Citrus Ringspot Diseases in Spain

J. Navas-Castillo, and P. Moreno

ABSTRACT. A 20-yr-old Newhall navel orange/Troyer citrange plot in Almussafes (Valencia) was inspected for psorosis-ringspot symptoms. This plot had been established with virus-free plants and later topworked with buds of uncontrolled origin. Twelve out of the 102 trees examined showed bark scaling and some chlorotic blotches in old leaves (Ps-ALM syndrome), 22 trees showed yellow spots and rings with sharp edges in old leaves, twigs and fruits, but no bark scaling (RS-ALM syndrome), and the remaining 68 trees did not show any of these symptoms. Inoculum from Ps-ALM trees induced shock and strong flecking and spotting on young leaves of sweet orange seedlings, and partially purified extracts from these plants were infective on *Chenopodium quinoa* and had a 48 kDa-protein that is associated with psorosis. On the contrary, inoculum from RS-ALM induced mild to moderate flecking on sweet orange seedlings but not a shock reaction. Extracts from these plants were not infective on *C. quinoa* and did not contained the 48 kDa-protein.

Further comparison of several ringspot isolates with psorosis isolate P-121 showed that isolates associated with bark scaling induced shock, protected against psorosis B, caused symptoms of similar intensity and were qualitatively indistinguishable from psorosis. These isolates and psorosis were transmissible to *C. quinoa* and contained a 48 kDa-protein. RS-BUR, another ringspot isolate with field symptoms similar to RS-ALM, did not induce shock, was less pathogenic than psorosis, did not protect against psorosis B, was not transmissible to *C. quinoa* and did not contain the 48 kDa-protein. RS-ALM and RS-BUR appear to be caused by a pathogen different from that causing psorosis and the other ringspot isolates.

Index words. electrophoresis, mechanical inoculation, psorosis, symptoms.

Citrus ringspot disease, as originally described by Wallace and Drake (29) in California, was characterized by the appearance of yellow blotches, vein banding and/or distinct rings in mature leaves of inoculated seedlings of several citrus species. Young leaves of these seedlings showed chlorotic flecking and spotting similar to those induced by different diseases of the psorosis group (28). Sometimes, inoculated seedlings suffered a shock reaction with leaf shedding and necrosis of the first flush after inoculation, whereas other times, soft stems developed necrotic lesions that occasionally could result in stem girdling and dieback of the part above the lesion. Ringspot symptoms were encountered in inoculations from field trees with leaf symptoms of psorosis, but in some instances from trees without these symptoms (29).

Reports on diseases similar to ringspot found in different countries (2,3,5,12,13,16,18,21,22,25,26) include leaf, flush, fruit or trunk symptoms, that were not given in the original description of the disease (29).

Ringspot isolates from different origins, some psorosis B isolates from California, and the original ringspot isolate described by Wallace and Drake (29), were later mechanically transmitted to *Chenopodium quinoa* (10), inducing similar local lesions. Furthermore, one of these isolates (11), triply cloned on *C. quinoa* by single lesion transfer and then inoculated back to citrus, induced all the leaf, flush and trunk symptoms of the original isolate. Since most ringspot isolates studied were associated to severe bark scaling (psorosis B-type) in field trees, it was suggested that ringspot and psorosis B could be the same disease and that mechanical transmissibility to *C. quinoa* could serve to identify this disease (23,24).

Infectivity on *C. quinoa* was later associated with two components separable by sucrose gradient centrifugation (6). These components contained a protein, about 48 kDa, that was suggested to be the capsid protein of the virus. Recently, infectivity on *C. quinoa* and the 48 kDa-protein have been found in

different ringspot and psorosis isolates, but not in other diseases of the psorosis group (4,9,14,16,17).

In a survey for psorosis-ringspot isolates in Spain, a citrus plot was found in Almussafes (Valencia) with two distinct syndromes. Trees in this plot were Newhall navel orange grafted on Troyer citrange, about 20 yr-old, that were initially planted virus-free and later were erroneously topworked with satsuma buds of uncontrolled origin. Though satsuma buds were removed a few weeks later, some of the trees were infected. Field symptoms and distribution of the infected trees suggested that at least two budwood sources had been used and that two different pathogens of the psorosis group had been introduced into the plot.

In this paper we present data supporting this suggestion and discuss the relationships between diseases of the psorosis-ringspot complex in Spain.

MATERIALS AND METHODS

Graft-inoculation on indicator plants. Indicator plants were grown on a steam sterilized artificial potting mix (50% sand and 50% peat moss) and fertilized following a standard system (1). The following species or hybrids were used: Pineapple sweet orange, Duncan grapefruit, Dweet tangor, rough lemon, Mexican lime, and Etrog citron (Arizona 861-S-1). Etrog citron was propagated on rough lemon and the other indicators were seedlings. Each inoculum source was graft-inoculated onto four plants of each indicator and four additional plants were self-inoculated as negative controls. Plants were pruned above the inoculation point and placed in a temperature-controlled greenhouse (18-26 C).

Symptom evaluation. Symptoms on individual inoculated plants were observed on at least two flushes and some were scored for intensity using a 0 to 3 scale in which 0 meant no symptoms and 3 very intense symptoms. Symptom expression in each indicator species was quantified by a pathogenicity index calculated by the following formula (15):

$$PI = (3xS + FxI + B + 2xD + 2xBFxI + 3xNExI + 3xDFxI + 3xPB + 4xSS + 4xSt) \times 100/176$$

In this formula, I equals the estimated intensity (0 to 3) for the affected symptom and the other letters corresponded to the number of plants that had shown the following symptoms: shock reaction in the first flush (S), chlorotic flecking (F), blotching (including oak leaf pattern and ringspots) (B), and deformation (D) in young leaves, chlorotic blotching (including ringspot) (BF), necrotic etching (NE) and deformation (DF) in fully expanded leaves, psorosis B lesions (PB) in old leaves, shock in secondary flushes (SS), and stunting of the infected plants (St). The coefficient for each addend (1 to 4) was the specific weight assigned to this symptom, and 176 was the maximum value possible for the whole factor in parenthesis.

A general pathogenicity index (GPI) was calculated as a weighed mean of the PI values obtained in different idicator species, using the formula:

$$GPI = [PI(Sw) + PI(DT) + PI(G) + PI(RL) + 2xPI(ML) + 2xPI(C)]/8$$

in which the characters in parenthesis indicate the host in which PI was calculated: Pineapple sweet orange (Sw), Dweet tangor (DT), Duncan grapefruit (G), rough lemon (RL), Mexican lime (ML) and Etrog citron (C) (15).

The isolates were grouped in three clusters according to their PI and GPI values, using the "Average" mode of the Statgraphics program (STSC, Inc., and Statistical Graphics Corporation).

Cross protection against psorosis B symptoms. Sweet orange seedlings, healthy or infected with the different psorosis or ringspot isolates, were challenge inoculated with two bark pieces of a plant infected with the PB-108 psorosis B isolate from the IVIA collection. The presence of psorosis B

leaf and twig symptoms (19,20,27) was assessed 3 months after challenge inoculation.

Isolates. A preliminary comparison was performed with trees of the Almussafes plot showing each of the two syndromes observed. A further comparison of biological characteristics and presence of an associated 48 kDa-protein in infected plants was done using eight Spanish ringspot isolates and the psorosis isolate P-121 from the IVIA collection (7). The origin and characteristics of these isolates have been described elsewhere (15). A summary of the field symptoms induced by these isolates is presented in Table 1.

Partial purification. Partial purification and detection of the 48 kDa-protein associated with some psorosis and ringspot isolates (4,6,9,14,16) was performed following the general procedure described by Derrick *et al.* (6) in conditions previously established (17). This procedure included extraction of young symptomatic tissue, clarification with Freon-113 followed by a low speed centrifugation, and concentration by high speed centrifugation. Concentrated extracts were then fractionated in a linear sucrose gradient. The fractions were assayed for infectivity on *C. quinoa*, concentrated by high speed centrifugation and further purified by agarose gel electrophoresis. Selected slices of the agarose gel (14,17) were analyzed for proteins by polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were stained with Coomassie blue and/or silver nitrate.

Mechanical inoculation on *Chenopodium quinoa*. *C. quinoa* plants, grown in a temperature controlled greenhouse (18-26 C) with a 16-hr period of supplemental light (Grolux, wide spectrum), were dusted with carborundum and mechanically inoculated with crude or concentrated extracts from symptomatic young leaves, prepared as indicated above. Inoculated plants were usually maintained in the laboratory.

RESULTS

Field symptoms and distribution of infected trees in the Almussafes plot. This plot was first inspected in 1989 and later in 1992, when the trees were about 20-yr-old. In both surveys, 12 of the 102 trees examined showed bark scaling in the trunk and psorosis B-type chlorotic blotches in the old leaves (this syndrome was named Ps-

TABLE 1
FIELD SYMPTOMS INDUCED BY A COLLECTION OF SELECTED PSOROSIS AND RINGSPOT ISOLATES

Isolate	Original host[z]	Field symptoms		
		Bark scaling	Old leaf symptoms[y]	Fruit symptoms[y]
RS-ALM	Sw.O/T.C.	–	YS	YS
RS-BUR	Sw.O/S.O	–	YS	YS
RS-ALC	Cl./S.O.	?[w]	CB	?[w]
RS-CV	Cl./C.M/Sw.O.	+	CB	–
RS-SOR	Cl./Sw.O./S.O.	+	CB	DR
RS-GR	Gf./S.O.	+	CB	?[v]
RS-SR	Gf./S.O.	?[w]	CB	YB
RS-INV	Sw.O./S.O.	+	CB	–
P-121	Cl./S.O.	+	–	–

[z]Sw.O. = sweet orange, T.C. = Troyer citrange, S.O = sour orange, Cl. = Clementine, C.M. = Common Mandarin, Gf. = grapefruit.
[y]YS = yellow spots with sharp edges, CB = chlorotic blotches, DR = depressed rings, YB = yellow blotchy rings.
[w]Young trees.
[v]The source tree was removed soon after discovery.

ALM), 22 trees showed yellow spots and rings with well defined edges in old leaves, twigs and fruits (Fig. 1), but no bark scaling (this syndrome was named RS-ALM), and the remaining 68 trees did not show any of these symptoms. The distribution of trees presenting each of these syndromes is shown in Fig. 2. Interestingly, trees with RS-ALM symptoms were always in even rows and those with the Ps-ALM syndrome were in odd rows (except the right end of the second row).

Preliminary comparison of isolates from trees with each syndrome. Bark samples from 4 Ps-ALM trees (nos. 1 to 4 in Fig. 2), 4 RS-ALM trees (nos. 5-8 in Fig. 2) and 3 symptomless trees (nos. 9-11 in Fig. 2) were graft-inoculated on sweet orange seedlings and young shoots from these plants used for partial purification and mechanical transmission to *C. quinoa*.

Sweet orange seedlings inoculated from trees 1 to 4 (Ps-ALM) showed shock and chlorotic flecking and extracts from symptomatic leaves induced local lesions on *C. quinoa* and contained a 48 kDa-protein associated with the infectious fractions of the sucrose gradient. Those inoculated from trees 5 to 8 (RS-ALM) and 9 (symptomless) only showed chlorotic flecking, and extracts from young leaves did not induce lesions on *C. quinoa* and did not contain the 48 kDa-protein. Finally, inoculum from trees 10 and 11 did not induce any symptom either on sweet orange seedlings or on *C. quinoa*.

Comparison of different Spanish ringspot isolates with a psorosis isolate. The original hosts and field symptoms induced by these isolates are summarized in Table 1. Source trees of isolates RS-ALM and RS-BUR differed from the other ringspot and psorosis isolates in that they did not show bark scaling, and they had yellow spots with sharp edges in old leaves and fruits instead of chlorotic blotches.

The biological characteristics and the presence of a 48-Kd protein associated with the different isolates are summarized in Table 2.

Psorosis P-121 and all the ringspot isolates, except RS-ALM and RS-

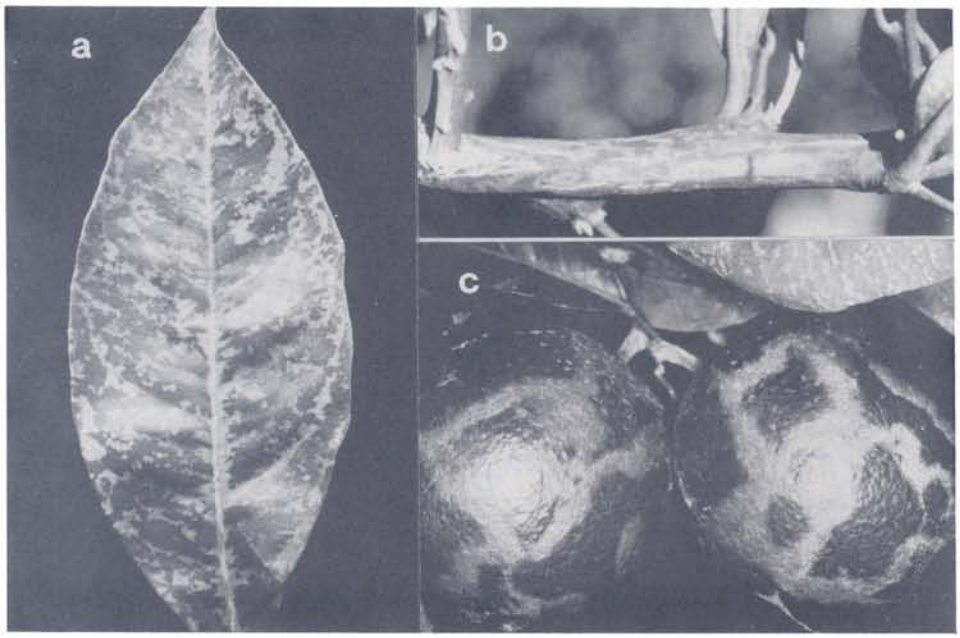

Fig. 1. Symptoms induced by isolate RS-ALM in leaves (a), shoots (b) and fruits (c) of a Newhall navel orange/Troyer citrange tree in Almussafes (Valencia).

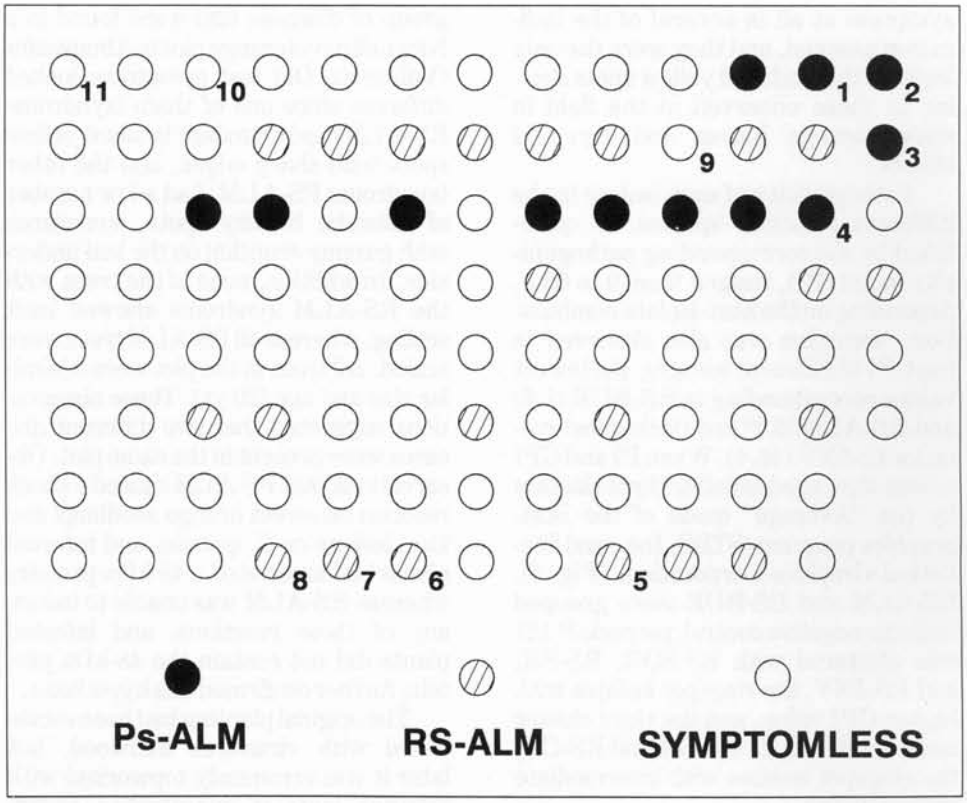

Fig. 2. Distribution of trees showing syndromes Ps-ALM and RS-ALM in a field plot in Almussafes (Valencia). Trees numbered were indexed in the greenhouse and assayed for mechanical transmission to *Chenopodium quinoa* and for the presence of a 48 kDa-protein in the infective fractions.

BUR, induced a shock reaction in the first flush of one or more of the indicators assayed. In the following flushes, symptoms caused by all ringspot isolates were qualitatively indistinguishable from those induced by the psorosis control (P-121). Nevertheless, RS-ALM and RS-BUR caused very mild or no

TABLE 2
BIOLOGICAL CHARACTERISTICS OF DIFFERENT RINGSPOT ISOLATES AND PRESENCE OF AN ASSOCIATED PROTEIN

Isolate	Shock reaction	Cross prot. against psorosis B	Transmission to *C. quinoa*	Associated ca. 48 kDa-protein
RS-ALM	–	–	–	–
RS-BUR	–	–	–	–
RS-ALC	+	+	+	+ (4)[z]
RS-CV	+	+	+	+ (4)
RS-SOR	+	+	+	+
RS-GR	+	+	+	Not done
RS-SR	+	+	+	+ (16)
RS-INV	+	+	+	+ (4)
Control (P-121)	+	+	+	+ (4)
Healthy control	–	–	–	–

[z]Parenthesis: References

symptom at all in several of the indicators assayed, and they were the only isolates that induced yellow spots similar to those observed in the field in sweet orange leaves and 1-yr old shoots.

Pathogenicity of each isolate in the different indicator species, as quantified by the corresponding pathogenicity index (PI), ranged from 0 to 68.8, depending on the host-isolate combination. Variation was also observed in the GPI of different isolates, the lowest values corresponding to RS-BUR (1.6) and RS-ALM (6.2) and the highest value to RS-INV (46.4). When PI and GPI values were used to define three clusters by the "Average" mode of the Statgraphics program (STSC, Inc., and Statistical Graphics Corporation) (Fig. 3), RS-ALM and RS-BUR were grouped with the negative control, psorosis P-121 was clustered with RS-SOR, RS-SR, and RS-INV, the ringspot isolates with higher GPI value, and the third cluster included RS-ALC, RS-GR and RS-CV, the ringspot isolates with intermediate pathogenicity.

Sweet orange plants uninoculated or inoculated with RS-ALM or RS-BUR did not show any protection against psorosis B 3 months after challenge-inoculation with the isolate PB-108, whereas those preinoculated with psorosis P-121 or any of the remaining ringspot isolates showed protection against psorosis B symptoms during the same period (Table 2). RS-ALM and RS-BUR were not mechanically transmissible to *C. quinoa*, whereas psorosis P-121 and the remaining ringspot isolates could be transmitted and induced typical necrotic lessions in the inoculated leaves (Table 2). Finally, purification of extracts from young symptomatic tissue enabled detection of a protein, about 48 kDa, in plants infected with five of the ringspot isolates (RS-GR could not be assayed) or with psorosis P-121, but not in those infected with RS-ALM or RS-BUR or in the healthy control (Table 2).

DISCUSSION

Two syndromes resembling those described for the psorosis-ringspot group of diseases (28) were found in a Newhall navel orange plot in Almussafes (Valencia). Old leaf symptoms looked different since one of them (syndrome RS-ALM) had abundant brilliant yellow spots with sharp edges, and the other (syndrome PS-ALM) had a low number of chlorotic blotchy spots, sometimes with gummy eruption on the leaf underside. In addition, none of the trees with the RS-ALM syndrome showed bark scaling, whereas all PS-ALM trees were scaled. All trees in the plot were of similar size and age (20 yr). These observations suggested that two different diseases were present in the same plot. Observations that PS-ALM caused a shock reaction on sweet orange seedlings and local lesions on *C. quinoa*, and infected plants had associated a 48 kDa-protein, whereas RS-ALM was unable to induce any of these reactions, and infected plants did not contain the 48-kDa protein, further confirmed this hypothesis.

The original planting had been established with virus-free budwood, but later it was erroneously topworked with satsuma buds of uncontrolled origin. Though these buds were removed a few weeks later, in the following years disease symptoms appeared in some trees. The trees showing each syndrome were separated in different rows suggesting that probably two operators took budwood from different trees and topworked parallel rows in the Almussafes plot following a zigzag pattern. Operator in row 1 carrying budwood with PS-ALM disease, apparently finished his row faster than the operator in row 2, and before moving from row 1 to row 3 topworked the tree at the right end of row 2 (Fig. 2).

In the last survey, a few trees in both odd and even rows were found showing mild concave gum in some branches, which would indicate that budwood carrying PS-ALM or RS-ALM also carried concave gum. Tree number 9 was symptomless but when inoculated onto sweet orange it induced only chlorotic flecking. Concave gum does not induce a shock reaction on sweet orange seedlings (20) and attempts to mechanically transmit it to *C. quinoa* and to detect an associated 48 kDa-protein were un-

successful (4). Thus, tree number 9 and perhaps some other symptomless trees in the plot might have been infected only with concave gum but have not shown trunk symptoms, or with a different pathogen inducing chlorotic flecking but not shock or local lesions on *C. quinoa*.

Further comparison of eight ringspot isolates, selected on the basis of the presence of chlorotic rings and/or spots

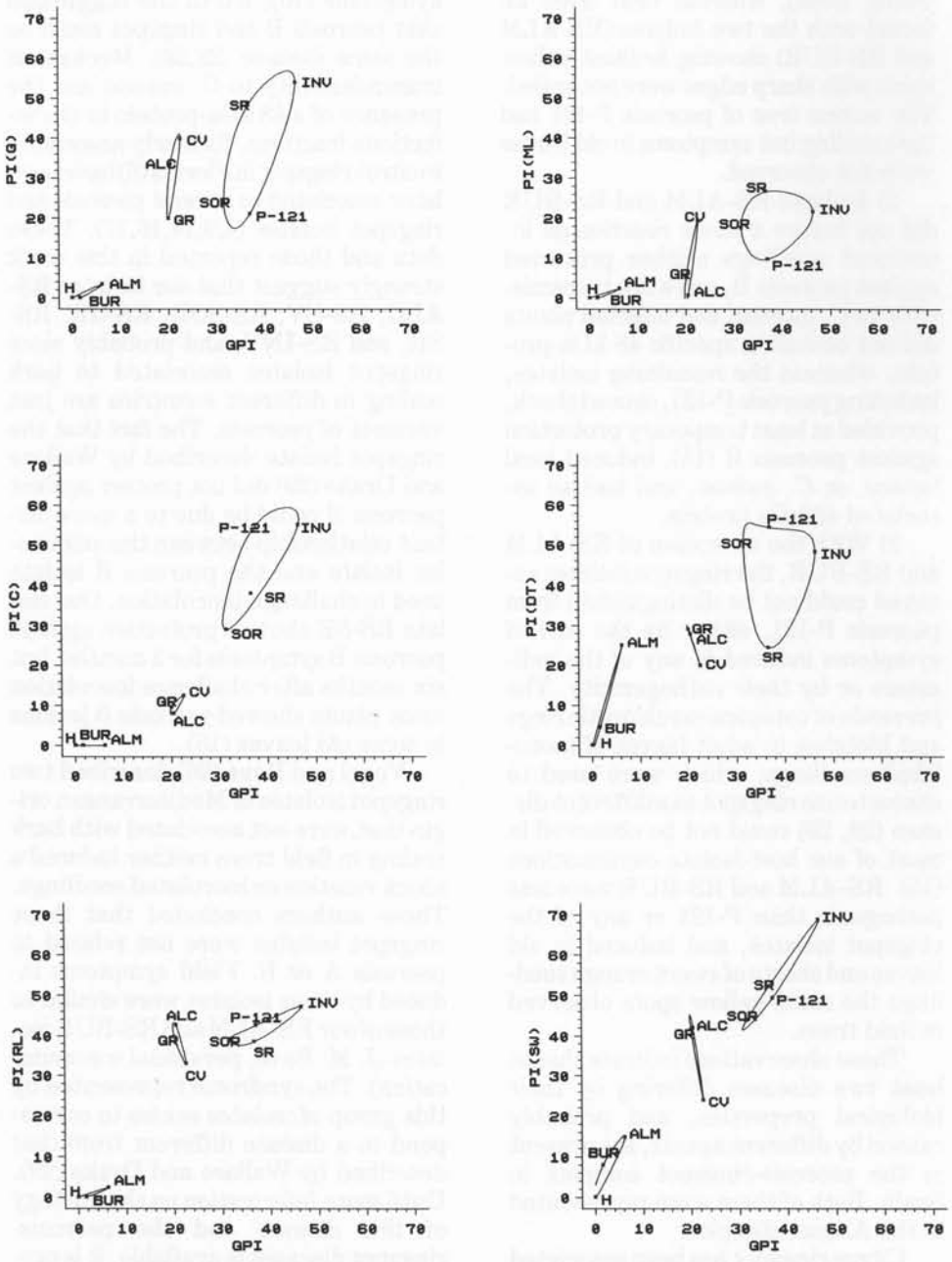

Fig. 3. Groups of isolates obtained when GPI (general pathogenicity index) and PI (pathogenicity index) values in sweet orange (Sw), Dweet tangor (DT), grapefruit (G), Rough lemon (RL), Mexican lime (ML), and citron (C), were used to define three clusters by the "Average" mode of the Statgraphics program (STSC, Inc., and Statistical Graphics Corporation). Isolates RS-ALM and RS-BUR were grouped with the negative control (origin).

in old leaves, and a well characterized psorosis isolate (7) resulted in the following observations:

1) Source trees of the isolates showing chlorotic blotches in old leaves showed bark scaling (except isolates RS-ALC and RS-SR that were taken from young trees), whereas field trees infected with the two isolates (RS-ALM and RS-BUR) showing brilliant yellow spots with sharp edges were not scaled. The source tree of psorosis P-121 had bark scaling but symptoms in old leaves were not observed.

2) Isolates RS-ALM and RS-BUR did not induce a shock reaction on inoculated seedlings neither protected against psorosis B, were not transmissible to *C. quinoa*, and infected plants did not contain a specific 48 kDa-protein, whereas the remaining isolates, including psorosis P-121, caused shock, provided at least temporary protection against psorosis B (15), induced local lesions on *C. quinoa*, and had an associated 48-kDa protein.

3) With the exception of RS-ALM and RS-BUR, the ringspot isolates assayed could not be distinguished from psorosis P-121, either by the sort of symptoms induced in any of the indicators or by their pathogenicity. The presence of conspicuous chlorotic rings and blotches in adult leaves of inoculated seedlings, which were used to characterize ringspot as a different disease (28, 29) could not be observed in most of our host-isolate combinations (15). RS-ALM and RS-BUR were less pathogenic than P-121 or any of the ringspot isolates, and induced in old leaves and shoots of sweet orange seedlings the same yellow spots observed in field trees.

These observations indicate that at least two diseases differing by their biological properties, and probably caused by different agents, are present in the psorosis-ringspot complex in Spain. Both of them were represented in the Almussafes plot.

Citrus ringspot has been associated with the presence of severe bark scaling in field trees and the ability to induce a shock reaction, chlorotic spots and ringspots on inoculated indicator plants (23,24). This association and the fact that different ringspot isolates from Florida and Texas, the original ringspot isolate described by Wallace and Drake (29), and several psorosis B isolates could be mechanically transmitted to *C. quinoa* and induced similar symptoms (10), led to the suggestion that psorosis B and ringspot could be the same disease (23,24). Mechanical transmissibility to *C. quinoa* and the presence of a 48 kDa-protein in the infectious fractions, formerly associated to citrus ringspot in Florida (6) have been later associated to several psorosis and ringspot isolates (4,9,14,16,17). These data and those reported in this work strongly suggest that our isolates RS-ALC, RS-CV, RS-SOR, RS-GR, RS-SR, and RS-INV, and probably most ringspot isolates associated to bark scaling in different countries are just variants of psorosis. The fact that the ringspot isolate described by Wallace and Drake (29) did not protect against psorosis B could be due to a more distant relationship between this particular isolate and the psorosis B isolate used in challenge-inoculation. Our isolate RS-SR showed protection against psorosis B symptoms for 3 months, but six months after challenge inoculation some plants showed psorosis B lesions in some old leaves (15).

Vogel and Bové (26) described two ringspot isolates of Mediterranean origin that were not associated with bark scaling in field trees neither induced a shock reaction on inoculated seedlings. These authors concluded that these ringspot isolates were not related to psorosis A or B. Field symptoms induced by these isolates were similar to those of our RS-ALM and RS-BUR isolates (J. M. Bové, personnal communication). The syndrome represented by this group of isolates seems to correspond to a disease different from that described by Wallace and Drake (29). Until more information on the etiology of this disease and the psorosis-ringspot diseases is available, it is preferable not to add a new name to the literature to avoid increasing the present confusion.

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