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Management of Citrus Canker in Argentina, a Success Story

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Citrus canker is an important bacterial disease of citrus in several regions of the world. Strains of *Xanthomonas citri* type-A (Xc-A) group are the primary pathogen where citrus canker occurs. After Xc-A entered the Northeast of Argentina in 1974, the disease spread rapidly from 1977 to 1980 and then slowed down and remained moving at slow pace until 1990 when it became endemic. Citrus canker was detected in Northwest Argentina in 2002. This paper presents the main steps in the fight of the disease and the management strategies that have been used to control citrus canker at this time. We think the process might be usefull to other countries with the same situation. Results from more than 40 years of research in Northeast (NE) Argentina indicate that we are at the limit of favorable environment for the disease. The severity of citrus canker is greatly affected by the environment and El Niño Southern Oscillation (ENSO) phenomenon which causes cyclic fluctuations on the disease intensity in the NE region. Weather-based logistic regression models adjusted to quantify disease levels in field conditions showed that the environmental effect was strongly modulated by the distance from a windbreak. Production of healthy fruits in citrus canker endemic areas is possible knowing the dynamics of the disease. A voluntary Integrated Plan to Reduce the Risk of Canker has been in place since 1994 and it allows growers to export unsymptomatic, uninfested fresh fruit to countries which are free of the disease and require healthy, pathogen free fruits. The experience from Argentina can be replicated in other countries after appropriate trials.

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In Argentina, citrus is an important regional fruit crop with two main production regions, the Northwest (NW) and the Northeast (NE) of the country. Current data indicate 140,000 hectares of commercial groves (Federcitrus, 2015). Citrus canker (CC) is caused by *Xanthomonas citri* which has spread internationally to new areas in the last decades. The bacterium binomial nomenclature of the CC pathogen varies depending on the author (Brunings and Gabriel, 2003; Bull et al., 2012; Graham et al., 2004).

Citrus canker exists since ancient times in Eastern Asia and is spreading to other citrus growing countries during the last 40 years. A previous expansion of the disease occurred at the beginning of the twentieth century, when eradication campaings were successful in USA, Australia, South Africa, and New Zealand, after several decades of host elimination. Eradication was supposed to be the best choice to control canker in new regions. However, worldwide movement of people and products and favorable environmental conditions due to climate change have made most difficult to eradicate the disease in modern times, except for isolated islands near Australia.

The probability of diseased plant movement from country to country is now high. The disease incidence and severity after introduction will vary depending on the environmental conditions and citrus varieties being grown. Experimental data were and will continue to be necessary for successful management of this disease where it is

established. Thus, management of the disease became a necessity almost everywhere, and all aspects of the disease triangle are very important to obtain adequate results. The eradication approach is no longer an alternative unless the disease can be detected at the earliest stage of introduction.

Citrus Canker in Argentina

The disease recognized as citrus canker (cancrosis in Spanish) existed in Argentina since 1928 as the B-type form (Canteros, 2001a, 2001b; Canteros de Echenique et al., 1985; Fawcett and Bitancourt, 1949). The low agressiveness and restricted host range confined the B-type strains for 40 years to a small area. These strains disappeared in 1978-90 after the introduction of the more aggressive A-type strains in 1974 (Canteros de Echenique et al., 1985; Goto et al., 1980). The replacement of the B-type strains with the A-type strains may have taken place due to the production of bacteriocin-like substance by the A strains against the B strains (Canteros et al., 2011a). The B-type strains of canker ocurred almost exclusively on lemon and adequate control was obtained by applications of copper containing bactericides.

The A-type of citrus canker, caused by *Xanthomonas citri* type A (Xc-A; syn. *Xanthomonas axonopodis* pv. *citri*) became endemic in NE part of Argentina after eradication efforts failed. The A-type entered the NE in 1974, it spread quickly from 1977 to 1980, and then at constant rate till 1990 when it became endemic in the region (Stall and Civerolo, 1991). The presence of canker in the NW of Argentina was apparent in 2002 (Rivadeneira et al., 2004a, 2004b).

End of Eradication and Application of Management Options

The eradication campaign which started in 1976 in the NE failed (Hogg, 1985; Rodriguez, 2012). A program of management and numeroud spray trials were initiated in 1976 based in the control of B-type canker with copper sprays. A-type canker was very severe on all young citrus plants and in grapefruit regardless of the age. Timing was the most important issue in controlling citrus canker. Numerous spray trials were performed in Argentina. The most appropriate timing for spraying copper-containing bactericides was determined. Trials of numerous chemical products, both commercial and experimental, were conducted. Based on these trials, management of the disease was achieved using integrated measures, among them protection with copper-containing bactericides at the exact leaf

growth stage, and the use of windbreaks around the plots (Stall et al., 1979).

Export Requirements

After quarantine was imposed by the European Union to keep the fresh fruit European market, an Integrated Plan to Reduce the Risk of Canker was carried out since 1994. Participation of growers is voluntary. Requirements are certification of selected, registered, symptom-free blocks of trees with permanent surveys of CC symptoms; windbreaks around 2-4 ha individual blocks areas; sanitation with disinfestants of harvested fruit, equipment and personnel; application of copper containing products to young leaf flushes and to developing fruits to prevent infection, and treatment of fruit in certified packing houses with sodium orthophenylphenate (SOPP) at 2% for 2 min and sodium hypochlorite at 0.02% for 45 s to ensure elimination of any living bacterial cell. Control is required of the insect leafminer (*Phyllocnistis citrella* Sta.) that entered Argentina in 1996 and became widespread in very short time. Damage by the leafminer is important for the bacteriium to enter the leaf through the cuticle and feeding in epidermal cells (Caceres, 1996). The occurrence of canker lesions on leaves with leafminer damage was evident only on infected trees. Disease-free plants did not show new lesions, even those heavily damaged by the insect. In plants with severe canker, heavy defoliation occurs after leaf-miner damage, and heavy sprouting will follow. Biological and chemical control are effective for leafminer.

Traceability and certification of citrus production during the entire process from trees to export-box is made by National Food Safety and Quality Service (Spanish: Servicio Nacional de Sanidad y Calidad Agroalimentaria, SENASA), the Animal and Plant Health Argentinian Service (Canteros, 2009).

Canker Intensity Variation

In the NE region of Argentina the ENSO causes cyclic variations in canker intensity. Disease intensity is lowest when spring and summer rainfall is low and highest when spring and summer rainfall is abundant. So, production of healthy fruits in endemic areas is possible knowing the dynamic of the disease. Populations of Xc-A on asymptomatics leaves and fruits surfaces of all citrus types are low in highly infected trees and undetectable in lightly infected tress. In years of low infection only grapefruit differs from other cultivars in canker severity on fruits. Important factors in disease intensity are: temperatures, relative humid-
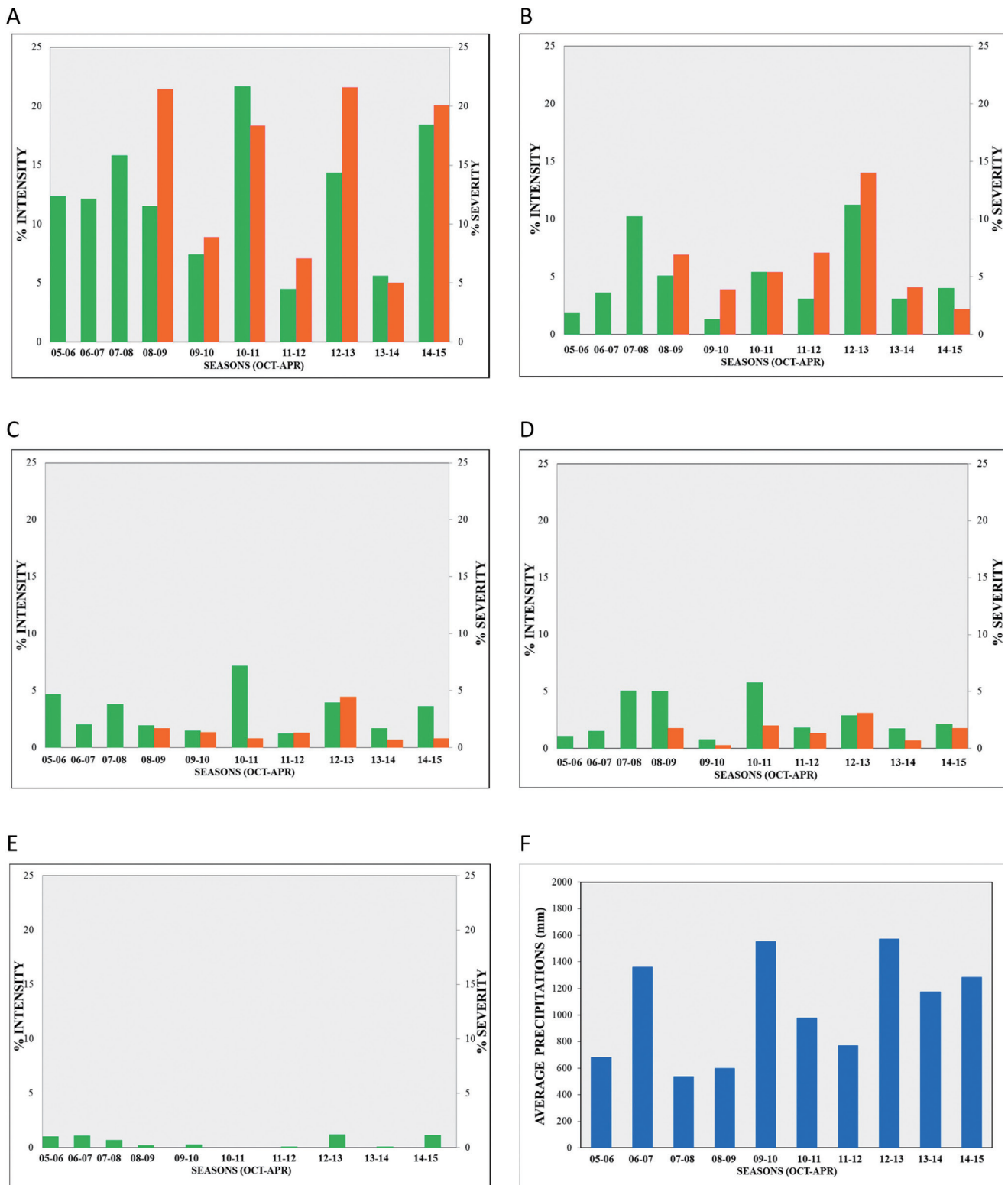
Fig. 1. Graphs indicate the intensity of infection (left y-axis) and severity (right y-axis) based on the three grades scale, calculated for both plant (green bars) and fruits (orange bars) at midseason (December-January from 2005 to 2015 years, x-axis) for grapefruit (A), lemon (B), Valencia orange (C), Murcott (D) and Satsuma (E) mandarins in Bella Vista (Corrientes, Argentina). (F) Average amount of rain is indicated in mm of precipitation for separates seasons (blue bars) delimitated from October to April for same period.

ity, and, most important, rain and wind, taken together (Canteros, 1998, 2005).

Disease Severity Variation

The relationship between disease and environment was determined based on data collected from 1991 to 2003, and still continue, on canker severity at mid-season in citrus varieties that differ in susceptibility to canker (Fig. 1). Disease incidence and severity in each tree was monitored every 1-2 weeks on a scale of 0-100% for more than 20 years. Severity of disease on fruits was taken at midseason and at harvest using a three grade scale; 0 (no symptoms); 1 (one large or three small lesions) and; 2 (more than one large or three small lesions per fruit). A formula was used to determine disease intensity. Disease intensity in fruits = (% fruits Grade 0×0) + (% fruits G1 $\times$ 1) + (% fruits G2 $\times$ 2)
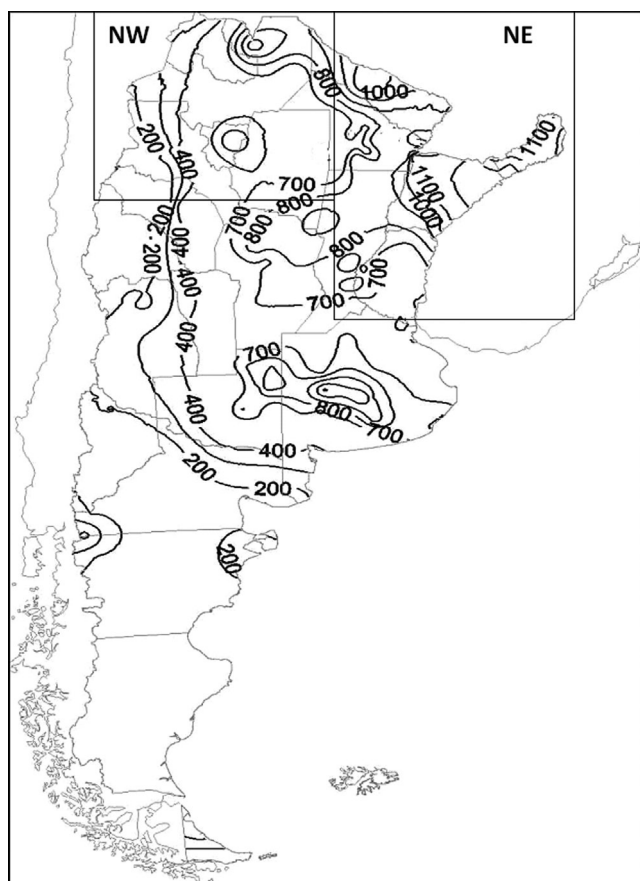
Precipitación del semestre cálido (OCT-MAR) 1991-2016

Fig. 2. Total precipitation for spring-summer in Argentina. Accumulation of average monthly precipitation values (in mm), from October to March, using 1991-2016 series. Northwest (NW) and Northeast (NE) regions are respectively indicated in left and right upper squares.

/ (number of grades = 3) (Canteros, 1998). Canker intensity in a given tree can vary when new healthy tissue occurs after defoliation caused by heavy infection. Intensity were measured every 2 weeks for several years. Variation occurs with the seasons and the years. This can be calculated already at midseason. An inverse relationship exists among disease intensity in plants at midseason and percentage of healthy fruits at harvest (high intensity gives low percentage), whereas a direct relationship occurs among intensity on fruits at midseason and intensity at harvest (high intensity gives high intensity). In years with low infection only grapefruit will differ from other cultivars in the severity of infection on fruits (Canteros, 1998). In Bella Vista, Corrientes., Argentina, a strong association was found among years with high frequency of spring rains and the severity of the disease (Fig. 2). Moisture variables associated with bacterial infection and thermal variables related to disease progress were identified. One general linear model included both variables (rainfall and temperature) discriminated by citrus variety shows a positive relationship (R^2 0.86) for CC severity (Canteros, 2006).

Inoculum and Sanitation

Pruning of infected tissue greatly decreases the available inoculum and is used in Argentina to ensure symptom-free trees. Herbicide defoliation can be used to start a program when the infection is high. Equipment, hands, clothing and gloves of laborers, collecting boxes, and any other tools should be treated with the appropiate disinfestants such as quaternary ammonium, phosphoric acid-iodine solutions, sodium hypochlorite or 70% ethanol (Canteros, 2000).

Susceptible Tissue

All young citrus tissue should be treated to prevent infection. Sprays are applied to leaf- flushes in their susceptible stage (10 to 14 days old) and to developing fruits every 40 days. Recommended chemicals are copper containing products. Soluble powders are preferred over liquid forms. The most important sprays are those applied from bloom to 4 months later (Supplementary Fig. 1). Timely application of copper sprays provided excellent control of the disease until 1994 when lack of control was noted in several groves and nurseries. Copper resistance within the bacteria population was demonstrated and evaluations of the mixture of copper + mancozeb showed that this mixture was the best control *in vitro* and in field trials. Current recommendations include the addition of mancozeb to the copper sprays in groves where resistance is found (Canteros, 1999).

Field Resistance

Inoculation of highly resistant citrus species like Satsuma tangerines (*C. unshiu*), 'Parana' grapefruit (*Citrus* sp.) (Canteros, 1992; Canteros and Gochez, 2008; Rinsdahl Canavosio et al., 2007), and kumquat (*Fortunella margarita* (Lour.) Swingle) (Canteros and Gochez, 2008) demonstrated that field resistance is due to a very short susceptible period for leaves and fruits. This occurs in Parana grapefruit as well in kumquat, similar to the previously described resistance in some tangerines and oranges (Canteros, 1992; Stall et al., 1981). These results could be used to select a source of quantitative resistance among the Rutaceae population. Compared with more susceptible varieties like 'Duncan' grapefruit or Key lime, the variable 'age of fruit/leaves' and 'inoculum concentration' were always selected as parameters to determine and describe canker resistance.

Resistance to Copper in Xc-A

Resistance to copper in several plant pathogenic bacteria has been reported worldwide since its discovery in Florida (USA) in 1983 in *Xanthomonas campestris* pv. *vesicatoria* (Xcv) (Marco and Stall, 1983). Copper resistant (Cu^R) strains of this bacterium were also found in Argentina in 1987 (Canteros et al., 1989). Timely application of copper sprays provided excellent control of citrus canker during 40 years. However, lack of control was noted in several groves and nurseries in autumn 1994. Resistance to copper was demonstrated for the first time in Xc-A in citrus groves that were sprayed with copper at least ten times per season. What was striking was the sudden and rapid widespread occurrence of the Cu^R strains throughout a large area, since strains isolated prior to 1994 from the same groves were all Cu^S (Canteros, 1996). Evidently, rapid selection and spread of the resistant strains occurred under the heavy pressure of numerous copper sprays applied in an attempt to control the high disease intensity.

In Florida, susceptible strains of *Xantomonas campestris* pv. *vesicatoria* (Xcv) were isolated even after several years of the first appearance of resistant strains (Canteros, 1990; Canteros et al., 1989, 1991; Pohronezny et al., 1992). In NE part of Argentina citrus, tomato, and pepper growing areas overlap but no proof exists that the Xc-A-Cu^R strains could have adquired the Xcv-CuR plasmid. Conjugation experiments demonstrated the transmission of a large plasmid from Xcv Cu^R to Xc-A Cu^S strains. Total DNA extracted from Xc-A and Xcv Cu^R and Cu^S strains, respectively from citrus and tomato, was restricted using enzymes and a Southern blot analysis performed. A probe containing fragments of Pseudomonas cop operon hybridized with all Xc-A and Xcv Cu^R strains from Argentina, and did not hybridize with any Cu^S strains. Polymorphism was observed in the hybridized fragments (Canteros et al., 2004).

An extensive survey was done from 1994 until 2009 to locate Cu^R strains in all citrus growing areas of Argentina. A total of 3350 strains were rated, about 10% of them were Cu^R. The resistant strains were only from a restricted region, several groves in the Paraná River área of Corrientes Province, several groves in Formosa province in 2004, and few groves in the SE of Corrientes since 2007. Some strains of *Pantoea agglomerans* and *Pseudomonas syringae* pv. *syringae* from citrus were Cu^R whereas numerous other saprophytes collected in groves were Cu^S or Cu^R. All other strains from groves located in the NW and NE of Argentina were Cu^S. The preventative use of the mix of mancozeb with copper at least once a year was enough to stop or delay the occurrence of Cu^R strains in groves that were infected with Cu^S strains. The addition of mancozeb to the copper products was recommended immediately after first detection of Cu^R in Xc-A. *In vitro* experiments and spray trials in the field were done with different copper compounds and several other products. Data from 18 years of spray trials on grapefruit seedlings infected with Cu^S or Cu^R strains were analyzed. In *in vitro* experiments and spray trials, addition of mancozeb to copper compounds eliminated Xc-A in water after 15 min and gave excellent control of canker in the field. Other mixtures with Cu products, such as ferric sulphate were effective but phytotoxic and mixtures with quaternary ammonium, sulfur, and several disinfectants, mineral salts or organic products did not give control. Presently, copper plus mancozeb is used in groves infected by Cu^R strains of Xc-A, whereas, Cu compounds can be used in plants infected by Cu^S strains (Canteros et al., 2010, 2013).

Xc-A Population in the Field

Quantification of population size, persistence, survival, and dispersal of Xc-A are important factors in canker management in endemic regions. We quantified Xc-A populations on leaves and fruits of grapefruit (*Citrus paradisi*), orange (*C. sinensis*), and lemon (*C. limon*) trees, in rain water, in dew, and on weeds at different distances from canker-infected citrus trees. Numbers of living bacterial cells (colony formation units, CFU) were calculated infiltrating the bacterial liquid suspension in the mesophyll of Duncan grapefruit and Key lime (*C. aurantifolia*); seedlings kept in a growth room and compared the number of canker lesions developed per square centimeter with a known infectivity

titration table. Sampling was made during four consecutive growing seasons, from 2004 to 2008. Results indicated that high concentration of Xc-A (10^5-10^7 CFU) was recovered by external washing of all of diseased fruits whereas 36% of symptomless fruits carried Xc-A at 10^2-10^6 CFU per fruit. Rainwater collected from diseased and symptomless leaves from an infected grove. The diseased leaves carried populations from 10^2-10^5 CFU whereas 55-87% of healthy ones had 10-10^4 CFU per leaf. Dew collected from similar leaves and fruits had Xc-A in 100% of them when they were diseased (10-10^4 CFU); 77% of the symptomless ones had 10-10^4 CFU. Weeds (mainly grass) were collected under the canopy and at 1, 5, 10, and 20 meters from diseased trees; samples positive for Xc-A decreased rapidly with distance from the infected grove. Positive samples were from 10-10^4 CFU. Dew, even it contains high Xc-A populations, apparently does not serve as a main inoculum source. Populations of Xc-A decreased sharply with increasing distances from the diseased trees even after rainstorms (Canteros, 1998, 2005, 2006; Canteros et al., 2011b; Stall et al., 1979, 1980).

Canker Resistance and Environment Variation

There are great variations in susceptibility among the cultivated varieties. The intensity also fluctuates according to the seasons and the years. Most epidemiological data that exist on citrus canker (Stall et al., 1993) are referred to the spread in time and space in new epidemics but little information existed on the disease after it became established in a new area. Field evaluations and inoculations have demonstrated that leaves of all cultivars of citrus are susceptible to the disease when they are very young (Stall et al., 1980, 1981) and they become increasingly resistant with age. Resistance to canker is expressed in leaves as lower number of infection resulting from a given amount of inoculum (Canteros, 1992; Stall et al.,1982). It was demonstrated previously (Stall et al., 1981) that reduction of the amount of disease in leaves will indirectly reduce the amount of disease in fruits. Knowing the period of susceptibility in fruits of the most economically important cultivars has improved the chance of controlling the disease (Supplementary Table 1). In a previous work (Canteros, 1992) it was found that, except for very young fruits, fruits of all cultivars became increasingly resistant with age. Infectivity titration experiments with 12-16 week-old fruits of grapefruit demonstrated that a minimum of 10^3 cells per milliliter was necessary to get lesions with the method of inoculation used. A positive linear relationship was found between inoculum concentration and number of lesions per square centimeter. Other important members of the environment are the bacteriophages that attack Xc-A cells. The numbers were very low at the beginning of the epidemic in 1978 but those numbers are now very high and may have contributed to the general decrease of disease intensity even in unsprayed plots in the last decades. In a survey of several plots of different citrus varieties in Argentina in the last years high numbers of phage particles were found after leafwashes (Balogh et al., 2008).

Weather and Citrus Canker

Studies of disease and environment relationship has helped greatly improving the citrus production in NE Argentina. Sprays at proper timing and change in citrus varieties helped in keeping disease incidence at very low levels. In this area, strong storms are very unusual and they occur only locally. Light winds are common (Canteros, 1998; Moschini et al., 2005). The spread of Xc-A from an infected tree might be at short distances only (Canteros et al., 2004). In 1979 we found in one occasion that Xc-A cells have spread at 30 m from a diseased grove (Stall et al., 1980). We present here more data that support the concept that Xc-A can be detected only under diseased trees and not on weeds collected at far distances. The quantification of Xc-A populations on leaves and fruits of grapefruit, orange, and lemon trees, in rain water, in dew, and on weeds at different distances from canker-infected citrus trees provides important information to improve canker management in groves where fruits for export are produced. Currently, healthy fruit without symptoms of canker and with no Xc-A cells on fruit surface can be produced and exported to countries with canker regulations.

The ENSO Phenomenon

The atmospheric conditions present irregular fluctuations on a wide range of time scales producing intraseasonal (26-60 days), interannual and interdecadal variability (Garreaud et al., 2009). El Niño Southern Oscillation (ENSO) is the most important oceanic-atmospheric phenomenon causing interannual climate variability. Walker and Bliss (1932) discovered the existence of an irregular interannual fluctuation called Southern Oscillation (SO), which involves changes in the rainfall and wind over the tropical Pacific and Indian oceans. Bjerknes (1969) associated the SO with fluctuations in the surface temperature of the eastern equatorial Pacific Ocean. ENSO phenomenon recognizes a neutral phase and two extreme phases: El Niño (warm sea surface in the central-eastern equatorial Pacific Ocean

and pressures greater than average in the Indian Ocean and Australia) and La Niña (processes in the opposite direction than in El Niño years). ENSO affects atmospheric circulation systems located at remote sites on the planet (teleconnections), causing thermal and rainfall anomalies. Based on the analysis of 12 years of incidence and severity data for citrus canker in several citrus species in Bella Vista (Corrientes), Canteros et al. (2004), demonstrated a positive correlation between CC severity and El Niño phase.

Later, Moschini et al. (2014) quantified the effect of weather variables on mid-season fruit canker intensity (1991-2010 growing seasons) in an experimental grove of grapefruit (Red Blush cultivar) in Bella Vista (Argentina), at two contrasting distances from a natural windbreak (closer or farther). One of weather variables that best correlated with disease levels (S: severe, M: moderate and L: light) at both windbreak distances was "Days with precipitation > 12 mm" (DPrec; Kendall Tau-b correlation coefficient = 0.60). The use of thresholds of precipitation less than 12 mm in DPrec produced a marked decrease in its correlation with the level of disease, based on the fact that light daily precipitations would not have sufficient energy to spread bacteria from the cankers. Weather variables were calculated in a time period beginning after accumulating 3372 degree days as from July 10, and finishing when the sum reached 985 degree days (base temperature = 12.5°C). The ordinal response logistic regression model that included DPrec and DDMaxT (sum of the exceeding amounts of daily maximum temperature from 33°C) and windbreak distance (strong or moderate wind protection), reached a prediction accuracy of 88.6%. From daily temperature and precipitation data collected from Corrientes and Monte Caseros weather stations (Argentina National Weather Service, http://www.smn.gov.ar/), the last selected logistic model was run over 82 years (1932-2013) (Moschini et al., 2015). Retrospective model predictions were use to analyze the effect of the climate variability associated to ENSO phases (defined by the Japan Meteorological Agency according to the sea surface temperature in El Niño 3 area: 5ºN-5ºS, 150ºW-90ºW) over CC levels. In both sites, very low probability of occurrence of severe disease levels was expected from the model in strong wind protection scenarios (close to the windbreak), contrasting with the increase number of year with severe CC levels at greater distance to the windbreak. In Monte Caseros, weather conditions of years with El Niño phase were strongly conducive to severe CC levels (14 out of 16 El Niño years observed severe disease levels). In Monte Caseros and also in Corrientes, a high proportion of La Niña years observed moderate to light CC levels. In periods of 60 days starting on 21 Setember and 12 October (mean date for the begining of the critical period for Corrientes and M. Caseros respectively) the differences between both extreme ENSO phases (19 La Niña and 16 El Niño years) in the mean number of days with precipitations > 12 mm were 2.5 and 4 for both sites. For the last very strong El Niño year (2015/16), a total of 11 days with precipitation > 12 mm were counted in M. Caseros and 8 days in Corrientes. In late spring (November and December), a greater influence of the warm and cold phases of ENSO phenomenom is observed in NE Argentina.

We understand that citrus canker could be controlled with approppiate management. After the introduction of the A-type strains in Argentina, and after a failed attempt of eradication an integrative approach was taken including studies of the host, the pathogen, and the environment. The classical disease triangle gave the anwers to a proper management which allowed the uninterrupted export to the European Unions. Thus, integral studies gave successful results in citrus canker in Argentina.

Acknowledgments

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