Nuclear magnetic resonance characterization of metabolite disorder in orange trees caused by citrus sudden death disease

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SUMMARY

Citrus sudden death (CSD) is a new disease of sweet orange and mandarin trees grafted on Rangpur lime and *Citrus volkameriana* rootstocks. It was first seen in Brazil in 1999, and has since been detected in more than four million trees. The CSD causal agent is unknown and the current hypothesis involves a virus similar to *Citrus tristeza virus* or a new virus named *Citrus sudden death-associated virus*. CSD symptoms include generalized foliar discoloration, defoliation and root death, and, in most cases, it can cause tree death. One of the unique characteristics of CSD disease is the presence of a yellow stain in the rootstock bark near the bud union. This region also undergoes profound anatomical changes. In this study, we analyse the metabolic disorder caused by CSD in the bark of sweet orange grafted on Rangpur lime by nuclear magnetic resonance (NMR) spectroscopy and imaging. The imaging results show the presence of a large amount of non-functional phloem in the rootstock bark of affected plants. The spectroscopic analysis shows a high content of triacylglyceride and sucrose, which may be related to phloem blockage close to the bud union. We also propose that, without knowing the causal CSD agent, the determination of oil content in rootstock bark by low-resolution NMR can be used as a complementary method for CSD diagnosis, screening about 300 samples per hour.

INTRODUCTION

Citrus sudden death (CSD) is a new graft-transmissible disease of sweet orange [*Citrus sinensis* (L.) Osbeck] and mandarin trees grafted on Rangpur lime (*Citrus limonia* Osbeck) and *Citrus volkameriana* rootstocks. It was first seen in Brazil in 1999, and has been detected in more than four million trees. It is a serious threat to the Brazilian citrus industry, as more than 85% of 200

million sweet orange trees are grafted on Rangpur lime rootstock (Bassanezi *et al.*, 2003; Derrick *et al.*, 2003). The causal agent of CSD is unknown. Several similarities to tristeza (quick decline) strongly suggest that a virus similar to *Citrus tristeza virus* (CTV) causes the disease (Bassanezi *et al.*, 2003; Román *et al.*, 2004). Several attempts have been made to characterize CTV isolates from CSD trees (Gomes *et al.*, 2008; Rivas-Valencia *et al.*, 2008); however, no differences were found between CSD-free and CSD-affected tree populations. Recently, Maccheroni *et al.* (2005) have suggested that CSD is caused by a new virus from the *Marafivirus* genus (family *Tymoviridae*), named *Citrus sudden death-associated virus* (CSDaV), or, alternatively, in a synergistic fashion with CTV.

The initial CSD symptoms include generalized foliar discoloration, partial defoliation, the presence of fewer than the normal number of new shoots and the absence of internal shoots. These symptoms intensify as the disease develops and culminate with tree death. The bark near the bud union of affected trees undergoes profound anatomical changes, such as size reduction of phloem cells, collapse and necrosis of sieve tubes, overproduction and degradation of phloem, accumulation of non-functional phloem (NFP) and invasion of the cortex by old NFP. The disease also causes root death, starting from the root tips and progressing to the main scaffold roots. Plant death normally occurs when the plant needs to absorb large amounts of water for the development of new shoots and to mature the fruits. One of the unique characteristics of CSD disease that has been used for diagnosis is the development of a yellow stain on the bark of Rangpur lime rootstock (Bassanezi *et al.* 2003; Román *et al.*, 2004).

To understand the major metabolic disorder caused by CSD in the bark of sweet orange grafted on Rangpur lime, we analysed the metabolites directly in the bark (*in situ*) and after extraction with solvents (*in vitro*) by nuclear magnetic resonance (NMR) spectroscopy. The results show major physiological disorder in the triacylglyceride (TAG) content, which can be detected rapidly by low-resolution NMR techniques. Therefore, NMR can be used as an alternative CSD diagnostic method.

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RESULTS AND DISCUSSION

The bark of Valencia sweet orange (scion) grafted on Rangpur lime (rootstock) with level 1 (CSD1) and level 2 (CSD2) CSD disease symptoms, as well as bark without symptoms (WSY), was analysed by magnetic resonance imaging (MRI) and NMR spectroscopy. These techniques were used to analyse the bark near the bud union of CSD-affected trees.

Figure 1 shows typical magnetic resonance images for CSD2 and WSY rootstock bark. The analyses were performed in six samples, and show that the rootstock bark of CSD2 is thicker (2.5 ± 0.5 mm) than that of WSY (1.5 ± 0.4 mm); they have similar functional phloem thickness (0.6 ± 0.1 mm), based on the assumption that it is indicated by the brighter areas in the images. This also indicates that NFP, the faded area in the images, is thicker in CSD2 rootstock bark (2 ± 0.3 mm) than in WSY bark (0.9 ± 0.2 mm). The presence of more NFP in the bark close to the bud union is one of the symptoms of CSD, as revealed by anatomical analysis (Román *et al.*, 2004). The detection of this disorder (thicker NFP) by MRI is rapid (several minutes), and can be performed with almost no sample preparation, but this method is too costly to be used as a large-scale screening technique.

Quantitative analysis

Figure 2 shows the percentage of hexane and alcoholic extracts obtained from WSY, CSD1 and CSD2 bark, close to the bud union. Figure 2A shows the amount of hexane extract (apolar lipids) obtained from rootstock and scion bark. The amount of hexane extract in rootstock bark is higher in CSD2 than in CSD1 or WSY plants. The amount of lipid in scion bark is lower than that in

Fig. 1 Magnetic resonance images of Rangpur lime rootstock bark without symptoms (WSY, top) and with level 2 citrus sudden death disease symptoms (CSD2, bottom). The images are 10 mm (height) by 28.5 mm (length).

Fig. 2 Percentage of hexane (A) and hydroalcoholic (B) extracts obtained from Valencia sweet orange grafted on Rangpur lime without symptoms (WSY), with level 1 citrus sudden death (CSD) disease symptoms (CSD1) and with level 2 CSD disease symptoms (CSD2). The rootstock (r) extracts are on the left and the scion (s) extracts on the right.

rootstock, and follows the same trend: CSD2 > CSD1 > WSY. These results clearly demonstrate the correlation between the lipid extract and CSD symptoms in both rootstock and scion bark.

The amount of alcoholic extract (Fig. 2B) is also higher in CSD2 rootstock bark than in CSD1 or WSY bark. The amount of alcoholic extract in scion bark is higher in CSD2 than in CSD1 or WSY plants; however, CSD1 and WSY extracts are similar. These results demonstrate the greater correlation of the amount of hexane extract (apolar lipids) than alcoholic extract with CSD symptoms. Hexane analysis is quite simple, but has not been used extensively in CSD screening because it is quite slow (a few samples per hour), even when the fastest extraction methodology is employed. To speed up the measurement of the lipid content in bark, we used low-resolution NMR techniques (Azeredo *et al.*, 2003).

In situ low-resolution NMR quantitative analysis

Low-resolution NMR has been used as a rapid method to analyse the oil content in seeds (Azeredo *et al.*, 2003), and was employed to measure the lipid content directly in dry bark. Low-resolution NMR analyses of lyophilized bark were performed by measuring the intensity of the free induction (FID) signal at 70 μ s after the pulse. In dry samples, only TAG and other lipid components with a transverse relaxation time longer than a few milliseconds can be measured by low-resolution NMR (Azeredo *et al.*, 2003). Proteins, carbohydrates and other solid materials, and bound water, have very short transverse relaxation times of about 10 μ s (Azeredo *et al.*, 2003), and do not contribute to the FID signal at 70 μ s.

Figure 3 shows a higher intensity FID signal in rootstock than in scion bark, with the order CSD2 > CSD1 > WSY in rootstock bark and CSD2 > CSD1 ~ WSY in scion bark. Figure 3 also shows a high correlation ($r = 0.89$) of the FID signal intensities of dry bark with the respective amounts of hexane extract. This result shows that low-resolution NMR can be used to measure the content of apolar lipids (oil) in rootstock bark. The method can easily discriminate between CSD2 and WSY citrus plants. However, the initial CSD1 stage is not well discriminated from other levels of the disease.

As the low-resolution NMR analysis can be automated (Colnago *et al.*, 2007; Prestes *et al.*, 2007), with more than 300 measurements per hour, it can be routinely used as a complementary tool to screen for CSD2 plants.

In vitro NMR qualitative analysis

The compositions of the raw extracts of Fig. 2 were analysed by high-resolution NMR spectroscopy. The ^{13}C NMR spectrum of the hexane extract of a CSD2 rootstock bark is shown in Fig. 4. The

Fig. 3 Correlation between the oil content in Rangpur lime rootstock bark (filled squares) and Valencia scion bark (filled circles) measured by low-resolution ^1H nuclear magnetic resonance (NMR) and hexane extracts.

Fig. 4 (A) ^{13}C nuclear magnetic resonance (NMR) spectrum of the hexane extract of CSD2 (level 2 citrus sudden death disease symptoms) rootstock bark in CDCl_3 . (B) Expansion of the spectrum in (A) in the region of unsaturated carbons from 131.0 to 126.0 p.p.m.

major peaks were assigned to unsaturated fatty acids of TAGs. The spectrum (Fig. 4A) shows carboxyl signals at 173.0 p.p.m., unsaturated carbons from 131.0 to 126.0 p.p.m., glycerol signals at 69.0 and 61.0 p.p.m., methylene carbons from 34.0 to 20.0 p.p.m. and a methyl carbon at 14.0 p.p.m. (Seidl and Colnago, 1987). The ^{13}C NMR spectrum of the hexane extract did not indicate the presence of free fatty acids in large amounts, as it did not show the characteristic free fatty acid carboxylic signal at about 180 p.p.m. (Forato *et al.* 2000, 2004).

The expansion of the spectrum (Fig. 4B) from 131.0 to 126.0 p.p.m. shows 12 peaks assigned to unsaturated carbons of oleic (129.9 and 129.7 p.p.m.), linoleic (130.2, 130.0, 128.0 and 127.9 p.p.m.) and linolenic (131.9, 129.9, 128.3, 128.2, 127.7 and 127.0 p.p.m.) acids (Forato *et al.*, 2000; Seidl and Colnago, 1987). The peak intensities show that linoleic and linolenic acids are the major unsaturated fatty acids in the sample analysed. The

^{13}C spectrum (Fig. 4A) also shows the presence of several small peaks related to other apolar compounds extracted by hexane, although in smaller amounts. Gas chromatography/mass spectrometry (GC/MS) analysis of the hexane raw extracts (data not shown) shows large amounts of TAG and the presence of several sterols, such as campesterol, stigmasterol and β -sitosterol. The characteristic ^{13}C NMR sterol signals (Kovganko *et al.*, 1999) are indicated as circles and arrows in Fig. 4A. The signals from 142 to 139 p.p.m. (a) and 121 to 119 p.p.m. (b) are assigned to the double-bonded carbons of sterols, at about 71 p.p.m. (c) to hydroxylated carbon 3, from 57 to 55 p.p.m. (d) to carbons 14 and 17 (five-membered ring), and at about 12 p.p.m. (e) to methyl carbons 18 and 29 of sterols (Kovganko *et al.*, 1999).

The spectra of the hexane extracts from other rootstocks, including CSD1 and WSY, and scions CSD2, CSD1 and WSY (data not shown) were similar to that of the CSD2 rootstock spectrum (Fig. 4), indicating similar fatty acid compositions.

Figure 5 shows the ^{13}C NMR spectrum of the alcoholic extract of CSD2 rootstock bark. The 12 intense peaks (*) at 104.0, 93.0, 82.0, 77.0, 75.0, 73.5, 73.0, 71.0, 70.0, 63.5, 62.0 and 61.0 p.p.m. were assigned to sucrose (Colnago and Seidl, 1983). The other major signals at 62.0, 47.0, 30.0 and 24.0 p.p.m. were assigned to proline (+), and at 56.0 p.p.m. to glutamine (x). The spectrum also shows some peaks at 69.0, 67.0 and 99.0 p.p.m. (#), which may be related to other sugars, such as raffinose and stachyose (Colnago and Seidl, 1983).

The ^{13}C spectra of the alcoholic extracts of the other bark samples (data not shown) were similar to that shown in Fig. 5, and the major signals were also assigned to sucrose and proline. The ^{13}C NMR spectrum of the alcoholic extract of the WSY rootstock (data not shown) showed a weaker proline signal than that observed in the spectrum of the CSD2 rootstock (Fig. 5). The large

Fig. 5 ^{13}C nuclear magnetic resonance (NMR) spectrum, from 110 to 10 p.p.m., of the alcoholic extract of CSD2 (level 2 citrus sudden death disease symptoms) rootstock bark in D_2O . The symbols '*', 'x', '#' and '+' indicate the signals of sucrose, glutamine, raffinose/stachyose and proline, respectively.

amount of proline observed in the spectrum of the CSD2 rootstock (Fig. 5) may be related to the plant response to water stress caused by phloem blockage (Bassanezi *et al.* 2003; Román *et al.*, 2004), which induces proline synthesis (Nolte *et al.*, 1997).

In situ NMR qualitative analysis

Figure 6 shows the high-resolution ^1H NMR spectra of fresh CSD2 and WSY rootstock bark. The spectra show very broad peaks as a result of the strong variation of magnetic susceptibility across the heterogeneous bark samples, but the resolution was sufficient to show a strong water peak at 4.7 p.p.m. and two weak signals at 4.0 and 1.0 p.p.m., which can be assigned to sucrose and methylenes of TAG fatty acids, respectively. The sucrose and methylene peaks are more intense in CSD2 than in WSY rootstock bark, indicating the higher concentration of sugar and lipids in symptomatic plants, in agreement with the results in Fig. 2. The same types of spectra were observed in scion bark (data not shown), showing a higher content of TAG and sugars in CSD2 plants.

This NMR experiment is rapid and can be performed directly on fresh bark; however, it has not been used as a CSD diagnostic because it is strongly dependent on the moisture content of the sample. The high-resolution ^1H NMR spectra of dry bark do not show the water and sugar peaks and can be used to quantify the oil content, in a few minutes, with about 20.0 mg of bark. However, the oil content analysis is much faster (few seconds) using the low-resolution ^1H NMR technique, as described in Fig. 3.

Figure 7 shows the ^{13}C NMR spectra of fresh rootstock bark of CSD2 and WSY samples. The ^{13}C NMR spectra of the bark are less susceptible than the ^1H spectra to the variation of magnetic susceptibility, and show sufficient resolution to identify most of

Fig. 6 ^1H high-resolution nuclear magnetic resonance (NMR) spectra of Rangpur lime bark with level 2 citrus sudden death disease symptoms (CSD2, top) and without symptoms (WSY, bottom).

Fig. 7 ^{13}C nuclear magnetic resonance (NMR) spectra of Rangpur lime rootstock bark without symptoms (WSY) (A) and with level 2 citrus sudden death disease symptoms (CSD2) (B).

the carbons of TAG, sucrose, glutamine and proline, as observed in the hexane (Fig. 4) and alcoholic (Fig. 5) extracts. The characteristic TAG signals are seen from 35.0 to 14.0 p.p.m. and from 131.0 to 126.0 p.p.m. The spectral resolution is not sufficient to measure the intensity of oleic and linolenic unsaturated carbons, as observed in the spectra of hexane extracts.

The 12 sucrose signals from 104.0 to 61.0 p.p.m. can also be assigned in most of the bark spectra. The signals at 182.0, 175.0 and 56.0 p.p.m. are assigned to glutamine, and at 175.0 and 47.0 p.p.m. to proline. Other glutamine and proline signals overlap with the triglyceride and sucrose signals. The major difference between the spectra in Fig. 7 is the higher intensity of the TAG signals in CSD2 than in WSY rootstock bark. This is certainly a result of the greater quantity of TAG in CSD2 rootstock bark, as also demonstrated in Figs 2 and 3. The ^{13}C NMR spectra show more intense proline and glutamine peaks in CSD2 than in WSY samples. As discussed above, the large amount of proline could be a result of phloem blockage or other plant stress, and is not specific to the CSD metabolic disorder.

Figure 8 shows the ^{13}C NMR spectra of fresh CSD2 and WSY scion samples of the same plant as analysed in Fig. 7. The scion spectra show identical signals to those observed in rootstock (Fig. 7), but with weaker TAG signals. The CSD2 scion samples also show higher intensity TAG signals than those observed in WSY scion samples, indicating a higher oil content in symptomatic plants, as also observed in Figs 2 and 3.

Although the ^{13}C spectrum can be used to identify the TAG disorder in symptomatic CSD plants with almost no sample preparation, it has not been used as a screening method, as it is quite slow (one analysis per hour) and is also dependent on the moisture content, as is the ^1H spectrum (Fig. 6).

The results (Figs 2–8) reveal a higher TAG content in rootstock than in scion bark, and a good correlation between CSD symptoms and the higher TAG and sucrose contents in both scion and rootstock bark. The high oil content in bark has also been observed

Fig. 8 ^{13}C nuclear magnetic resonance (NMR) spectrum of Valencia scion rootstock bark without symptoms (WSY) (A) and with level 2 citrus sudden death disease symptoms (CSD2) (B).

in citrus tristeza disease (Zhou *et al.*, 2002), indicating a correlation between the two diseases, as already noted in the case of anatomical pathology (Román *et al.*, 2004) and epidemiological patterns (Bassanezi *et al.*, 2003). The high oil content found in both diseases would not represent a problem for CSD diagnosis, as tristeza quick decline symptoms are not observed in sweet orange grafted on Rangpur lime or *C. volkameriana* rootstocks, which are tolerant for tristeza but intolerant for CSD. Tristeza quick decline symptoms are observed only in sweet orange plants grafted on sour orange rootstock.

Without a knowledge of the causal agent of CSD, the analysis of the oil content in rootstock bark by low-resolution NMR can be employed as a complementary CSD diagnostic method, which can screen about 300 samples per hour using about 200.0 mg of dry bark.

EXPERIMENTAL PROCEDURES

Plant material

The bark of Valencia sweet orange plants (*C. sinensis* Osbeck) grafted on Rangpur lime (*C. limonia* Osbeck) rootstock was collected from orchards in Comendador Gomes county, Minas Gerais, Brazil. The trees were planted in 1995, and the samples were collected in December 2006. The bark, close to the bud union, was 10.0 cm in length and 3.0 cm wide. The bark was collected from 20 plants without symptoms (WSY) and 20 CSD symptomatic plants, eight CSD1 and 12 CSD2. The CSD1 samples were obtained from plants with initial symptoms, characterized by light yellow rootstock bark and foliar discoloration, and the CSD2 samples were obtained from plants with severe symptoms and strong yellow rootstock bark.

Methods

Extraction with hexane

Ten grams of tree bark were dried, ground and extracted with hexane for 24 h in a Soxhlet extractor. After removing the solvent, the residual mass was considered to be the total lipid.

Extraction with ethanol–water solution

The insoluble material from the hexane extractions was dried and stirred in a 100.0 mL alcoholic solution (80% ethanol and 20% water, v/v) for 24 h at room temperature (Chum *et al.*, 2003). The soluble materials were separated by filtration and the insoluble materials were used in two further alcoholic extractions. The extracts were lyophilized and the resulting mass was considered to be the total alcoholic extract.

GC/MS analysis

The raw hexane extracts, containing sterols and TAG, were analysed by GC using a Shimadzu GC-17A combined with a QP 5050A MS detector. The GC experimental conditions were as follows: 30 m × 0.25 mm LM5 (B 591197) fused silica capillary column with a film thickness of 0.25 µm; initial oven temperature of 280 °C, raised to 300 °C at 10 °C/min and held for 50 min; helium carrier gas at a flow rate of 44 cm/s; injector temperature, 320 °C; injection amount of 3 µL at split mode (1 : 30) with a purge-off time of 1 min; temperature of the GC/MS transfer line, 280 °C; mass spectrometer operated at 250 °C in the electron impact mode (70 eV). The mass spectral identification of sterols was carried out by comparison with the mass spectral library of the spectrometer.

NMR analysis

The high-resolution NMR spectra were acquired using a Varian Inova 400 spectrometer, with a 9.4 T magnet, corresponding to resonance frequencies of 400 and 100 MHz for ¹H and ¹³C, respectively.

The ¹H and ¹³C analyses were performed with a 5.0-mm probe with 90° pulse widths of 9.5 and 6.5 µs, acquisition times of 2 and 1 s, and 16 and 2000 scans, respectively. The hexane and alcoholic extracts were dissolved in CDCl₃ and D₂O, respectively. For the *in situ* measurements, 2.0 cm × 0.3 cm fragments of fresh bark were inserted directly into the NMR tube, without solvent.

MRI of fresh bark was acquired using a Varian Inova 80, 2.1 T Oxford magnet, with a spin echo sequence: time of echo (TE) = 30 ms; recycle delay (RD) = 2 s; eight scans. Data sets were collected as 256 × 512 points and zero-filled to 512 × 512. The slice thickness was 2 mm with 0.1 mm of spatial resolution.

The low-resolution NMR measurements were performed in a 2.1 T Oxford magnet, using a Tecmag CAT100 console. For low-resolution NMR analyses, the bark samples (about 200 mg) were ground, lyophilized and weighed. They were stored and analysed at 22 ± 1 °C. The intensity of the free FID signal of the samples was collected 70 µs after the pulse.

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REFERENCES

- Azeredo, R.B.V., Colnago, L.A., Sousa, A.A. and Engelsberg, M. (2003) Continuous wave free precession: a practical analytical tool for low resolution NMR measurements. *Anal. Chim. Acta*, **478**, 313–320.
- Bassanezi, R.B., Bergamim, A., Amorim, L., Gimenez-Fernandes, N., Gottwald, T.R. and Bové, J.M. (2003) Spatial and temporal analysis of citrus sudden death as a tool to generate hypothesis concerning its etiology. *Phytopathology* **93**, 502–512.
- Chum, L.Y., Weiss, D. and Goldschmidt, E.E. (2003) Effects of carbohydrate starvation on gene expression in citrus root. *Plant*, **217**, 11–20.
- Colnago, L.A. and Seidl, P.R. (1983) Application of carbon-13 nuclear resonance to the germination of soybean seeds *in vivo*. *J. Agric. Food Chem.* **31**, 459–461.
- Colnago, L.A., Engelsberg, M., Souza, A.A. and Barboza, L.L. (2007) High-throughput, non-destructive, determination of oil content in intact seeds by continuous wave free precession nuclear magnetic resonance. *Anal. Chem.* **79**, 1271–1274.
- Derrick, K.S., Beretta, M.J. and Barthe, G.A. (2003) Identification of strains of citrus tristeza virus by subtraction hybridization. *Plant Dis.* **87**, 1355–1359.
- Forato, L.A., Colnago, L.A., Garratt, R.C. and Lopes-Filho, M.A. (2000) Identification of free fatty acids in maize protein bodies and purified zeins by ¹³C and ¹H nuclear magnetic resonance. *Biochim. Biophys. Acta*, **1543**, 106–114.
- Forato, L.A., Yushmanov, V.E. and Colnago, L.A. (2004) Interaction of two prolamins with 1-¹³C oleic acid by ¹³C NMR. *Biochemistry*, **43**, 7121–7126.
- Gomes, C.P.C., Nagata, T., Jesus Jr, W.C., Borges Neto, C.R., Pappas Jr, G.J. and Martin, D.P. (2008) Genetic variation and recombination of RdRp and HSP 70h genes of Citrus tristeza virus isolates from orange trees showing symptoms of citrus sudden death disease. *Virology J.* **5**, 9.
- Kovganko, N.V., Kashkan, Zh. N., Borisov, E.V. and Batura, E.V. (1999) ¹³C NMR spectra of β-sitosterol derivatives with oxidized rings A and B. *Chem. Nat. Compounds*, **35**, 646–649.
- Maccheroni, W., Alegria, M.C., Greggio, C.C., Piazza, J.P., Kamla, R.F., Zacharias, P.R.A., Barjoseph, M., Kitajima, E.W., Assumpção, L.C., Camarotte, G., Cardozo, J., Casagrande, E.C., Ferrari, F., Franco, S.F., Giachetto, P.F., Girasol, A., Jordão, H., Silva, V.H.A., Souza, L.C.A., Aguilarvildoso, C.I., Zanca, A.S., Arruda, P., Kitajima, J.P., Reinach, F.C., Ferro, J.A. and Dasilva, A.C.R. (2005) Identification and genomic characterization of a new virus (*Tymoviridae* Family) associated with citrus sudden death disease. *J. Virology*, **79**, 3028–3037.
- Nolte, H.D., Hanson, A.D. and Gage, D.A. (1997) Proline accumulation and methylation in citrus: implications for genetic engineering of stress resistance. *J. Am. Soc. Hort. Sci.* **122**, 8–13.
- Prestes, R.A., Colnago, L.A., Forato, L.A., Vizzotto, L., Novotny, E.H. and Carrilho, E. (2007) A rapid and automated low resolution NMR method to analyze oil quality in intact oilseeds. *Anal. Chim. Acta*, **596**, 325–329.
- Rivas-Valencia, P., Kuk, E.L., Mora-Aguilera, G., Febres, V., Martínez, D.O.,

- Espinosa, A.G., Jesus-Junior, W.C., Malvas, C.C. and Wulff, N.A. (2008) Estructura poblacional de aislamientos del Citrus tristeza virus y su asociación con la Muerte Súbita de los Cítricos en Brasil. *Agrociencia*, **42**, 85–93.
- Román, M.P., Cambra, M., Juárez, J., Moreno, P., Duran-Vila, N., Tanaka, F.O.A., Alves, E., Kitajima, E.W., Yamamoto, P.T., Bassanezi, R.B., Teixeira, D.C., Jesus Junior, W.C., Ayres, A.J., Gimenes-Fernandes, N., Rabenstein, F., Girotto, L.F. and Bové, J.M. (2004) Sudden death of citrus in Brazil: a graft-transmissible bud union disease. *Plant Dis.* **88**, 453–467.
- Seidl, P.R. and Colnago, L.A. (1987) Carbon-13 NMR spectroscopy of oilseeds. In *Recent Advances in Organic NMR Spectroscopy* (Lambert, J.B. and Rittner, R., eds), pp. 143–151. New York: Norell Press.
- Zhou, C.L.E., El-Desouky, A., Sheta, H., Kelley, S., Polek, M. and Ullman, D.E. (2002) Citrus tristeza virus ultrastructure and associated cytopathology in *Citrus sinensis* and *Citrus aurantifolia*. *Can. J. Bot.-Rev. Can Bot.* **80**, 512–525.