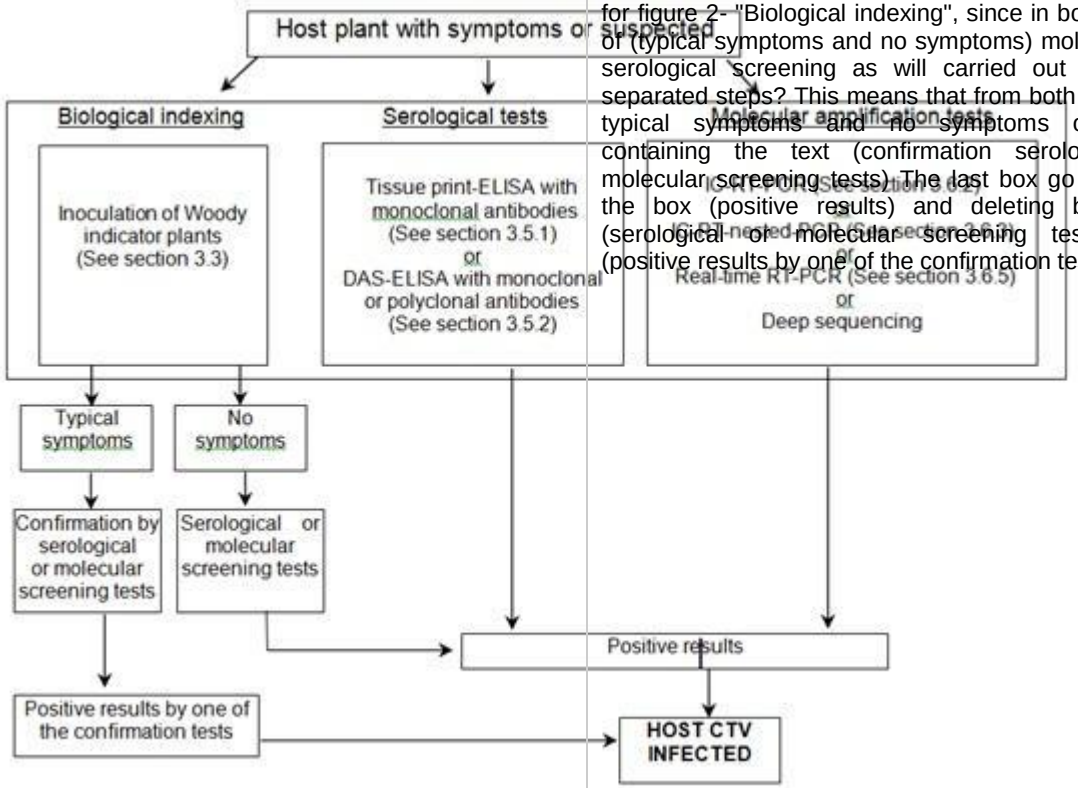
Comm. no.	Para. no.	Comment type	Comment	Explanation	Country	SC Responses
			115 bp amplicon. Failure of the samples to amplify with the internal control primers suggests for example that the RNA extraction has failed, RNA has not been included in the reaction mix, compounds inhibitory to RT-PCR are present in the RNA extract or the RNA has degraded.			on primer sequence Menzel and Mais, 2002): Sense GATGCTTCTTGGG GCTTCTTGTT 181 bp antisense CTCCAGTCACCAA CATTGGCATAA (final product of 181 bp) We included in the text as China suggested.
60.	*122*	Technical	**3.7 ~~Ring test~~ ~~v~~Validation by a test performance study**	Replaced by : "Validation by a test performance study".	European Union	**INCORPORATED** We agree and the term was included in the current text.
61.	*123*	Technical	In a DIAGPRO ring test (Cambra *et al*., 2002) conducted by ten laboratories using a set of ten coded samples including CTV-infected and healthy tissue samples from the Valencian Institute of Agrarian Research (IVIA) collection, tissue print-ELISA using 3DF1 + 3CA5[2] monoclonal antibodies was 99% accurate (the number of true positives and true negatives diagnosed by the technique/number of samples tested). This accuracy was greater than that achieved with DAS-ELISA (98% accurate), IC-RT-PCR (94% accurate) and IC nested RT-PCR in a single closed tube (89% accurate). The sensitivity (Vidal *et al*., 2012) of tissue print-ELISA was 0.98 while the sensitivity of the other above-mentioned techniques was 0.96, 0.96 and 0.93, respectively. The diagnostic specificity of tissue print-ELISA was 1.0 while the diagnostic specificity of the other techniques was 1.0, 0.91 and 0.82, respectively. The positive predictive value with a positive test that actually have the disease; Sackett *et al*., 1991) of tissue print-ELISA was 1.0 while the positive predictive value of the other	Important : Specify diagnostic sensitivity/specificity.	European Union	**INCORPORATED** We agree and “diagnostic” term was included in the current text.

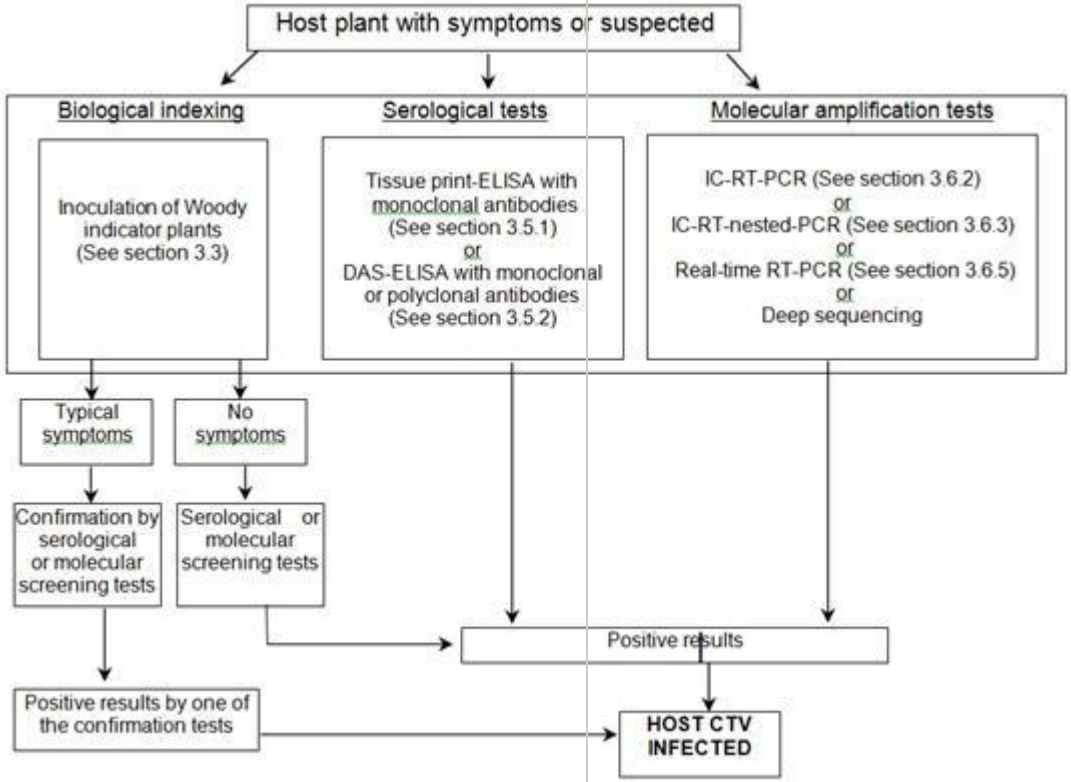
Comm. no.	Para. no.	Comment type	Comment	Explanation	Country	SC Responses
			techniques was 1.0, 0.94 and 0.89, respectively. The negative predictive value (Sackett *et al*., 1991) of tissue print-ELISA was 0.97 while the negative predictive value of the other techniques was 0.95, 0.94 and 0.88, respectively (Harju *et al*., 2000).			
62.	*128*	Technical	Given the genetic and biological variability of CTV, techniques other than sequencing may provide erroneous results when attempting to identify CTV strains. The use of deep sequencing, also referred to as next generation sequencing, could rapidly supply information about the genomic sequence. However, the nucleotide sequence of CTV cannot yet be related to the biological properties and behaviour of the strain (i.e. aggressiveness and transmissibility). Even though CTV strains have been classified and grouped by their phenotype, virulence, host range, epitope composition and, more recently, by sequence ~~homology~~identity of one or more genes (Moreno *et al*., 2008), no accurate correlation with the biological behaviour has been found (Harper, 2013).	Homology replaced by identity.	European Union	**INCORPORATED** We agree and "identity" was introduced in the current text.
63.	*131*	Technical	i. The part of differentiation by MCA13 should be cancelled.Reactivity against the monoclonal antibody MCA13 (Permar *et al*., 1990), which recognizes an epitope that is well conserved in severe (aggressive) CTV strains but lacking in mild (less aggressive) strains (Pappu, *et al*., 1993). The reaction with MCA13 is strongly associated with the capacity to induce the decline of trees grafted on sour orange or lemon rootstocks. The majority of CTV strains that produce stem pitting in grapefruit or in sweet orange are MCA13 positive.	MCA13 has been proved to be useful only within those isolates in the US, but not stem-pitting tristeza in other countries such as in China, Australia, South Africa etc.	China	**CONSIDERED, BUT NOT INCORPORATED** We do not agree. MCA13 monoclonal antibody mainly reacts against CTV isolates able to cause decline on trees grafted on sour and with more severe CTV isolates able to cause stem pitting on sweet and in grapefruit worldwide. Of course that some exceptions (basically severe CTV isolates testing

Comm. no.	Para. no.	Comment type	Comment	Explanation	Country	SC Responses
						negative) were described to this general rule, nevertheless, we support the inclusion in the text of the paragraph: "The reaction with MCA13 is strongly associated with the capacity to induce the decline of trees grafted on sour orange or lemon rootstocks. The majority of CTV strains that produce stem pitting in grapefruit or in sweet orange are MCA13 positive", that summarizes correctly the international experience concerning MCA13 reactivity, supported by the latest review of Moreno et al. (2008).
64.	*137*	Technical	Membranes that have been tissue printed (recommended size: approximately 7 × 13 cm) are placed in an appropriate container (tray, hermetic container or plastic bag), covered with a 1% solution of BSA in distilled water and incubated for 1 h at room temperature or overnight (about 16 h) at 4 °C (the latter is recommended). Slight agitation is beneficial during this step. The albumin solution is discarded but the membranes are kept in the same container. A solution of CTV-specific MCA13 monoclonal antibody linked to	It would be less repetitive if a reference to 3.5.1 was added and only the differences indicated here.	European Union	**MODIFIED** We agree with the suggestion. Please see the optional text significantly shortened.

Comm. no.	Para. no.	Comment type	Comment	Explanation	Country	SC Responses
			alkaline phosphatase (about 0.1 µg/ml in PBS) is prepared and poured onto the membranes, covering them, and the membranes are incubated for 3 h at room temperature, with slight agitation. The conjugate solution is then discarded. The membranes and the container are rinsed with washing buffer (PBS, pH 7.2–7.4, with 0.05% Tween 20; see section 3.4.3 for the composition of PBS), and washed by shaking (manually or mechanically) for 5 min. The washing buffer is discarded and the washing process is repeated twice. The substrate for alkaline phosphatase (Sigma[7] Fast BCIP/NBT tablets) is then poured over the membranes and the membranes are incubated until a purple-violet colour appears in positive controls (about 10–15 min). The reaction is stopped by washing the membranes with tap water. The membranes are spread on absorbent paper and allowed to dry. The prints are examined using a low-power magnification (×10 to ×20). The presence of purple-violet precipitates in the vascular region of plant material reveals the presence of a CTV strain of increased aggressiveness.			
65.	*140*	Technical	An appropriate dilution of polyclonal or monoclonal antibodies 3DF1 + 3CA5[2] (usually 1-2 µg/ml immunoglobulins) is prepared in carbonate buffer pH 9.6 (Na_2CO_3, 1.59 g; $NaHCO_3$, 2.93 g; distilled water, 1 l). A volume of 200 µl is added to each well, and incubation is carried out at 37 °C for 4 h or at 4 °C for 16 h. The wells are washed three times with washing buffer (PBS, pH 7.2–7.4, with 0.05% Tween 20). Then add 200 µl per well of the plant extract (see Section 3.4). Two wells of the plate is used for each sample or positive controls, and at least two wells for negative controls. Incubation is carried out at 4 °C for 16 h. Then wash the plates three times, using the washing process described before. Prepare the specific monoclonal antibody MCA13 linked with alkaline phosphatase at appropriate dilution (about 0.1 µg/ml in PBS with 0.5% bovine serum albumin-BSA added). A volume of 200 µl of the monoclonal antibody solution is added to each well, and incubation is carried out at 37 °C for 3 h.	It would be less repetitive if a reference to 3.5.1 was added and only the differences indicated here.	European Union	**MODIFIED** We agree and an optional text is proposed in the current version of the manuscript. The text refers to 3.5.2. (instead of to 3.5.1. that was suggested).

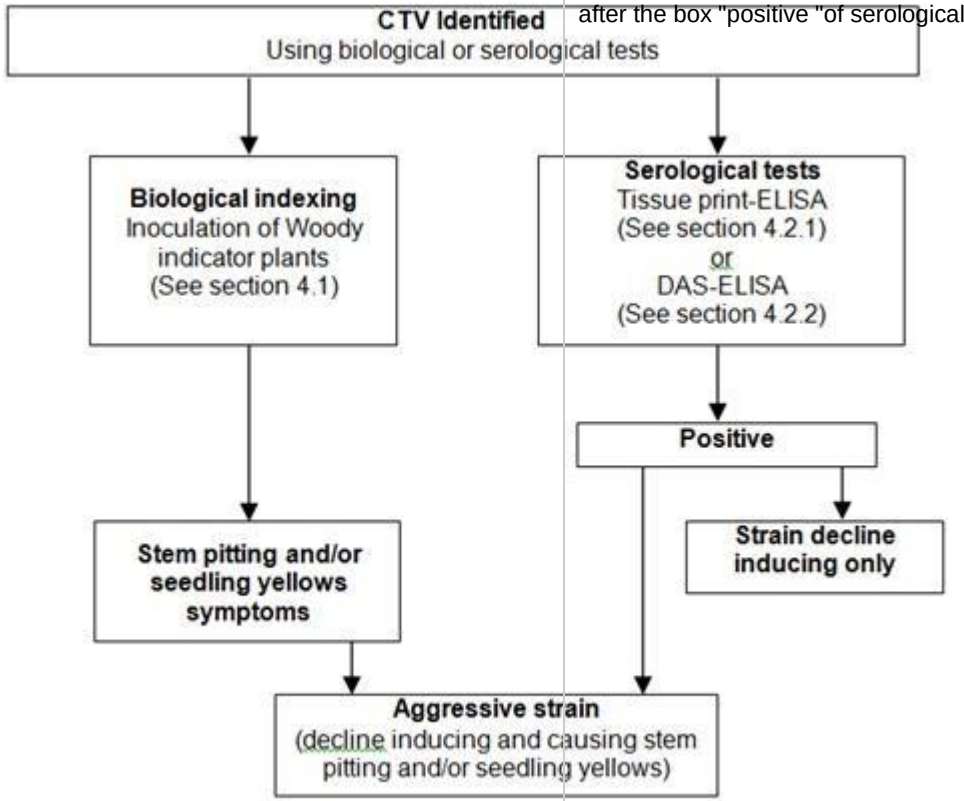
Comm. no.	Para. no.	Comment type	Comment	Explanation	Country	SC Responses
			Wash as before. Prepare 1 mg/ml alkaline phosphatase substrate (p-nitrophenyl phosphate) in substrate buffer (diethanolamine 97 ml; diluted in 800 ml of distilled water, adjusted to pH 9.8 with concentrated HCl and made up to 1,000 ml with distilled water). Then add 200 µl of the substrate solution to each well. This is then incubated at room temperature and read at 405 nm after 30, 60 and 90 min. The ELISA test is negative if the absorbance of the sample is less than twice the absorbance of the healthy control, and positive if the absorbance of the sample is equal to or greater than twice that value.			

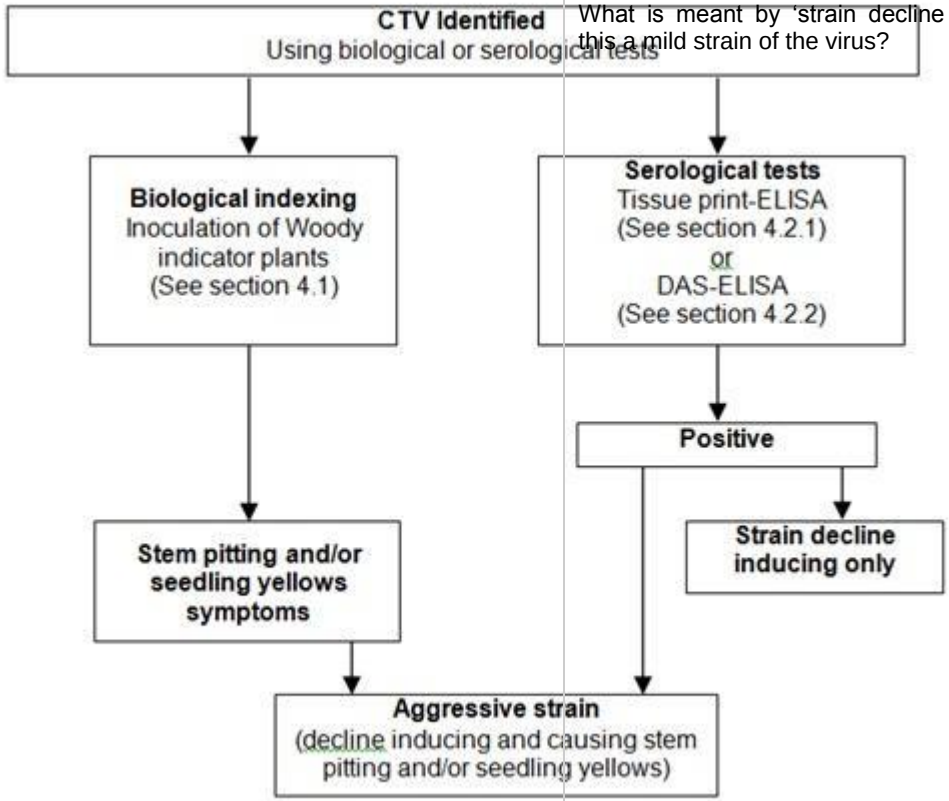
Comm. no.	Para. no.	Comment type	Comment	Explanation	Country	SC Responses
66.	*204*	Editorial		for figure 2- "Biological indexing", since in both cases of (typical symptoms and no symptoms) molecular or serological screening as will carried out Why it's separated steps? This means that from both boxes of typical symptoms and no symptoms one box containing the text (confirmation serological or molecular screening tests) The last box go direct to the box (positive results) and deleting boxes of (serological or molecular screening tests) and (positive results by one of the confirmation tests)	Bahrain	**MODIFIED** Please see 204 below. The flow diagram was modified to include negative results as suggested by China.
67.	*204*	Substantive	The flow diagram should add negative flow, two results of three methods are negative indicated the virus is not present.	More reliable.	China	**Considered and incorporated**

Comm. no.	Para. no.	Comment type	Comment	Explanation	Country	SC Responses
						
68.	*205*	Substantive	**Figure 2.** Flow chart for the detection and identification of *Citrus tristeza virus* (CTV).	The use of deep sequencing is not described in the protocol consequently it should not appear in the flow chart. More generally since analysis of these NGS data is critical and not much standardization has been done so far, inclusion of this technique in the protocol/figure at this stage is too early and not desirable.	European Union	The use of NGS is not described in the protocol because we think is not necessary and will be long and complicated; because we agree

Comm. no.	Para. no.	Comment type	Comment	Explanation	Country	SC Responses
						that the technique is not yet validated. Different commercial companies currently apply NGS as an available service. In practice there are not big differences between conventional sequencing (that also is not described hear nor in other protocols). Currently a COST EU Action is devoted to this technology that is based on PCR amplification, automatic sequencing by specific machines and finally bioinformatics analysis of the results. We hope than soon will be available validation. This interesting and powerful option will be very popular

Comm. no.	Para. no.	Comment type	Comment	Explanation	Country	SC Responses
						soon, adopted in many laboratories and in a short period will probably replace or substitute indexing. We think that in a standard protocol prepared in 2015, this technique merit to be included as an alternative to conventional sequencing that is today commonly used. We remember the same discussions and objections concerning ELISA in 1977-1980, and about PCR in 1994-2000. The panel has to conclude on this particular subject. In the current protocol we deleted from the flow chard following the European Union suggestion.
69.	*205*	Technical	**Figure 2.** Flow chart for the detection and identification of *Citrus tristeza virus* (CTV).	Options for negative results should be included.	European Union	See 204 comments.

Comm. no.	Para. no.	Comment type	Comment	Explanation	Country	SC Responses
70.	*206*	Editorial		For figure 3- why there is no box "Biological indexing" after the box "positive "of serological tests" ?	Bahrain	**CONSIDERED, BUT NOT INCORPORATED** Because we propose optionally biological tests or serological ones, no both simultaneously.

Comm. no.	Para. no.	Comment type	Comment	Explanation	Country	SC Responses
71.	*206*	Technical		1: Options for negative results should be included. 2: What is meant by 'strain decline inducing only'? Is this a mild strain of the virus?	European Union	**CONSIDERED, BUT NOT INCORPORATED** We think that is not necessary to include a negative flow. The flow diagram we proposed is for confirmed CTV infection. The goal is to identify the putative ability of CTV strains to cause decline on sour or decline+stem pitting in citrus cultivars. "Strain decline inducing only" is a CTV strain able to cause tristeza syndrome on trees grafted on sour orange or lemon, but unable to cause stem pitting on grapefruit and/or sweet orange. These CTV strains are common in many citrus industries, especially in the Mediterranean basin and Mexico. Is very important to differentiate from the CTV strains that in addition are able to cause stem pitting

Comm. no.	Para. no.	Comment type	Comment	Explanation	Country	SC Responses
						(aggressive isolates on sweet orange and grapefruit). These last strains are present in some citrus industries such as in Asia, South America, Australia and South and Central Africa (see Moreno et al., 2008 review)..