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RESEARCH PAPERS

Improved method for assessing incidence of *Citrus tristeza virus* in large scale monitoring

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Summary. *Citrus tristeza virus* (CTV) induces typical quick decline when citrus trees are grafted onto the sour orange. The virus is efficiently vectored by different aphid species. Large scale CTV monitoring and eradication requires a precise sampling and testing method for assessing virus incidence. The sampling procedure described by Hughes and Gottwald (1998) was adapted in the official monitoring for the mandatory control of CTV in the Apulia Region of Southern Italy. However, instead of the hierarchical sampling (HS) of grouped samples tested by Enzyme Linked Immunosorbent Assay (ELISA), a systematic sampling of individual trees tested by Direct Tissue Blot Immunoassay (DTBIA) was applied by the Mediterranean Agronomic Institute of Bari (MIAB-S). In this study, the actual CTV incidence assessed in the eight citrus commercial groves by DTBIA was used for evaluating the efficacy and precision of MAIB-S and HS methods. The simulation of different sampling scenarios using advanced spatial (automatic tree extraction) and statistical (Standard Error, Root Mean Square Error) analyses showed that in all groves the infection rates were always underestimated using the HS method, while most infection rates were overestimated and closer to the actual CTV incidence when the MAIB-S method was performed. For the MAIB-S method, no effect of high temperature in the detection of virus incidence occurred during July monitoring. Unlike MAIB-S, noticeable reduction in the precision of CTV incidence estimation was observed with HS.

Key words: sampling, DTBIA, DAS-ELISA, automatic tree extraction, standard error, root mean square error.

Introduction

Citrus tristeza virus (CTV), the most destructive virus of citrus, is present in almost all citrus growing countries worldwide, reaching different infection levels. This virus induces typical quick decline when the trees are grafted onto the susceptible sour orange rootstocks. In the Euro-Mediterranean area, the virus is mainly transmitted by *Aphis gossypii*, while *Toxoptera citricidus*, the most efficient virus vector, has been reported only from Portugal and Spain (Iharco *et al*., 2005; Moreno *et al*., 2008). Because most infected trees may remain symptomless for years, large scale virus monitoring and eradication is fundamental, but requires a precise sampling and testing method for assessing CTV incidence.

The sampling procedure described by Hughes and Gottwald (1998) was used in the official monitoring for the mandatory control of CTV in the Apulia Region, Southern Italy. However, instead of the hierarchical sampling (HS) of grouped samples tested by Enzyme Linked Immunosorbent Assay (ELISA), a systematic sampling of individual trees tested by Direct Tissue Blot Immunoassay (DTBIA) was applied by the Mediterranean Agronomic Institute of Bari (MAIB-S). DTBIA is as reliable as the ELISA in CTV detection but it is cheaper, more rapid and user-friendly (Garnsey *et al*., 1993; Cambra *et al*., 2000).

An efficient sampling and testing procedure is fundamental for the estimation of the presence, inci-

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dence and distribution patterns of CTV. Automated image interpretation methods, utilizing the multispectral information from very high spatial resolution aerial and satellite images, can reduce the effort required to extract spatial information in the areas to be monitored for the presence of the pathogen (Caprioli and Tarantino, 2006; Tarantino and Figorito, 2012).

The Apulian monitoring and eradication programme relies totally upon CTV incidence in each citrus grove for the elimination of single infected trees or of whole infected groves. The MAIB-S and HS methods were investigated therefore assessed for efficacy and precision in the monitoring of CTV on a large scale.

Materials and methods

Area of study

A survey was conducted in June 2012 in the CTV outbreak area of Apulia region. Eight commercial citrus groves were selected and all trees were sampled and tested for CTV by DTBIA for assessing the actual virus incidence (Figure 1).

A second survey was carried out in July in grove A in Taranto Province (Figure 1), when the temperatures were non-optimal for CTV titre in plants, in order to evaluate the effect of high ambient temperatures on the sensitivity of ELISA versus DTBIA.

Sample collection

In order to assess efficacy and precision of the MAIB-S and HS methods on a statistical basis, the actual incidence of virus infection was detected in the eight citrus groves by testing 3520 trees using DTBIA. Afterwards, the two methods were simulated on the map of each grove, following the scheme of quadrants (four trees/quadrant) between two rows, as described by Hughes and Gottwald (1998). In both methods, 25% of the trees were sampled beginning from the same starting point which was chosen randomly (Figure 2). In each quadrant, grouped trees (four trees/sample) were prepared when HS was applied (Figure 2A) while individual samples were considered when the MAIB-S method was applied (Figure 2B). Grouped samples were processed by DAS-ELISA.

Laboratory assays

For CTV detection by DTBIA, each individual sample consisted of eight leaves collected from the four cardinal points of the tree. Two prints from the fresh cross section of each leaf petiole were made onto the nitrocellulose membrane, including the negative and positive controls. The printed membrane was processed following the procedure of Garnsey *et al.* (1993). After blocking in 1% bovine serum albumin (BSA) solution, the printed membrane was incubated for 2 h with a solution of CTV monoclonal antibodies conjugated with alkaline phosphatase (Agritest srl) and developed using the substrate BCIP-NBT (Sigma fast tablets). The processed membrane was observed using a microscope (×10 to ×20 magnification) for the detection of the purple-violet precipitation inside the sieve tube elements of each test sample and experimental controls.

As for ELISA, 238 grouped samples were tested by DAS-ELISA (Bar-Joseph *et al*., 1989) using a specific polyclonal antiserum for CTV, according to the manufacturer's instructions (Agritest srl). Each grouped sample/quadrant was prepared by collecting a piece of leaf midribs from the four individual tree samples analysed by DTBIA. The wells of polystyrene plates were coated with IgG and incubated. After incubation with crude extracts, the IgG conjugate was added. Following the incubation, the p-nitrophenylphosphate was added, and plates were left at room temperature (for 1–2 h) for photometrical measures at 405 nm. Results were considered negative when the absorbance value was less than three times the mean absorbance value of the healthy controls, and positive when the absorbance value was equal to, or greater than, three times the mean absorbance value of the healthy controls.

Processing of remote sensing data

During the survey, a satellite image WorldView-2 (PAN and multispectral) of the study area was acquired. WorldView-2 satellite sensor provides a cost-effective combination of sufficient spectral and spatial resolutions, with nine spectral bands (1.8 m) and a panchromatic band with very high spatial resolution of 0.5 m (Figorito *et al*., 2012; Dalezios *et al*., 2013).

The image was processed in ArcGIS (ESRI, 2008) using the technique of *spatial feature extraction* for the automatic extraction and spatial identification of all citrus trees from the eight citrus groves (Santoro *et*

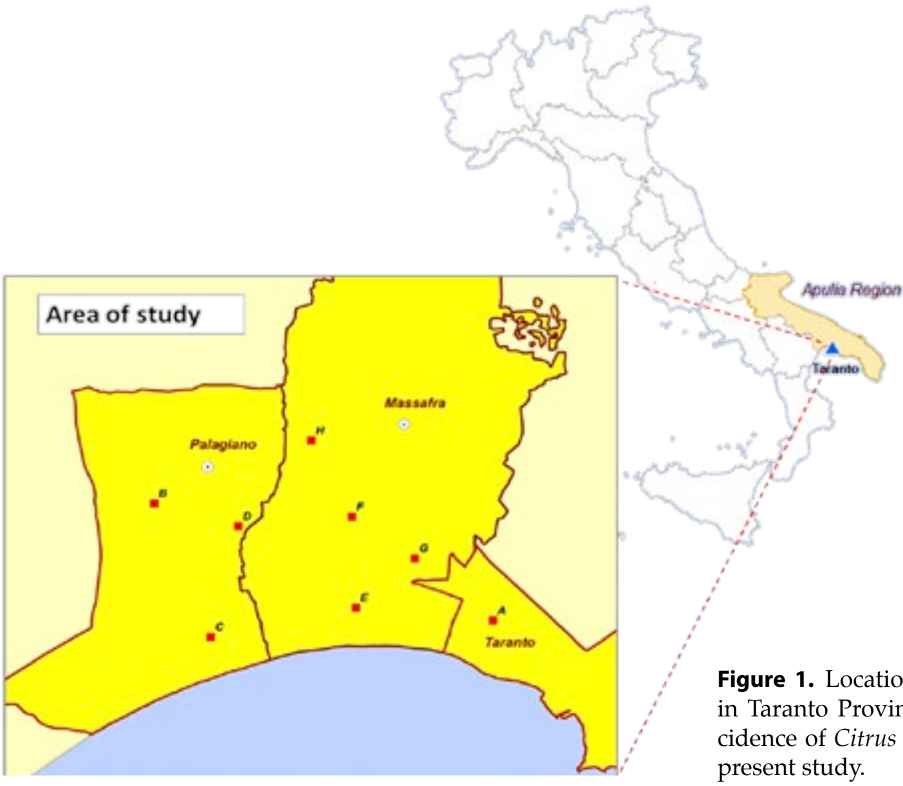

Figure 1. Location of the eight citrus groves (A-H) in Taranto Province of Southern Italy, where the incidence of *Citrus tristeza virus* was monitored for the present study.

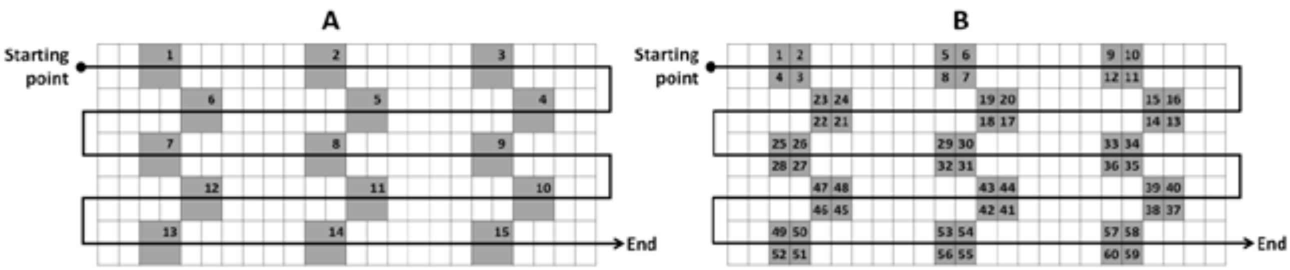

Figure 2. Representation of the sampling scheme by Hughes and Gottwald (1998): grouped samples of four trees each (grey squares) in HS method (A), and samples of individual trees (in grey squares) in the MAIB-S method (B). In both methods un-sampled trees are in white squares and the black arrows represent the sampling path through the grove from the same starting point.

al., 2013). Monitoring data attributes (citrus species, sampling scheme, sampled trees, infected trees and missing trees) were inserted into each geo-localized tree as spatial attributes.

Statistical and spatial analyses

The process flow described in Figure 3 generated a dataset for each citrus grove studied, and these datasets were used in statistical and spatial analyses (Figure 4).

The grove basic datasets were used as inputs in order to statistically compare the two different procedures. Statistica 7 software was used to calculate the mean estimates of CTV incidence in each grove by HS and MAIB-S methods. Standard Error (SE; Equation 1) was scored for each method so as to compare the two procedures.

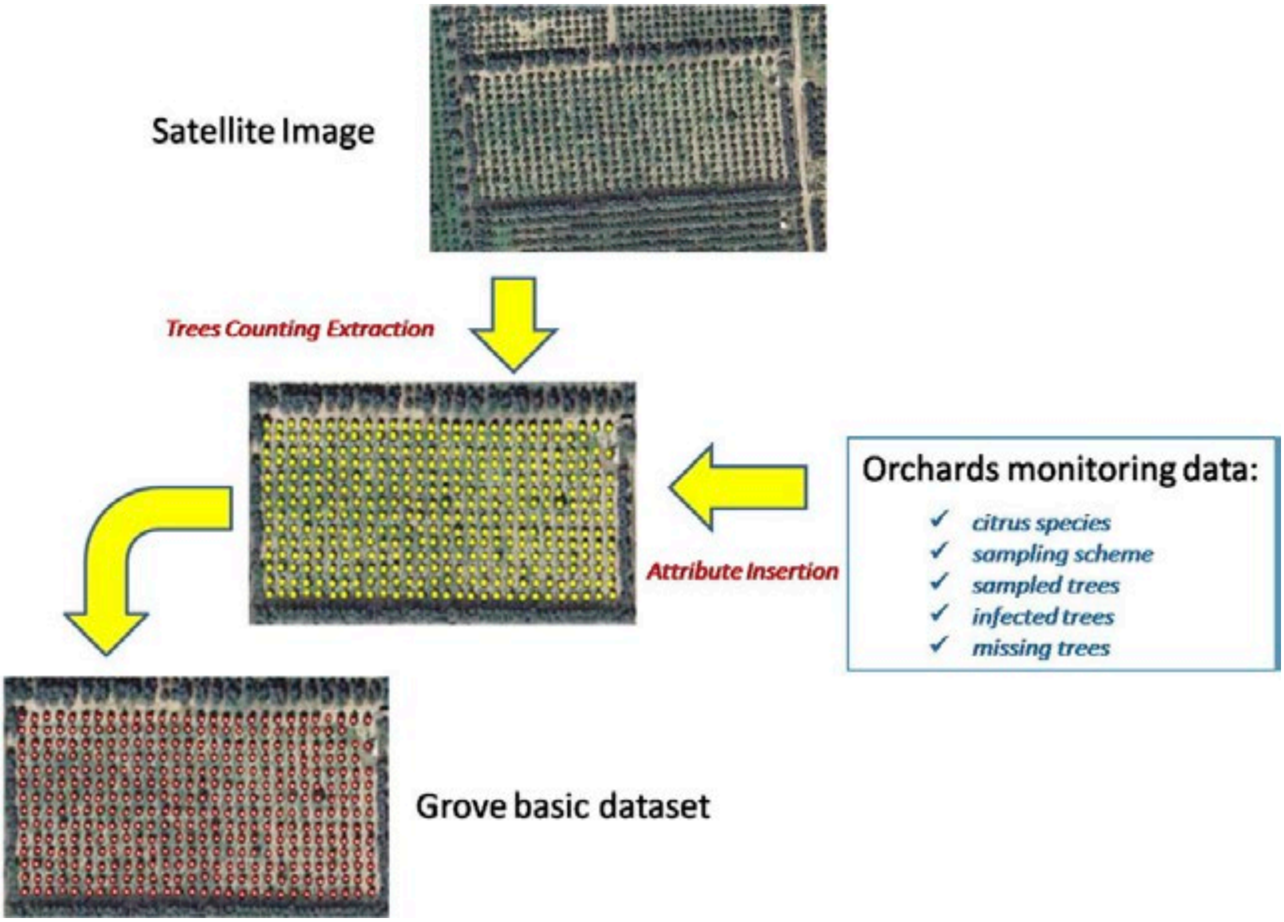

Figure 3. Scheme of data processing from the satellite image WorldView-2 of the study area to the grove basic dataset.

$$\mathbf{SE} = \sqrt{\frac{p(1-p)}{N_{CTV-infected}} \times \frac{(N_{Sample} - N_{CTV-infected})}{N_{Sample}}} \quad \text{(Equation: 1)}$$

where
N_{sample} = Number of sampled trees
$N_{CTV\text{-}infected}$ = Number of CTV-infected trees, and
p = Estimated CTV incidence.

Considering that the estimation of CTV incidence by HS and MAIB-S was based on 25% of the trees, a study was also carried out using the computer simulation, for each of the two methods. The simulation of CTV incidence was made within the range 0 to 100%, and the positions of the CTV-positive trees were allocated at random in each simulation. The precision of the compared methods was evaluated by Root Mean Square Error (RMSE) using the following equation:

$$\mathbf{RMSE} = \sqrt{\frac{\sum_{i=1}^{n}(P_i - O_i)^2}{N}} \quad \text{(Equation: 2)}$$

where

N = Number of scenarios of infection (300)
P_i = Simulated virus incidence (from 0 to 1), and
O_i = Observed virus incidence (in 300 scenarios of infections)

A simulation procedure developed in Matlab allowed the precision of both methods to be estimated, providing the error RMSE obtained between the actual virus incidence and the simulated virus incidence (from HS and MAIB-S), in all possible scenarios of virus spread in the monitored groves.

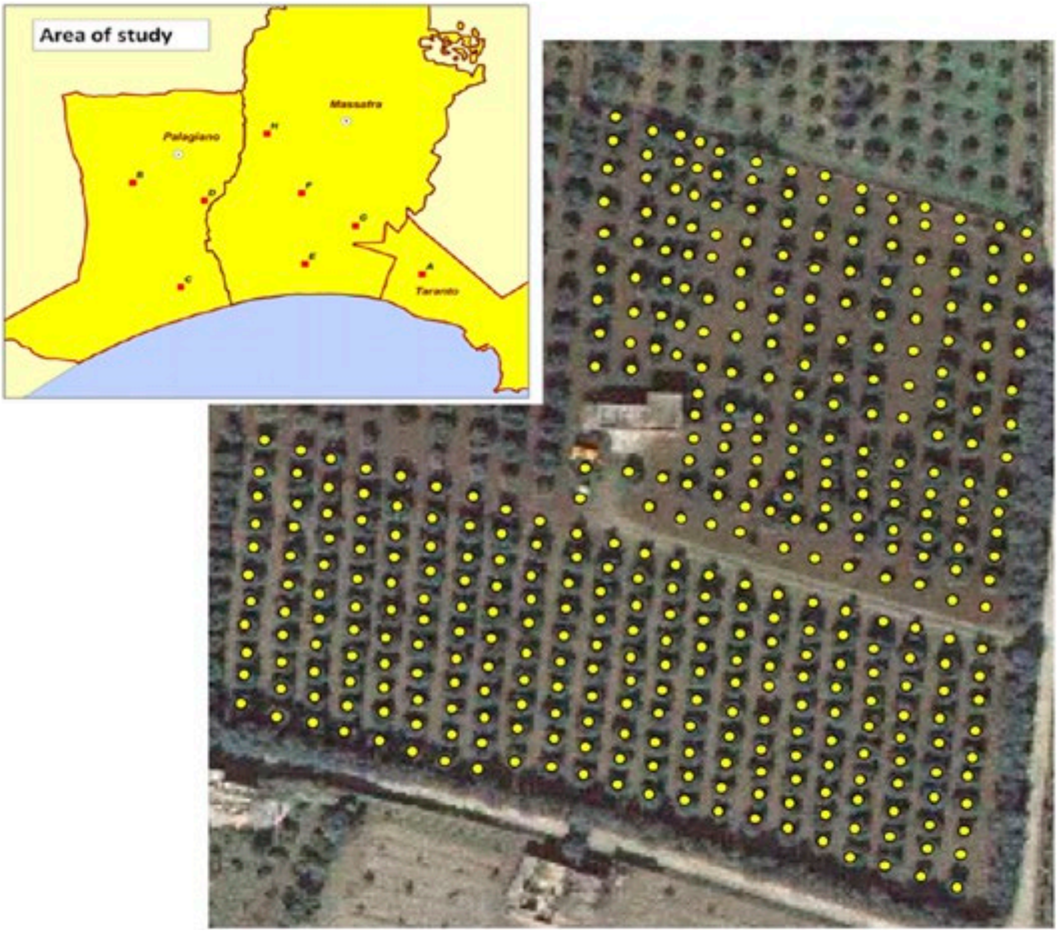

Figure 4. Tree extraction *dataset* which shows the location and the number of the citrus trees in grove A in Taranto Province.

Results and discussion

The actual CTV infection rates, as assessed by testing of 100% of the trees using DTBIA, ranged from 0.46% to 16.21% in the different orchards studied (Table 1).

Based on the spatial spread of the actual infection assessed in each grove (Figure 5), the standard error was calculated in all monitored groves simulating the two scenarios obtained by HS and MAIB-S methods on 25% of the trees, following the same monitoring sampling scheme (Table 2).

In Table 2, the statistical data obtained by Statistica 7 from the eight groves is reported. The CTV incidence estimated by MAIB-S method with respect to the estimation obtained by HS was the closest to the actual CTV incidence in most of the groves. However, the actual virus incidence was always underestimated by HS, while the MAIB-S method underestimated CTV incidence in only three groves (D, G, H). As for SE, this value was always less from the MAIB-S method, due to the individual sampling system adopted in this method. This implies a greater number of samples compared with the grouped sampling of HS.

Concerning the testing period in grove A, approximately similar results to the actual virus incidence (16.21%) were provided in June by MAIB-S (17.39%) and HS (14.68%) methods. In July the actual CTV incidence remained the same as that provided by MAIB-S method (17.31%). Conversely, using the HS method gave reduced estimated infection rate in July of only 6.02%. These data clearly show that, under non-optimal higher temperature conditions,

Table 1. The actual incidence of *Citrus tristeza virus* (CTV) in eight groves (A-H) monitored in this study. CTV incidence was studied by sampling from all trees included in the study and individual testing by using Direct Tissue Blot Immunoassay (DTBIA).

Location	Grove Code	Trees No.	Citrus species	Actual CTV incidence (%)
Taranto	A	401	Common clementine	16.21
Massafra	B	169	Valencia orange	2.37
	C	308	Common clementine	4.87
	D	330		7.88
Palagianello	E	1307	Navelina orange	0.46
Palagiano	F	387	Common clementine	11.11
	G	381		2.10
	H	237		5.91

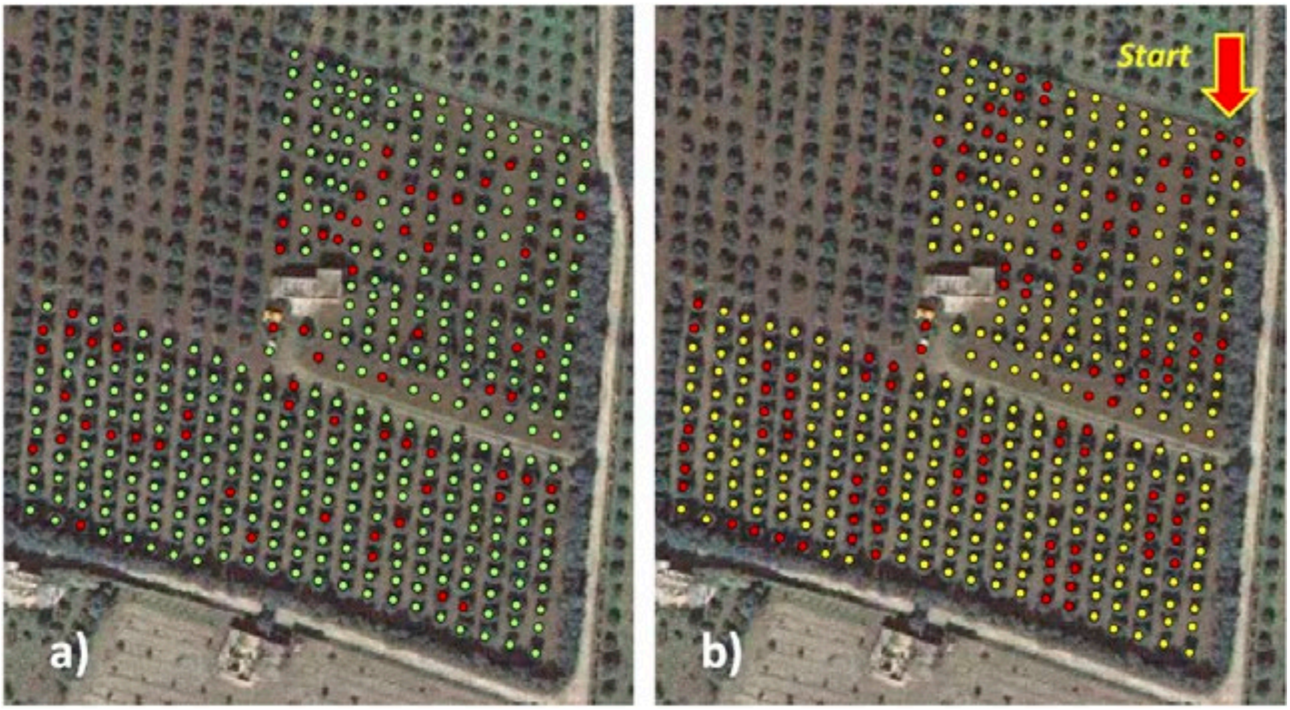

Figure 5. Spatial spread of the infection (a) and monitoring sampling scheme (b) in grove A. The red points show (a) the *Citrus tristeza virus* infected trees obtained by testing all of the trees by Direct Tissue Blot Immunoassay (DTBIA); while the green points are DTBIA-negative trees; (b) the 25% of sampled trees following the sampling scheme by quadrants used for the Mediterranean Agronomic Institute of Bari sampling method (MAIB-S) and the Hierarchical Sampling method (HS). The red arrow indicates both the starting point and the orientation of the sampling scheme.

Table 2. The actual incidence of *Citrus tristeza virus* (CTV) in eight groves (A-H) and the results obtained from the Mediterranean Agronomic Institute of Bari sampling method (MAIB-S) and the Hierarchical Sampling method (HS). The statistical data provided by Statistica 7 are shown.

Grove	Method	Samples (No).	CTV incidence (%)	SE
A	MAIB-S	103	17.39	3.14
	HS	26	14.68	14.55
	Total sampling	401	16.21	
B	MAIB-S	39	2.56	3.87
	HS	13	1.98	12.65
	Total sampling	169	2.37	
C	MAIB-S	77	6.49	3.45
	HS	25	3.14	13.42
	Total sampling	308	4.87	
D	MAIB-S	81	5.94	2.09
	HS	22	4.89	10.14
	Total sampling	330	7.88	
E	MAIB-S	329	0.61	0.37
	HS	85	0.30	2.7
	Total sampling	1307	0.46	
F	MAIB-S	98	12.24	1.48
	HS	23	6.19	8.38
	Total sampling	387	11.11	
G	MAIB-S	97	1.03	0.88
	HS	28	0.91	4.68
	Total sampling	381	2.10	
H	MAIB-S	61	3.64	1.40
	HS	16	1.60	6.15
	Total sampling	237	5.91	

results obtained by DTBIA of individual trees using the MAIB-S method were more reliable than testing by ELISA of pooled samples by the HS method.

Comparison of mean actual virus incidence in the eight groves with mean estimated virus incidence by two methods compared in this study showed that the MAIB-S method was more efficient in assessing CTV incidence than the HS method (Figure 6). Moreover, the mean of Standard Error (SE) from the HS method was significantly greater than from the MAIB-S method. This also implies low precision of the HS method for estimating CTV incidence.

The simulation procedure developed in Matlab showed that when the simulated CTV incidence was 0.1%, the two methods gave similar RMSE values (Figure 7).

As the simulated incidence increased above the previous value, the HS method became less precise compared to the MAIB-S method. At CTV incidences

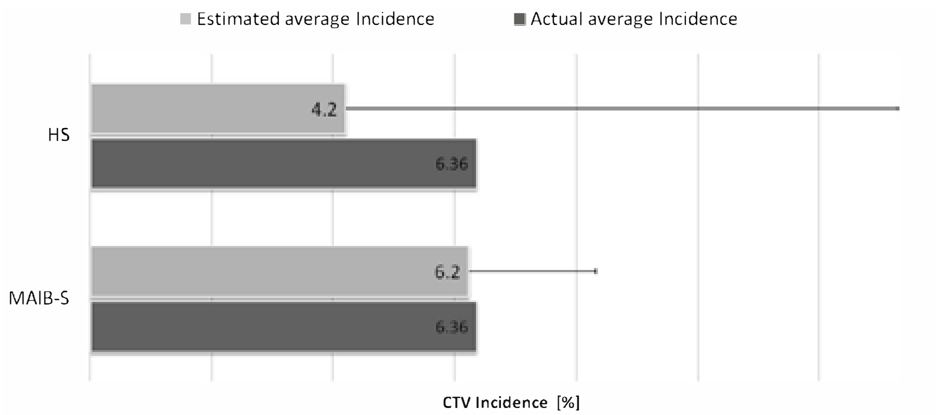

Figure 6. Mean *Citrus tristeza virus* (CTV) incidence in all eight groves assessed in this study, estimated by the Hierarchical Sampling method (HS) and the Mediterranean Agronomic Institute of Bari sampling method (MAIB-S), each compared to the actual incidence of CTV as estimated by testing of all trees in study by Direct Tissue Blot Immunoassay (DTBIA) method. The standard errors introduced by each method are shown.

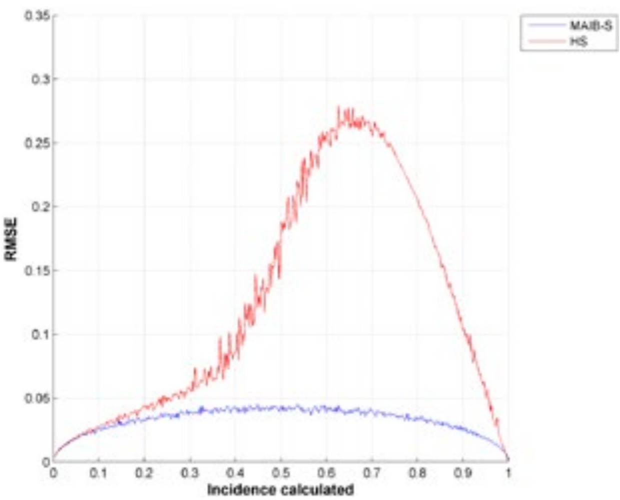

Figure 7. The Root Mean Square Error chart obtained from the average differences between the *Citrus tristeza virus* (CTV) calculated incidence (x100) and the CTV observed incidence, evaluated by applying each sampling method.

of 0.3 the HS method introduced error of ≈ 0.05 (5%) or more, whereas the error for the MAIB-S method decreased slightly to ≈ 0.04 (4%) and did not exceed this value regardless of the increase in CTV incidence. On the other hand, the error incurred with the HS method tended to increase greatly at greater levels of virus incidence.

Following the sampling same scheme indicated by Hughes and Gottwald (1998), the MAIB-S method, which was adapted in the Apulian CTV monitoring programme, performed better than the HS method for precise estimation of CTV incidence in citrus orchards. Even if the estimation by MAIB-S in the groves was either more or less than the actual infection incidence, the mean estimated value was very close to the actual infection incidence. This was confirmed by the simulation study performed through the Matlab procedure, which showed that RMSE produced from the HS method was greater than that from the MAIB-S method. The HS was therefore less precise than MAIB-S method for determining infection rates greater than about 0.1. This could be explained by the virus content in the grouped samples (HS), which was less than that in the individual samples (MAIB-S), the uneven virus distribution in the trees and/or the high sensitivity of the DTBIA compared to ELISA. Accordingly, no effect of high ambient temperatures in virus detection was noticed during July testing using DTBIA. Considering that monitoring methods must be practical in terms of time, labour and costs, the MAIB-S method could be easily adopted in very large citrus-growing areas, reducing the errors in assessing CTV incidence for the elimination of infected trees.

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