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***Citrus junos* as a host of citrus bacterial canker**

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Abstract

Following a request from the European Commission, the European Food Safety Authority (EFSA) Plant Health (PLH) Panel analysed a dossier submitted by the Japanese authorities in order to clarify the host status of *Citrus junos* with regard to *Xanthomonas citri* pv. *citri* and *Xanthomonas citri* pv. *aurantifolii*, causal agents of citrus bacterial canker, and to indicate whether *C. junos* fruit could represent a pathway for the introduction of citrus bacterial canker into the European Union. In a previous opinion in the year 2014, the EFSA PLH Panel concluded that commercial fresh citrus fruit is generally pathway and that no commercially important *Citrus* species or variety can be considered as immune to citrus bacterial canker. In the current assessment, the EFSA PLH Panel analysed the two scientific papers provided by the Japanese authorities, as well as 16 additional papers identified through a systematic literature review. The PLH Panel considered that the conclusions of its previous opinion remain valid and that convergent lines of evidence provide sufficient demonstration that *C. junos* is a host of *X. citri* pv. *citri* and *X. citri* pv. *aurantifolii*. Therefore, there is no reason to consider the *C. junos* fruit differently from other citrus species. Consequently, the assessment of the general citrus fruit pathway from the 2014 opinion still applies. Uncertainties on these conclusions are a result of the scarce scientific evidence published on this subject in addition to the methodological and reporting limitations of the published papers.

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1. Introduction

1.1. Background and Terms of Reference as provided by the requestor

The European Food Safety Authority (EFSA) is requested, pursuant to Article 22(5.b) and Article 29 (1) of Regulation (EC) No 178/2002¹, to provide a scientific opinion in the field of plant health.

The Japanese phytosanitary authorities consider that *Citrus junos* is not a host of *Xanthomonas citri* pv. *citri* and *Xanthomonas citri* pv. *aurantifolii* or citrus bacterial canker. Therefore, Japan has requested that *C. junos* fruits are excluded from the specific requirements listed in Point 16.2 of Annex IVAI of the Directive 2000/29/EC in relation to citrus bacterial canker. Japan has provided the Commission with two scientific papers on this issue, which are attached for consideration:

- Koizumi M, 1979. Ultrastructural changes in susceptible and resistant plants of *Citrus* following artificial inoculation with *Xanthomonas citri* (Hase) Dowson. Annals of the Phytopathological Society of Japan, 45, 635–644 (Appendix 1 of the request);
- Koizumi M and Kuhara S, 1982. Evaluation of *Citrus* plants for resistance to bacterial canker disease in relation to the lesion extension. Bulletin of the Fruit Trees Research Station of Japan D, 4, 73–92 (Appendix 2 of the request)

Therefore, EFSA is requested to prepare a scientific opinion to clarify the host status of *C. junos* with regard to *X. citri* pv. *citri* and *X. citri* pv. *aurantifolii* or citrus bacterial canker, and to indicate whether *C. junos* fruits could represent a pathway for the introduction of citrus bacterial canker into the Union.

1.2. Interpretation of the Terms of Reference

The taxonomy of the species *X. citri* was recently revisited by Constantin et al. (2016). *Xanthomonas citri* (ex Hase, 1915) Gabriel et al., 1989 emend. Ah-You et al., 2009 is now proposed to comprise 14 different pathovars (pv. *anacardii*, pv. *aracearum*, pv. *aurantifolii*, pv. *citri*, pv. *fuscans*, pv. *glycines*, pv. *malvacearum*, pv. *mangiferaeindicae*, pv. *punicae*, pv. *rhynchosiae*, pv. *sesbanae*, pv. *thirumalacharii*, pv. *vignaeradiatae* and pv. *vignicola*). Among these, only two pathovars are associated with citrus bacterial canker disease: *X. citri* pv. *citri* and *X. citri* pv. *aurantifolii* (EFSA PLH Panel, 2014). Different pathotypes have also been proposed within these two pathovars, namely pathotype A, A* and A^w linked to Asiatic citrus bacterial canker and *X. citri* pv. *citri* (Sun, 2004; Vernière et al., 1998), and pathotype B and C for the South American canker and *X. citri* pv. *aurantifolii*.

EFSA have previously published several opinions related to citrus bacterial canker (EFSA, 2007; EFSA PLH Panel 2011, 2013, 2014). Commercial fresh fruit in general (for direct consumption or for industrial uses) is shown to be a pathway.

Directive 2000/29/EC considers '*Xanthomonas campestris* (all strains pathogenic to *Citrus*)' in its present wording. Changes in taxonomy led to reclassification of that group of bacteria that are now separated as *X. citri* pv. *citri* and *X. citri* pv. *aurantifolii*. This does not change the conclusions of previously issued EFSA opinions in anyway because those taxonomic evolutions were already taken into consideration.

In directive 2000/29/EC, the term 'fruit of *Citrus*' includes fruit from plant genera *Citrus* (to which *C. junos* belongs), *Poncirus* and *Fortunella*. That list shall be expanded to genera *Microcitrus*, *Swinglea*, *Naringi* and their hybrids (see later), as already indicated in the previous EFSA opinion (EFSA PLH Panel, 2014). In the present opinion, 'fruit of *Citrus*' means (except if specified differently) fruit from a plant belonging to the genera *Citrus*, *Fortunella*, *Poncirus*, *Microcitrus*, *Swinglea*, *Naringi* and their hybrids.

Import into, and circulation within, the Community of fruit from all plants belonging to genera *Citrus*, *Fortunella*, *Poncirus* and their hybrids, is regulated by Directive 2000/29/EC, which prescribes measures to be applied by exporting countries in order to mitigate the risk of introduction into the European Union (EU) of *X. citri* pv. *citri* and *X. citri* pv. *aurantifolii*. The import of fruit from *C. junos* shall therefore be in accordance with regulation.

The current legislation is presented in Appendix A of this opinion.

Given the request, in this opinion, the Plant Health (PLH) Panel considers only the fresh citrus fruit trade pathway (citrus fruit, commercial trade as assessed in the previous opinion under pathway I, (EFSA PLH Panel, 2014). Other pathways, including citrus fruit and/or leaves import by passenger

¹ Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. OJ L 31, 1.2.2002, pp. 1–24.

traffic, referred as pathway II in the previous EFSA opinion (EFSA PLH Panel, 2014), are not taken into consideration.

2. Data and methodologies

2.1. Data

Search strategies were undertaken in order to identify scientific literature on *C. junos* and citrus bacterial canker or *Xanthomonas*. It has been noted that the specific species *C. junos* might not be present in the title and/or abstract of the article but only in the full text. Therefore, two searches were combined in the main bibliographic databases: a tailored search to identify *C. junos* and *Xanthomonas* or citrus canker; a general search to identify studies including information of *Citrus* species and resistance or tolerance to *Xanthomonas* or citrus canker.

The following databases were searched (Table 1):

Table 1: Overview of searched databases

Database	Platform
Web of Science Core Collection: <ul style="list-style-type: none"> • Science Citation Index (1975 to present) • Conference Proceedings Citation Index- Science (1990 to present) • Emerging Sources Citation Index (2015 to present) 	Web of Science
BIOSIS Citation Index (1926 to present)	Web of Science
CABI: CAB Abstracts® (1910 to present)	Web of Science
Chinese Science Citation Database (1989 to present)	Web of Science
KCI-Korean Journal Database (1980 to present)	Web of Science
SciELO Citation Index (1997 to present)	Web of Science
Crop Protection Compendium (inception to present)	CABI
AGRICOLA (inception to present)	National Agricultural Library

Additional searches, limited to retrieve documents on *C. junos* and *Xanthomonas* or citrus canker, were run in Google Scholar via Publish or Perish version 4 software.

The searches were run on 7 March 2017. No language, date or document type restrictions were applied in the search strings. The search strategies were adapted according to the configuration of each resource of information. Full search strategies are listed in Appendix B.

The outputs from all the databases except AGRICOLA were exported to an Endnote x8 file and the duplicate results were removed. AGRICOLA outputs were sent via email and the results not yet identified in other databases were added manually into EndNote x8. The final number of results of the database searches after removing duplicates was 238.

The results of the searches in Google Scholar were exported in a different EndNote x8 file. The duplicate results of the different queries were removed; results already identified in the databases searches were also removed. The final number of unique results identified in Google Scholar after deduplication was 140.

2.2. Methodologies

The assessment was conducted in line with the principles described in the EFSA Guidance on transparency in the scientific aspects of risk assessment (EFSA, 2009). The present document is structured according to the Guidance on the structure and content of EFSA scientific opinions and statements (EFSA Scientific Committee, 2014).

For a thorough evaluation of *C. junos* as a possible host of *X. citri* pv. *citri* or *X. citri* pv. *aurantifolii*, as well as to indicate whether *C. junos* fruits could represent a pathway for citrus bacterial canker introduction into the Union, the Panel considered all the data and information provided by the Japanese authorities, the previous EFSA opinions on the topic (EFSA PLH Panel, 2014) and relevant literature.

3. Assessment of *C. junos* as a host for *X. citri* pv. *citri* and *X. citri* pv. *aurantifolii*

3.1. Background information

Citrus junos Sieb ex Tanaka (*Citrus ichangensis* x *Citrus reticulata* var. *austere*), called Yuzu in Japanese, Yuja in Korean and Xiangchéng in Chinese, is a citrus species native to China (Ferguson and Grafton-Cardwell, 2014). It is mostly cultivated in South Korea and Japan where fresh fruits are used for juice, traditionally for preparing vinegar and seasoning. Yuzu essential oil is increasingly used in food, beverages, cosmetics, perfumery and aromatherapy (Sawamura and Lan-Phi, 2010). In Japan and China, several varieties are recorded and have been studied for the variation of the volatile constituents of their cold-pressed peel oil (Sawamura and Lan-Phi, 2010). There is also an emerging demand outside of Asia for yuzu fresh fruit.

Although *C. junos* is considered to originate from China, Japan and South Korea are considered as the main *C. junos* producing areas. In 2014, yuzu acreage in Japan was evaluated as 2,216 ha (3% of the Japanese citrus acreage) (Omura and Shimada, 2016). Kim et al. (2015) report an area of 2,237 ha equivalent to a production of 19,127 metric tonnes in South Korea. Presently, at least one Japanese company is exporting *C. junos* fresh fruits to Europe (Sasu Olivier Derenne, online). There are some indications about EU importation of *C. junos* from California. *C. junos* fruits are also produced in Spain (Old, 2016; Stoffels, online).

There is no precise data on quantity of *C. junos* importation into the EU. In the import statistical data in EUROSTAT (online), only the main citrus species are individually reported (such as 'orange', 'mandarins', 'grapefruit', 'lemons', 'limes'); therefore, data on *C. junos* are not presented as such but are comprised within the term 'Citrus other'.

EFSA issued a scientific opinion on the risk to plant health of *X. citri* pv. *citri* and *X. citri* pv. *aurantifolii* for the EU territory in 2014 (EFSA PLH Panel, 2014). Previous EFSA opinions also considered citrus bacterial canker and the fruit pathway (EFSA, 2007; EFSA PLH Panel 2011, 2013).

The latest opinion considered in detail the commercial fresh fruits pathway, with the following main conclusions (EFSA PLH Panel, 2014):

- '*Xanthomonas citri* pv. *citri* and *X. citri* pv. *aurantifolii* are likely to be associated with citrus fruit, with a medium uncertainty because of (i) incomplete data on the presence of *X. citri* pv. *citri* or *X. citri* pv. *aurantifolii* strains in the country at origin; (ii) the variation in cultivar susceptibility; (iii) the differences in the pest management measures set up according to the countries exporting citrus fruit; and (iv) differences in packinghouse operational procedures;
- *Xanthomonas citri* pv. *citri* and *X. citri* pv. *aurantifolii* are very likely to survive during transport and storage of fruit, with a low uncertainty;
- *Xanthomonas citri* pv. *citri* and *X. citri* pv. *aurantifolii* are very likely to survive the existing pest management procedures, with a low level of uncertainty;
- the probability of transfer to a suitable host is unlikely, but with a high uncertainty because of (i) the paucity of literature and (ii) the lack of extensive information on transfer under natural conditions'.

Considering consequences of introduction of those bacteria into the Community, the previous EFSA opinion (EFSA PLH Panel, 2014) stated that:

- 'all citrus-growing areas in the EU can be considered as the endangered area;
- where citrus bacterial canker occurs, the quantity and quality of the fruit production is impaired owing to defoliation, premature fruit drop, dieback, blemishes on fruit, and general tree decline;
- once introduced, citrus bacterial canker cannot be controlled without regulation, although those measures hardly permit to control the disease'.

Considering risk reduction options, with regard to the already implemented measures for the fruit pathway (including measures that may help to keep fruit free from bacteria in the field) and the request from Japan, the previous EFSA opinion (EFSA PLH Panel, 2014) reached the conclusions that:

- 'the effectiveness of the prohibition of leaves and peduncles is rated as low, with a very high technical feasibility and a low uncertainty;

- the prohibition of fruit from certain plant genotypes (which means the authorisation for others) appears not to be applicable;
- the effectiveness of guarantying pest freedom of consignments through inspections and testing is rated as moderate, with moderate technical feasibility and medium uncertainty;
- the effectiveness of measures taken while preparing the consignments in the country of origin (cleaning of harvesting material, separate facilities for fruit coming from known healthy fields, culling and cleaning of fruit, storage at cold temperatures, etc.) is rated as moderate, with very high technical feasibility and medium uncertainty;
- the effectiveness of specific treatments of fruit to reduce pest prevalence in consignments is rated as moderate, with very high technical feasibility and low uncertainty;
- the effectiveness of integrated pest management procedures in citrus growing fields and their environment is rated as moderate, with very high technical feasibility and low uncertainty;
- the use of resistant or less susceptible varieties, as claimed by the Japanese authorities for *C. junos* (providing it would be proved that that species is resistant or less susceptible), is rated high to moderate, with a low technical feasibility and low uncertainty;
- the effectiveness of limiting import to fruit originating from pest-free areas is rated as very high, with a high technical feasibility and medium uncertainty;
- the effectiveness of limiting import to fruit originating from pest-free production places is rated as high, with a high technical feasibility and high uncertainty;
- the effectiveness of systems approaches integrating individual risk reduction options is rated as moderate, with high technical feasibility and medium uncertainty'.

Some other risk reduction options, not implemented in EU regulation and not included into the request from the Commission for this opinion, were nevertheless evaluated:

- 'the effectiveness of restriction on distribution of fruit within the EU is rated as high, with low technical feasibility and low uncertainty;
- the effectiveness of restriction on end use of fruit is rated as high, with high (for non-endangered areas) or low (for endangered areas) technical feasibility, as a result of difficulties in implementation, with low uncertainty'.

Relying on those conclusions from the previous EFSA opinion (EFSA PLH Panel, 2014), fresh fruit of *Citrus* sp. was clearly considered as a pathway for *X. citri* pv. *citri* and *X. citri* pv. *aurantifolii*.

3.2. Assessment of data provided by the Japanese authorities

To support their request to exclude *C. junos* from the specific requirements listed in Point 16.2 of Annex IV Part A Section I of the Directive 2000/29/EC in relation to citrus bacterial canker, the Japanese phytosanitary authorities provided two papers by Koizumi (1979), and Koizumi and Kuhara (1982).

The two studies refer to previous work published by Koizumi, in a subsequent way. First, Koizumi developed a way of assessing the bacterial multiplication using serial dilutions of lesions and pin-prick inoculation on a susceptible host (Koizumi, 1971). In *C. junos*, the bacterial multiplication in attached, pin-pricked inoculated, leaves was evaluated under controlled conditions (glasshouse) at 21°C, to reach levels of 10⁶ cells per lesion, as determined by the needle-prick inoculation of tenfold dilutions from lesion crushes (Koizumi, 1976). Water-soaked lesions appeared 7 days post-inoculation on *C. junos*, whereas they were recorded 5 days post-inoculation in hosts that are more susceptible. Finally, the level of bacterial multiplication was also evaluated over a 4-month period using either the needle-prick inoculation method (Koizumi, 1971, 1977) or an indirect detection/enumeration technique based on CP1 citriphage multiplication from lesion extracts (Koizumi et al., 1966; Koizumi, 1969a,b, 1972). Indirect *X. citri* pv. *citri* population size estimates by this citriphage technique from symptoms on yuzu were similar to those on other resistant hosts; for example, Ponkan mandarin (*C. reticulata*) or calamondin (*Citrus madurensis*) (Koizumi and Kuhara, 1982).

The paper by Koizumi (1979) is based on macroscopical and ultrastructural observations following leaf infiltration (2-year-old *Citrus* spp. potted plants) by a hypodermic syringae of a high concentration of inoculum (10⁹ cells/mL) of the virulent isolate QN7501 of *X. citri* (name of the bacterium at the time this paper was published – see Section 1.2), performed under controlled conditions at 20°C.

The author states that: 'In the resistant *C. junos* leaves, ultrastructural changes were similar to those observed on the susceptible host until 3 days after inoculation. Four days after inoculation, however, the liberated fibrils became concentrated around the bacterial cell and filled the intercellular

spaces. These enveloped bacteria became electron-dense indicating deterioration of the cells. By 6 days after inoculation, the spaces between separated plasmalemma and the cell wall were filled with numerous granules mixed with small vesicles and the cytoplasm was markedly electron-dense. This was associated with necrosis or a hypersensitive reaction'. The authors stress also the fact that less hypertrophy and translucence were observed on the symptoms of Yuzu plants compared to the Natsudaikai susceptible ones.

Although the paper does not provide the reader with detailed information on the inoculation conditions (no indication about the precise line or variety of *C. junos* used, nor about any different night-day regime), it is reported that the experiments were conducted at 20°C, whereas the optimum temperature for *X. citri* is between 25°C and 30°C (Dalla Pria et al., 2006).

The experiment was performed with a single isolate (QN5701), for which no detailed information is available. Kitagawa et al. (1992) provide with the information that the strain was isolated from the Kuchinotsu fruit tree research station (Nagasaki prefecture) from sweet orange (*Citrus sinensis*).

Finally, the symptoms described by Koizumi (tissue hypertrophy and watersoaking) appear to be rather different from those observed following a true hypersensitive reaction on non-host plants as stated by Dunger et al. (2005).

In the second paper provided by the Japanese authorities, Koizumi and Kuhara (1982) investigated the susceptibility of citrus lines following (i) outdoor artificial inoculations (*C. junos* included) and (ii) natural infections in field conditions (*C. junos* not included). The authors classified *C. junos* as class F (lowest susceptibility) together with calamondin on the basis of a lack of bacteriophage CP1 multiplication (used as an estimator of the density of *Xanthomonas* cells present in the lesion) from lesions produced from inoculations on *C. junos*. The authors did not clearly report the morphology of the lesions assayed from *C. junos* but mention lesions sized between 0.6 and 0.8 mm² on potted plants placed in the field. The reported field studies were performed between 1968 and 1970. The authors provide a table classifying the different resistance levels, probably also based on the former paper by Koizumi (1978), which itself is based on field observations made by three different observers in 1973 at Kuchinotsu Fruit Tree Research Station, when the disease was 'severely prevalent'.

3.3. Assessment of additional literature data

Based on an extensive literature search, 16 additional peer-reviewed papers provide data on *C. junos* as a host for citrus bacterial canker. Most of these papers focus on *X. citri* pv. *citri*, except for two papers dealing with *X. citri* pv. *aurantifolii* (Goto et al., 1980; Malavolta et al., 1984).

An overview of additional literature data is provided in Table 2.

Table 2: Assessment of additional literature data

Paper	Aim	Procedure	Status of yuzu as a host species (as cited in the paper)	Clear-cut description of reaction type (lesion morphology)	Population size assessed
Peltier and Frederick (1920)	Susceptibility of citrus lines and rutaceous relatives	Spray-inoculations in a greenhouse and in open-field (one yuzu accession assayed)	'Somewhat resistant'	No (small leaf lesions with epidermis rupture; no twig canker observed)	No
Obata (1974)	Variability of <i>X. citri</i> pv. <i>citri</i> strains from Japan based on susceptibility to citraphages CP1 and CP2	Phage-typing assay	<i>X. citri</i> pv. <i>citri</i> strains collected from natural infections of <i>C. junos</i> in Japan (exact location not mentioned)	No	No
Koizumi (1976)*	Assess <i>in planta</i> multiplication of <i>X. citri</i> pv. <i>citri</i> and histological changes on a range of citrus lines	Needle-prick inoculation of leaves from potted plants placed in a greenhouse (one yuzu accession assayed)	'More resistant species'	Yes (watersoaking and hypertrophy, i.e. compatible interaction); hypertrophy was less pronounced in more resistant cultivars (including yuzu)	Yes: reaches approximately 1×10^6 cfu/lesion at an incubation temperature suboptimal for <i>Xcc</i> multiplication (21°C)
Koizumi (1977)*	Assess <i>in planta</i> multiplication of <i>X. citri</i> pv. <i>citri</i> and histological changes on a range of citrus lines	Needle-prick inoculation of leaves from potted plants placed in open-field (one yuzu accession assayed)	'Resistant'	Yes (hypertrophy with late defence reactions developing); hypertrophy was less pronounced in more resistant cultivars (including yuzu)	Yes: low population size (approximately 1×10^4 cfu/lesion recorded) were determined by needle-prick inoculation method of serial dilutions; environmental conditions not precisely documented
Goto et al. (1980)	Comparative characterisation of <i>X. citri</i> pvs. <i>aurantifolii</i> (pathotype B) and <i>citri</i> by phenotypic tests including pathogenicity	Needle-prick and spray-inoculation of leaves from potted plants placed in a greenhouse (one yuzu accession assayed)	'Resistant'	Yes (symptoms produced from spray-inoculations of several citrus lines including yuzu with <i>X. citri</i> pvs. <i>aurantifolii</i> and <i>citri</i> similar to that observed in field situations). Weak pathogenicity of pv. <i>citri</i> strains on some citrus lines (e.g. yuzu) hypothesised to be related to strain maintenance system used by authors	No

Paper	Aim	Procedure	Status of yuzu as a host species (as cited in the paper)	Clear-cut description of reaction type (lesion morphology)	Population size assessed
Malavolta et al. (1984)	Host range of <i>X. citri</i> pv. <i>aurantifolii</i> (pathotype C) assayed through pathogenicity tests	Two inoculation procedures: (i) needle-prick inoculation and (ii) infiltration into leaf mesophyll on 21 citrus lines including yuzu	'Hypersensitivity reaction'	Yes (hypersensitive reaction)	No
Matsumoto and Okudai (1990)*	Analysis of ACC resistance inheritance from crosses (F1) compared to evaluation on adult trees	Disease assessments based on canker lesion size from seedlings and from adult trees <i>Citrus natsudaikai</i> (moderately susceptible used as a reference) (one yuzu accession assayed)	'Highly resistant'	No	No
Jiao et al. (1992a)	Evaluation of citrus leaf polar lipid, phospholipid contents and fatty acid unsaturation as a proxy of resistance level	Biochemical analyses	Text: 'More resistant than citrumelo, lemon, rough lemon, key lime and grapefruit' Table 1: 'Highly resistant' (from literature; no pathogenicity assays performed)	No	No
Jiao et al. (1992b)	Enzymatic activities in citrus leaves as a proxy of resistance level	Biochemical analyses	Text: 'More resistant than citrumelo, lemon, rough lemon and grapefruit' Table 2: 'Highly resistant' (from literature; no pathogenicity assays performed)	No	No
Kitagawa et al. (1992)	Application of an immunoassay (SAIEA) for evaluating canker resistance in citrus plants	Population size estimation using SAIEA from wound-inoculations in a detached leaf assay over 1 week (one yuzu accession assayed)	'Highly resistant'	No	Yes: quantification performed over 1 week only by an SAIEA immunoassay having a poor ability to detect small population sizes; data suggest a slower growth rate in yuzu compared to more susceptible species (e.g. Valencia orange or clementine)

Paper	Aim	Procedure	Status of yuzu as a host species (as cited in the paper)	Clear-cut description of reaction type (lesion morphology)	Population size assessed
Shiotani et al. (2000)	Phenotypic, genetic and pathological diversity of <i>X. citri</i> pv. <i>citri</i> strains from Japan interacting with <i>Citrus grandis</i> and other <i>Citrus</i> species	Citriphage susceptibility, ERIC-PCR, needle-prick inoculations on leaves from potted plants in a greenhouse (28°C day; 25°C night) (one yuzu accession assayed)	Not stated	No, but no mention of distinct symptomatology on some assayed host species; lesion size from inoculation similar to that produced on Mexican lime, a species highly susceptible to <i>X. citri</i> pv. <i>citri</i>	Yes, but not on yuzu
Hyun et al. (2003)	<i>Citrus</i> species and cultivar evaluation for susceptibility to citrus bacterial canker	Pathogenicity tests based on (i) spray inoculations of young flushes from 3- to 4-year-old grafted potted plants (one yuzu accession assayed) in a greenhouse (temperature ranging from 18°C to 32°C) and (ii) natural infection of 3- to 4-year-old grafted potted plants (one yuzu accession assayed) placed underneath heavily diseased <i>C. natsudaidai</i> grove trees	'Highly resistant'	Yes. Greenhouse experiment: no difference mentioned in lesion morphology but lesion development delayed by 4 days on yuzu compared to the susceptible control (sweet orange); disease severity (percentage of diseased area) recorded on yuzu not statistically different from Satsuma mandarin (i.e. a partially resistant cultivar) 30 days after inoculation Field experiment: disease incidence recorded on yuzu not statistically different from Satsuma mandarin	No
Myung et al. (2003)	<i>Citrus</i> species and cultivar evaluation for susceptibility to citrus bacterial canker	Two inoculation procedures on potted plants incubated under environmentally controlled conditions (28°C C 75% RH): (i) spray inoculations of young flushes from 2- to 3-year-old grafted potted plants (one yuzu accession assayed) and (ii) infiltration into leaf mesophyll	'Resistant'	Yes. No difference in lesion morphology but lesion development delayed by 3 days on resistant cultivars (including yuzu); disease severity recorded on yuzu not statistically different from Satsuma mandarin (i.e. a partially resistant cultivar) 18 days after inoculation (dai) but lower 30 dai	Yes, growth rate recorded on yuzu not statistically different from Satsuma mandarin

Paper	Aim	Procedure	Status of yuzu as a host species (as cited in the paper)	Clear-cut description of reaction type (lesion morphology)	Population size assessed
Lee et al. (2009)	Comparative ultrastructure of nonwounded susceptible and resistant leaves infected with <i>X. citri</i> pv. <i>citri</i>	Transmission electron microscopic observations of spray-inoculated juvenile Mexican lime and yuzu leaves	'Resistant'	Yes (typical raised canker lesions with a 2-day delayed lesion development on yuzu); ultrastructural analysis consistent with partial resistance developing on yuzu (differences in dynamics of structural barriers through host cell wall modifications, differences in biofilm development)	No
Amaral et al. (2010)	<i>Citrus</i> species and cultivar evaluation for susceptibility to citrus bacterial canker	Actively growing flushes from potted plants (in a greenhouse) spray-inoculated with a virulent strain (IAPAR306; 10^8 cells/mL) Rating based on diseased areas (one yuzu accession assayed)	'Highly susceptible'	No (but no mention of citrus lines producing lesions with an atypical morphology)	No
Deng et al. (2010)	<i>Citrus</i> species and cultivar evaluation for susceptibility to citrus bacterial canker	Three inoculation procedures were used: (i) detached leaf assay (five yuzu cultivars assayed); (ii) needle-prick inoculation of fully expanded leaves; and (iii) spray inoculations of young flushes from 1-year-old grafted plants settled in a diseased sweet orange grove (two yuzu cultivars assayed)	Variable depending on cultivars assayed	Yes (detached leaf assay: typical callus-like symptoms on all five cultivars assayed; attached leaf assay: only two less susceptible cultivars were assayed ('Shenju' and 'Zhencheng'); they showed typical canker lesions (example in Figure 3d) with a disease incidence ranging (i) from 3% to 15% following needle-prick inoculation and (ii) 9% to 13% following spray inoculation depending on year and cultivar; some non-inoculated <i>C. junos</i> plants also exhibited typical canker lesions	No

*: Paper in Japanese; abstract and tables available in English. Translation in English of full papers implemented for EFSA by Translation Centre for the Bodies of the EU.

From the review of these additional papers, the PLH Panel emphasises that:

- *Citrus junos* inoculated with *X. citri* pv. *citri* exhibits typical canker-like lesions, although the extent of watersoaking, tissue hypertrophy and lesion increase in size was less than those observed on susceptible cultivars. Population sizes in leaf lesions as high as 1×10^6 *X. citri* pv. *citri* per lesion were detected, although this experiment was conducted at a temperature suboptimal for *X. citri* pv. *citri* multiplication (Koizumi, 1976). Similarly, natural infections of *C. junos* in production groves were reported from Japan and *X. citri* pv. *citri* strains were readily isolated from these lesions (Obata, 1974). *C. junos* is therefore considered to be a host of *X. citri* pv. *citri* (low uncertainty).
- Although very little literature is available (which leads to high uncertainty), *C. junos* can be considered as a putative host of *X. citri* pv. *aurantifolii*. Two major groups of strains (i.e. referred to as pathotypes B and C) have been reported within this pathovar (EFSA PLH Panel, 2014). Goto et al. (1980) stated that 'Strains of canker B organism from Argentina were weakly virulent on all *Citrus* spp. tested, producing similar symptoms as those due to natural infection'. The host list used in this study included *C. junos*. Conversely, Malavolta et al. (1984) submitted a range of *Citrus* species/cultivars (including *C. junos*) to a pathotype C strain. Typical canker lesions were solely recorded on Galego lime, syn. Mexican lime (*Citrus aurantifolia*), whereas a hypersensitive reaction was recorded on all other citrus lines assayed.
- Analysed papers suggest that *C. junos* exhibits partial resistance but no immunity to *X. citri* pv. *citri*. A single exception listed yuzu as highly susceptible to *X. citri* pv. *citri* (Amaral et al., 2010) but assignment of the assayed cultivar to *C. junos* was considered doubtful (personal communication, 10 March 2017, Dr Sérgio Alves de Carvalho, Centro de Citricultura Sylvio Moreira – Instituto Agronômico, Cordeirópolis, SP, Brazil; replying to a specific query with regard to susceptibility rating of yuzu to *Xanthomonas citri* subsp. *citri*).
- Most papers investigating the susceptibility of *C. junos* to *X. citri* pv. *citri* used a single, genetically uncharacterised strain for pathogenicity assays. The genetic structure of *X. citri* pv. *citri* in *C. junos* production areas is not precisely known. Shiotani et al. (2007) showed the occurrence of genetic and pathological diversity among Japanese strains of *X. citri* pv. *citri* in relation to their TALE (transcription activator-like effector) gene content, yielding a differential susceptibility of some strains to some pummelo (*C. maxima*) cultivars. *C. junos* has, to our knowledge, not been challenged with strains representative of such a diversity. In addition to the distinct experimental procedures used for characterising levels of susceptibility of citrus lines to *X. citri* pv. *citri*, the lack of fully congruent conclusions (Table 2) may be a result of such pathological diversity.
- Most papers investigating the susceptibility of *C. junos* to *X. citri* pv. *citri* used a single, uncharacterised *C. junos* accession for pathogenicity assays. A single study (Deng et al., 2010), investigating the susceptibility level of five Chinese *C. junos* accessions, suggested that levels of partial resistance are cultivar-dependent.

Studies presenting a pathological characterisation based on a single host cultivar/strain interaction cannot be viewed as a final proof of resistance of *C. junos*. Based on available literature review, the PLH Panel concludes that the studies reviewed herein suggest an immunity of *C. junos* solely towards *X. citri* pv. *aurantifolii* pathotype C and rather suggest that *C. junos* is partially resistant to *X. citri* pv. *citri*. Based on the levels of partial resistance provided in table 4 of the scientific opinion on the risk to plant health of *Xanthomonas citri* pv. *citri* and *Xanthomonas citri* pv. *aurantifolii* for the EU territory (EFSA PLH Panel, 2014), *C. junos* may be classified as being resistant to highly resistant. This is because the only study assessing several yuzu cultivars suggests cultivar-dependent levels of partial resistance in *C. junos*. Such citrus cultivars/species that are classified as resistant or highly resistant are known to display natural infections when exposed to high inoculum pressure (e.g. the presence of highly diseased plants in the vicinity).

3.4. Uncertainties

The literature available on *C. junos* as a host for citrus bacterial canker is very limited (Table 2).

Often, the data provided are given for a single uncharacterised *C. junos* accession (Table 2), although different accessions are well known and characterised (Rahman et al., 2001). A single study (Deng et al., 2010), investigating the susceptibility level of five Chinese *C. junos* accessions, suggested that levels of partial resistance are cultivar-dependent. Additionally, *Citrus* spp. are often grafted in

commercial orchards and rootstock. Such a practice can significantly modify the susceptibility of the grafted cultivar to the aboveground pest (Bruessow et al., 2010; Agut et al., 2014, 2016), including *X. citri* pv. *citri*, for which a higher disease severity was observed for rootstock associations conferring a higher tree vigour (Agostini et al., 1985).

Most papers investigating the susceptibility of *C. junos* to *X. citri* pv. *citri* used a single, genetically uncharacterised strain for pathogenicity assays.

Additional pathogenicity data would be useful for confirming the non-host status of *X. citri* pv. *aurantifolii* pathotype C suggested by Malavolta et al. (1984).

There is also a lack of quantitative data on the bacterial population size in citrus bacterial canker lesions found on *C. junos*, both from naturally occurring infections or following inoculations. Only five papers report quantitative assessment over the 16 available, and often by the use of indirect assessment methodologies (using phage population as quantitative estimator, immunoassay, indirect needle-prick inoculation of serial dilutions obtained from lesion crush), which are considered as being less suitable for the estimation of small bacterial population. The use of bacteriophages for estimating the bacterial cell densities was established in the 1960s in Japan. Such an indirect enumeration technique only provides an approximate estimation of *X. citri* pv. *citri* population densities. Obata (1974) stated that, over multiplication cycles, *X. citri* pv. *citri* subpopulations can switch towards a citriphage-resistant phenotype, further questioning the reliability of this technique for quantification of population densities.

No data assessing the susceptibility level of *C. junos* fruits are presently available.

There is also a lack of data on *C. junos* production outside South Korea and Japan.

4. Conclusions

In answer to a request of the European Commission, the EFSA PLH Panel performed an analysis of the two scientific papers submitted by the Japanese authorities and of additional relevant scientific literature, aiming to reach a conclusion on the host status of *C. junos* to *X. citri* pv. *citri* and *X. citri* pv. *aurantifolii*. Taking into account the current lack of data on *C. junos* fruit as a pathway, the PLH Panel also checked that the conclusions of its previous opinion (EFSA PLH Panel, 2014) remain valid and up to date regarding fruit as a pathway and the ongoing update of Directive 2000/29/EC.

The Panel acknowledges the difficulty in providing compelling evidence for non-susceptibility of a particular plant species to infection with a pathogenic organism.

The Panel recognises that the available lines of evidence, although not fully demonstrative on their own, complement and reinforce each other. Therefore, in reaching its conclusions, the Panel systematically considered all information available, as well as the associated uncertainties and the possible synergies between the different lines of evidence.

From the two papers provided by the Japanese authorities and the 16 additional ones obtained from the literature review, the PLH Panel notes that:

- *C. junos* artificially inoculated with *X. citri* pv. *citri* exhibits typical canker-like lesions, although the extent of watersoaking, tissue hypertrophy and lesion increase in size was less than on susceptible cultivars;
- natural infections of *C. junos* were reported from Japan in production groves in the 1970s (although the precise locations in Japan have not been provided) and *X. citri* pv. *citri* strains had been isolated from those lesions;
- the analysed scientific papers suggest that *C. junos* exhibits partial resistance but not immunity to *X. citri* pv. *citri*;
- based on limited scientific papers, *C. junos* can also be considered a putative host of *X. citri* pv. *aurantifolii*.

The Panel also emphasises the limited literature available, and especially that:

- most scientific papers investigating the susceptibility of *C. junos* used single genetically uncharacterised bacterial strain for inoculations;
- the genetic structure of the population of *X. citri* pv. *citri* in the *C. junos* production zones is not precisely known. Up to now, *C. junos* has not been challenged with strains representative of the *X. citri* diversity;
- a single scientific paper investigating the susceptibility level of five Chinese *C. junos* accessions suggested cultivar-dependent partial resistance levels among these.

From this, the PLH Panel considers that the conclusions of its previous opinion remain valid and that convergent lines of evidence provide sufficient demonstration that *C. junos* is a host of *X. citri* pv. *citri* and *X. citri* pv. *aurantifolii*.

Therefore, there is no reason to consider the *C. junos* fruit differently from other citrus species; consequently, the assessment of the general citrus fruit pathway from the 2014 opinion still applies.

Documentation provided to EFSA

- 1) Request to provide a scientific opinion on the risk of *Citrus junos* fruits for the introduction of citrus canker. SANTE.GI/GVC/as (2016) 7209370. 15/12/2016. Submitted by European Commission, DG SANTE, Directorate-General for Health and Food Safety.
- 2) Koizumi M, 1979. Ultrastructural changes in susceptible and resistant plants of *Citrus* following artificial inoculation with *Xanthomonas citri* (Hasse) Dowson. Annals of the Phytopathological Society of Japan, 45, 635–644 (Appendix 1).
- 3) Koizumi M and Kuhara S, 1982. Evaluation of *Citrus* plants for resistance to bacterial canker disease in relation to the lesion extension. Bulletin of the Fruit Trees Research Station of Japan D 4, 73–92 (Appendix 2).
- 4) Tokushima Prefecture. Yuzu and citrus canker disease. Power Point presentation handed out during a meeting between European Commission, DG SANTE, Directorate-General for Health and Food Safety and Japan representatives in March 2016.

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Abbreviations

cfu	colony forming unit
dai	days after inoculation
PLH Panel	Plant Health Panel

Appendix A – EU regulation related to the import of citrus fruit from outside the Community

Xanthomonas campestris, all strains pathogenic to *Citrus*, are bacteria presently listed in Annex II, Part A of Directive 2000/29/EC as harmful organisms whose introduction into, and spread within, all Member States shall be banned if it is present on certain plants or plant products. They belong to Section I, harmful organisms not known to occur in the Community and relevant for the entire Community.

Fruit of *Citrus* are also considered in Section I, Part A of Annex IV of Directive 2000/29/EC (version in force), as plants, plant products and other objects originating outside the Community for which special requirements must be laid down by all Member States for the introduction and movement within all Member states.

- According to Point 16.1, fruits of *Citrus*, *Fortunella*, *Poncirus* and their hybrids, originating in third countries shall be free from peduncles and leaves, and the packaging shall bear an appropriate origin mark.
- According to Point 16.2, fruits of *Citrus*, *Fortunella*, *Poncirus* and their hybrids, originating in third countries, without prejudice to the provisions applicable to the fruits in Annex IV(A)(I) (16.1), (16.3), (16.4) and (16.5), official statement is that:
 - a) the fruits originate in a country recognised as being free from *X. campestris* (all strains pathogenic to *Citrus*), in accordance with the procedure referred to in Article 18(2)
 - or
 - b) the fruits originate in an area recognised as being free from *X. campestris* (all strains pathogenic to *Citrus*), in accordance with the procedure referred to in Article 18(2) and mentioned on the certificates referred to in Articles 7 or 8 of that Directive,
 - or
 - c) either,
 - in accordance with an official control and examination regime, no symptoms of *X. campestris* (all strains pathogenic to *Citrus*) have been observed in the field of production and in its immediate vicinity since the beginning of the last cycle of vegetation
 - and
 - none of the fruits harvested in the field of production has shown symptoms of *X. campestris* (all strains pathogenic to *Citrus*),
 - and
 - the fruits have been subjected to treatment such as sodium orthophenylphenate, mentioned on the certificates referred to in Articles 7 or 8 of Directive 2000/29/EC,
 - and
 - the fruits have been packed at premises or dispatching centres registered for this purpose,
 - or
 - any certification system, recognised as equivalent to the above provisions in accordance with the procedure referred to in Article 18(2) of Directive 2000/29/EC, has been complied with.

Fruit of *Citrus*, *Fortunella*, *Poncirus* and their hybrids, are then considered in Point 3, Section I, Part B of Annex V of Directive 2000/29/EC (version in force), as plant products originating in territories other than the Community, which are potential carriers of harmful organisms of relevance for the entire Community, and which must be subject to a plant health inspection before being moved within the Community, in the country of origin or the consignor country, before being permitted to enter the Community.

Apart from Directive 2000/29/EC, Commission Decision 2006/473/EC recognises certain third countries and certain areas of third countries as being free from *X. campestris* (all strains pathogenic to *Citrus*). Japan is listed neither in Article 1, nor in Article 2 of that decision, dealing with *X. citri* pv. *citri* or *X. citri* pv. *aurantifolii*, and cannot therefore be considered as partly or entirely free from those bacteria.

Appendix B – Search strategies

Date of the searches 7/3/2017

Web of Science Core Collection:

- Science Citation Index
- Conference Proceedings Citation Index- Science
- Emerging Sources Citation Index

Set	Query	Results
# 8	#7 OR #3 <i>Indexes=SCI-EXPANDED, CPCI-S, ESCI Timespan=1975-2017</i>	74
# 7	#6 AND #5 AND #4 <i>Indexes=SCI-EXPANDED, CPCI-S, ESCI Timespan=1975-2017</i>	73
# 6	TS=(resistan* OR toleran*) <i>Indexes=SCI-EXPANDED, CPCI-S, ESCI Timespan=1975-2017</i>	1,737,023
# 5	TS=(xanthomona* OR canker OR cankers) <i>Indexes=SCI-EXPANDED, CPCI-S, ESCI Timespan=1975-2017</i>	13,223
# 4	TS= ((citrus NEAR (genotype* OR "species" OR "varieties")) OR "citrus plants") <i>Indexes=SCI-EXPANDED, CPCI-S, ESCI Timespan=1975-2017</i>	3,185
# 3	#2 AND #1 <i>Indexes=SCI-EXPANDED, CPCI-S, ESCI Timespan=1975-2017</i>	4
# 2	TS=(Xanthomona* OR canker OR cankers) <i>Indexes=SCI-EXPANDED, CPCI-S, ESCI Timespan=1975-2017</i>	13,223
# 1	TS=("Citrus junos" OR "C junos" OR yuzu OR yuzus OR yuja OR yujas) <i>Indexes=SCI-EXPANDED, CPCI-S, ESCI Timespan=1975-2017</i>	207

BIOSIS Citation Index

Set	Query	Results
# 8	#7 OR #3 <i>Indexes=BCI Timespan=All years</i>	71
# 7	#6 AND #5 AND #4 <i>Indexes=BCI Timespan=All years</i>	65
# 6	TS=(resistan* OR toleran*) <i>Indexes=BCI Timespan=All years</i>	1,227,514
# 5	TS=(xanthomona* OR canker OR cankers) <i>Indexes=BCI Timespan=All years</i>	20,789
# 4	TS= ((citrus NEAR (genotype* OR "species" OR "varieties")) OR "citrus plants") <i>Indexes=BCI Timespan=All years</i>	4,277
# 3	#2 AND #1 <i>Indexes=BCI Timespan=All years</i>	9
# 2	TS=(Xanthomona* OR canker OR cankers) <i>Indexes=BCI Timespan=All years</i>	20,789
# 1	TS=("Citrus junos" OR "C junos" OR yuzu OR yuzus OR yuja OR yujas) <i>Indexes=BCI Timespan=All years</i>	281

CABI: CAB Abstracts

Set	Query	Result
# 8	#7 OR #3 <i>Indexes=CAB Abstracts Timespan=All years</i>	152
# 7	#6 AND #5 AND #4 <i>Indexes=CAB Abstracts Timespan=All years</i>	147
# 6	TS=(resistan* OR toleran*) <i>Indexes=CAB Abstracts Timespan=All years</i>	979,845

Set	Query	Result
# 5	TS=(xanthomona* OR canker OR cankers) Indexes=CAB Abstracts Timespan=All years	37,027
# 4	TS= ((citrus NEAR (genotype* OR "species" OR "varieties")) OR "citrus plants") Indexes=CAB Abstracts Timespan=All years	8,902
# 3	#2 AND #1 Indexes=CAB Abstracts Timespan=All years	12
# 2	TS=(Xanthomona* OR canker OR cankers) Indexes=CAB Abstracts Timespan=All years	37,027
# 1	TS=("Citrus junos" OR "C junos" OR yuzu OR yuzus OR yuja OR yujas) Indexes=CAB Abstracts Timespan=All years	488

Chinese Science Citation Database

Set	Query	Results
# 8	#7 OR #3 Indexes=CSCD Timespan=All years Search language=Auto	8
# 7	#6 AND #5 AND #4 Indexes=CSCD Timespan=All years Search language=Auto	8
# 6	TS=(resistan* OR toleran*) Indexes=CSCD Timespan=All years Search language=Auto	171,787
# 5	TS=(xanthomona* OR canker OR cankers) Indexes=CSCD Timespan=All years Search language=Auto	1,212
# 4	TS= ((citrus NEAR (genotype* OR "species" OR "varieties")) OR "citrus plants") Indexes=CSCD Timespan=All years Search language=Auto	343
# 3	#2 AND #1 Indexes=CSCD Timespan=All years Search language=Auto	1
# 2	TS=(Xanthomona* OR canker OR cankers) Indexes=CSCD Timespan=All years Search language=Auto	1,212
# 1	TS=("Citrus junos" OR "C junos" OR yuzu OR yuzus OR yuja OR yujas) Indexes=CSCD Timespan=All years Search language=Auto	21

KCI-Korean Journal Database

Set	Query	Results
# 7	#6 AND #5 AND #4 Indexes=KJD Timespan=All years	0
# 6	TS=(resistan* OR toleran*) Indexes=KJD Timespan=All years	33,260
# 5	TS=(xanthomona* OR canker OR cankers) Indexes=KJD Timespan=All years	273
# 4	TS= ((citrus NEAR (genotype* OR "species" OR "varieties")) OR "citrus plants") Indexes=KJD Timespan=All years	53
# 3	#2 AND #1 Indexes=KJD Timespan=All years	0

Set	Query	Results
# 2	TS=(Xanthomona* OR canker OR cankers) Indexes=KJD Timespan=All years	273
# 1	TS=("Citrus junos" OR "C junos" OR yuzu OR yuzus OR yuja OR yujas) Indexes=KJD Timespan=All years	94

SciELO Citation Index

Set	Query	Results
# 5	#4 AND #2 AND #2 Indexes=SCIELO Timespan=All years	4
# 4	TS=(resistan* OR toleran*) Indexes=SCIELO Timespan=All years	19,663
# 3	TS=((citrus NEAR (genotype* OR "species" OR "varieties")) OR "citrus plants") Indexes=SCIELO Timespan=All years	252
# 2	TS=(Xanthomona* OR canker OR cankers) Indexes=SCIELO Timespan=All years	324
# 1	TS=("Citrus junos" OR "C junos" OR yuzu OR yuzus OR yuja OR yujas) Indexes=SCIELO Timespan=All years	0

Crop Protection Compendium

Query	Results
((resistan* OR toleran*) AND (xanthomona OR xanthomonas OR canker OR cankers) AND ((citrus AND (genotype* OR species)) OR "citrus plants")) OR ((Xanthomona OR xanthomonas OR canker OR cankers) AND ("Citrus junos" OR "C junos" OR yuzu OR yuzus OR yuja OR yujas))	99
(resistan* OR toleran*) AND (xanthomona OR xanthomonas OR canker OR cankers) AND ((citrus AND (genotype* OR species OR varieties)) OR "citrus plants")	96
resistan* OR toleran*	104,250
xanthomona OR xanthomonas OR canker OR cankers	11,823
(citrus AND (genotype* OR species OR varieties)) OR "citrus plants"	6,617
(Xanthomona OR xanthomonas OR canker OR cankers) AND ("Citrus junos" OR "C junos" OR yuzu OR yuzus OR yuja OR yujas)	5
Xanthomona OR xanthomonas OR canker OR cankers	11,823
"Citrus junos" OR "C junos" OR yuzu OR yuzus OR yuja OR yujas	34
Xanthomona OR xanthomonas OR canker OR cankers	6,483

AGRICOLA

Search string 1

Query	Results
(Xanthomona? OR canker OR cankers) AND ("Citrus junos" OR "C junos" OR yuzu OR yuzus OR yuja OR yujas)	2

Search string 2

Query	Results
(resistan? OR toleran?) AND (xanthomona OR xanthomonas OR canker OR cankers) AND ((citrus AND (genotype? OR species OR varieties)) OR "citrus plants")	48

Total results in AGRICOLA after deduplication 48.

Google Scholar (via Publish or Perish v. 4)

Queries	Results
"citrus junos" canker	45
"c junos" canker	51
Yuzu canker	102
Yuja canker	0
"citrus junos" xanthomona	39
"c junos" xanthomona	49
yuzu xanthomona	79
Yuja santhomona	0
Yuja canker	0

Total results in Google Scholar after deduplication: 173.