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ABSTRACT

Stubborn disease causes important economic losses in most of the citrus growing regions, namely in the Mediterrranean area. Its causal agent is a mycoplasma-like-organism (MLO), Spiroplasma citri, and its vectors are leafhoppers of the Circulifer/Neoaliturus complex.

Two variants of the Circulifer tenellus complex were found in "stubborn" affected areas, in Israel, Circulifer tenellus-A on Atriplex halimus and Circulifer tenellus-P on Portulaca oleracea, in the Jordan Valley and coastal plain, respectively.

A study was carried out in order to characterize the two leafhoppers based on morphology, host preference and vectorial capability. Morphologically they were similar, except for color and the presence of a denticle on the 7th sternum of the female. Host preference was quantified by the longevity and fecundity on several host plants. Both leafhoppers were found to prefer Cruciferae and Chenopodiaceae but differences were observed between the two leafhoppers, namely with Brassica oleracea, Brassica rapa, Beta vulgaris and Matthiola incana. The latter was the most suitable and chosen as the host plant to maintain the cultures and for transmission studies.

The rate of transmission by C. tenellus P after being injected with a Spiroplasma citri isolate was 5.0%, but when the pathogen was acquired by feeding it was 2.6%. With the variant C. tenellus A, the rate of transmission was 42.2% and 11.9% respectively.

An effect of the pathogen on the longevity and fecundity of the variant C. tenellus-A was observed.

Crosses between the two variants did not produce descendents.

Attempts were also made to characterize S. citri isolates found in different geographical regions of Israel. Serological and electrophoretic methods and DNA-DNA hybridization were used. Isolates appeared to be similar, however, those collected from Valencia orange in the coastal plain, showed differences when a Western blot technique was used.

RESUMO

A doença "stubborn" causa perdas económicas importantes na maioria das regiões citrícolas, nomeadamente na area Mediterranea. O agente causal, Spiroplasma citri, e um "mycoplasma-like-organism" (MLO) e os seus vectores são cicadelídeos do complexo Circulifer/Neoaliturus.

Foram encontradas duas variantes do complexo Circulifer tenellus em áreas afectadas pela doença "stubborn", em Israel, uma C. tenellus-A colectada em Atriplex halimus e outra, C. tenellus-P em Portulaca oleracea, no Vale do Jordão e na planície costeira, respectivamente.

Foi realizado um estudo de modo a caracterizar os dois cicadelídeos, baseado na morfologia, preferencia de hospedeiro e capacidade vectorial.

Morfologicamente as duas variantes sao similares, excepto na cor e na presença de um denticlo no sétimo esternito da fêmea. A preferencia de hospedeiro foi quantificada através da longevidade e fecundidade em diferentes plantas hospedeiras. Verificou-se que os dois cicadelídeos tinham preferência por plantas das famílias Cruciferae e Chenopodiaceae embora se tenham observado diferenças, nomeadamente com as especies botanicas Brassica olearacea, Brassica rapa, Beta vulgaris e Matthiola incana. Esta última foi escolhida para manutenção das culturas e experiencias de transmissão.

A taxa de transmissão por C. tenellus-P após ter sido injectada com S. citri foi de 5.0% e 2.6% após aquisição do agente patogenico por injestão. Com a variante C. tenellus-A a taxa de transmissão foi de 42.2% e 11.9% respectivamente.

Verificou-se que S. citri afectava a longevidade e fecundidade de C. tenellus-A.

Cruzamentos entre os dois cicadelídeos não produziram descendentes.

Foi também feito um estudo preliminar a fim de caracterizar isolamentos de S. citri colhidos em diferentes regiões de Israel, utilizando métodos serologicos, analise de proteinas por

electrophorese e hibridizaçao de DNAs. Apenas um isolamento, extraido de laranja Valencia colhida na planicie costeira, apresentou diferenças quando a técnica de "Western blot" foi aplicada.

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Abreviations

AAP	-	Acquisition access period
AAF	-	Acquisition access feeding
BSA	-	Bovine serum albumin
EDTA	-	Ethylendiaminetreta-acetic acid (disodium salt)
KD	-	Kilo dalton
IP	-	Incubation period
IAP	-	Inoculation access period
O.D	-	Optical density
PBS	-	Phosphate buffer saline
PVP	-	Polyvinylpyrrolidone
Ro	-	Net reproductive rate
rpm	-	Rotations per minute
SDS	-	Sodium dodecyl sulfate
SDS-PAGE	-	Polyacrylamide gel electrophoresis
TEMED	-	Tetramethylenediamine
Tc	-	Generation time
U.V.	-	Ultra violet

INTRODUCTION

The citrus stubborn disease (CSD) (=little leaf disease of citrus) causes reduction in yields and fruit quality in major citrus varieties. Although it does not kill trees, it often affects them so severely that they become economically nonprofitable. Young orchards are particularly vulnerable to infection. The little leaf syndrome, described in Israel, was the first to be reported by Reichert and Perlberger (1931). However the term "stubborn" was coined in California to describe the syndrome which was seen in 1915 (Fawcett et. al., 1944). Thereafter it has been recorded in most citrus producing countries in the Mediterranean. Igwegbee and Calavan (1970) associated the disease with mollicutes (mycoplasma-like-organisms [MLOs]) which were found in sieve tubes of stubborn affected citrus from California. Saglio et al. (1971) and Fudl-Allah et al. (1972) isolated and cultured a mollicute from young leaves of stubborn affected citrus seedlings and, in 1973, Saglio et al., characterized the isolated agent and named it Spiroplasma citri.

Historically, S. citri was the first mollicute infecting plants that could be cultured and for which Koch's postulates were fulfilled (Daniels et al., 1973; Markham et al., 1974). Today, about 30 spiroplasmas species are known, classified in 23 different serotypes (Tully et al., 1987) and a family Spiroplasmataceae established. However, the only ones known as plant pathogens are S. citri, S. kunkelii, the corn stunt spiroplasma , and S. phoeniceum, the Vinca spiroplasma. Spiroplasmas have the characteristics of MLO's but are distinguished by a spiral forming structure from which the name Spiroplasma is derived.

Since 1973, when Lee et al. first cultured S. citri from Circulifer tenellus in California, leafhoppers have been shown to be the vectors of this spiroplasma. It was transmitted by Scaphitopius nitridus from infected citrus to Vinca rosea

(Kaloostian et al., 1975 and Oldfield et al., 1976). Oldfield et al., (1977) reported citrus-to-citrus transmission by S. nitridus and C. tenellus. However, Bove (1979) found that many leafhopper species harboring S. citri (as determined by ELISA) could not transmit it to test plants. Fos et al. (1986) found Neoaliturus haematoceps transmitted a Syrian S. citri isolate, but not an Israeli isolate, from Vinca rosea to citrus.

In the Near East, distinction between morphologically similar leafhopper species, in the C. tenellus complex, is difficult to establishe, hence the problem in identifying natural vectors. But, information on leafhopper vectors, the role of each vector on the epidemiology of S. citri as well a potential natural hosts is necessary to develop more efficient control measures.

S. citri is known to be harbored by many herbaceous host in the U.S.A. (Oldfield and Calavan, 1982; Oldfield et al., 1977). Evidence supports the idea that transmission occurs from herbaceous hosts to citrus rather than within groves from citrus to citrus.

S. citri isolates may differ in their transmissibility by different leafhopper vectors species and by their pathogenicity. Oldfield et al., 1984, were unable to transmit an isolate from S. citri (originally obtained from citrus) by Macrosteles fascifrons.

In Israel two variants of N. haematoceps, trapped on the coastal plain of Israel on Salsola kali and on the Golan Heights on Chenopodium album respectively, failed to transmit the agent (Klein and Raccah, 1992). However, transmission was successful from an S. citri infected Matthiola incana plant by a variant of C. tenellus, trapped on Atriplex halimus in the Jordan Valley (Klein et al., 1988). A second variant of the C. tenellus complex was trapped in the same area and in the coastal plain, on Portulaca oleracea. This variant was morphologically similar, but its vectorial capacity had not been evaluated.

The objectives of this study were the following:

1. To characterize variants of C. tenellus and S. citri in Israel and their role in S. citri transmission.
2. To study the interrelationships between vector and pathogen.

3. To relate differences between leafhopper variants and pathogen isolates to transmissibility and pathogenicity.

The characterization of the leafhoppers will include a morphological comparison with the genus Circulifer and a comparison within the two variants. Host preference will be studied mainly with plants of the Cruciferae and Chenopodiaceae families.

Transmission experiments of various S. citri isolates by the two variants of the C. tenellus complex will provide data which can help in the characterizition of the insects as well as of the isolates and verify interrelationships between vector and pathogen.

Additional techniques will be applied to caracterize the isolates including serology and hybridization which can give a more clear indication of differences between isolates, and their role in transmissibility.

LITERATURE REVIEW

The leafhopper

History: The leafhopper *Circulifer tenellus* (Homoptera: Cicadellidae) was first described as *Thamnotettix tenella* by Baker (1896) and later placed in the genus *Euttetix* by Forbes and Hart (1900). Between 1900 and 1948 it was referred as *Euttetix tenellus* in all the literature.

Extensive studies have been done on this insect, since 1899, when a disease appeared on sugar beet, in California. It was referred to the "California Blight", "Western Blight" or "curly leaf". The following year it had reached all beet growing areas in the Western United States, causing a serious threat to the industry.

Gillette and Baker (1895) had recorded the leafhopper's presence on sugar beet. Ball (1907) associated the feeding of *E. tenellus* with the "curly leaf" disease. Shaw (1910) confirmed Ball's findings and demonstrated that the pathogenic agent was a virus (which is now known as Beet Curly Top Virus - BTCV) and its vector the beet leafhopper.

Detailed studies of *E. tenellus* followed, concerning its preferential hosts, breeding areas, migrations, life cycle and distribution.

The beet leafhopper was proved to be a vector of other plant pathogens i.e. *Spiroplasma citri*, causal agent of citrus stubborn disease (Lee *et al*.,1973, Oldfield *et al*., 1976), a Mycoplasma-like organism (MLO) virescence agent to periwinkle (BLTVA) (Oldfield *et al*., 1977) and Virescence of horseradish (Eastman *et al*., 1984).

Distribution

In North America *C. tenellus* is widely distributed in most of the western states (Young and Frazier, 1954). Oman (1936) found morphological similarities between *Thamnotettix indivisus*

Haupt (1927), from Palestine and T. ignavus Matsumura collected in Sicily, to those of E. tenellus, and in 1948 determined that tenellus should be associated with a number of Mediterranean species included in the genus Circulifer Zakhvatkin (1935). The author, thereby established, the presence in the Mediterranean region of Circulifer tenellus.

The Mediterranean distribution was later confirmed by Frazier (1953) and Young and Frazier (1954), who made a preliminary survey of the region to locate populations of Circulifer tenellus and obtain information on distribution, host plants and natural enemies. They reported that, the genus Circulifer, was found to be common over all the areas surveyed, i.e. the Mediterranean basin, and was collected in Algeria, Tunisia, (Tripolitania), Egypt and Spain.

However, the Mediterranean collections made by Frazier indicated that this species was not found on sugar beets in this region and more evidence to identify the species was desirable. However, Freitag et al. (1955) conducted successful crossbreeding tests between C. tenellus males from Morocco and females from California. The Morocco males and F1 individuals were vectors of curly top virus which was additional evidence that the African leafhoppers were the same species as the North American. This prooved that C. tenellus occurs in the Old World and, being the only species of the genus known to occur in the Western Hemisphere, Freitag suggests that the beet leafhopper is a native species of the Mediterranean area where its close relatives occur and was probably introduced into Western North America.

It has been recorded from South Africa and Southwest Africa (Young and Frazier, 1954, Linnavuori, 1961), British Columbia, Mexico and Jamaica, (summarized by Young and Frazier, 1954), Puerto Rico (Caldwell and Martorell, 1952), Florida (DeLong, 1925) and India (Bindra et al., 1970).

Since then, other authors have considered the genus Circulifer as synonym or as a subgenus of Neoaliturus (Linnavuori, 1962) and (Emel'yanove, 1961). However, Oman (1970) suggested the name Circulifer tenellus should be applied to the beet leafhopper until some understanding of the genetics of the C. tenellus complex clarifies the taxonomic problems which still

exist. However, he subdivides all known species belonging to this genus under two genera: Neoaliturus for species with their aedeagal rami in a semi-circle, and Circulifer for species with their aedeagal rami in a full circle, although male genitalia can no longer serve as the only characteristic for classifying these species, as specimens which have similar male genitalia differ from each other.

Host Plants

C. tenellus is by nature a desert and semi-desert leafhopper. Before the intervention of man in the leafhopper's natural habitat, this insect maintained a certain pattern of dispersal and migration in search of food and breeding sites in accordance to climatic conditions. When natural habitat is disturbed by man, new adaptations take place, i.e. the search for new hosts which include economic crops. On the other hand when alkali lands are placed under cultivation in the arid and semi-arid regions, and the alkali salts are brought to the surface the Atriplexes and other plants adapted to this type of soils will increase, offering opportunities for enormous multiplication of the insect (Severin, 1922).

The host plants on which the leafhopper breeds are almost entirely annuals which will germinate whenever sufficient heat and moisture are available and grow rapidly for a short time, before maturation and death. The leafhopper is however well equipped to cope with this conditions, and has become adapted to a wide range of host plants (Cook, 1967).

Knowlton (1932) listed 108 species of plants on which the leafhopper was captured. Of these, 36 were breeding hosts, 17 were holdover hosts, and the remainder were plants on which the leafhopper was found only during migrations. Severin (1933) listed 39 species. Douglass and Hallock (1957) report 43 host plants were leafhoppers were captured. Cook (1967) summarized all the host records for C. tenellus and combined them into a tripartite list of winter and spring (7 families), summer (4 families) and holdover hosts (4 families) that are important in

the Western States. The most important of the listed families are Chenopodiaceae, Cruciferae and Compositae. The latter being predominant in the holdover hosts group.

Cultivated plants

The onset of extensive crops, such as sugar beet, in the United States, in the beginning of the century, caused an outbreak of the sugar beet leafhopper, which affected other cultivated plants. Severin and Henderson (1928b) describe that, when in 1918-1919 thirty thousand acres of sugar beet were destroyed by curly top, cantaloupe (Cucurbitaceae) and spinach (Chenopodiaceae), were also found to be infected by the same virus. In the following years, beans (Carsner, 1925) and squash (McKay and Dykstra ,1927) were added to the list of affected crops.

Severin (1927) published a list of crops which are naturally infected by the BCTV in California among which were solaneceous and cruciferous plants. The following year, Severin, establishes the etiological relationship of E. tenellus to tomato yellows, which in turn, is also caused by BCTV (Severin, 1928a). The same author, in association with Henderson, publishes a list of field and garden plants, in California, of three plant families (Chenopodiaceae, Leguminosae and Cucurbitaceae), and a year later adds an additional list of economically important plants.

Number of generations

Ball (1917) studied the life history of the beet leafhopper as it occurs in the sugar beet fields, in Utah, and found a single annual generation on beets. Lawson and Piemeisel (1943) reports that in the San Joaquim Valley the beet leafhopper passes the winter in the adult stage and one or two generations develop in the spring on host plants which form part of the winter-annual cover. When these annuals dry in April or May, movements to other weeds and cultivated crops occur, where they transmit the virus.

During the summer, the insect breeds for several generations on annual weeds and sugar beets and the overwintering generation, is produced, on these plants in the autumn. The insects move back to the plains and foothills and survive on any available green plants until the rains come and germination of winter annuals takes place.

Cook (1967) refers to the life history in much the same terms, mentioning however that the females are fertilized in the autumn and the males die during winter, with no true hibernation. The insect is active whenever the weather is warm.

Several authors (Ball, 1917; Stahl, 1920; Knowlton, 1932; Harries and Douglass, 1948; Hills, 1937) studied the number of generations per year and the conclusions were diverse. Cook (1967) summarizes this information by stating: the leafhopper apparently has three broods annually in the northern part of the San Joaquim Valley, four to five broods in central California and the southern desert, and possibly an additional brood near and south of the Mexican border.

Migration and disssemination of C. tenellus

Numerous studies which have been done on the migratory behaviour of C. tenellus. Ball (1917), suggested that leafhoppers migrate from spring breeding grounds to sugar beet fields. This was confirmed by Severin (1921). Dorst and Davis (1937) followed long distance movements of the insect by establishing small plots of host plants along the presumed flight paths in Utah.

Severin (1933) concluded flight was stimulated by host senescence; Cook (1945) believed it was associated with brood maturity.

Lawson and Chamberlin (1951) studied the dissemination of the beet leafopper in California and verified that the major factors that control the magnitude of leafhopper flights are as follows: Number of adults present, host-plant conditions, physiological condition of the leafhoppers, temperature, light or time of day, and wind direction. But, they added that, the way in which the factors combine, may often be more important than any

single influence.

Douglass (1954) studying the occurrence of C. tenellus or BCTV in areas far removed from the permanent breeding grounds concluded that the beet leafhopper is a migratory insect which occasionally extends its normal range and possibily can become acclimated to new areas. During long periods of drought, migration may extend over thousands of miles.

A series of environmental and biological parameters were investigatetd by Yokomi (1979) to determine the factors affecting spring dispersal of the beet leafhopper in the foothills of the San Joaquim Valley. The author concluded that oviposition was not dependent on photoperiod and temperature. Flight occurred when host plant conditions were appropriate. However, both maturity and host plant senescence are influenced by temperature, photoperiod, and availability of water. These factors change concurrently in the environment.

In a recent study (Gorder, 1990) characterized flight of field populations of beet leafhoppers. Behavior was related to physiological status of the insect. At certain times of the year flight is migratory rather than trivial, supporting the hypothesis that this flight is driven by environmental imperatives rather than by maturity status. Thus, the insects must be capable of moving and reproducing in several different sites. It was also showed that the tendency for the insects to leave stressed plants suggests that moisture stress in host plants may serve as important stimulus for flight. These results support findings from field studies (Yokomi, 1979) that correlated soil moisture which is available to host plants, with the onset of flight. The author also showed that leafhoppers reared on different host plants differ significantly in their lipid content, that is, in the amount of flight fuel reserves.

Biology of C. tenellus

Reproduction - This species is bisexual. Perkes (1970) found that the optimum temperature for fertilization, under laboratory conditions, was between 26.7 and 32.2°C. The largest rate of

fertilization (95%) was recorded at 29.5°C. One maiting is sufficient to fertilize all the eggs of a female during her life time. The male is polygamous. Mating is common during migration, and the number of gravid females usually increases from near 10, to 85 or 90 percent (Lawson and Chamberlin, 1951).

Preoviposition - The mean incubation periods of the egg stage, at a constant temperature, ranged from 5.8 days (at 31.9°C) to 34.4 days (at 16.7°C) and the minimum thermal threshold for embryonic development , a reported by Yokomi, 1979, was 13.0°C.

Carter (1930) and Severin (1933) observed that ovarian development halted in the fall.

Harries and Douglass (1948) noted that the preoviposition period could range from 5 to 6 days (in midsummer) to 6-7 months (in winter).

Oviposition - Eggs are inserted, in a row, beneath the epidermis of leaves or stems of the plant, in slits made by the ovipositor. The maximum fecundity recorded for a female of C. tenellus was 675 eggs, the average overwintering female depositing 300 to 400 eggs (Harries and Douglass, 1948). Harries and Douglass (1948) determined that the duration of the egg stage ranged from 5.5 days at 37.8°C to 43.8 days at 15.6°C.

Severin (1931) reported that, during the summer months, the number of days necessary for incubation will average about 10 days. In the case of overwintering eggs, the incubation period often ranges between one to several months.

Nymphal development - The nymphal stage of C. tenellus consists of 5 instars. Severin (1921) studying the life history of the beet leafhopper, in California, observed that the nymphal periods varied from 23-37 days from April to October and the egg and nymphal periods varied from 37-99 days.

Carter (1930) compared egg and nymphal development at different temperatures and showed that with the diurnal fluctuations obtained, the time for the egg period and the nymphal stages was approximately equal to the time required for these same stages at 23.4°C constant temperature, and reported 12

days for the average egg period and 22 days for the nymphal period.

Harries and Douglass (1948) determined that total duration of the nymphal stage ranged from 13.0 days at 35°C to 75.4 days at 18.3°C. Their data also indicate that the minimum threshold for development was 14.4°C and calculated that the egg stage required 111.1 degree-days while nymphal development required 250.0 degree-days. Yokomi (1979) obtained similar results reporting the rates of embryonic and nymphal development to be related linearly to constant temperatures between 16.7 and 33.4°C.

Citrus stubborn disease

Distribution in the United States - Stubborn disease of citrus was observed in California in the early 1900s and first described by Fawcett in 1944 (Fawcett et al., 1944), in " Washington" navel orange (Citrus sinensis L.) trees.

The term "stubborn" was first used as a disease name, in 1929, by J.C. Perry (Fawcett et al., 1944) who verified that some influence was transmitted from stubborn trees into apparently good buds used in topworking.

By 1944, Tidd estimated that the disease reduced production of naval oranges by 30-50%. During 1945-1953 the importance of "stubborn" increased, especially in navel oranges and grapefruit. Research was then accelerated by the University of California and the U.S. Department of Agriculture in 1954. Calavan (1969) reports that the number of stubborn affected trees , in the citrus-growing areas of California, had increased to about one million.

In the Mediterranean area - Chapot (1959) reports that in 1949, a citrus grower in Morocco brought to the attention the fact that "Washington" navel orange trees had a tendency to produce blossoms all the year through. Fruits were distorted and were of no comercial value. By 1951 symptoms had shown up on other trees. From 1952 to 1957 a survey was done in the Mediterranean

area and was found that the disease was present in all groves examined, namely in Algeria, Tunisia, Lebanon, Syria, Turkey and Corsica.

Garcia et al. (1976) reports that "stubborn" was detected in citrus groves, in Spain. Severe symptoms of citrus disease have been observed in Cyprus on sweet orange trees (Kyriakou and Ioannou, 1989).

In Israel, the disease then called " little leaf of citrus ", has been known since 1928 (Reichert and Perlberger, 1928). A survey done in 1969, by Pappo and Baumann, showed that incidence of stubborn, on citrus groves, ranged between 5 and 50%.

The causal agent of stubborn

The citrus stubborn agent was believed to be a virus. However mycoplasma-like bodies were discovered in phloem tissues of plants, with stubborn symptoms, by Doi et al. (1967). In 1970, the same type of microorganism was observed independently by Igwegbe and Calavan, in California, and by Lafleche and Bove in France. It was then cultured on solid and liquid media from young shoots and leaves of stubborn-affected trees (Saglio et al., 1971; Fudl-Allah et al., 1971; Saglio et al., 1973) as well as from aborted seeds of affected fruits (Fudl-Allah et al., 1972), and from the leafhopper C. tenellus (Lee et al., 1973).

In 1972, the organism was fully characterized as being a mollicute with helical morphology and motility and was given the name of Spiroplasma citri (Saglio et al., 1973).

S. citri was the first mollicute of plant origin obtained in culture, and which could fulfill Koch's postulates, i.e., experimental inoculation of this culture agent into healthy citrus, production of disease, and recovery of S. citri from experimentally infected plants (Daniels et al., 1973 and Markham et al., 1974); both researchers, working with the leafhopper Euscelis plebejus (Fallen), microinjected S. citri culture and demonstrated transmission and reisolation of S. citri to white clover and citrus, respectively. These reports provided the first

real evidence that the causal agent of stubborn and little leaf is not a virus but a phloem-confined microorganism.

Biology of Spiroplasmas

Classification and Phylogeny - Spiroplasmas are classified as mycoplasmas, Class Mollicutes, Order Mycoplasmatales, Family Spiroplasmataceae. The 5S rRNA sequences of eubacteria and mycoplasmas have been analyzed and a phylogenetic tree constructed (Rogers et al., 1985). Mycoplasmas form a phylogenetic group that , with Clostridium innocuum, arose as a branch of the low G+C Gram-positive tree. The initial event in mycoplasma phylogeny was formation of the Acholeplasma branch; hence, loss of cell wall probably occurred at the time of genome reduction to approximately 1000 MDa. A subsequent branch produced the Spiroplasma. This branch appears to have been the origin of sterol-requiring mycoplasmas. The gene structure of spiroplasmas can be expressed in a bacterium, even though the spiralin gene (the major membrane protein of S. citri) has remained, up to now the only mollicute gene that could be fully expressed in E. coli (Mouches et al., 1985). The reason resides in the fact that, in the spiroplasmas, the codon UGA has progressively replaced the codon UGG, which is the unique codon for tryptophane in the genetic code designated "Universal" (Yamao et al., 1985). However, the codon UGA is not a termination codon, as in bacteria, bu codes for tryptophan. Therefore, a UGA codon specifying "tryptophan" in spiroplasmas, will be read as a stop codon in bacteria and will result in premature termination of protein synthesis. Only spiroplasmas genes without UGA codons will be fully expressed in E. coli which is probably the case with the spiralin gene since spiralin contains no tryptophan (Bove, 1988). Due to the fact that the Guanine plus Cytosine (G+C) content of the DNA of spiroplasmas is low, ranging from 24-30 mol%, and the persistence within spiroplasmas of the codon UGG as well as the codon UGA may reflect the fact that spiroplasmas and Acholeplasmas are the first mollicutes which have evolved from the Gram-positive bacteria (Woese, 1987).

Since the first culture of S. citri, in the 1970s, 30 different spiroplasmas are known and probably many more are still to be discovered. Spiroplasmas are classified on the basis of their serological relationships, the G + C content of their DNA, one and two dimensional analysis of their proteins on polyacrylamide gels, DNA-DNA hybridization and biological properties. Tully, et al. (1987) classifies the known spiroplasmas into 23 groups. Group I is subdidived into 8 subgroups, containing the three known plant pathogenic spiroplasmas: S. citri (citrus stubborn), S. kunkelii (corn stunt disease) (Whitcomb et al, 1986) and S. phoeniceum (periwinkle disease) (Saillard, et al., 1984).

S. citri was placed in subgroup I-1 and five strains have been listed and approved by the International Committee for Systematic Bacteriology: the Morocco R8A2T (27556) citrus strain, the California C189 (27665) orange strain, the Israel NCPPB (27565) orange strain (Tully et al., 1987), the A2-103 (33723) orange strain (Allen and Donndelinger, 1982) and the Illinois HR-101 (33451) horseradish brittle root strain (Raju et al., 1981).

Structure and Function - Spiroplasmas are unique among wall-less microorganisms in their capacity to maintain helical shape and to exhibit several types of motility (including translational movement, flexing and rotation about the longitunal axis) during log-phase growth, in viscous liquid media, as well as when observed in sieve tubes of infected plants. By contrast, the morphology of S. citri, observed in leafhoppers, is not helical, but appear roughly spherical.

Lack of cell wall in spiroplasmas implies absence of peptidoglycane, hence insensitivity to penicillin, which inhibits one of the last steps in peptidoglycane biosynthesis (Bove, 1988). On solid medium, S. citri produces fried egg-shaped colonies which is related to the absence of cell wall. Spiroplasmas display chemotaxis i.e., they are attracted by certain substances and repelled by others. This characteristic may be involved in the movement of S. citri in the phloem elements (Daniels et al., 1980). It grows from short elementary helices into longer parental helices. The latter divide by

constricton, producing elementary helices. During elongation of an elementary helix, growth seems to start at one end of the helix (polar growth) (Garnier, 1984). Regarding the movement of these organisms, no flagella, periplasmic fibrils or other organelles of locomotion are present, but intracellular fibrils have been demonstrated. Towsend and Plaskitt (1985) have seen in transmission electron microscope of thin sectioned cells of the honeybee spiroplasma revealed evidence of a helically twisted ribbon closely associated with the cytoplasmic surface of the plasma membrane and concluded that spiroplasma fibrils are arranged in a single helically twisted ribbon rather than forming a contractile sheath or branched axial fibre. However the authors conclued that the maintenance of helical morphology by a single twisted ribbon implies that an alternative system is responsible for maintaining the filamentous form of the cell. Furthermore, it is difficult to envisage how the fibril ribbon could generate the rotary twisting movements.

Transmission of S. citri

Vectors in the U.S.A. - Following the identification of S. citri as the causal agent of stubborn disease in citrus and the subsequent isolation of the organism from field-collected C. tenellus, from "Madam Vinous" sweet orange seedlings at Riverside, Califronia, (Lee et al., 1973) two other leafhoppers were found harboring S. citri, i.e. Scaphytopius nitridus (DeLong) and Scaphytopius acutus delongi (Young) (Kaloostian et al., 1975; Oldfield et al., 1976) and both reproduce on citrus, but proved to be minor vectors. C. tenellus does not reproduce on citrus but reproduces on a wide range of other plants including several brassicaceous weeds that occur commonly in citrus-growing areas and which were identified as natural hosts of S. citri (Oldfield and Kaloostian, 1979). Furthermore, this organism was isolated from C. tenellus, collected from areas far removed from citrus plantings, which suggested the existence of non-citrus natural host plants and a more complex epidemiology for S. citri (Oldfield et al., 1975). Transmission to single citrus plants by

C. tenellus and S. nitridus was demonstrated after the insects had ingested S. citri by feeding through parafilm membranes (Rana et al. 1975). In the same year, Kaloostian et al. provided the first evidencefor plant to plant transmission by S. nitridus, to Madagascar periwinkle, after feeding on a stubborn diseased citrus plant (Kaloostian et al, 1975). The following year reports of transmission of S. citri by C. tenellus from field collected insects to Madagascar periwinkle (Oldfield et al., 1976) and the isolation of S. citri from crucifers, Sisymbrium irio and Brassica rapa (both hosts of C. tenellus) (Calavan et al., 1976), contributed to the incrimination of this leafhopper as a natural vector.

Among the various leafhoppers known to transmit S. citri in the laboratory and, of those collected and assayed for its presence, from 1974 to 1982, only C. tenellus was frequently found to be naturally inoculative (Oldfield et al., 1984).

Vectors in the Mediterranean countries - The fact that in 1977, S. citri was for the first time isolated, in the Mediterranean region, from a Madagascar perinwinkle plant, found in Morocco (Bove et al., 1978), made it imperative that a thorough survey should be made to determine which vector/s were transmitting S. citri. Bove et al. (1979) reports that from 34 species of leafhoppers collected in Morocco, 7 found positive by ELISA. However the organism could only be cultured from Neoaliturus haematoceps (Mulsant and Rey).

Fos et al. (1986) surveyed several regions i.e. Morocco, Turkey, Syria and Corsica between 1978 and 1985 and from 78 species of leafhoppers collected and tested, culture of S. citri was only obtained from N. haematoceps. Transmission tests were done and proved behyond doubt that this species was a natural vector in the Mediterranean area. The gendre Scaphytopius was never found in this region (Fos et al., 1986) and only small numbers of (Circulifer) Neoaliturus tenellus were collected in Morocco, Syria, Turkey and Corsica in a survey made in 1973 by Frazier. However, the author reports abundance of (Circulifer) N. tenellus in Egypt and N. haematoceps in all areas surveyed. The latter species has also been reported as being connected with

the transmission of S. citri to sweet orange trees, in Cyprus, were severe symptoms of the disease has been reported (Kyriakou and Ioannou, 1989).

In 1985-6 a search for the vectors of S. citri, was done in Israel (Klein and Raccah, 1986). Species of the Circulifer/Neoaliturus complex were collected, two belonging to the C. tenellus complex (Klein and Almeida, 1990), one to the C. haematoceps complex and the fourth to the C. opacipennis complex (Klein and Raccah, 1991). After acquisition feeding on Matthiola incana plants infected by S. citri (a red grapefruit isolate), only two populations of the C. tenellus complex were able to transmit the organism to healthy seedlings of various plants including oranges (Klein et al., 1988; Rasooly, 1988; Klein and Almeida, 1990).

Hosts of Citrus Stubborn Disease Pathogen - Stubborn disease is prevalent in California and Arizona and in the southern and eastern Mediterranean areas, on sweet orange (Citrus sinensis), grapefruit (Citrus paradisi), tangelo (Citrus reticulata x C. paradisi), pomelo (Citrus grandis), pomelite (C. grandis x C. paradisa) and some kinds of mandarin (C. reticulata), but is rare on lemon (C. limon) trees. C. tenellus, the main vector of S. citri in the USA, does not survive long on citrus (Oldfield, et al., 1976) but existing evidence indicates that a large reservoir of S. citri is to be found in herbaceous hosts, many of which are recognised hosts of the beet leafhopper.

Sisymbrium irio was the first wild plant shown to be a natural host of S. citri and demonstrated to be a source from which C. tenellus could acquire the pathogen (Calavan et al., 1976; Kaloostian et al., 1976). Reports followed of other brassicaceous weeds hosts of C. tenellus and found to be naturally infected with S. citri (Calavan et al., 1976; Oldfield and Calavan, 1982; Allen and Donndelinger, 1982; Oldfield and Kaloostian, 1979) and many cultivated hosts, mainly from the Cruciferous family (Calavan et al., 1979; Oldfield et al., 1977; Oldfield et Calavan, 1981; Oldfield and Kaloostian, 1979). Plantago ovata, which occurs widey in desert areas of California is a host of C. tenellus and is commonly infected with S. citri

and gether with several brassicaceous weeds can play a important role as winter and spring host for the beetleafhopper as well as source of infection (Flock, 1977). Salsola kali, an important summer and and fall host for C. tenellus has not been found infected with S. citri nor has experimental transmission to this botanical species been demonstrated (Oldfield, et al. 1987).

Horseradish (Armoracia rusticana L.) was found naturally infected with S. citri in northern California (Raju et al., 1981) and in Illinois (Fletcher et al., 1981). It is transmitted by C. tenellus, causing brittle root disease of horseradish (Fletcher et al., 1981).

In the Mediterranean areas, S. citri has been isolated from citrus infected trees as well as from ornamental plants, such as Matthiola incana, in Syria (Fos et al, 1986) and from Madagascar periwinkle, in Morocco (Bove et al., 1978) In Corsica the only species of leafhopper found to be infected in nature was N. haematoceps collected in the "maquis", along the costal area. Plants representing the main species growing in this type of environment were tested for the presence of S. citri, by ELISA, but all gave negative results.

Vector - Pathogen Relationships

Transmissibility - Feeding of insects infected with plant mycoplasmas does not necessarily result in plant infection. Both inter- and intraspecific variation in susceptibility can be observed. Some differences may cause the inability of vector insects to feed on some plants for periods of sufficient length to assure transmission. Inherent nutritional requirements differ among spiroplasma species, so some differences in the degree of infection are probably due to the composition of the phloem sap. Plants have a variety of compounds such as phytoalexins and polyphenols which act in some capacity to limit infections by other types of pathogens although specific defense against mycoplasmal plant pathogens has not been demonstrated. The role of insects as biological reservoir of plant pathogens must also

be considered (Whitcomb and Bove, 1983). Extracts from plants show that anti-spiroplasma activity is present in primary cultures of the microorganism and it is probably due to anti-spiroplasma factors which may temporarily bind to cell membranes and subsequentely imbalance the process of cell replication (Liao and Chen, 1980). Daniels (1979) reporting a simple technique for assaying microbial phytotoxins, in which he tests S. citri, describes its toxin as an unstable, acidic, polar compound of low molecular weight.

Transmission of four S. citri isolates by C. tenellus was studied by Liu et al. (1983). Three methods of acquisition were used: feeding on plants infected by S. citri, injection and membrane feeding. The beet leafhopper could transmit S. citri regardless of the acquisition method, the minimum acquisition feeding period was 6 hr and the minimum transmission period was 2 hr. The latent period, vary with the mode of acquisition, from 10 days,using injected insects, to 24 days by membrane feeding. Rates of transmission were 2-4% when leafhoppers were caged singly on test plants; rates of 70-80% were reached when groups of 20 leafhoppers per plant were used. Experiments to determine transovarial transmission of S. citri gave negative results.

As mentioned previously, the beet leafhopper is also a vector of a virescent agent (BTLVA) which causes disease on many important crops: Oldfield et al. (1977) have shown that when periwinkle plants were infected with both BTLVA and S. citri the normally lethal effects caused by the latter were prevented. The survival of S. citri in herbaceous plants, which are also infected by BLTVA and, are reproductive hosts of C. tenellus, may play an important role in the epidemiology of citrus stubborn disease.

Golino et al. (1987) carried out a study on vector relationship of BLTVA with the beet leafhopper, relative to acquisition, latency and inoculation, and reported that minimum AAP was 5 min. and more than 50% of test plants developed symptoms after exposure to insects that had undergone a 4-hr acquisiton access period (AAP). The minimum latent period in C. tenellus was 12 days and Inoculation Access Periods (IAPs) of 2 days provided high rates of infection in test plants.

Characteristics of transmission, of a horseradish brittle root (BR) isolate of S. citri from Illinois, by C. tenellus were determined by Eastman et al. (1988). Nymphs given AAPs as brief as 45 minutes were inoculative. The minimum incubation period (IP) was 7-9 days, whereas the minimum inoculation access period (IAP) was 15 minutes. The variability in the maximum rates of transmission ranged from 41-90%, which are higher than those reported by Liu et al. (1983) who tested 10 insects per plant compared to individually caged insects used in these experiments.

Transmission of the corn stunt spiroplasma (CSS) by its vector Dalbulus maidis (DeLong and Wolcott) was highly efficient, with a transmission rate of 4.4%(9 days after of starting acquisition) to a maximum of 93% (after 22 days) as compared to transmission of S. citri by its most common natural vector, C. tenellus (2-4%) (Alivizatos and Markham, 1986). These two pathogens are serologically realted, and it has been suggested that whereas CSS has become a specialist with respect to both plant and vectors, S. citri has remained a generalist (Whitcomb, 1981). As a result, S. citri has many plant hosts and can be transmitted by more than one vector.

Pathogenicity

Organisms in the course of multiplication in the insect may cause reduced longevity and/or fecundity. Spiroplasmas must pass through, if not multiply in, at least two cell types - the gut epithelial cells and the salivary acini, however the largest accumulations of spiroplasmas occur in the hemolymph (Whitcomb and Williamson, 1975). The effect of S. citri on its vectors may or may not be lethal. However, its pathogenicity is important as it may elucidate the role of the insect species in the perpetuation of the disease.

Experimental evidence that spiroplasma isolates multiply in their leafhopper vectors has been provided by various researchers: Whitcomb et al. (1973) verified that the multiplication of S. citri in Dalbulus elimatus (Ball), the corn stunt vector, was usually characterised by a decrease in titer the first day after injection and thereafter the titer rose

slowly and reached a peak between 12 and 18 days after injection, but longevity of the insect was not significantly affected. However, Granados and Meehan (1975) reports the pathogenicity of the corn stunt agent to the vector D. elimatus by a decrease in longevity and fecundity. Macrosteles fascifrons (Stal) the vector of aster yellows (MLOs), after being injected with an S. citri isolate showed a decrease in longevity which was associated with a rapid increase in titer of the spiroplasma (Whitcomb et al., 1973). Pathogenicity of S. citri to C. tenellus was demonstrated by Liu et al. (1983). The author reports that simultaneous occurrence of maximum multiplication, maximum transmission and high mortality suggested that the titer of S. citri in its insect vector plays a major role in transmission and mortality.

Alivizatos and Markham (1986) report that the corn stunt spiroplasma multiplies in D. maidis and that spiroplsmas could be isolated from the haemolymph and salivary glands 1 h after injection and at any time subsequently, but longevity of the insects was not significantly affected. Similar patterns of multiplication were observed by results Towsend et al. (1977) when injecting the leafhopper Euscelis plebejus (Fallen) with an S. citri isolate, but spiroplasmas had little effect on the longevity of the leafhoppers.

Spiroplasmas - Identification

Since the taxonomic description of S. citri by Saglio et al. (1973), 30 different spiroplasmas have been discovered (Bove, 1988). Classification of helical, wall-less procaryotes (members of the genus Spiroplasma [class Mollicutes]) has changed since the genus was first described. When genomic and other molecular techniques were applied to spiroplasmas, they confirmed that the genus Spiroplasma consisted of a number of distinct species. Serological and molecular genetic data was proposed as a basis for spiroplasma classification by Junca et al., (1980), and later revised to accomodate isolation and characterization of new spiroplasmas (Whitcomb et al., 1982; Whitcomb et al.,1983). Techniques which have been established are as follows: growth

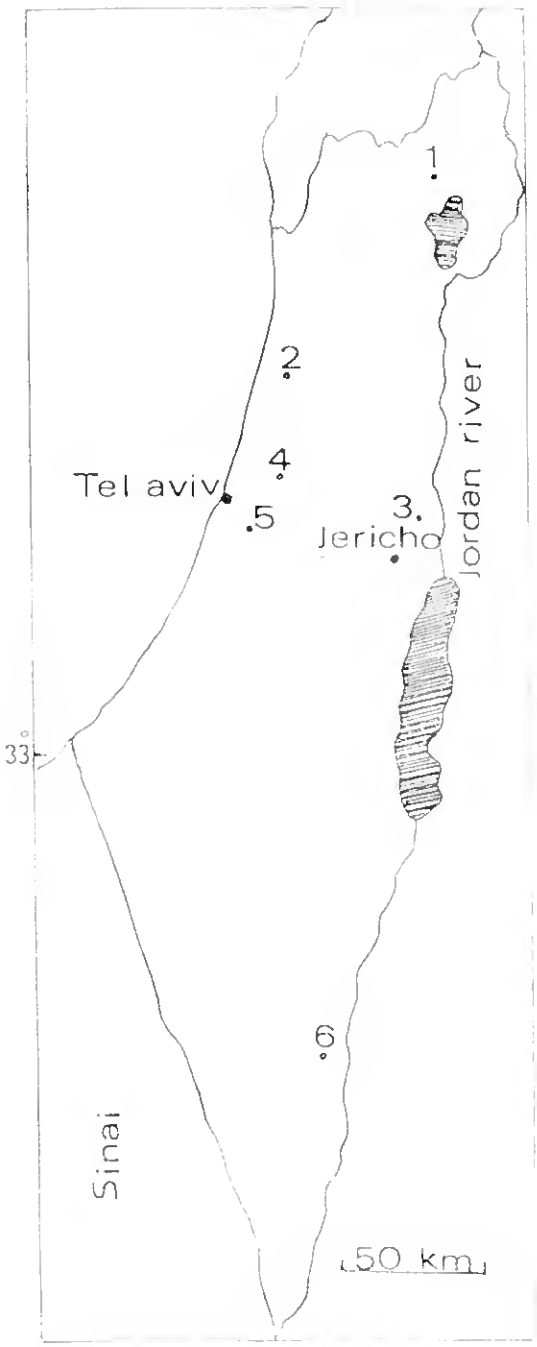
inhibition (Whitcomb et al., 1982); deformation test (Williamson et al., 1978); metabolic inhibition (Davis, et al., 1979); ring interface precipitin tests (Tully et al., 1977); enzyme-linked-immunosorbent assay (ELISA) (Bove and Saillard, 1979); immunodifusion test (Coomaraswamy and Gumpf, 1984); one and two dimensional gel electrophoresis (Daniels et al., 1980; Mouches et al., 1979, Mouches et al., 1982); analysis of the guanine-plus-cytosine contents (Bove et al., 1982) and DNA-DNA homologies (Junca et al., 1980).

Five groups (I to V) and eight subgroups (I-1 to I-8) have been defined. More groups and subgroups are being proposed in order to accomodate new spiroplasmas being characterized.

1. Climatic conditions in Israel. Citrus areas affected by stubborn disease.

Israel is situated between the subtropical arid and subtropical wet zones. This location helps to explain the great climatic contrast between the light rainfall in the south and the heavy rainfall in the north, in all four orographic belts: coastal plain, western mountain ridge, Jordan Valley and Galilea.

Citrus growing areas can been found in the coastal plain, from the north to the south of the Jordan Valley and in the Galilea, meaning a wide range of temperatures and precipitation. However, stubborn disease has developped in all these regions causing significant losses, especially in young orchards, which have been planted in new lands. This implies that vectors are adapted to different climatic conditions or migrate from other regions. Areas from were leafhoppers were collected as well as mean temperatures and precipitation throughout the year are represented in Figs. 1 - 4.



1 - Amiad
2 - Hadera
3 - Tomer (J. Valley)
4 - Modiin
5 - Bet Dagan
6 - Arava

Fig. 1 - Locations in Israel were S. citri isolates were collected and C. tenellus found

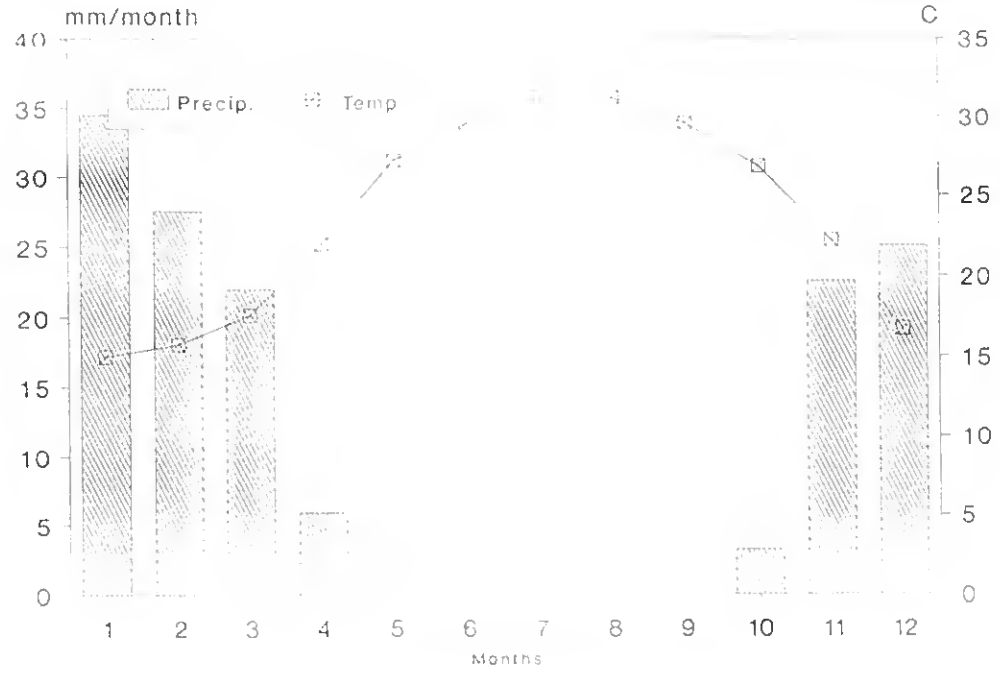


Fig. 2 - Precipitation and average temperature in Jericho, between January and December.

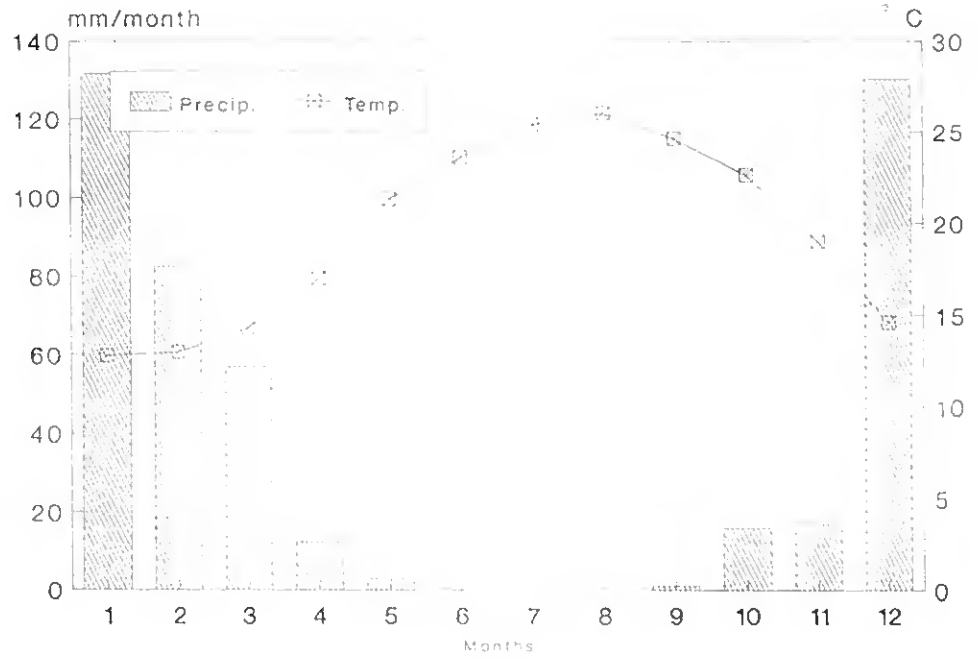


Fig. 3 - Precipitation and average temperature in Bet Dagan, between January and December.

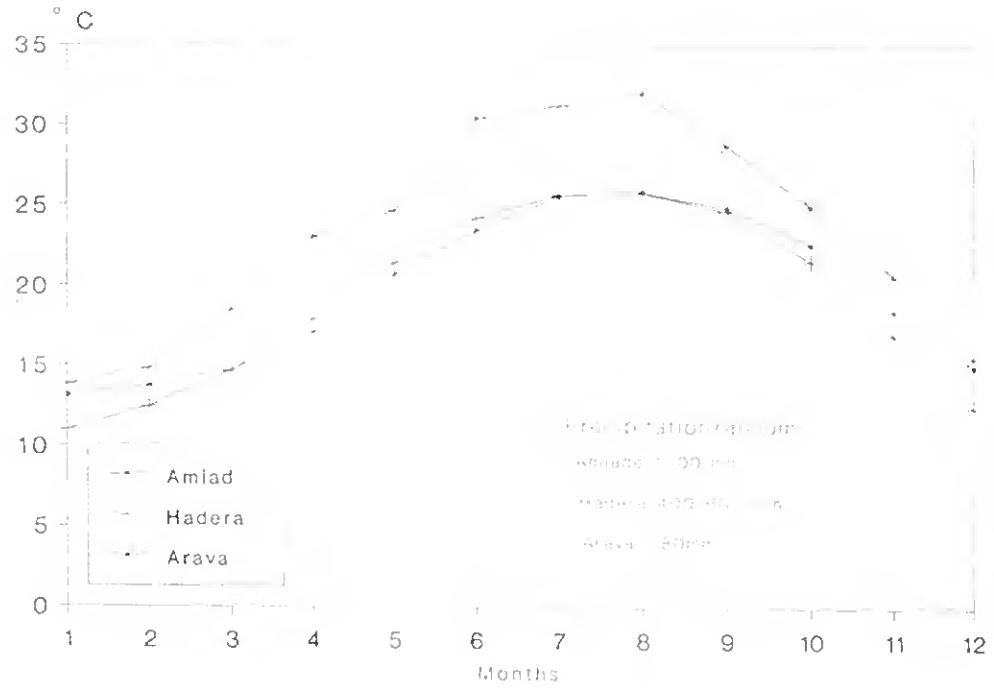


Fig. 4 - Precipitation and average temperature in Amiad, Arava, and Hadera , between January and December.

2. Insects - Collection

The C. tenellus-A variant was found in Israel, breeding on Atriplex halimus L. (Chenopodiaceae). This bush is abundant in the Jordan Valley area.

Leafhoppers which were used to initiate the laboratory colony, were collected in this area, on A. halimus bordering a pomelo grove, adjacent to a dattes plantation. The collecting was done with a portable suction trap (build in the workshop of the Volcani Center), over and around host plants. Insects were sorted out immediately after being collected and placed in glass jars together with some leaves of the host plant. Upon arrival at the laboratory, leafhoppers were sorted again and caged on Matthiola incana plants, in groups of approximately 10 insects. Selection of leafhopers, in the laboratory, was done in a dark box (1mx1mx0.5m), with one glass side, in front of a fluorescent light source to attract the insects.

The second C. tenellus-P variant, found breeding on Portulaca oleracea, in a field neighbouring an orange (Valencia) grove, located in the grounds of the Volcani Center. The method used for collection was the same as for variant C. tenellus-A.

The colonies were augmented during additional trips to the same location.

The leafhoppers were caged on M. incana plants and transferred weekly to new plants, (Cages type A and B), and kept in growth chambers at a temperature of 25 ± 2°C , with constant illumination.

3. Cages

3.1. Rearing Cages

3.1.1. - The wooden rearing cage is shown in Fig. 5a. It is made of a 16cm x 16cm x 40cm frame whith a wooden bottom. A nylon screen was placed at the top and three sides and the front was fitted with a glass plate. A 5mm hole was cut on the top to permit the introduction of the insects with a glass aspirator.

Plants for use in this type of cage were grown individually in plastic pots (15cm diameter), in the greenhouse. When using these cages, insects were transferred to new plants inside the wooden cage described in A.

3.1.2. - The plastic rearing cage (Fig. 5b) was made of polivinyl bottles (7.5 cm diameter x 19 cm height) where the top and bottom were cut out to form a tube. Two rectangular holes (5cm x 3cm) were made on opposite sides and covered with nylon netting. The cage was placed over plants in plastic pot, 10cm diameter The top of the cage was covered with a nylon net held by a rubber band. Insects were introduced through a small hole at the top.

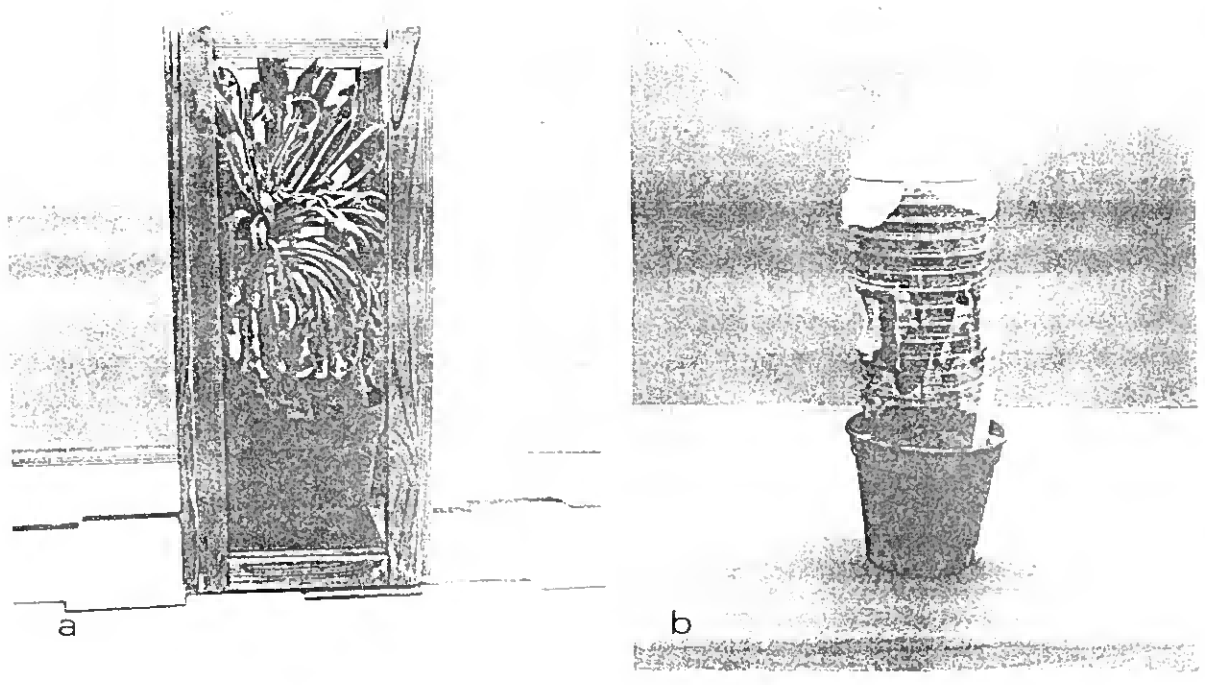


Fig.5 - Rearing cages: (a) The cage is with a wooden frame, netting and glass on the sides. (b) The cage is made of polivinyl bottles.

3.2. Cages used for transmission experiments

3.2.1. - For transmission experiments, cages to cover small M.incana plants (4 to 6 leaves) were used. These cages were made of plexiglass cups (6.5cm x 9cm) with the bottom part cut out and the top covered with nylon netting (Fig 6).

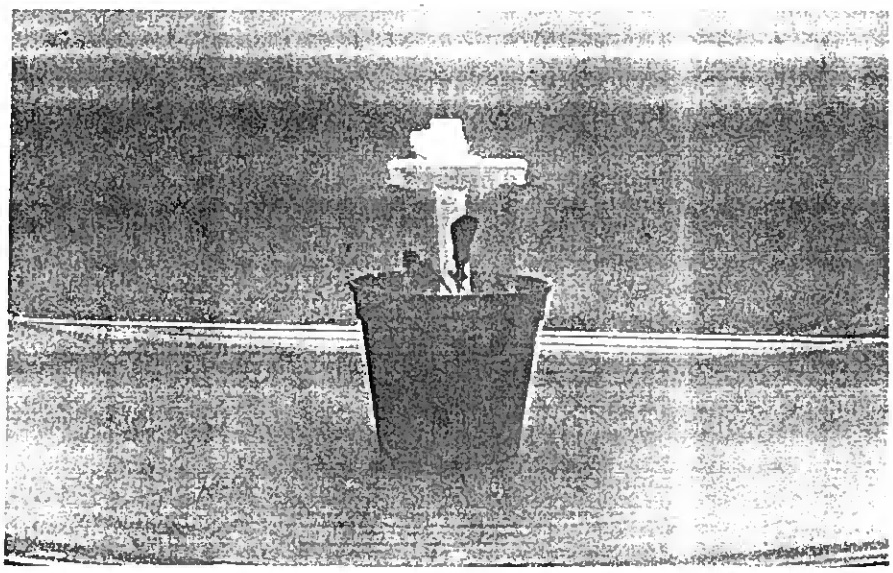


Fig. 6 - Transmission cage made of plexiglass cups.

3.3. Mating and oviposition cages

3.3.1 - These cages consist of a plexiglass vial (7.5 cm x 3.0 cm) with a screw top (Fig. 7a). The bottom was replaced with wire netting (60 mesh). The top part of the screw was cut and replaced with a spring operated lid, lined with foam in order not to damage the leaf and to ensure adequate adhesion.

3.3.2 - Mating and oviposition cage (Fig. 7b) - Made of a plexiglass tube (9.0cm x 3.5 cm) with one end covered with nylon netting. The whole leaf would be introduced into the tube and the top closed with a strip of foam rubber involving the petiole.

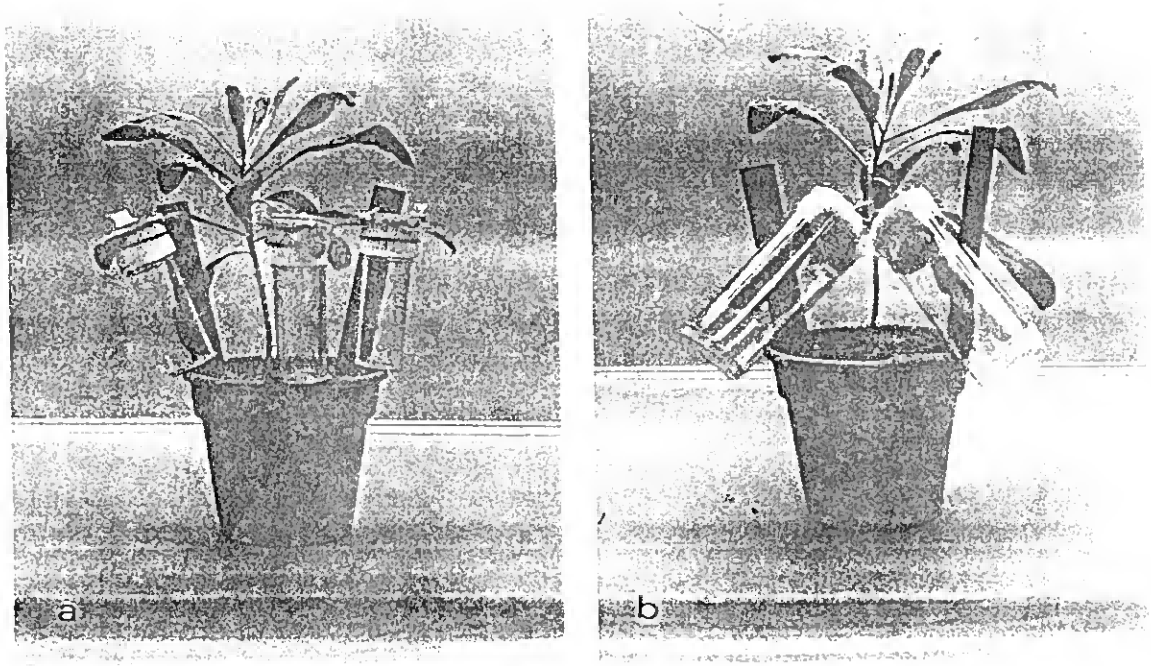


Fig.7 - (a) Cage for oviposition and mating made of plexiglass (b) Mating and oviposition cage of plexiglass

4. Isolation of Spiroplasma citri

S. citri was isolated from aborted seeds of citrus fruits. Several Valencia orange, grapefruits, pomelo, pomelite and clementina were picked, from field grown trees, for isolation. Fruits were taken from trees which showed symptoms of stubborn disease (assimetry in the shape of the fruits - Fig. 8).

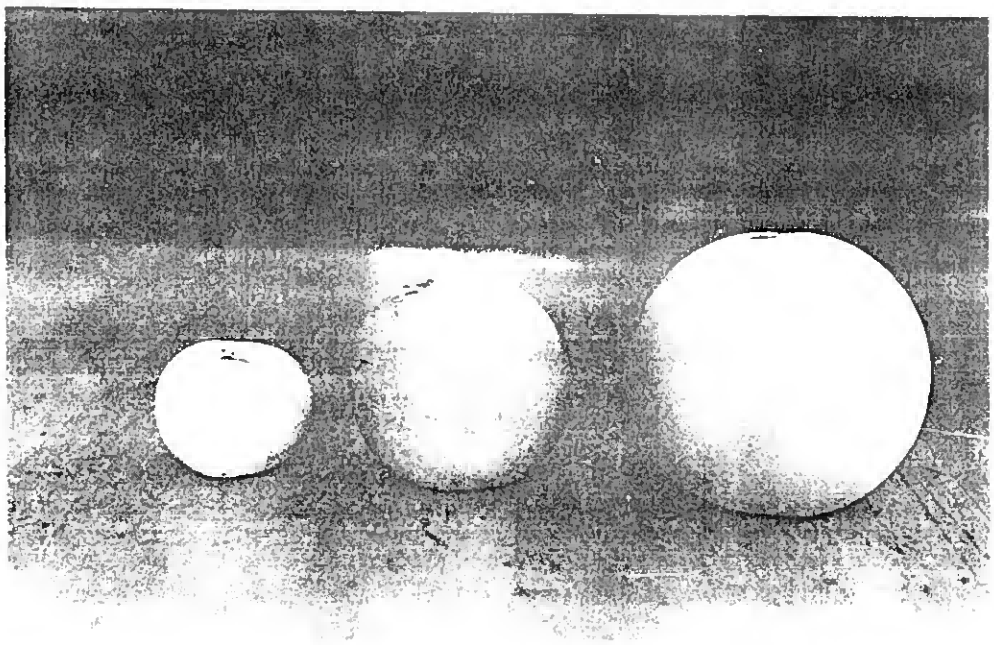


Figure 8. Grapefruits (Citrus paradisi). Right - normal; Left - S. citri infected showing typical symptoms.

4.1 - Isolation procedure:

a) Fruits were surface-sterilized in 70% ethanol, rinsed in sterile water

b) Seeds were asseptically removed from the fruits and rinsed in sterile water, 70% ethanol (1-2 minutes) and again rinsed in sterile water.

c) The seeds were finely chopped on a sterile glass slide, in a small amount of liquid medium (BSR modified) which was then

pipetted to a tube containing 5 ml of the same medium and incubated at 30°C.

The procedure described above was followed for each separate seed of each fruit. Blank controls were incubated from each batch of medium to detect possible contaminants. Growth was detected by the change of colour (red to yellow) due to metabolic by-products (mainly lactic acid) causing a drop in pH. The presence of S. citri was confirmed by examining a sample under dark field microscopy. Spiroplasmas in culture medium have a sigmoid type of growth pattern. In the exponential (logarithmic) phase, the growth rate reaches the constant maximal value which may have a duration of 3-4 days. After the stationary phase, helices become very slender, loose their helical configuration and there is a rapid decrease in the number of organisms (Liao and Chen, 1977). For this reason passages of the cultures were done regularly, by pipetting 1 ml of the culture into 4 ml of medium, incubated at 30°C and following the procedure described above. Cultures were then kept at -80°C after reaching log phase.

4.2 - Culture medium - Modified BSR medium contained 2.5% (w/v) heart infusion broth (DIFCO, Michigan), 0.1% (w/v) glucose (SIGMA), 0.1% (w/v) fructose (SIGMA), 1% (w/v) sucrose (Frutarom, Israel),7% (w/v) sorbitol (BDH Chemicals Ltd. Poole, England), 5ml phenol red (SIGMA) (stock solution: 100 mg phenol, 2.8 ml NaOH 1M, 25 ml distilled water), 440 ml distilled water. Before autoclaving at 120°C for 20 minutes the pH was adjusted to 7.8. After cooling, 50 ml of horse serum (Biological Industries, Israel) which had been previously inactivated at 50°C for 30 minutes, was added to the solution together with penicillin (TEVA, Israel) (300 units/ml). The medium was stored at 4°C.

5. Enzyme-linked Immunosorbent Assay (ELISA)

ELISA was first applied for detection of plant viruses by Clark and Adams (1977). The technique, was modified, for detecting organisms of the class Mollicutes (mycoplasma and spiroplasma) from leaves and seed extracts from stubborn infected trees (Clark et al., 1978). Other authors reported use of ELISA to detect S. citri from infected periwinkles (Vinca rosea) (Saillard et al., 1978; Bove et al., 1979; Clark et al., 1983) and leafhoppers (Bove et al., 1979a). Its usefulness in determining serological relationships between spiroplasmas of different origins has also been documented (Bove and Saillard, 1979; Tully et al., 1980).

ELISA is carried out in plastic polystyrene microtiter plates. The assay is based on the fact that proteins firmly bind to polystyrene (Voller et al., 1976). Firstly, the specific immunoglobulins (IgGs) directed against the corresponding antigens (S. citri, in the present case) are added to wells of the plate. The IgGs will bind to the internal surface of the wells (=coating). These samples containing the antigens are added. If present, the antigen will react and form a complex with the found IgGs . Thirdly, the same IgGs used in the first step are used again, but they have been previously linked to the enzyme alkaline phosphatase (APase) to form a IgG-APase complex (conjugate) (Avrameas, 1969). If antigen is present in a well, it will bind the IgG-APase complex. Hence, the presence of antigen results in the presence of APase. The last step,the substrate p-nitrophenyl phosphate (colorless) is added and is hydrolyzed by the enzyme to form the yellow p-nitrophenol and phosphate. The colour yellow is the evidence for presence of the antigen. The p-nitrophenol produced is directly related to the amount of antigen present. Their color intensity can be determined spectrophotometrically, yielding a quantitative estimate of the amount of antigen available in the sample.

Buffers used in ELISA:

- Coating buffer: pH 9.6. Na_2CO_3, 1.59 g; $NaHCO_3$, 2.93 g; NaN_3, 0.2 g; dissolved in 1 liter of water.
- PBS buffer, pH 7.4. NaCl, 8.0 g; KH_2PO_4, 0.2 g; $Na_2HPO_4.12\ H_2O$, 2.9 g; KCL, 0.2 g; NaN_3, 0.2 g; dissolved in 1 liter of water.
- Washing buffer (PBS-Tween). PBS, 999.5 ml; Tween 20, 0.5 ml.
- Sample buffer (PBS-Tween-PVP). PBS-Tween containing 2% polyvinylpyrrolidone (PVP) MW 25,000 (Kollodon 25, Fluka, AG).
- Enzyme-IgG buffer (PBS-Tween-PVT-ovalbumin) (conjugate). PBS-Tween-PVP containing 0.2% ovalbumin (Sigma Chemicals).
- Substrate buffer. Diethanolamine, 98 ml; H_2O, 800 ml.

5.1 - Preparation of antiserum against S. citri

Antiserum against S. citri was prepared using an isolate of S. citri deriving from a stubborn infected Valencia orange at Bet-Dagan. 0.5 ml of medium containing S. citri were incubated in a medium in which the horse serum was replaced by rabbit serum. S. citri was then fractionated from the medium by a series of differential centrifugations. A formaldehyde (0.2%) treated preparation of the agent was mixed with an equal volume of Freund's incomplete adjuvant. The series of subcutaneous and intramuscular injections were carried out at two weeks intervals. The rabbit was bled one week after the last injection.

5.2. - Purification of σ-globulin (IgG)

Antiserum to S. citri (isolate from Bet-Dagan) was diluted in distilled water (1:10). 10 ml of saturated ammonium sulphate solution was added. Left 60 minutes at room temperature. Centrifuged at 10,000 r.p.m. for 15 minutes and the precipitate was dissolved in 2 ml of 1/2 strength PBS. The serum was dialyzed three times against 500 ml of 1/2 strength PBS at 4°C. Filtration was done through a 3-5 ml DE 22 cellulose pre-equilibrated in 1/2 strength PBS, and filtrate was washed through DE 22 with 1/2 strenght PBS. Effluent was monitored at 280 nm and the first protein fractions were collected. The concentration of IgG was adjusted to approximately 1.4 OD (about 1 mg/ml) at OD 280.

5.3. - Conjugation of Alkaline Phosphatase with IgG

A solution containing 5,000 unites (ca. 0.5mg/ml) of the enzime in ammonium sulfate was centrifuged at 10,000 r.p.m. for 20 minutes to precipitate the enzyme. The precipitate was dissolved in 2 ml of purified IgG and dialyzed three times against 500 ml PBS. Fresh glutaraldehyde solution was added to a final concentration of 0.06% and left four hours at room temperature. The solution was dialyzed three times against 500 ml PBS to remove glutaraldehyde; 0.5 ml of 0.02% sodium azide solution was added to last dialysis cycle to prevent contamination of the conjugate.

CHAPTER I - BIOLOGY OF THE BEET LEAFHOPPER COMPLEX

1. General characteristics of Circulifer tenellus (Baker) (Homoptera: Cicadellidae)

The genus Circulifer was described by Zakhvatkin (1935:111) based on the species Thamnotettix (Jassus) haematoceps (Mulsant and Rey), 1855. Some of the characteristics, as interpreted by Young and Frazier (1954) are as follows: the genus includes all deltocephaline leafhoppers with a biramous aedeagal shaft, the rami of which form a circle (Fig. 9A and Fig. 9B). Specimens range in size from 2.2 to 3.8 mm. Seventh sternum of the female is medially emarginated on the posterior margin; the emargination may or may not bare a small tooth. Males plates are provided with a uniseriate group of macrosetae which are marginal near the base and discal near the apices of the plates. The species Circulifer tenellus ssp. tenellus (Baker) is described by the same authors: color extremely variable from yellow, greenish yellow to green. Length of male 2.8-3.5 mm, female 3.3-3.8 mm. Crown slightly produced, rounded at apex, median length usually two thirds or less the distance between eyes. Female 7th sternum with median excision quite variable even among individual progeny of a single female, usually without a median denticle. Male plates quadrilateral, appearing truncate posteriorly, with a small apointed apex. Style with apical extension digitiform and typically not expanded on inner or outer margin, apex rounded (Fig. 11A and Fig. 11B).

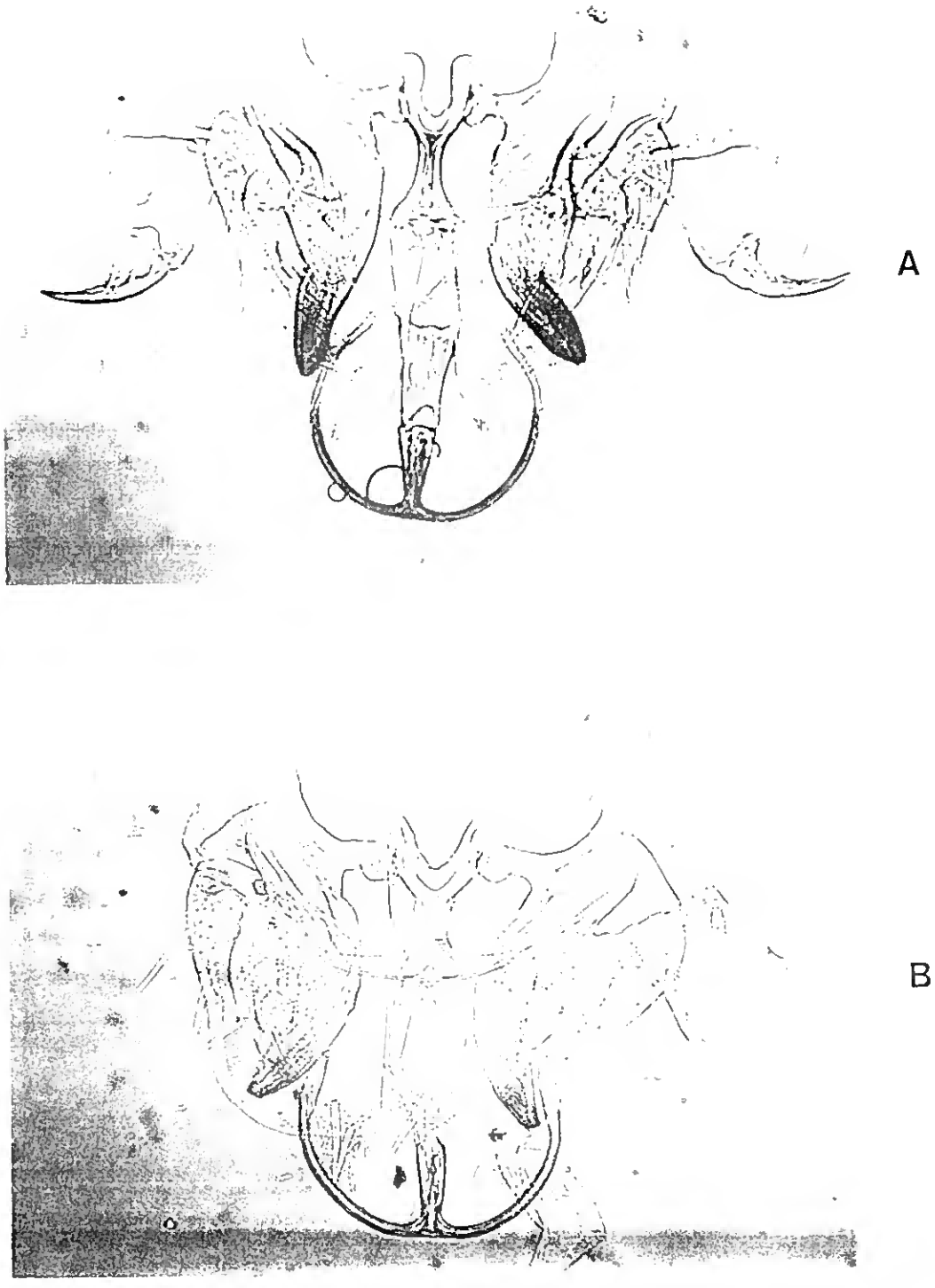


Fig.9 - Males genitalia of Circulifer tenellus from Atriplex (A); and Circulifer tenellus from Portulaca (B). × 400

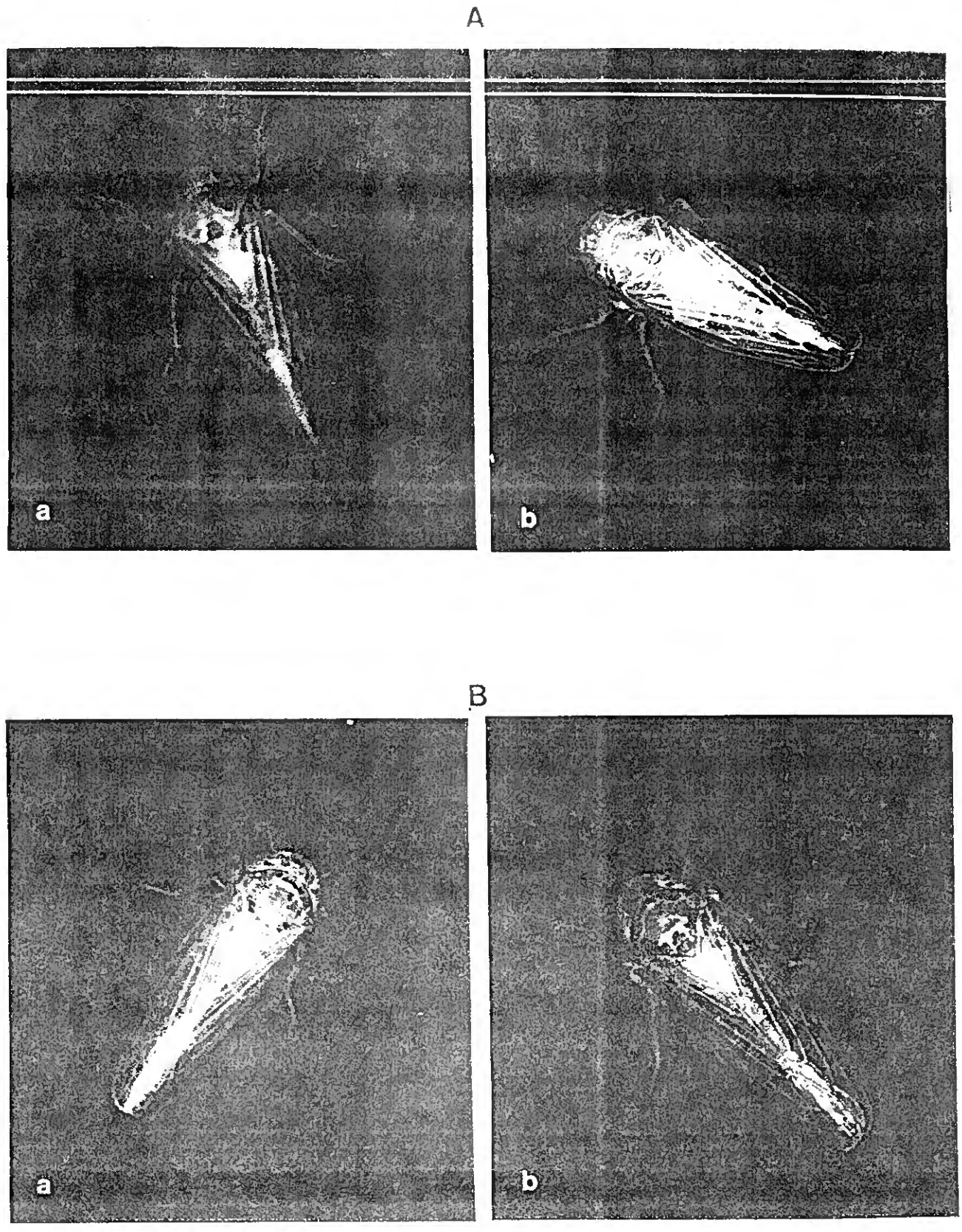


Fig.10 - Male (a) and female (b) of Circulifer tenellus from Atriplex (A); and male and female of Circulifer tenellus from Portulaca (B) (c and d respectively). × 20

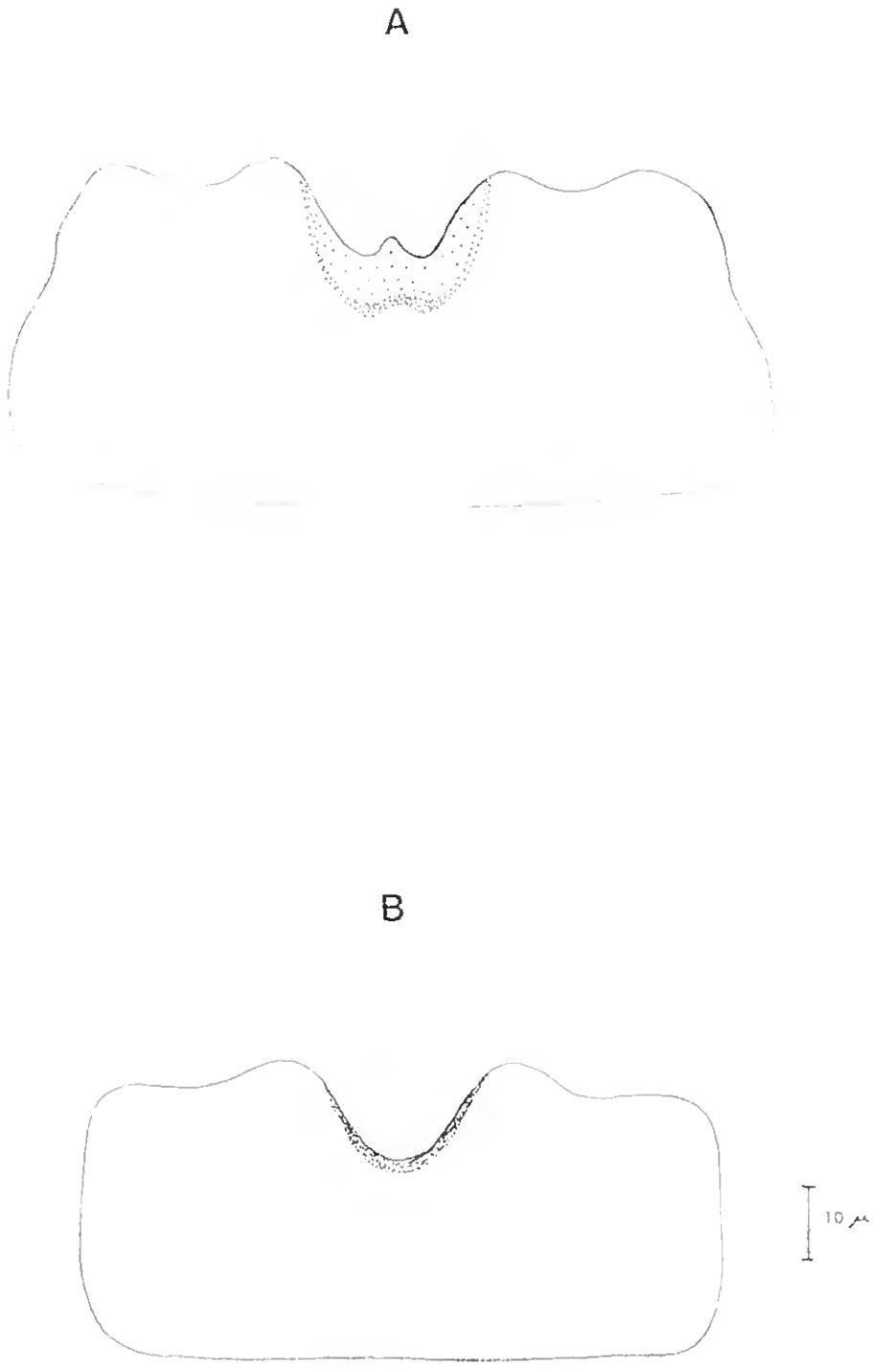


Fig. 11 - Female's 7th sternite of Circulifer tenellus from Atriplex (A); and Circulifer tenellus from Portulaca (B).

2. Description of two variants of the Circulifer tenellus complex

The two C. tenellus variants studied in this work have the general characteristics above mentioned. They differ morphologically in color: in C. tenellus-A they are pale green while the C. tenellus-P are yellowish (Fig. 10A and 10B, respectively). They also differ in the 7th female sternum (Fig. 11A and 11B, respectively), with a denticle protruding from the 7th sternum median excisionin females of C. tenellus-A (Fig. 11A) while in females of C. tenellus-P the denticle absent (Fig. 11B). Courting signals comparison revealed that male calls are different between the variants (Klein, personal communication). Host preference and transmission capacity of S. citri, will be discussed in later chapters.

3. Host - insect relation of the two Circulifer tenellus variants

3.1. Introduction

Suitability of host plants for both variants: Two variants of leafhoppers, of the C. tenellus complex, were collected in the vicinity of citrus groves in two different geographical areas of Israel. One in the Jordan Valley on Atriplex halimus another in Bet Dagan on Portulaca oleracea. In order to establish cultures of the insects, a range of plant species were tested to determine which would be suitable as hosts. The choise of plants was based on previous observations, reported by Klein et al. (1982) and on surveys carried out during the summers of 1984 and 1986 (Klein and Raccah, 1991).

3.2. Methods

3.2.1 Insects: Leafhopper were taken from the stock culture (see Material and Methods). The first generation of insects was then used to test their adaptation to various host plants.

3.2.2. Plants: Crop and ornamental plants were tested, as well as spring and winter weeds (Table 1). The first were sown and grown in the greenhouse. Wild plants were uprooted, transplanted and kept in the greenhouse for three weeks to one month.

In both areas, from were the leafhoppers were collected, wild winter vegetation is similar. A. halimus, a spring weed, is found mainly in the Jordan Valley.

Table 1 - Selected list of wild, cultivated and ornamental plants tested as hosts of two variants of leafhoppers of the C. tenellus complex

Common Name	Species	Plant Family
Madagascar periwinkle	* Vinca rosea L.	Apocynaceae
Geranium	Pelargonium spp.	Geraniaceae
Sugar beet	* Beta vulgaris L.	Chenopodiaceae
Wild beet	Beta vulgaris	"
Orach	Atriplex halimus	"
Radish	* Raphanus sativus L.	Cruciferae
Cauliflower	* Brassica oleracea L. var. botrytis	"
Turnip	* Brassica rapa L.	"
Common stock	* Matthiola incana L.	"
Common purslane	Portulaca oleracea L.	Portulacaceae
Daisy	Chrysanthemum spp.	Compositae
Common plantain	Plantago spp.	Plantaginaceae
Thorn-Apple	Datura spp.	Solanaceae

* Crop and ornamental plants obtained from seeds and grown in the greenhouse.

Wild weeds transplanted from the field and kept in the greenhouse from 3 weeks to one month.

3.3. Experimental procedures: Four experiments were carried out. Plants which proved not suitable were excluded from the following experiment. The methodology was similar except for the number of insects tested.

3.3.1. Experiment 1 - Ten couples, from both variants, were caged (one couple/plant) on several different botanical species and transferred weekly to new plants until the last insect died . Every ten days all plants were checked for appearence of nymphs. Adults of the F1 generation were coupled and placed on new plants. The process was repeated as previously described. Records of longevity and fecundity were kept until nymphs of the F2 generation reached the third or fourth instar, except in the cases were plants did not survive in captivity.

Nymphs from the 4 - 5th instar were also used with some of the plant species. In this case, groups of 25 nymphs were placed on one plant and the emerging adults were transferred to a new plant (one couple per plant). Cages were held in a growth chamber at a constant temperature of 25±2°C and constant illumination.

3.3.2. Experiment 2 - Twenty couples, of each variant , were tested on the following host plants: Matthiola incana, Beta vulgaris, Brassica oleracea and Brassica rapa. In the case of B. oleracea only females were used, throughout the experiment, as males were removed after one week. Insects were transferred every 9-10 days to new plants and the number of descendents of the F1 and F2 generation counted.

3.3.3. Experiment 3 - Leafhoppers of both variants were caged on B. vulgaris (5 males and 5 females of each variant). The method used was similar, with the exception that records on descendants was kept from the F1 generation only, and the net

reproductive rate and generation time determined.

3.3.4. Experiment 4 - In this experiment, only the C. tenellus-A variant was tested. Males and females of 4-5th instar nymphs were placed on M. incana individual plants (one couple/plant) and the same method used as above (Exp. 3).

3.4. Results

Preliminary experiments have shown that host plants, from Chenopodiaceae and Cruciferae appeared to be the most suitable. Results of longevity and fecundity are shown in Table 2 and represented as (+) for leafhoppers that survived for at least one month and reproduced on the host plant and as (-) for leafhopers which survived only a few days and did not reproduce.

From the second experiment, where four host plants were tested, the results on the performance of the leafhoppers are summarised in Table 3 and Fig. 12 for M. incana. Fig. 13, for Beta vulgaris, fig. 14 for Brassioa oleraceae and Fig. 15 for Brassica rapae. Longevity was determined from the day young adults were coupled and caged, on the various host plants.

Females of the two variants of leafhoppers survived longer on B. oleracea followed by males and females on B. vulgaris, but with both plants, fecundity of C. tenellus-P was lower than on M. incana. In the case of B. rapa the C. tenellus-P variant had a high number of descendents but, the variant C. tenellus-A did not adapt to this host plant as no descendents were produced and most adults had died after two weeks.

Table 2 - Survival and reproduction of field leafhoppers of the C. tenellus complex reared in different host plants in the laboratory

Plant species	Survival and fecundity C. tenellus-A	C.tenellus-P
Vinca rosea	-	-
Pelargonium sp.	-	-
Beta vulgaris	+	+
Beta spp.	+	+
Atriplex halimus	-	-
Raphanus sativus	-	-
Brassica oleracea	+	+
Brassica rapa	+	+
Matthiola incana	+	+
Portulaca olearacea	-	+
Chrysanthemum spp.	-	+
Plantago spp.	-	+
Datura spp.	-	-

(+) Insects survived for at least one month and produced nymphs
(-) Insects survived for a few days and did not reproduce

Table 3 - Survival and fecundity of two variant the *C. tenellus* complex on four different host plants

Host Plant	*C. tenellus* variant	Mean longevity(days±SD)(c) Males	Females	Descendents (F1 + F2)
Matthiola incana	CTA	18.8 ± 17.0c	18.6 ± 16.2	323
	CTP	16.8 ± 11.7c	16.0 ± 13.6	375
Beta vulgaris	CTA	25.4 ± 19.9b	23.2 ± 18.5	299
	CTP	17.2 ± 14.9c	18.3 ± 14.5	14
Brassica oleracea	CTA	-	b40.0 ± 25.2 (a)	251
	CTP	-	a37.8 ± 24.0 (a)	86
Brassica rapa	CTA	-	- (b)	-
	CTP	14.2 ± 15.0c	16.7 ± 15.4	339

CTA = *C. tenellus*-A; CTP= *C. tenellus*-P;

(a) Only females were tested, after being allowed coupling with males for one week.

(b) Test was ended as survival and fecundity were very low.

(c) Longevity determined from the day young adults were coupled and caged on the various host plants.

Means with the same letter are not significantly different (Duncan's multiple test) ($P > 0,05$)

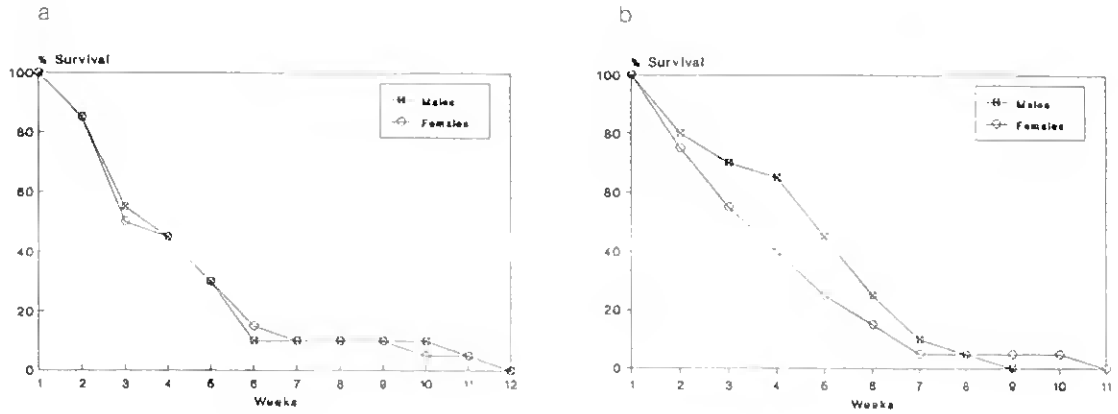


Figure 12. Longevity of Circulifer tenellus-A (a) and C. tenellus-P (b) on the host plant Matthiola incana.

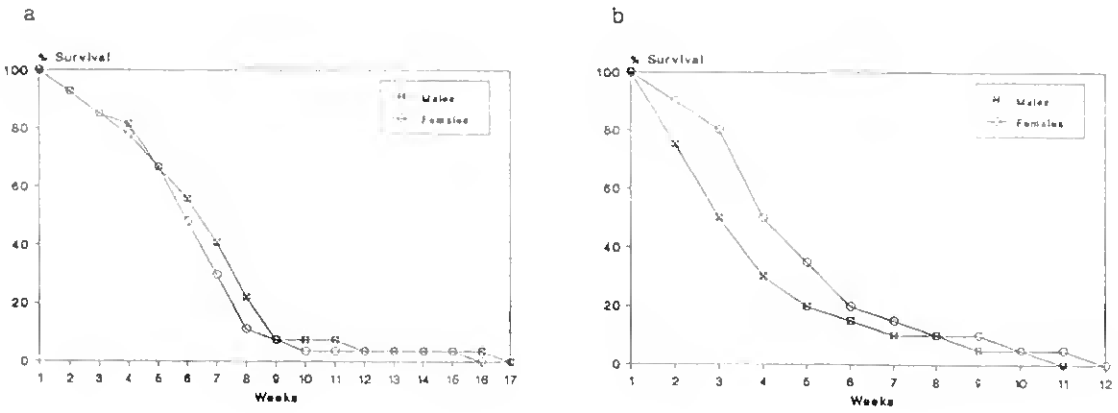


Figure 13. Longevity of Circulifer tenellus-A (a) and C. tenellus-P (b) on the host plant Beta vulgaris.

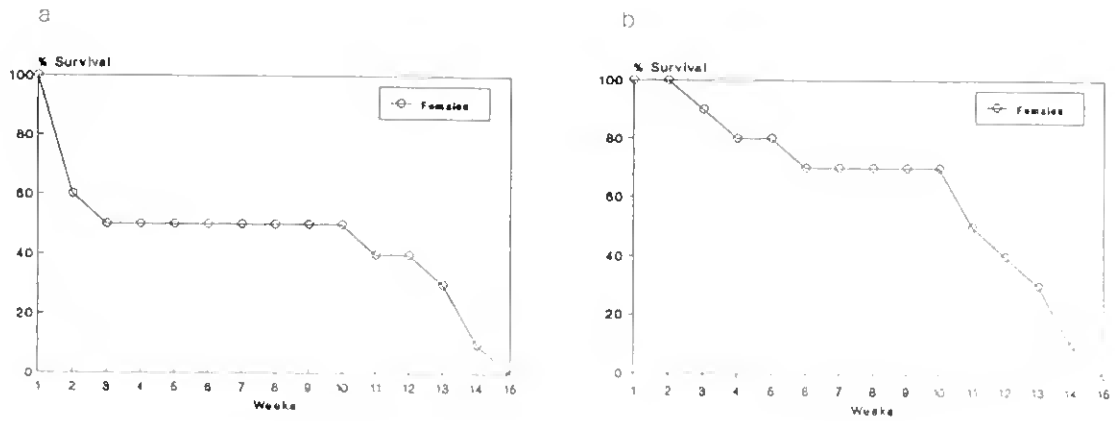


Figure 14. Longevity of Circulifer tenellus-A (a) and C. tenellus-P (b) on the host plant Brassica oleracea.

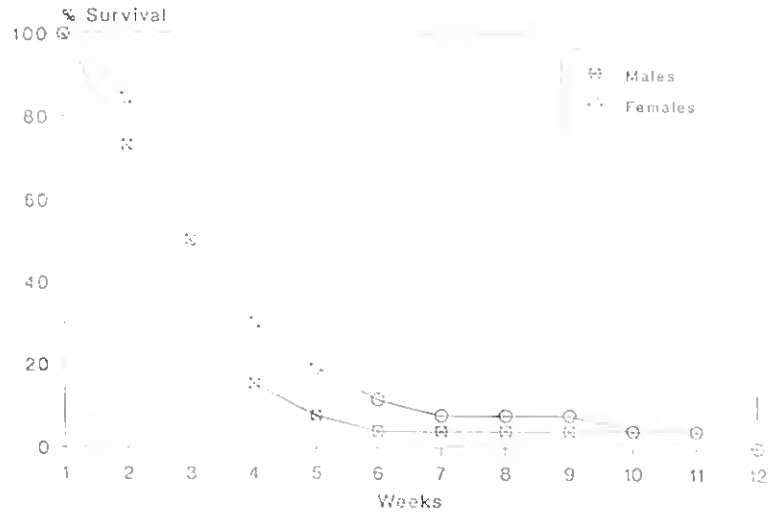


Figure 15. Longevity of Circulifer tenellus-P on the host plant Brassica rapa.

In this last experiment with B. vulgaris as host plant longevity for C. tenellus-P. exceeded in 16 days (males) and 17 days (females) as compared with the previous experiment. There may be a possibility that there was an adaptation to B. vulgaris by this variant (Table 4).

Taking into account survival and fecundity for both variants M. incana proved to be the most adequate host plant. The third experiment was carried out in order to verify whether B. vulgaris, which is the cro most affected by C. tenellus in the western states of the U.S.A., could serve as a host plant. Results (Table 4) showed that, although the variant C. tenellus-A had a mean longevity of 28 days (males) and 15 (females) and the variant C. tenellus-P 33 days (males) and 35 (females) the mean number of offspring born to a leafhopper during its lifetime (Ro) was 40 for the variant C. tenellus-A and 9 for the variant C. tenellus-P. These data confirmed former results which were obtained in the previous experiment. B. vulgaris allowed extended longevity but fecundity on this plant was low.

Table 4 - Longevity and fecundity of two variants of the C. tenellus complex reared on B. vulgaris

Leafhopper variant	Mean longevity (days ±SD)		Ro	Tc
	Males	Females	(a)	(b)
C. tenellus-A	27.84 ± 26.81	14.5 ± 11.24	39.9	19.3
C. tenellus-P	32.96 ± 23.40	35.0 ± 27.12	8.95	24.38

No. of insects = 10 (for each variant)

(a) Net reproductive rate (Ro) equal to $l_x m_x$, where l_x is the age-specific rate of survival, m_x is the age-specific fecundity.

(b) Generation time (Tc) represents $l_x m_x X / l_x m_x$, where X is the age in days.

In view of these results, M. incana was chosen as host plant for the stock cultures of both variants of leafhoppers, and as test plant in transmission experiments. Another consideration was the resistance of M. incana to aphids which did not require chemical treatments in the greenhouse, where the plants were kept, previously to being used in the experiments. Furthermore, this host plant was more suitable to environmental conditions in the growth chamber. Once the choice of the host plant was established, a fourth experiment was carried out to determine longevity and the net reproductive rate (Ro) for the C. tenellus-A variant, when reared on M. incana, and the results are summarised in Table 5.

Table 5 - Longevity and fecundity of a variant of the C. tenellus complex reared on M. incana

Leafhopper variant	Mean longevity (days±SD)		Ro	Tc
	Males	Females		
C. tenellus-A	42.4 ± 34.4	37.4 ± 27.7	83.6	21.0

No. of insects = 20

4. Life cycle of two variants of the Circulifer tenellus complex

4.1. Introduction

The duration of egg and nymphal periods of C. tenellus has been studied by several authors. Two variants of the C. tenellus complex were found to be, in preliminary experiments, vectors of S. citri, in Israel. This has served as a basis of a thorough study of the leafhoppers in order to be able to understand the interrelations vector/pathogen. The life cycle of both variants of leafhoppers was investigated under laboratory conditions.

4.2. Methods: C. tenellus-A and C. tenellus-P. derived from a laboratory stock colony (see material and methods). The hosts were M. incana was used as host plant.

4.3. Procedure: Insects were sexed at the nymphal stage, and caged on M. incana plants until becoming adults. Couples were then placed on a leaf which was covered with a cage (Fig. 7b). After three days, the insects were removed, and three days later the leaves were cut with a razor and, to keep them moist, a wet cotton wool was wrapped around the petiole, covered with "Parafilm" and placed in petri dishes.

The leaves were observed daily under the binocular,and the date of eclosion and subsequent nymphal stages were recorded until appearance of the adult. Throughout the experiment, insects were kept in a rearing chamber, under constant illumination, at 25 ± 2°C. Each test was composed of 25 couples for each variant.

4.4. Results

From a total of 50 females of the two leafhopper variants, 22 insects of C. tenellus-A completed the whole cycle while four of C. tenellus-P reached adulthood. The average duration of the life cycle for the C. tenellus-A leafhoppers was 26.7 days and for the C. tenellus-P 33.7 days (Table 6).

Table 6 - Average duration (days) of eclosion and nymphal stages for two variants of the C. tenellus complex

Leafhoppers (variant)	Egg	Nymphal stages I	II	III	IV	V	Total
C. tenellus-A	7.8	3.7	3.3	3.2	3.3	3.7	26.7
C. tenellus-P	8.5	5.2	5.5	5.7	4.2	4.5	33.7

5. Crossbreeding two variants of leafhoppers from the Circulifer tenellus complex

The two Circulifer variants, which were morphologically similar, were different in the rate by which they transmitted S. citri and their preference for host plants in nature and in greenhouse conditions. Crossbreeding between the two variants was necessary to complement information on the taxonomic position of variants of te C. tenellus complex.

5.1. Procedure:

5.1.1. Experiment 1: Fifth instar nymphs were sexed and caged on separate M. incana plants, for one week. One male and one female, each from a different variant, were placed in a cage (Fig. 7a), and attached to a leaf of M. incana. Insects were transferred weekly to new leaves. After the insects have been removed, the leaves were covered with a cage (Fig. 7b), closed

with a strip of foam rubber, and left for an additional week, during which daily checks were made for appearance of nymphs. Leaves were then cut and cleared by immersion, in a slow boiling saturated solution of chloral hydrate, for ± 30 seconds, in order to expose the eggs which had been deposited, mainly, along the mid rib. Eggs were examined for developing embryos. A developing embryo is characterised by the presence of two red eye spots near the surface of the leaf. This experiment was repeated three times, with 12 males and 12 females from each variant.

As control, a similar experiment was done, with the exception that, the male and female placed in each cage, were of the same variant.

5.1.2. Experiment 2: In a second experiment, the same procedure was followed but after the second transfer insects were caged with a male or female of the same variant for one more week.

5.1.3. Experiment 3: Another method was used, which consisted in caging 20 virgin adults, in two plants (males and females of different variants). After two weeks, insects were transferred to two new plants but with males and females of the same variant, where they remained for one week.

5.2. Results

There were no descendents from the 97 couples tested. From examination of the eggs, no embryos were found, and no nymphs observed in the cages. However, after males and females of the same variant were coupled (Exp. II), nymphs appeared (6/22 couples) and couples placed together in the same plant (Exp.III) produced 20 nymphs, after being coupled with the same variant (Table 7). Of the 25 couples (C. tenellus-A an C. tenellus-P) tested as control, 9 produced nymphs. From the results obtained it appears that crossbreeding is not possible between the two variants of the C. tenellus complex.

Table 7 - Crossbreeding leafhoppers from C. tenellus-A and C. tenellus-P variants

Experiment	No. couples M P x F A	No. couples F P x M A	Fecundity (a)	Fecundity (b)
I	12	12	-	
	12	12	-	
	12	12	-	
II	12	12	-	6
III (c)	5	5	-	20 n.
	C. tenellus-A.	C. tenellus-P		
Control	11	14		9

(a) No. of couples producing descendents when coupled with an insect of another variant.
(b) No. of couples producing descendents when coupled with an insect of the same variant
(c) 5 couples/plant
M = males; F = females; A = C. tenellus-A; P = C. tenellus-P

CHAPTER II - TRANSMISSION OF S. citri BY TWO VARIANTS OF THE C. tenellus COMPLEX

1. Introduction

Although both variants of the C. tenellus complex which were found in Israel, were capable to transmit S. citri, only C. tenellus-A had shown to be an efficient vector. There was however, a need for more detailed information on the vector-pathogen relationships. At the start of this study, C. tenellus-P had not been tested for its vectorial capacity to transmit S. citri. Therefore, it was necessary to establish the role of both variants in the epidemiology of S. citri in Israel. Data obtained from this study could also provide further information towards the understanding of their taxonomic position in the Circulifer tenellus complex.

2. General methods adopted in S. citri transmission experiments:

2.1. Injection of disease agents: young adults and late-instar nymphs of the two variants of leafhoppers were anesthetised with carbon dioxide and placed, on ice, between two layers of "Parafilm", with the ventral side facing upwards. The injection intra-abdominal, averaging 0.2 µl of the culture of spiroplasma, was done under the binocular microscope.

2.2. Acquisition access feeding (AAF) on infected plants: Groups of 20-30 young adults and 5th instar nymphs fed on S. citri infected plants for pre-determined periods (depending on the experiment).

2.3. Incubation of the disease agent in the leafhoppers: Leafhoppers which were injected or completed the AAF were caged on 2-3 months old healthy M. incana plants for an incubation period.

2.4. Incoculation access feeding: Upon termination of the incubation period, the insects were tranferred individually or in groups to young M. incana plants, (4 to 6 leaves) where they remained for the inoculation acces feeding (IAF) for the required period (depending on the experiment).

2.5. Monitoring infection in plants exposed to infective leafhoppers: Each group of plants which was exposed to infective leafhoppers, was kept to record appearance of stubborn symptoms (consisted of a darkening of the older leaves and rosette forming of the younger leaves due to a shortening of the internodes). After removing the infective insects, the plants were kept in growth chambers until all nymphs hatched from eggs which were laid by infective leahoppers. Then, the plants were sprayed with a 1% nicotine solution and taken to the greenhouse (kept at 30°C) with constant illumination. Five to eight weeks later, plants would start showing symptoms.
Random samples of infected plants were tested by ELISA to verify the presence of S. citri. Healthy M. incana plants and liquid medium were used as negative control and S. citri culture as positive control.

3. Inoculation of leafhoppers by injection of four isolates of S. citri

3.1. Methods:

In these experiments, the follwoing S. citri isolates were used: Sc 25, Sc 30, Sc 50, Sc 83 and Sc 136 (see Appendix I). Each isolates was injected to at least 100 insects. Insects were caged, in groups of 20-30, on healthy M. incana plants for an incubation period of 14 days.

Following injection of the different isolates and an incubation period of 14 days, insects were transferred individually to small M. incana plants (4-6 leaves) for an inoculation period of 3 days and placed again in groups, on

bigger plants, for 7 days. This procedure was repeated until the last insect died.

Both variants of leafhoppers were injected a second time with the Sc 25 and Sc 83 isolates of S. citri (see material and methods). After an incubation period of 14 days, insects were caged in groups of 3 per plant and transferred every 3-4 days to new plants until the survival of the last insect.

3.2. Transmission by leafhoppers which acquired S. citri by feeding:

Plants infected by the Sc 25 and Sc 30 isolates of S. citri (see Appendix I) were used as source plants for acquisition. Groups of 10-20 leafhoppers of the two variants were caged on infected plants, for an AAF of 3 days, and then transferred to healthy plants for a 14 days incubation period. Individual insects were caged on small healthy plants and transferred weekly to new plants.

3.3. Results

Survival of the leafhoppers, after being injected with the different S. citri isolates, ranged from 17.7 to 53.2% for the variant C. tenellus-A while for the variant C. tenellus-P it ranged from 10.0 to 37.7% (Table 8).

Table 8 - Survival of the two variants of leafhoppers after injection and incubation period

Isolate Passage level	Plant source	Insect survival			
		C. tenellus-A		C. tenellus-P	
		(a)No.	%	No.	%
Sc 25 (P1)	grapefruit	23/130	17.7	30/130	23.0
Sc 25 (P1)		37/115	32.1	30/125	24.0
Sc 25 (P4)(b)	"	29/60	48.3	-	-
Sc 30 (P1)	"	-	-	28/100	28.0
Sc 50 (P2)	pomelite	-	-	11/100	10.0
Sc 83 (P1)(b)	"	50/94	53.2	10/50	20.0
Sc136 (P2)	grapefruit	17/73	23.2	34/90	37.7

(a) Insects survived/Insects injected after incubation period of 14 days
(b) 3 insects/plant

Transmission results show (Table 9), the number of insects that became infective, according to the appearance of typical symptoms of stubborn on the M. incana plants on which the insects fed, was higher for the C. tenellus-A variant (52.1%), than for the variant C. tenellus-P (11.5%). In both cases the highest infectivety was reached when insects were injected with the Sc 25 and Sc 30 isolates from grapefruit collected in Amiad. The fact that one insect of the variant C. tenellus-P infected two plants, after being injected with the isolate Sc 50 could be an indication of a potential vector in this region and explain the existence of stubborn in areas were the variant C. tenellus-A has never been collected. Hence the repetition of the experiment with isolate Sc 83 (from Modiin). However, there was no transmission by C. tenellus-P leafhoppers. On the other hand, variant C. tenellus-A (from the Jordan Valley) showed a rate of infection of 22.32%.

Table 9 - Rate of infection for the two variants of leafhoppers C. tenellus-A and C. tenellus-P following acquisition by injection of S. citri

Isolate	Variant	No. of Insects			No. of Plants		
		Used	Infective	(%)	Exposed	Infected	(%)
Sc 25	CTA	37	14	37.8	79	28	35.4
Sc 25		23	12	52.1	71	28	39.4
Sc 136		17	0	0.0	32	0	0.0
Sc 25		(a)29	-	-	95	61	64.2
Sc 83		(a)50	-	-	112	25	22.3
Sc 25	CTP	30	1	3.3	73	5	6.8
Sc 25		30	2	6.6	98	5	5.1
Sc 30		26	3	11.5	57	5	8.8
Sc 50		11	1	9.0	39	2	5.1
Sc 136		34	0	0.0	68	0	0.0
Sc 83		(a)10	0	0.0	33	0	0.0

(a) Groups of 3 insects/plant
CTA = C. tenellus-A ; CTP = C. tenellus-P

When infected plants were obtained, preliminary experiments were carried out to verify the transmission capability of the two variants of leafhoppers after acquiring the pathogen by feeding on those plants. The results are expressed in (Table 10) and compared with the efectiveness of transmission by insects that acquired the pathogen by injection. The highest percentage of plants showing symptoms occurred after being inoculated with injected insects, 42.2% for the variant C. tenellus-A and 5.0% for C. tenellus-P as compared with 11.9% and 2.6% respectively, when insects acquired the pathogen by feeding. Greater number of C. tenellus-A variant became infective by both methods (51.8% and 16.2%) as compared with C. tenellus-P (5.3% and 2.6% respectively). (^{2}= 65.70 between injected C. tenellus A and C. tenellus P which is highly significant at $P < 0.01$)

Table 10 - Acquisition and transmission of S. citri by the two variants of leafhoppers after injection and acquisition feeding

	C. tenellus-A				C. tenellus-P			
	Insects		Plants		Insects		Plants	
(a)	No.	(%)	(b)No.	(%)	No.	(%)	No.	(%)
Injection	55/106	51.8	117/277	42.2	7/131	5.3	17/335	5.0
Acquisition feeding	21/129	16.2	25/280	11.9	2/76	2.6	4/153	2.6

(a) Insects infective/Insects injected or feeding on infected plant
(b) Plants infected/plants exposed

The data allowed to compare longevity of C. tenellus-A injected with S. citri (25) with those acquiring the Sc 25 by feeding.

Mean longevity for insects injected with Sc 25 was 17.75 ± 14.50 days as compared with a longevity of 22.47 ± 16.26 days for those acquiring the pathogen by feeding (Table 11, Fig.16). Although mean longevity was different by only 3 days, one should consider the effect of injection as mortality after insects had been injected was 51.67% while after acquisition feeding the mortality was only 10.19%.

Table 11. Longevity of C. tenellus after acquisiton of S. citri by injection and acquisitiion feeding

	No. of insects	Mean longevity (days±SD)
Injected	60	17.8 ± 14.5
Feeding (a)	108	22.5 ± 16.3

(a) Insects had an AAP of 3 days on a M. incana plant infected with S. citri (Sc 25).

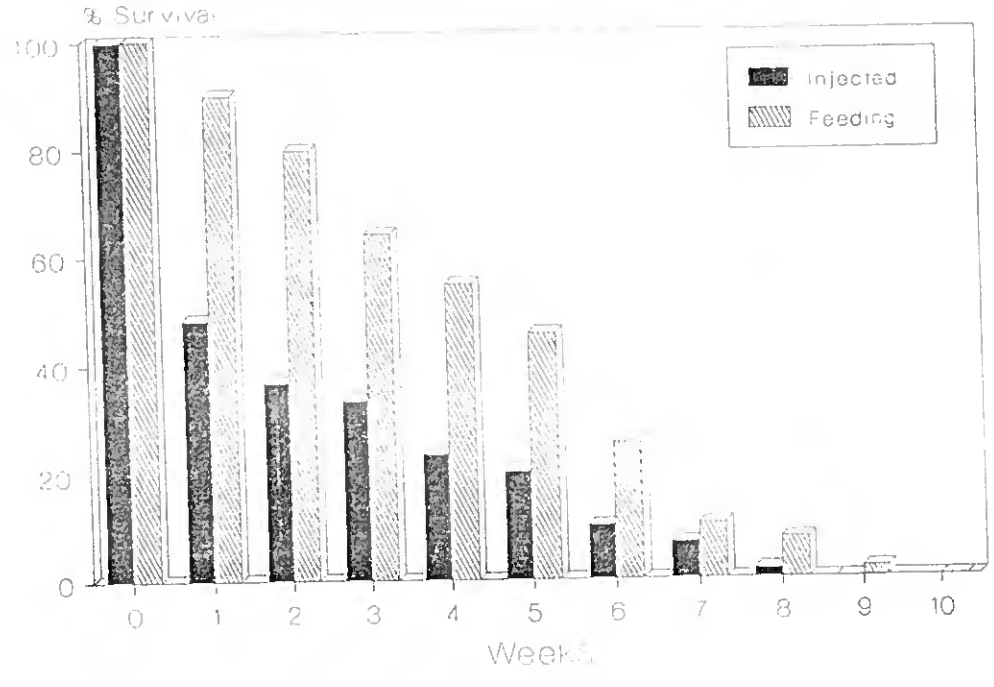


Fig. 16 - Longevity of C. tenellus-A after acquiring S. citri by injection and by acquisition access feeding on infected plants.

A possible cause for the low rate of transmission of S. citri by C. tenellus-P could be the inability of the agent to be established in the salivary glands. Either by not being able to enter or by being inactivated there. Comparisom between the two leafhoppers variants for the ability to infect after injection (Table 10) revealed that C. tenellus-A transmitted at a rate of 51.8% and C. tenellus-P at a rate of 5.3%. In a second experiment, where insects were injected with Sc 83, none of the leafhoppers from the variant C. tenellus-P transmitted the pathogen, while C. tenellus-A infected 22.3% of the plants exposed.

In an attempt to verify if the S. citri titer increased after a determined period of incubation a preliminary experiment was carried out: Leafhoppers were given an AAP of 3 days and then transferred to an healthy M. incana plant. After 3, 6, 10, 13 and 19 days, between 3 and 5 insects were removed and kept at -20°C. One week after (so that all insects had been frozen and thawed) ELISA was used to determine the presence of S. citri. The experiment was done twice with insects of C. tenellus-A and a third time with both variants. Healthy insects were used as control.

ELISA show (Fig. 17), in C. tenellus-A, a decrease in absorbance values after 16 days of incubation and at 22 days a sharp increase. With C. tenellus-P values continued to decrease after 16 days.

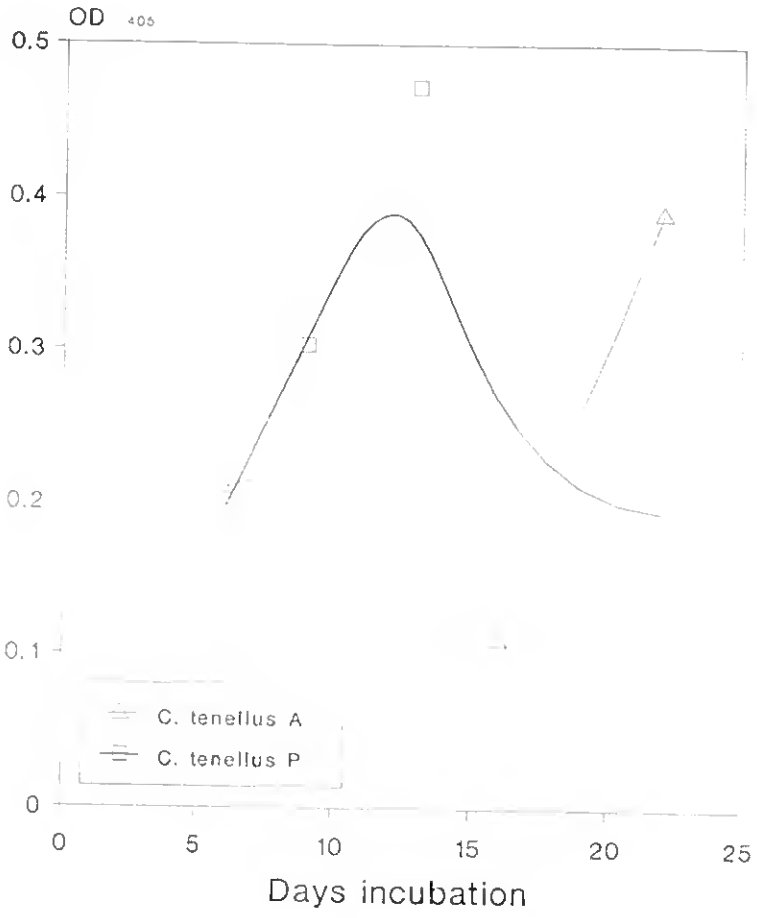


Fig. 17 - Absorption rates (A_{405}) by ELISA in leafhoppers infected with S. citri after various incubation periods.

These results confirm the hypothesis that low or non-transmissibility of S. citri by C. tenellus-P is related to stability of the pathogen in the salivary glands. However, the number of insects tested was not sufficient to draw a definite conclusion. Also, additional confirmation of ELISA data is necessary as ELISA my result sometimes with false positives with healthy leafhoppers (Saillard et al., 1980), or high concentrations of insects (20 /ml) can give similar results to a concentration of only 2 insects/ml (Eden-Green, 1982).

Liu et al. (1983) investigated the multiplication pattern of S. citri in C. tenellus after different methods of acquisition (injection, acquisition through membranes and feeding) and verified that the time and degree to which the pathogen affects vector longevity varies with the form of acquiring spiroplasma. S. citri injected into the abdominal cavity of the leafhopper moves, directly into the hemolynph and is transported to the salivary glands within 10 days causing a high mortality rate during this period. On the other hand, when acquisition is by feeding mortality increases during the insect lifespan and it might be attributed to an inhibitor or toxin produced in the plant as a result of infection with S. citri. The same author comments that this subtance may be directly produced by the spiroplasma or by the plant in response to infection with S. citri and is toxic to C. tenellus.

Other authors (Purcell, 1982; Chiykowski, 1981; Whitcomb et al., 1966) also verified that needle inoculation generally increases the transmission efficiency and reduces the latent period compared with feeding acquisition. Based on these results the following information was obtained:

1. Leafhoppers from the variant C. tenellus-A transmitted S. citri isolates more efficiently than leafhoppers from the variant C. tenellus-A.
2. S. citri isolates from the Upper Galilea as well as from Modiin (coastal plain) were the only ones which were transmitted by both variants.
3. Insects which became infectious by injection, were better vectors, despite having a shorter longevity as compared with insects which acquired S. citri by acquisition access feeding

4. Vector - pathogen relationships

4.1. Determination of minimal Acquisition Access Period (AAP)

Following the results previously reported, where two variants of leafhoppers of the C. tenellus complex transmitted S. citri isolates from grapefruit collected in the Upper Galilee region, acquisition access period, latent period and inoculation period were determined for the two potential vectors with one of the isolates.

4.1.1. Methods:

The AAPs were determined by allowing young adults or 5th instar nymphs to feed on M. incana plants infected by S. citri. Groups of 130 to 150 insects were starved for 1 hour and then exposed to several AAFs: 5, 15 minutes; 1, 2, 4, 6, 8 and 12 hours and 1, 2, 3 and 5 days. After an incubation period of 15 days, groups of 3 insects were placed on young healthy plants and transferred at 10-day intervals until the insects died. For C. tenellus-P up to 6 insects/test plant were used (instead of 1- 3 insects as with C. tenellus-A), due to the fact that in preliminary tests, single pathogen injected leafhoppers were capable to cause no more than 5% transmission.

4.1.2. Results

There was no transmission of the S. citri isolate (Sc25) from Amiad, by the leafhoppers of the variant C. tenellus-P after the various acquisition access periods (AAPs).

As for the variant C. tenellus-A results are presented in Table 12, showing the number of plants which became infected after being inoculated by leafhoppers given different AAPs.

The minimum acquisition access period required for variant C. tenellus-A to become infective was 1 hour. Transmission

after periods of 5, 15 minutes was inefficient. With 1 hour AAP transmission rate was 37.9%. AAPs of 6, 8, 12, 24, 48, 72 and 120 hours resulted in transmission rates of 21.8% , 45.9%, 18.6%, 32.0%, 11.4%, 13.0% and 29.8% respectively.

Results showed that after a minimum of 1 hour AAP (acquisition threshold) the proportion of insects transmitting was not affected by the increasing of acquisition access periods (Table 12). In addition to the factors above mentioned which can influence transmission, it can be assumed , with regard to short AAPs, that for acquisition of spiroplasma, restricted to specific tissues such as the phloem, feeding must progress to the point of ingestion from the infected tissue. The transfer of insects could be another factor as it usually disturbs normal feeding behavior. The concentration of S. citri present in the different infected source plants, can probably explain why the rate of transmission varied between repetitons where insects were given the same access feeding period.

Table 12 - Effect of different acquisiton access periods on the transmission of S. citri by a variant of the C. tenellus-A

AAP	No.symptomatic plants/ No. exposed plants	% Infected
5 min	0/26	0.0
15 min	0/83	0 0
30 min	0/54	0.0 *
1 hr	67/177	37.9 *
2 hr	0/119	0.0 *
4 hr	0/38	0.0 *
6 hr	17/78	21.8 *
8 hr	28/61	45.9 *
12 hr	19/102	18.6 *
24 hr	39/122	31.96
48 hr	15/132	11.36
72 hr	15/117	12.82
120 hr	34/114	29.82

* Insects tested individually throughout.

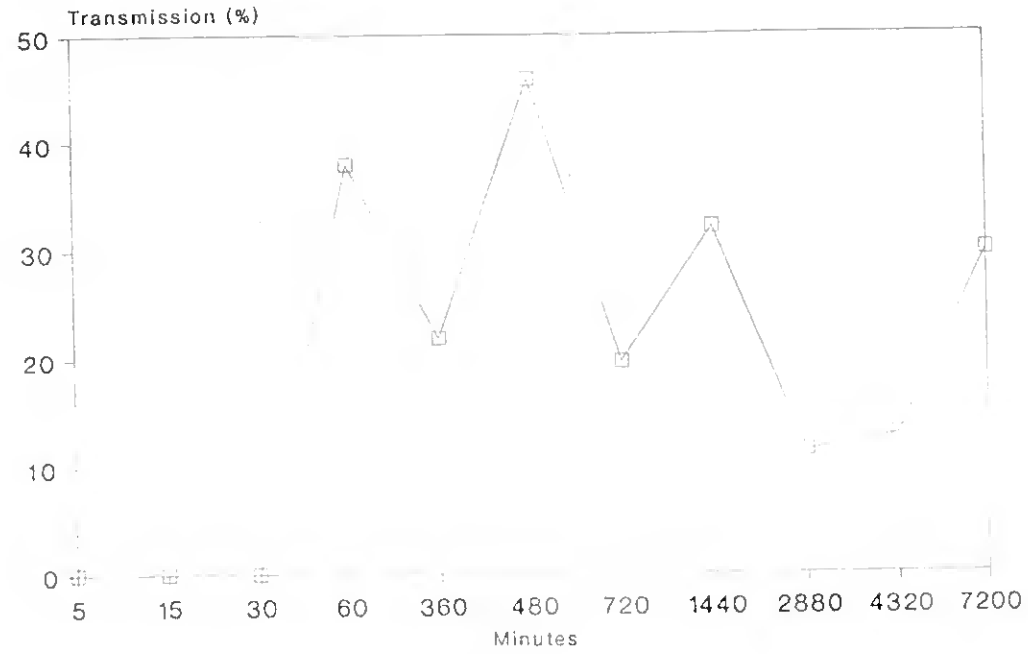


Fig. 18 - Transmission of S. citri by C. tenellus-A after various acquisiton access periods on infected plants.

The acquisition threshold of one hour, determined in these experiments, differs from the minimum acquisition feeding period of 6 hours reported by Liu et al. (1983). These authors also report rates of transmission of 2-4% when leafhoppers were caged singly on test plants and rates of 70-80% when groups of 20 leafhoppers per plant were used, as compared to our results where the maximum rate of transmission was 45.9% after 8 hours acquisition feeding by individual insects.

Characteristics of transmission of a horseradish brittle root (BR) isolate of S. citri from Illinois, by C. tenellus were determined by Eastman et al. (1988). Nymphs given AAPs as brief as 45 minutes were inoculative. The variability in the maximum rates of transmission ranged from 41-90%, using individual insects.

4.2. Transmissibility by males and females of variant C. tenellus-A

In some of the experiments already described, in which transmissibility was studied after leafhoppers had been injected with the pathogen or given acquisition access feeding to infected plants, insects were separated according to sex in order to determine wether males or famales had different transmission capability. Results show a similar percentage of transmission for both sexes, i.e., 27.5% for males and 29.0% for females (Table 13). After verifying that leafhoppers of the variant C. tenellus-P did not transmitte the S. citri isolate (Sc25), experiments to determine the Latent Period (LP) and Inoculation Period (IP) were carried out, but only for the variant C. tenellus-A.

Table 13 - Vector ability between the sexes of C. tenellus-A

	Insects		Total	%
	Infective/ injected	Infective/ feeding		Transmission
Males	11/20	17/82	28/102	27.5%
Females	12/20	20/90	32/110	29.0%

4.3. Incubation Period (IP)

The route of S. citri in the leafhopper passes through the gut lumen were most cells of S. citri are digested or destroyed. Some of the surviving cells enter the gut wall into the epithelial cells where they multiply. They move further toward the basement membrane of the intestine and finally the organism pass to the hemocoel where further multiplication occurs. Once in the hemocoel, S. citri is transported to the salivary glands where the leafhoppers can then inject the spiroplasma via salivary secretions during the feeding process (Liu et al., 1983). The period between ingestion and inoculation is considered THE INCUBATION PERIOD or LATENT PERIOD in the insect.

4.3.1. Methods

Leafhoppers of the C. tenellus-A variant were allowed an AAF of 3 days acquisition access period on M. incana plants infected with the Sc 25 isolate. The insects were then caged individually on test plants. Then, transferred daily up to 10 days after the AAF. Therefater, they were transferred every 2-3 days.

4.3.2. Results

Leafhoppers began transmitting the pathogen between the 6th and 7th day after acquisition feeding with a percentage of transmission of 7.8% and 10.8% respectively. Transmission was 31.5% at 12 IP and continue to increase, reaching a maximum of 43.4% after 35 days. By 60 days post-acquisition, only 7 insects remained alive of which 5 were still infective. The latent period (LP_{50}) was calculated at 26.18 days after acquisitiion feeding (determined from a linear regression between time and number of first infected plants) ($y=0.019x-0.004$; $r^2=0.9638$ (Fig. 19).

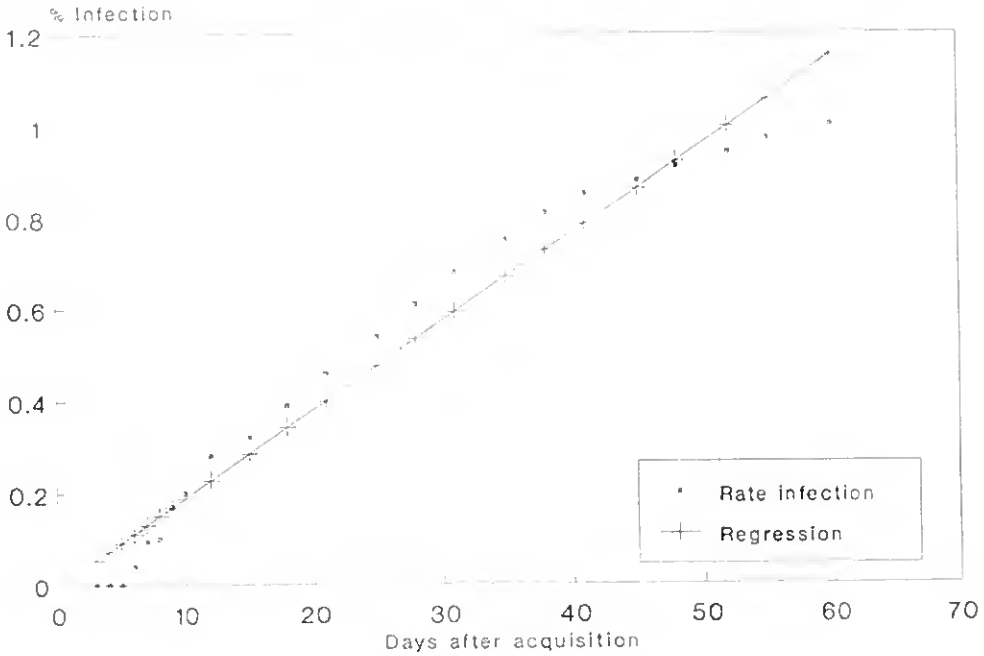


Fig. 19 - Transmission of S. citri by C. tenellus-A after various incubation periods. LP_{50} = 26.18 by linear regression ($y = 0.019x - 0.004$; $r^2 = 0.9638$)

4.4. Inoculation Access Period

Once the Acquisition Access Period (AAP) was established for the C. tenellus-A variant, experiments were carried out to determine the Inoculation Access Period (IAP) (time required for infected leafhoppers to transmit the pathogen to healthy plants).

4.4.1 Methods

Groups of 30 to 50 insects were allowed an AAF for 3 days. After the incubation period (14 days), groups of 1 to 3 leafhoppers were placed on test plants for several inoculation access periods (IAPs): 30, 45 and 60 minutes and 4, 24 and 48 hours. Finally, the insects were caged for 5 days periods, in order to ascertain whether lack of transmissionis due to the short duration of AAP or to the inability of the leafhoppers to infect.

4.4.2. Results

The minimum IAP observed was 60 minutes (Table 14). Plants which did not show typical symptoms of stubborn disease, when exposed to insects for less than 60 minutes became infected after 5 days IAP, by the same leafhoppers (Table 14 and Fig. 20). Longer IAP did not always result in higher rates of transmission i.e. 41% transmission after 60 minutes and 38.1% transmission after 48 hours (Table 14 and Fig. 20).

Table 14 - Effect of Inoculation Access Period (IAP) on transmission of *S. citri* by *C. tenellus* A

IAP	(a) Plants	%	(b) Plants	%
30 min	0/26	0.0	2/19	10.5
45 min	0/28	0.0	4/22	18.2
60 min	12/29	41.4	5/25	20.0
4 hr	4/26	15.3	9/26	34.6
24 hr	20/56	35.7	12/40	30.0
48 hr	21/55	38.1	26/48	54.1

(a) Plants infected/Plants exposed
(b)Plants infected/plants exposed after an additional inoculation access feeding of 5 days

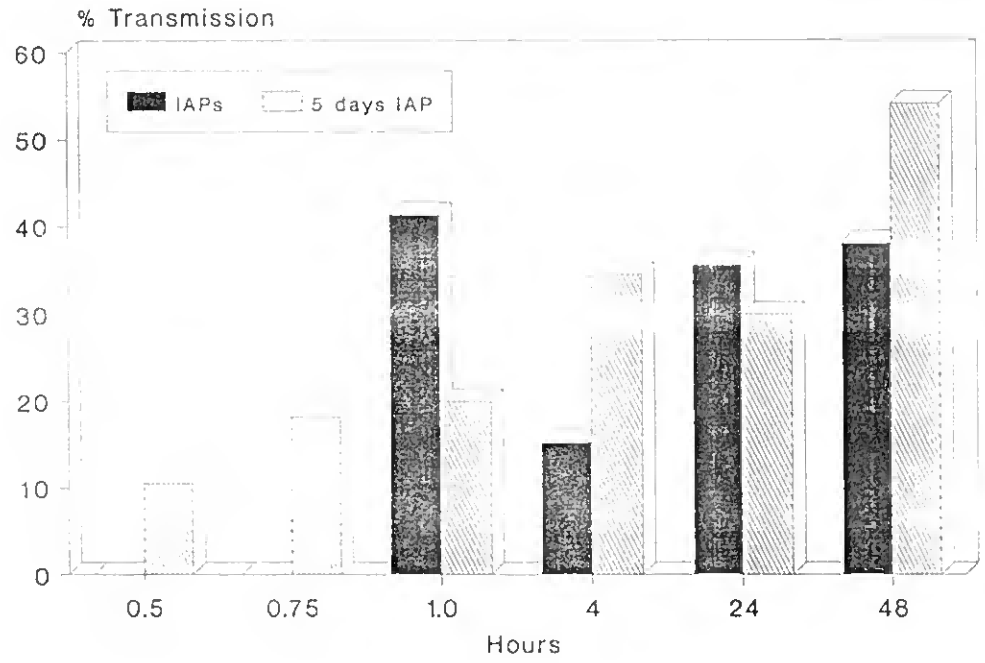


Fig. 20 - Transmission of S. citri by C. tenellus-A after various inoculation access feeding periods.

The minimum AAPs and IAFs resulting from these experiments are probably longer than the ones occurring in nature due to the fact that insects were disturbed when transferred and did not resume feeding immediately. It was also observed in some cases that insects stayed on the side of the cages for some time before feeding while others remained there throughout the whole experiment.

4.5. Relation between presence of S. citri in an infected plant and rate of transmission by the vector C. tenellus-A.

4.5.1. Introduction

Determination for the presence of spiroplasmas in plant material by enzyme-linked immunosorbent assay (ELISA) is well documented. Bove et al. (1987) reports that by using this technique to detect S. citri in citrus trees, Catharantus roseus and leafhoppers in Iraq and Syria results indicated that there was a good correlation between symptom expression and the presence of S. citri.

While studying te interrelationships between S. citri and one of its vectors, C. tenellus-A., it was observed that leafhoppers, from the same colony, would transmit the pathogen after access feeding on a stubborn infected plant but, would not transmit when given access feeding to a similar plant also showing symptoms of stubborn infection. If the cause of these negative results resided in the nonexistence of S. citri in the plant then the data could be misinterpreted. On the other hand, if we obtained positive results by ELISA , the cause for non-transmission could then be attributed to the vector.

4.5.2. Procedure

A. Application of ELISA to the detection of S. citri in plants

New leaves (ca. 1g) of 10 infected plants and of one healthy plant were homogenized, in 1.5 ml of phosphate saline buffer (PBS) containing 0.05% tween 20 and 2% polyvinylpyrrolidone, using a mortar and pestle. The homogenate was gently squeezed through four layers of cheesecloth, and 200 µl of filtered homogenate was placed in each well of the microtiter plate (2 wells/sample). ELISA was carried out as described previously (Saillard et al., 1978). IgG was from existing stock which had been prepared against S. citri from Bet-Dagan and was used at a 1000-fold dilution as well as the stock

solution of alkaline phosphatase linked IgG (enzyme conjugate). Absorbance values (OD405) were determined using the autoreader micro-ELISA.

B. Transmission of S. citri by C. tenellus-A

Leafhoppers (21 per plant) were given 24 hours access feeding to the 10 infected plants. As a control, a similar group of leafhoppers were fed for the same time on healthy plants. Following an incubation period of 15 days, insects were allowed IAF for a period of 10 days. Thereafter insects were transferred, (3 per plant) to healthy plants every 10 days.

4.5.3. Results

Absorbance values, at 405 nm, for all samples was low (<0.100) except in one case were it reached 0.109 . However, there was transmission from seven of the plants tested (Table 15 and Fig. 21).

The low values obtained by ELISA was probably due to a low number of spiroplasmas to be detectable. However, plants proved to be infected, after transmission by insects to healthy plants. ELISA could not, in this case, be reliable for the detection of S. citri in M. incana plants, prior to transmission tests.

Table 15 - Detection of S. citri in plants. Relation between ELISA values and rate of transmission by C. tenellus-A

Plant No.	* Absorbance values (A_{405})	Plants infected/ Plants exposed	Infection (%)
1	0.008	7/13	53.8
2	0.049	5/15	33.3
3	0.049	2/4	50.0
4	0.033	5/14	35.7
5	0.077	0/4	0.0
6	0.109	4/6	66.6
7	0.077	6/13	46.2
8	0.022	0/10	0.0
9	0.056	0/6	0.0
10	0.047	2/6	33.3

* After subtraction of control values

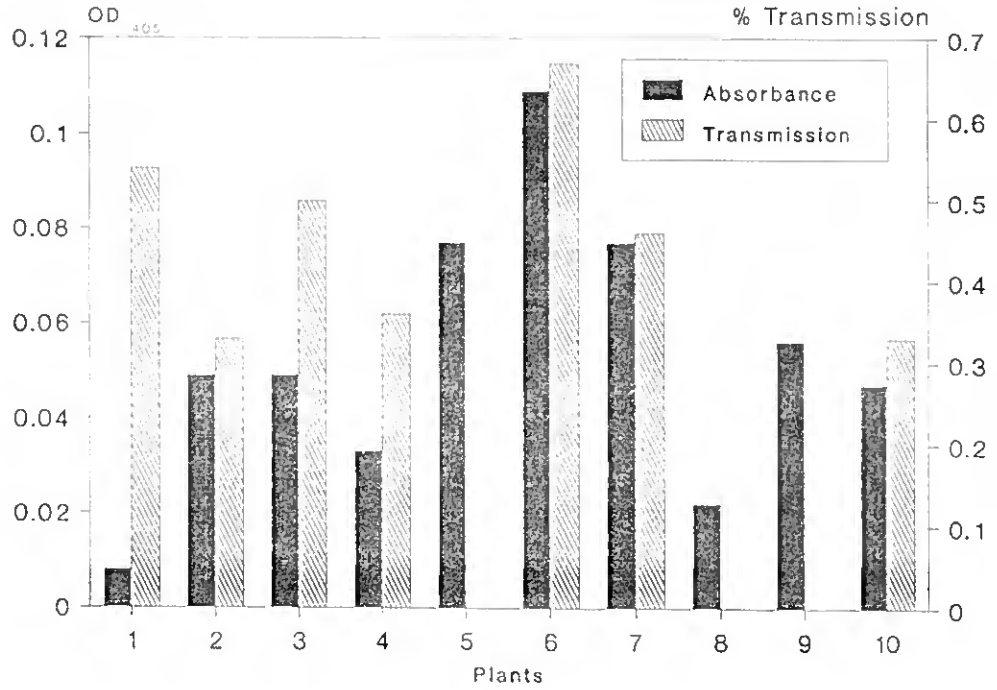


Fig. 21 - Absorption rates (A_{405}) by ELISA in plants infected by S. citri and the rates of transmission for leafhopper which acquired the agent from these same plants.

5. Pathogenicity of S. citri to C. tenellus-A

5.1. Introduction

Vector - born plant pathogens are divided into two groups (persistent and nonpersistent) based on duration of their retention by vectors. Persistent pathogens are further divided into propagative and circulative dependingon replication in the vector.

Reports on persistent and propagative pathogens (Granados and Meehen, 1975) has shown that a strain of Corn Stunt Agent (CSA) induced premature death, reduced fecundity in the leafhopper vector, Dalbulus elimatus, and found cypathological changes in the brain and salivary glands of diseased insects; Chen and Liao (1975) and Whitcomb and Williamson (1975) showed that CSA was pathogenic to D. elimatus. S. citri was reported to reduce the longevity of M. fascifrons (Whitcomb et al.,1974). Other procaryotes have been shown to affect their vectors, such as, clover phyllody MLOs which was pathogenic to M. fascifrons (Sinha and Paliwal, 1970) and increased mortality of E. plebejus (Giannotti et al.,1968); Western X mycoplasmas (WXMs) reduced drastically fecundity of the leafhopper Colladonus montanus (Jensen , 1971) and was shown to cause premature death of its vector (Jensen, 1959). In the present study we intend to verify the effect of S. citri on survival and fecundity in the C. tenellus-A variant.

5.2. Procedure:

Groups of 25 nymphs, at the 4th and 5th instar, of C. tenellus-A, were caged on each of eight S. citri (Sc 25) infected plants, for 4 days of AAF. Males and females were separately transferred to healthy plants for an IP of 14 days. Thereafter, the leafhoppers were caged individually on test plants for four days of IAF. This allowed to verify which of the insects were infective (both males or females). Couples were then caged on older plants (two months after transplanting), for a week, after

which the males were removed. The individual females were transferred sequentially every 7-10 days to new plants until the survival of the last insect was recorded. The plants which served for eggs deposition were kept for at least one month after removal of adults. Every ten days nymphs were removed and counted.

The same procedure was applied for leafhoppers, used as control, which were fed on healthy plants throughout the experiment (spiroplasma free). A total of 200 insects were allowed acquisition feeding on infected plants and the same number were fed on healthy plants.

5.3. Results

5.3.1. Effect of S. citri on the longevity of C. tenellus-A

Fifty females were fed on diseased plants, of which, 12 transmitted the pathogen and showed a mean longevity of 15 days while 38 females were noninfective and had a showed a mean longevity of 17 days. Insects which fed on healthy plants had a mean longevity of 20 days (Table 16 and Fig. 22)

Table 16 - Effect of S. citri on the longevity of C. tenellus-A following 4-days of acquisition feeding period on diseased M. incana plants.

Test insects	Total insects	Insects transmitting	Longevity in days * Range	Mean ± SD
Infective	50**	12	10-43	15.76 ± 10.96
Noninfective		38	2-53	17.11 ± 12.15
Control	49**	-	2-79	20.34 ± 14.26

*Longevity is measured from last day of acquisition access feeding followed by a 14-days incubation period.
** Females
(Applying the Student test to compare longevity of infective insects with control, t = 2.14 which is significant at P < 0.05)

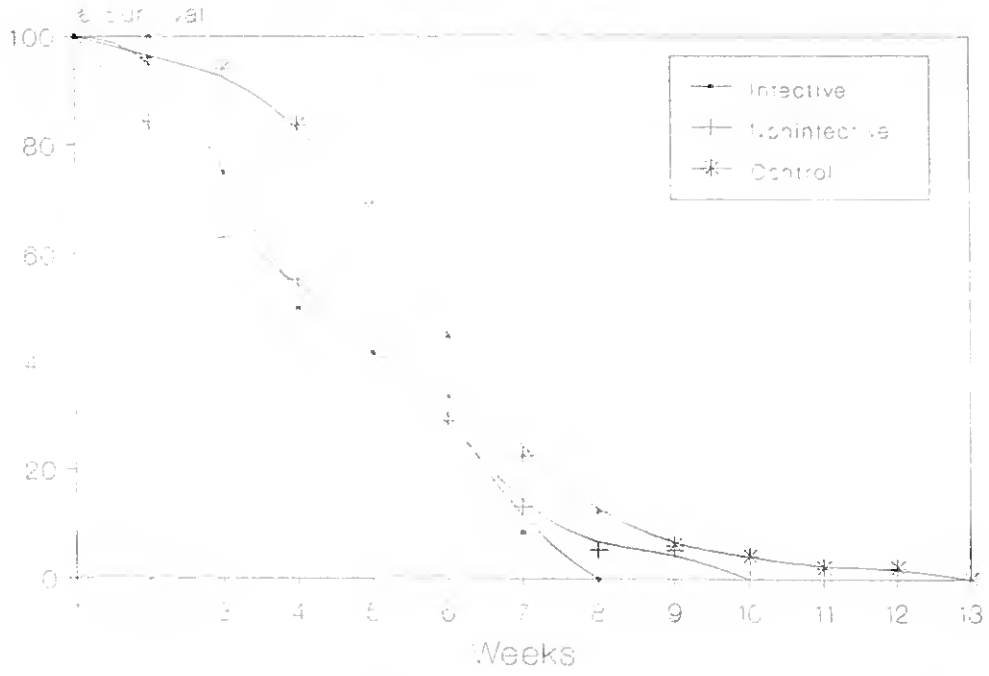


Fig. 22 - Effect of S. citri on the longevity of C. tenellus-A following 4-days AAF on diseased M. incana plants.

The rate of mortality during the first 3 weeks after completion of the incubation period (14 days), was lower for infective leafhoppers as compared with control. However, spiroplasma-free insects survived 12-13 weeks as compared to those which acquired the agent and survived 6-7 weeks (see Table 17).

Table 17 - Rate of mortality of C. tenellus after an acquisition access feeding on S. citri

	Mortality (%) after (weeks):										
***	1	2	3	4	5	6	7	8	9	10	11 12
Infected*	-	-	8	42	58	67	100				
Non-infected*	24	40	48	56	60	80	90	96	100		
Control**	16	37	45	62	78	90	96	96	96	98	98 99

* Nymphs of C. tenellus fed on S. citri infected M.incana plants.
**Insects fed on healthy M. incana plants.
*** Mortality recorded after completion of 14 days incubation period.

5.3.2. Effect of S. citri on fecundity of C. tenellus-A

As seen in Table 18 and Fig. 23 the net reproductive rate (NRR) of infective insects was 28.25 females/female/generation. The noninfective insects have shown a NRR of roductive rate of 44.50 females/female/generation and the NRR in the control 44.93 females//female/generation. On the other hand, the generation time differs slightly more than one day between infective and noninfective females, 6.37 days and 7.58 days respectively, and in both it was much lower as compared with spiroplasma-free insects whose generation time was 13.14 days.

Liu et al. (1983) found significant differences in the survival of infected and noninfected leafhoppers ranging between 1-7 weeks. These authors consider that the difference may be

attributed tothe presence of an inhibitor or toxin produced in the plant as a result of infection with S. citri. This substance may be directly produced by the spiroplasma or by the plant in response to infection with S. citri and is toxic to C. tenellus. As descendents appear during the first 4 weeks, mortality had little effect on the total number of nymphs produced.

Table 18 - Life-table statistics for infective, noninfective and control C. tenellus, the vector of the stubborn disease agent(a)

Number leafhoppers tested	(b) Net reproductive rate (Ro)	(c)Generation time (days) (Tc)	(d)Intrinsic rate of increase
Infective (12)	28.25	6.37	1.48
Noninfective (38)	44.50	7.58	1.76
Disease-free (49)	44.93	13.14	1.23

(a) Infective insects are considered those that infected plants at least once; noninfective insects those which fed on infected plants
but did not transmit. Control,insects which fed on healthy plants.
(b) Net reproductive rate (Ro) is equal to $l_x m_x$, where
l_x
is the age-specific rate of survival, m_x is the age-specific fecundity.
(c) Generation time (Tc) represent $l_x m_x X / l_x m_x$ (see in b), X is the age in days.
(d) The intrinsic rate of increase is equal approximately to ln $l_x m_x / Tc$

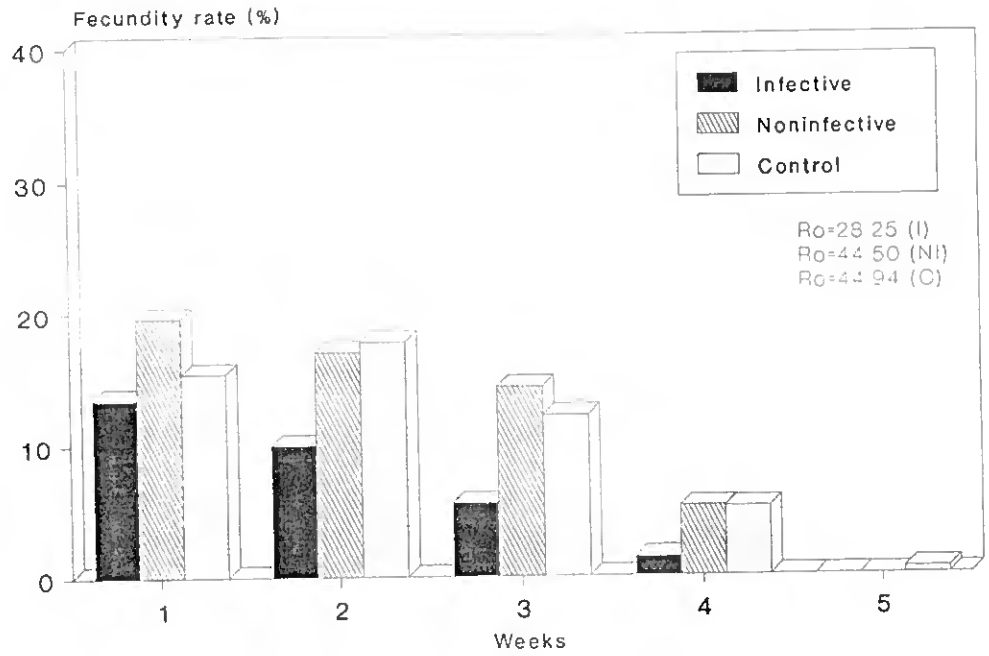


Fig. 23 - Effect of S. citri on the fecundity of C. tenellus-A after 4-days AAF on S. citri infected M. incana plants (Ro = net reproductive rate).

CHAPTER III- CHARACTERISTIC OF S. citri ISOLATES

1. Serological relatedness of Spiroplasmas by Enzyme-linked Immunosorbent Assay (ELISA)

In the present study, spiroplasma isolates represented different geographicl locations in Israel. Two of those were poorly or non insect transmissible. The serological test was aimed at relatednesse between serological determinants and insect transmissibility.

1.1. Material and Methods

1.1.1. S. citri isolates: the isolates of S. citri to be tested by ELISA, were were presented in details in the chapter of Materials and Methods (see also Table 18). Cultures of S. citri were renewed by passages to fresh media. Presence of spiroplasma was confirmed by dark-field microscopic observation.

1.1.2. Preparation of spiroplasma antigens

The method utilised followed the procedure by Saillard, et al. (1978). Spiroplasmas were harvested from 15 ml of a log broth culture by centrifugation at 20,000 g for 20 minutes. Organisms were washed by resuspending the pellet in phosphate buffer (0.10 M sodium phosphate, pH 7.4, containing 0.33 M NaCl). This washing step was repeated three times. The final pellet was resuspended in 1 ml of phosphate-buffered saline (PBS) (0.02 M phosphate plus 0.15 M NaCl at pH 7.4) containing 0.05% Tween - 20 and 2% polyvinylpyrrolidone.

An aliquot of 0.1l from each sample was diluted in distilled water (1:8) before testing. Optical density at 260 nm and 280 nm allowed to determine proteins and nucleic acids concentration (with the use of a Nomograph table).

1.2. Scheme of the assay

1.2.1. Calibration test

In order to standardize the serological assay, a calibration test was done. Antigen (Sc 50) was prepared as previously described, with the exception that organisms were harvested from 50 ml of culture. IgG was tested at the following dilutions: 1:250; 1:500; 1:1000 for coating the plates. After an incubation of 4 hours at 32°C, the plates were washed 3 times, and the samples at 1:5, 1:10 and 1:20 concentrations. BSR medium was used as control. After incubation for 20 h at 4°C, the wells were washed again. Conjugate was added at 1:500 and 1:1000 dilutions and incubated at 32°C for 4 h before washing plates oncce more. Finally, substrate was added at 0.6 mg ml-1. Each sample was applied in two wells (0.2 ml/well).

1.2.2. ELISA test of S. citri isolates

Five S. citri isolates were compared using a volume of 25 ml of culture for each one. The selected ELISA conditions were the following: IgG 1:1000, sample 1:5 and conjugate 1:500. In three tests, samples were placed in 6 wells (0.2ml/well), at random, and in a fourth samples were applied to four wells. BSR medium was used as control in all tests and applied to the same number of wells as the samples. Protein concentration was determined spectrophotometrically at 405 nm using the autoreader micro-ELISA.

1.3. Results

1.3.1. Calibration tests

Absorbance values for various concentrations of Sc 50 isolate was determined using the antiserum against the S. citri isolate from Bet Dagan (Table 19).

Table 19 - ELISA absorbance values* for S. citri (Sc 50)
- Calibration test -

Conjugate	Samples	IgG concentrations		
		1:250	1:500	1:1000
1:500	1:5	0.467	0.315	0.276
	1:10	0.251	0.261	0.219
	1:20	0.261	0.223	0.180
1:1000	1:5	0.172	0.137	0.136
	1:10	0.137	0.121	0.103
	1:20	0.111	0.092	0.091

* After subtraction from control values

1.3.2. Comparison of S. citri by ELISA

All isolates reacted with antiserum to S. citri (isolate from B. Dagan). The highest absorbance values were observed for isolates Sc 50 (A_{405}775), Sc 83 (A_{405}632) and Sc 210 (A_{405}727), showing therefore a strong affinity to - globulin (Table 20).

Table 20 - Absorbance values * of S. citri isolates tested by ELISA

Isolate	* Absorbance values (A_{405}) (Mean ± SD)	Protein content (g/c.c.)	Ratio
Sc 50	0.775 ± 0.410$_a$	1.04	0.745
Sc 20	0.411 ± 0.109$_c$	1.44	0.285
Sc 83	0.632 ± 0.181$_b$	0.64	0.988
Sc 210	0.727 ± 0.287$_{ab}$	0.64	1.137
Sc 25	0.379 ± 0.287$_c$	1.00	0.379

* After subtraction of control values
Means with the same letter are not significantly different (Duncan's multiple range test.

Absorbance values were higher, for fresh cultures as compared with one week old cultures which were kept at -20°C. In the case of Sc 25, reaction was considerably reduced (up to x 10). This may explain the variation of the data.

The ratio between ELISA values and protein concentration shows discrepancies. Sc50 and Sc210 although with similar absorbance values differ in 34.5% in their ratios.

From these results can be concluded that affinity of all isolates to antiserum (Bet-Dagan) exists but relatedness between them could not be established. Other serological tests, PAGE-SDS analysis and hybridization were carried out in order to obtain further information.

2. Immuno-double-diffusion serological relationships among Spiroplasma isolates

2.1. Introduction

Several serological methods can be used to characterize and compare the diverse isolates of Spiroplasma, such as: ELISA (referred in the previous section), growth inhibition, organisme deformation, metabolic inhibition and ring interface precipitin tests. The immuno-double-diffusion is another technique which was first used by Coomaraswamy and Gumpf (1984) to compare Spiroplasma isolates. The principle in this procedure consists in the diffusion of antibody and antigen into the medium and the appearance of a precipitation zone where they meet in optimum proportions. If no affinity exists between the antigen and a particular isolate no precipitation occurs.

In order to verify the cross reactivity between the various S. citri isolates this technique was applied.

2.2. Material and Methods

2.2.1 Preparation of antigens - Samples of 1 ml, from a logarithmic growth phase culture were used as the inoculum for each 4 ml of liquid medium. Cultures were incubated aerobicaly at 32°C until a decrease in pH was observed, as indicated by a colour change in the medium, from red to yellow. The cultures were centrifuged at 15.000 r.p.m. for 20 minutes, to sediment the organisms. The pelleted organisms were suspended in 0.1 ml of SDS buffer at a concentration of 0.1%.

2.2.2 Antiserum and medium - Antiserum against S. citri (isolated from Valencia orange - Bet Dagan) was prepared previously in the Department of Virology by Mr. R. Rasooli. The medium consisted of agar gel (1%) supplemented with sodium chloride (0.85%) and 0.2% (wt/vol) sodium azide.

2.3. Procedure - Petri dishes (50mm in diameter) containing 8 ml of medium, 6 wells (5 mm in diameter) equidistant from a central well, were punched and the plugs removed by slight suction. 100 ul aliquots of each sample was added to the peripheral wells and 100 ul aliquot of serum added to the central well. The distance between the centers of the central well and the peripheral wells was 20 mm. One well contained SDS buffer, as control. Plates were incubated at room temperature in a moist environment. After 2 days, precipitation lines started to appear and on the 3rd day they were well defined.

2.4. Results

Results from the immuno-difusion test show that all S. citri isolates tested have at least one common antigenic component, with the exception of Sc 102 [Fig 24 (a) for well 3] of which no precipitin lines were formed. Spiroplasma sp. [Fig 24 (a) for well 4] did not cross react with antiserum . Although no precipitin lines were formed by Sc 102 (isolate from Arava) common antigenic components may exist but not detected due to low concentrations.

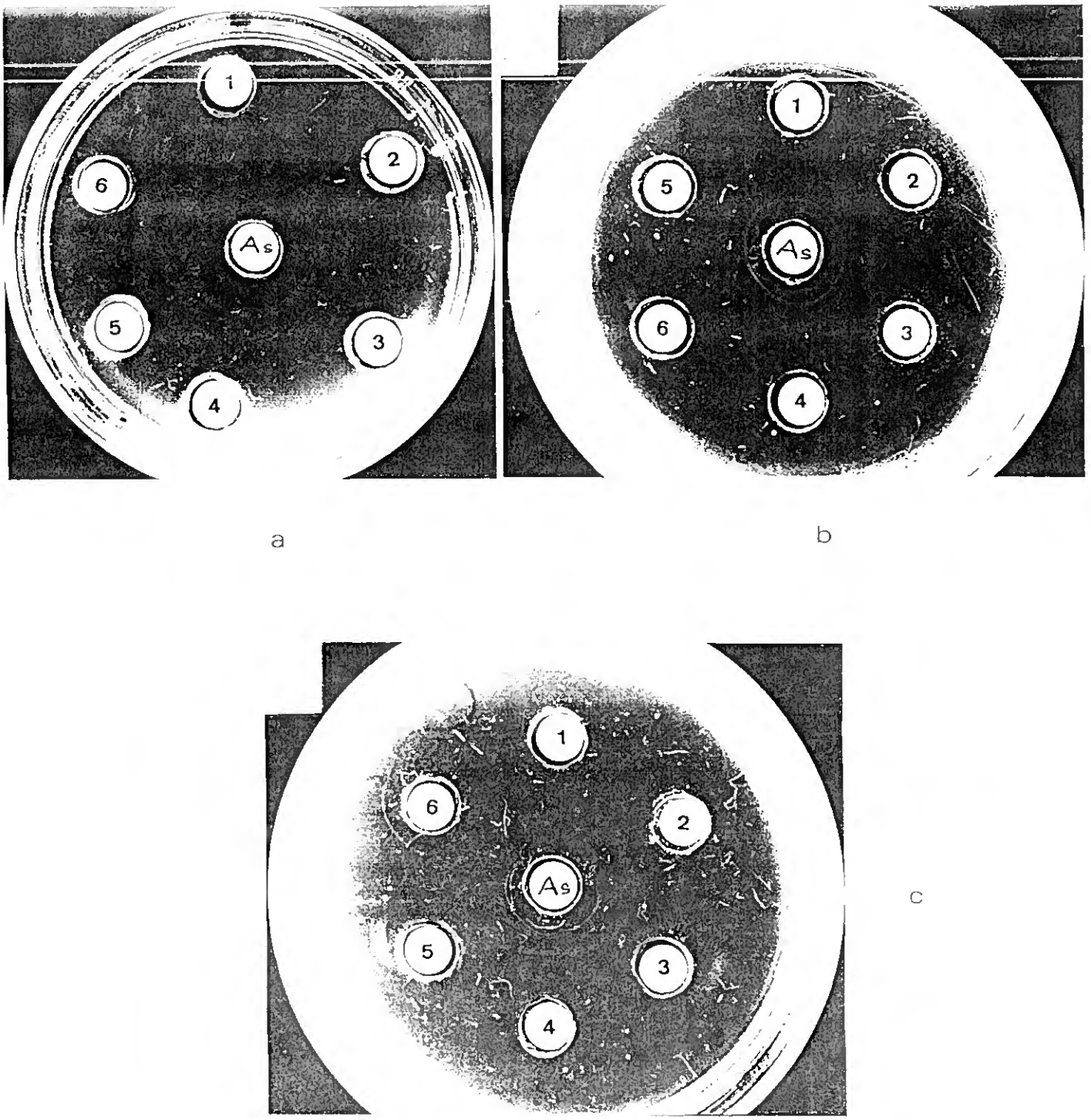


Fig.24 - Photographs of immuno-double-diffusion plates showing serological realtionships among S. citri isolates. (a) As, S. citri antiserum Bet-Dagan); well 1, Sc 136; well 2, Sc 25; well 3, Sc 102; well 4 Spiroplasma sp.; well 5, Sc 20; well 6, SDS (control). (b and c) well 1, Sc 136; well 2 Sc 25; well 3, Sc 200; well 4, Sc 84; well 5, Sc 20; well 6, SDS (control) .13

3. Characterization of S. citri isolates by one-dimensional protein analysis on polyacrylamide slab gels (PAGE)

3.1. Introduction

Five S. citri isolates reacted positively when tested by ELISA showing affinity to the antiserum prepared against the Bet-Dagan isolate, indicating some relatness. Absorbance values and ratios between these and protein content differed. However, from these results, S. citri isolates could not be distinguished, differences in pathogenicity or insect transmissibility could not be explained. Both leafhopper variants were capable to transmit the S. citri isolate from Amiad. Only one leafhopper variant (C. tenellus-A) transmitted the S. citri isolate from Modiin and both variants were not capable to transmit the isolate from Jordan Valley. In order to compare the protein pattern of the various isolates a protein analysis was done by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). Finally the Western blot, which is a combination of PAGE and serology was also applied. This technique consists on the electrophoretic transfer of proteins from polyacrylamide gel to nitrocellulose membrane and immunological detection of specific proteins.

3.2. Material and Methods

3.2.1. S. citri isolates - The isolates tested were the same as in the previous section except for Spiroplasma sp. which was not included in this experiment. These isolates were grown in BSR (modified) medium and harvested at the end of the exponential phase of growth.

3.2.2. Preparation of protein samples - Organisms were harvested from 5 ml cultures by centrifugation at 30,000 x g for 30 min. at 4°C. The pellet was washed three times by resuspension in 2 ml 0.1 M sodium phosphate (pH 7.4) - 0.33 M sodium chloride buffer, and finally resuspended in 0.1 ml of 0.06 M tris-HCl

buffer, pH 6.8, containing 5% [vol/vol] 2-mercaptoethanol, 3% [wt/vol] SDS, 10% [vol/vol] glycerol, and a few grains of bromophenol blue disruption buffer) and heated for 3 min in boiling water bath. After cooling it was stored at -20°C.

3.3. One-dimensional electrophoresis - Twelve microliters of the SDS-disrupted S. citri isolates were applied to 12% polyacrylamide minigels (10 x 7.5 cm, 0.45 mm thick) and separated by electrophoresis using a Bio-Rad Mini Protean II apparatus (Bio-Rad Laboratories, Richmond, CA). The 12% solution of acrylamide contained 1 ml Tris-hydrochloride (pH 8.8), 1.64 ml of 30% acrylamide , 2 µl TEMED , 10 µl ammonium persulfate and 1.3 ml bi-distilled water (according to Laemmli, 1970. After 1 hour the stacking gel was added and had the following composition: 0.5 ml Tris-hydrochloride (pH 6.8), 0.33 ml of 30% acrylamide, 2 µl TMED , 15 µl ammonium persulfate and 1.17 ml of bi-distilled water. A slot former, providing fifteen 0.25-cm-wide tracks, was placed into the stacking gel mixture. The stacking acrylamide solution was allowed to polymerise at room temperature for 1 h, and the slots were washed with several changes of water. The mold, with the gel in between, was clamped onto the electrophoresis apparatus. The two buffer reservoirs were filled with 200 ml of SDS-electrophoresis buffer (3g Tris-[base], 14.4 g glycine, 10 ml SDS 10% solution) and twice-distilled water to a final volume of 1 liter; final pH 8.8). Electrophoresis performed was at room temperature for 1-2 h at constant voltage (150 V). The slab was removed, stained for 1 h in Coomassie blue (0.2% [w/v] Coomassie blue, 50% [v/v] metanol, 45% [v/v] acetic acid) and then destained in 25% metanol and 7% acetic acid. The following marker proteins (Bio-Rad Laboratories) were used: lysosyme (14,400 KDa), soybean trypsin inhibitor (21,500 KDa), carbonic anhydrase (31,000 KDa), ovalbumin (45,000 KDa), bovine serum albumin (66,200 KDa) and phosphorylase B (92,500 KDa).

3.4. Immunological detection of proteins on nitrocellulose

An electrophoretic gel was prepared as described above but using a prestained SDS-PAGE marker proteins (Bio-Rad Laboratories), lysozyme (18,500 KDa), soybean trypsin inhibitor (27,500 KDa), carbonic anhydrase (32,500 KDa), ovalbumin (49,500 KDa) bovine serum albumin (80,000 KDa) and phosphorylase (106,000 KDa) were electrophoretically transferred onto nitrocellulose membranes as described by Towbin et al. (1979): a sheet of nitrocellulose (0.45 µm pore size, Millipore) was briefly blotted with transfer buffer(25 mM Tris, 192 mM glycine, 20% [vol/vol] methanol at pH 8.3) and laid on a scouring pad (Scotch-Brite) which was supported by a plastic grid. The gel to be blotted was put on the nitrocellulose sheet and care was taken to remove all air bubbles. A second pad and plastic grid were added and rubber bands were strung around all layers. The assembly was put into an electrophoretic apparatus with electrode buffer as described previously. The voltage applied was 275 volts for 45 minutes. The membrane was soaked in 1% bovine serum albumin in saline (0.9% NaCl, 10mM Tris-HCl, pH 7.4) for 2 hours at room temperature, to saturate additional protein binding sites. It was dried and incubated for 30 minutes with antiserum against an S. citri isolate (Sc 20 from Bet-Dagan) diluted in bovine serum albumin (1:500 dilution) and then kept at ± 6°C overnight. Washed in saline (3 changes during 10 minutes) and incubated, for 2 hours, with the second antibody (indicator), directed against the immunoglobulins of the first antiserum. As indicator antibodies we used horseradish peroxidase-goat-anti rabitt IgG which was diluted in bovine serum albumin (1:500 dilution). Washed with bovine serum albumin for 3 minutes and twice in PBS-Tween. Immunoreactive bands were visualised by incubating the membrane in 50 mM sodium acetate, pH 5.0, containing 0.4 mg/ml of 3 amino-9-ethylcarbazole and 0.015% (v/v) H2O2 until color developed. The reaction was stopped by washing the membrane in deionized water.

3.5. Results

Proteins from the cell membrane of S. citri have been resolved into more than 29 bands by SDS-PAGE, with apparent molecular sizes ranging from 12 to 170 kDa (Wroblewski, 1981). Spiralin, a polypeptide with an apparent molecular size of 26 to 28 kDa, is the most abundant protein in the S. citri membrane (Wroblewski et al., 1977) and is the main antigen of this mollicute. Our results (Fig. 25) show similar protein pattern for the six S. citri isolates with molecular weights (MWs) from about 14 kDa to over 94 kDa. However, bands between (MWs) of 25.5 kDA and 31 kDa, which should correspond to spiralin show differences in their mobility, especially isolate Sc 20 (lane d, [a]). Confirmation was obtained , using the Western blot technique, showing (Fig. 25 [b]) a strong affinity between S. citri antigens and τ-globulin against S. citri (Bet-Dagan), and a higher MW for Sc 20. Another difference which became apparent refers to isolate Sc 100 (Arava) where affinity to antiserum was very weak. This fact could be due to low concentration or lack of antigenic sites. Two S. citri isolates (Sc 84- lane 6 and Sc 25- lane 7) tested for their transmissibility by two variants of the C. tenellus complex have similar protein profiles. Isolate (Sc 90- lane 2) also tested for its transmissibility, showed a slightly lower MW.

The relatdness between isolates Sc 84 and Sc 25 , confirms the differences earlier observed between the two C. tenellus variants in their capability of transmitting the pathogen. Regarding isolate Sc 90, which was not transmitted by either variants, may prove to be a different S. citri strain. DNA sequencing would have to be done to determine whether differences existe, at molecular level, between these isolates.

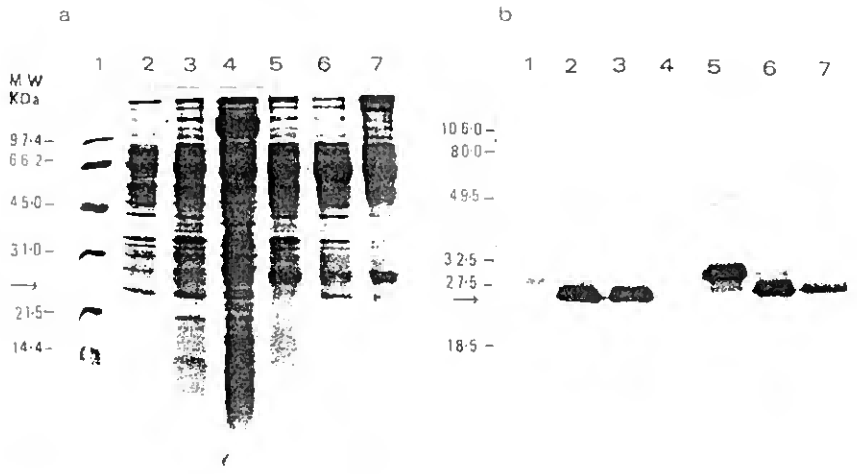


Fig. 25 - SDS-polyacrylamide gel electrophoresis (a) and immunoblotting (b) of S. citri isolates. Conditions and molecular weight standards are described in Material and Methods section. Lane 1 (a and b), molecular weight standards; lane 2, Sc 90; lane 3, Sc 210; lane 4, Sc 100; lane 5, Sc 20; lane 6, Sc 84 and lane 7, Sc 25.

4. Characterization of S. citri isolates by DNA-DNA hybridization

The use of cloned "random" fragments of MLO DNA and MLO genes as molecular probes in nucleic acid hybridizations, has permited sensitive and specific detection of MLOs in infected plant or insect tissues. It has also permited investigation of the genetic relatedness among MLOs from various sources (Davis et al., 1988).

Relatedness of the various S. citri isolates were determined by serology and electrophoresis of S. citri protein. An additional test for relatdness was made by dot hybridization using probes from cloned DNA derived from isolate Sc 90 (Jordan Valley). This clone is designated herein as pSc-90.

4.1. Material and Methods

The S. citri isolates were Sc 90 (Jordan Valley), Sc 25 , Sc 84 (Modiin), Sc 20, Sc 200 (Hadera) and Sp Jer (honeybee). 5 ml from each culture were centrifuged at 13,000 x g for 20 minutes. Pellets were resuspended in 0.5 ml of BSR medium. DNA was extracted from Sc 90, Sc 25 and Sp Jer as described below.

a) DNA extraction: DNA was extracted from washed spiroplasma cell pellets according to Marmur (1961). Spiroplasmas were collected from 500 ml of culture, at late log phase, by centrifugation at 20,000 x g for 30 minutes. The cell pellet was washed in 2,5 ml of phosphate-NaCl buffer (0.1 M phosphate, pH 7.4, in 0.33 M NaCl) and recentrifuged at 20,00 x g for 30 minutes at 4°C. DNA was extracted from the resulting pellets by the technique of Gross-Bellard et al.(1973), as described by Carle et al. (1983): pellets were resuspended in 10 ml of pH 8 buffer (0.1 M EDTA disodium salt at pH 8.0 in 0.15 M NaCl). Cells were lysed by addition of 25%sodium dodecyl sulfate (SDS) to a final concentration of 2.5%. Incubation was carried out at 60°C for 10 minutes. After cooling, 5 M sodium perchlorate was added to a final concentration of 1 M. Deproteinization was done as follows:

an equal volume of chlorophorm:isoamyl alcohol solution (24:1) was added and mixed for 30 minutes. The resulting emulsion centrifuged for 15 minutes at 4000 x g. The aqueous phase containing the nucleic acids was submitted to a second deproteinization with chlorophorm:isoamyl alcohol for 15 minutes and the emulsion centrifuged for 15 minutes at 4,000 x g. The nucleic acids from the last aqueous phase were precipitated by the addition of two volumes of cold ethanol, by centrifugation at 12,000 x g for 10 minutes. Dried for 5 minutes, by vacuum, and dissolved in 0.5 ml of distilled water. Concentration obtained was 1 µg/1µl. RNA elimination was done by adding RNase (20µl/ml) at 37°C for 30 minutes. DNA was again purified by phenol extraction by adding 0.5 ml of phenol:chlorophorm (1:1), vortex 1 minute and centrifuge for 2 minutes at 14.000 x g. Supernatant was collected and precipitated with 2 volumes of 70% ethanol (containing 1/10 sodium acetate 3M, pH 5.5) by centrifugation at 12,000 x g for 10 minutes. The pellet washed with 70% ethanol, dried by vacuum and dissolved in 0.5 ml of distilled water.

b) DNA Digestion: DNA was digested by restriction endonuclease EcoRI by adding to 5 µl DNA 3 µl restriction buffer (500 mmol Tris-HCl, 10 mmol MgCl2, 1000 mmol NaCl, 10 mmol Dithioerythritol [DTE]), 20 µl distilled water and 2 µl EcoRI (20 U) (Boehringer Mannheim, Germany). Incubated overnight at 37°C. Digested DNA was electrophoresed on 1% agarose gel at 100 volts for 90 minutes.

Agarose gel consisted of: 1% solution of agarose in TAE (0.04 M Tris-acetate, 0.001 M EDTA [Ethylenediaminetetraacetic acid]) buffer, boiled for 40 seconds in a microwave oven. After cooling, poured into a BIO-RAD (wide mini-sub cell) apparattus. A volume of 5 µl of DNA solution was mixed with 1 µl bromophenol dye and 9µl of distilled water and alliquots (15 µl each) of this nucleic acid poured in each well. The gel was transferred onto a solution of 0.1-0.5 µg of ethidium bromide for 20 minutes and washed in water for another 20 minutes. The gel was examined under a UV light were the bands of DNA could be observed (Fig. 26).

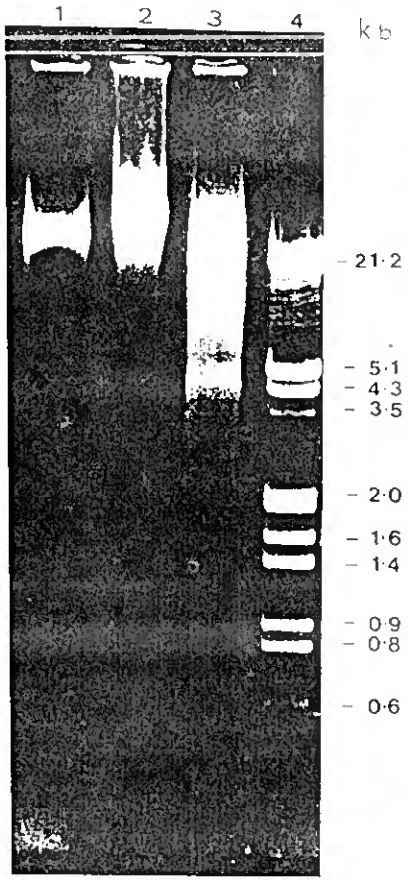


Fig. 26 - Electrophoretic analyses on 1% agarose gel of purified DNA extracted from S. citri culture Amiade (lane 1), Jordan Valley (lane 2), Jordan Valley DNA digested by EcoRI restriction enzyme (lane 3) and DNA digested by EcoRI/HindIII molecular marker (lane 4).

c) Cloning EcoRI fragments into a bluescript plasmid: Bluescript plasmids (phagemids) (Stratagen) are cloning vectors that have been designed to simplify commonly used cloning. They will replicate autonomously as plasmids, therefore, colonies are obtained following transformation. The plasmid has a large polylinker with 20 restriction sites (including EcoRI site). Plasmids which have no inserts in the polylinker will grow as blue colonies on the appropriate strains of bacteria. Plasmids with inserts will grow as white colonies on the same strain, since the inserts interrupt the coding region of the LacZ gene fragment. These strains allow blue-white color selection for bluescript plasmids containing inserts when plated on LB (Luria-Bertani Medium) plates containing 100 µg/ml ampicillin, 40µg/ml X-gal, 5 mM IPTG. [Luria-Bertani Medium: 10 g Bacto-tryptone, 5 g

Bacto yeast extract, 10 g NaCl per liter (pH 7.5)].

Digestion of the vector by restriction enzyme EcoRI folowed the same procedure described above for DNA.

d) Purification of the digested DNA and the EcoRI digested plasmid: To 30 µl of digested DNA ,70 µl of distilled water were added and 100 µl of phenol-chloropform solution (1:1). After mixing by vortex for 1 minute it was centrifuged for 2 minutes. The supernantant was collected and then precipitated by adding 2 volumes of ethanol with 1/10 of the volume of 3M sodium acetate (pH 5.5). Incubated at -20°C for 2 hours and precipitated by centrigugation at 14.000 r.p.m. for 10 minutes, washed with 70% ethanol (1 ml) and dried for 5 minutes by vacuum and resuspended in 30 ul of distilled water.

e) Ligation of EcoRI DNA fragments to digested EcoRI bluescript vector was done by mixing: 50 ng EcoRI linearized plasmid, 150 ng EcoRI digested spiroplasma DNA , 2 µl Ligation buffer (10x), 2 units of T4 DNA Ligase, in a volume of 20 µl. It was incubated overnight at 16°C. (Ligation buffer [x10]: 0.5 M Tris hydrochloride [pH 7.4], 0.1 M MgCl , 0.1 M dithiothreitol, 10 mM spermidine, 10 mM ATP, 1 mg/ml BSA).

f) Transformation of the plasmids obtained after ligation: 0.2 ml of E. coli JM 101 competent cells (derived from a stock kept at -80°C which had been prepared by the method described in Maniatis et al., 1982) was added to the ligation reaction and kept on ice for 30 minutes. Tubes containing the mixture were placed in water for 2 minutes at 42°C and immediatelly transferred to ice (heat-shock treatment). LB medium (0.6 ml) was added and incubated at 37°C for 40 minutes, in a shaker, (for expression of the ampicilin resistance
β-lactamase gene). 200 µl of wet cells transformed with ligation reaction were platted into each of four Amp plates containing 40 µg/liter X-Gal, 0.1mM/ml IpTG and 0.1 g/l ampicillin. Plates were incubated overnight, upside down, at 37°C. Colonies with intact KS become blue. Colonies with inserts in β-galactosidase gene become white.

g) Screening for colonies which contain the insert of the spiroplasma: A plasmid Mini-Prep (protocol from PROMEGA) was done in order to isolate the insert carrying plasmids. 5 ml of LB broth containing 100 µg/ml of ampicillin was inoculated with a single bacterial colony. Incubated at 37°C overnight with vigorous shaking. An alliquote (1.5 ml) of culture was placed into a microcentriguge tube and centrifuge at 12,000g for 1 minute. Medium was removed by aspiration leaving the bacterial pellet. The pellet was resuspended by vortexing in 100µl of an ice-cold solution containing 50 mM glucose, 10 mMEDTA and 25 mM Tris-HCl, pH 8.0. RNase was added to a final concentration of 10 µg/ml. Incubated for 5 minutes at room temperature. A volume 200 µl of a freshly prepared solution containing 0.2N NaOH, 1% SDS was added and mixed by inversion for 10 seconds. Incubated on ice for 5 minutes. Added 150 l of ice-cold potassium acetate, pH 4.8 (3M potassium and 5M acetate). Mixed by inversion for 10 seconds and incubated on ice for 5 minutes. Centrifuged at 12,000g for 10 minutes and the supernatant transferred to a fresh tube, and centrifuged again for an additional 5 minutes. Supernatant transferred to a fresh tube and added 1.0 volume of phenol:chlorophorm saturated with TE buffer, vortexed for 1 minute and centrifuged at 12,000g for 5 minutes. The upper aqueous phase was transferred to a fresh tube and 2.5 volumes of absolute ethanol added, mixed and preciptated for 5 minutes at room temperature. Centrifuged at 12,000g for 15 minutes, rinsed with 70% ethanol (prechilled) and the pellets dried and dissolved in 16 µl of deionized water. To verify wether clones of spiroplasma DNA had been formed, 5 µl of the solution was digested with EcoRI, as described above, and then separated by electrophoresis in 1% agarose gel (Fig. 27).

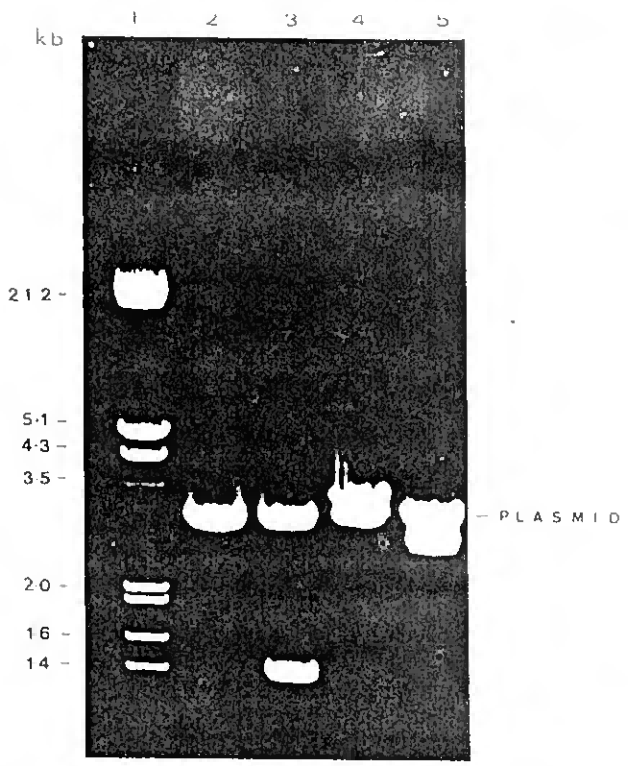


Fig. 27 - Electrophoretic analyses on 1% agarose gel of S citri clones 5, 9, and 3 (lanes 2, 3 & 4 respectively) digested by EcoRI restriction enzyme linear KS plasmid (lane 5), DNA digested by HindIII/EcoRI, molecular marker in MWs (bp) (lane 1). Arrow indicates DNA plasmid. The gel was stained in ethidium bromide for visualization.

h) Random primed DNA labeling: Using the method of Feinberg and Vogelstein (1983) which is based on the hybridization of a mixture of all possible hexanucleotides to the DNA to be labeled. The random primed reaction mixture contained the following: 100 ng of denaturation plasmid DNA, 5 µl of hexanucleotide mixture in 10 x reaction buffer, 2 µl 0.5 mM of dATP, dGTP,dTTP, 100 µCi of [$\alpha 3^2P$] dCTP, 2 units of Klenow enzyme (PROMEGA) and completed with water to a volume of 50 µl. The reaction was carried out at 37°C for 30 minutes.

i) Spotting of the samples on a Hybond-N membrane: Preparation of S. citri samples: Concentration of the S. citri isolates, in BSR medium, was done by centrifugation at 15.000g of 5 ml of culture . The pellet was resuspended in 0.5 ml of medium and SDS added to final concentration of 0.5%. Alliquots of 3 µl were deposited on the membrane, from 6 S. citri isolates (culture) and from DNA (200-500 ng) extracted from 3 of those isolates. The membrane was dried for 30 minutes and the DNA fixation to the membrane was done by exposing it to UV radiation for 3 minutes.

j) Pre-hybridization (saturation of the membrane with a non-specific DNA for blocking the membrane): The membrane was pre-hybridise for 2 hours at 42 °C in a pre-hybridisation solution: 6xSSC, 5xDenhart's solution (0.1% polyvinylpyrrolidone [PVP], 0.1% Ficol, 0.1% bovine serum albumin [BSA]), 50mM sodium phosphate buffer, 2 mg/100 ml Salmon sperm DNA.

l) Hybridization: The random priming probe (50 µl) was denatured by boiling for 5 minutes and immediately transferred to ice . It was added to the pre-hybridization solution covering the membrane and incubated for 16 hours at 42°C. After the hybridization was completed the solution was discarded and the membrane washed for 5-10 minutes twice, in a large volume (300-500 ml) of 2 x SSC (saline-citrate buffer) containing 0.1% SDS at room temperature, and then washed again twice with 0.2 x SSC containing 0.1% SDS for 10 minutes at 50°C. The membrane was air dried on a sheet of Whatman 3MM paper at room temperature. The Whatmann paper and membrane were covered in Saran Wrap and applied to X-ray film (Kodak XR) and exposed overnight at -70°C with an intensifying screen.

m) Development of the film: The film was soaked in Kodak liquid X-ray developer - 5 minutes; washed in a water bath - 1 minute; fixed with Kodak rapid fixer - 5 minutes and washed in running water for 15 minutes (Fig. 28).

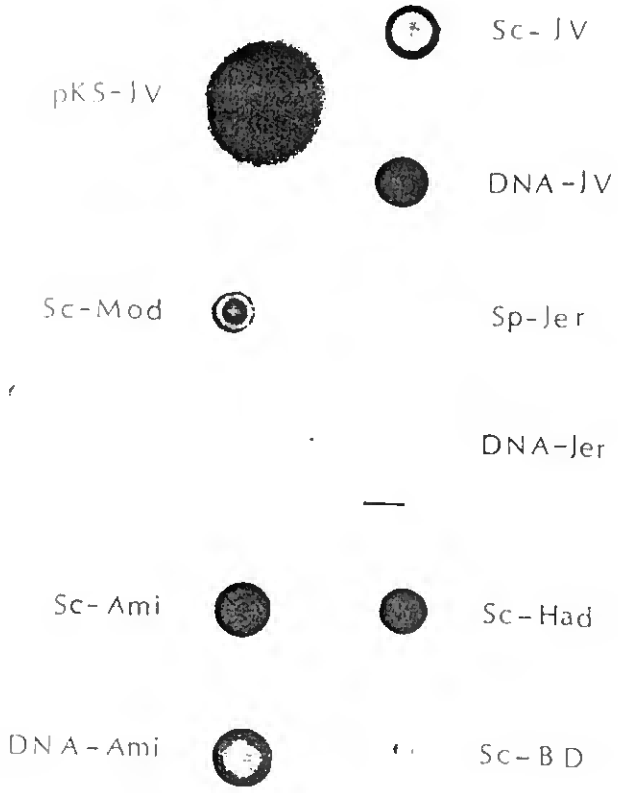


Fig. 28 - Hybridization of S. citri DNA probe with DNA of various spiroplasma isolates. Lysed cultures of: Sc 90 (J.Valley) (Sc-JV); Spiroplasma (honeybee) (Sp-Jer); Sc 200 (Hadera) (Sc-Had); Sc 20 (B. Dagan) (Sc-BD); Sc 83 (Modiin) (Sc-Mod) and Sc 25 (Amiad) (Sc-Ami). DNA purified from cultures of: Sc 90 (DNA-JV); spiroplasma (honeybee) (DNA-Jer) and Sc 25 (DNA-Ami).

4.2. Results

The DNA preparations from Sc 90 and of DNA clones in agarose gel electrophoresis is shown in Fig. 25 and Fig. 26. The probe from clone pSc-90 hybridized with spiroplasma cultures of isolates Sc-90, Sc-200, Sc-20, Sc-83 and Sc-25 and also with spiroplasma DNA extracted from Sc-90 and Sc-25. No hybridization occurred with Spiroplasma sp. (honeybee), which was used as a negative control and proves thespecificity of the probe to S. citri (Fig. 27).

These results confirm the relatedness between all isolates being studied. It does not prove, however, why Sc-90 (from Jordan Valley) was not transmitted by either of the variants. Further tests, at molecular level may help to determine the cause, which can reside in one or more point mutations, and cannot be detected serologically or by hybridization methods.

DISCUSSION AND CONCLUSION

When characterizing morphologically the two variants of the complex, both leafhoppers present the typical characteristics of the species C. tenellus. Differences between them reside in the color (green and yellowish) and presence or absence of a denticle in the females 7th sternum. These are not very significant as they can vary within the same population.

With regard to host range, the highest longevity for the two variants, was observed on Brassica oleracea followed by Beta vulgaris and M. incana respectively. The differences were, however, not significant. It should be mentioned, however, that in relation to M. incana , C. tenellus-P did not adapt easily to this host and only after approximately one year was the culture established. The highest number of descendents was produced when fed on M. incana, which was then the host plant chosen to rear the insects and carry out experiments. Other reasons contributing for the use of this plant was its adaptation to laboratory climatic conditions and a resistance to aphids when plants were kept in the greenhouse.

With the fourth plant tested, Brassica rapa, C. tenellus-P was the only one which could survive and reproduce on this plant.

The preference for the Cruciferae and Chenopodiaceae is in accordance with the findings of several authors when studying the beet leafhopper in the U.S.A. (see Literature Review).

Cross-breeding the two variants did not produced any offsprings. Although this information may not be conclusive it provides an important factor to consider the distinction between them.

Leafhoppers of both variants transmitted S. citri to M. incana plants after being injected with the pathogen. There was, however, a significant difference in the rates of transmission. The highest observed, for a single insect, following injection was by C. tenellus-A (39.4%) while by C. tenellus-P was only 8.8%. The isolates were both from Amiad (Sc25) and (Sc30) respectively. When leafhoppers were injected with isolates (Sc 50

and Sc 83), from the coastal plains, the rate of transmission by the first variant of leafhoppers was 22.3% while the second variant had a rate of transmission of 9.0% in a first experiment (with Sc 50) and did not transmit when tested a second time (with Sc 83).

When insects acquired the pathogen by feeding, similar results were obtained, with C. tenellus-A transmitting (11.9%) and C. tenellus-P (2.6%). When further experiments were carried out i.e. to determine AAPs, no infected plants were obtained when the second variant was used as a vector.

The acquisition threshold, determined for C. tenellus-A, was one hour. The rate of transmission did not always increase with a higher AAP. This can be expected as selection of a particular genetic line with high transmission rates was not chosen for these experiments. Therefore, transmission was dependent on the vectorial capacity of the individual leafhoppers as well as in the S. citri titer in the plant. Results are very similar to the ones obtained with C. tenellus in the U.S.A. where S. citri siolates from the Western States were used (Klein, personal communication). The minimum incubation period was between 6 and 7 days. The latent period (LP_{50}) or time required for 50% of the population to transmit the pathogen was calculated at 26 days. The minimum time required for innoculative leafhoppers to feed on healthy test plants and transmit the pathogen was one hour.

The periods determined for acquisition and inoculation are most probably longer than what occurs in nature due to the handling of the insects during the experiments.

There was no significant difference between transmission by males and females.

It can be concluded that the two variants of the C. tenellus complex in Israel varied in host preference and vectorial capability for the S. citri isolates tested.

The fact that stubborn disease exists in the coastal plains of Israel implies the presence of a vector. At this stage we know that one variant of the two C. tenellus studied here is indeed a vector of S. citri. This particular variant has not been found in this area. However, C. tenellus-P has been found in the costal

plain but transmits the pathogen at a very low rate, which cannot explain the spread of stubborn disease in this area. The latter leafhopper could possibly be an efficient vector but for a specific isolate which has not yet been identified. Another possibility is that a different leafhopper acts as a vector in this area, i.e. C. haematoceps as the case in Syria (Fos et al., 1985). Therefore this is a study which should be carried out. A third possibility is that C. tenellus-A migrates from its natural habitat in search for more suitable hosts, but does not establishe itself due to the lack of a reproduction host.

Pathogenicity of S. citri to C. tenellus-A showed that longevity of the leafhoppers was significantly different between those which were infected after acquisition feeding on diseased plants and those feeding on healthy ones with mean longevities of 15 and 20 days respectively. Fecundity was also affected with a net reproductive rate of 28 females/female/generation as compared to 45 in control.

To characterize S. citri isolates obtained from different geographical regions in Israel and from various citrus varieties, several tecnhiques were used.

The immuno-diffusion test showed that the isolate from (pummelo in Arava) did not cross react with antiserum obtained from a Bet-Dagan isolate (Valencia orange), but presented a similar protein pattern by SDS-PAGE protein analysis and by the western blot technique. The dot hybridization showed that probes from cloned DNA derived from isolate Sc 90 (grapefruit -Jordan Valley) hybridized with spiroplasma cultures from all isolates as well as with DNA extracted from Sc 90 and Sc 25 (grapefruit - Amiad). It cannot be conclusive, however, as further tests, at molecular level, could detecte one or more point mutations which could explain the specificities in the vectors.

These results indicate a close relation between all isolates and adds information towards the characterization of the two leafhoppers. Assuming that isolates Sc- 25 and Sc-83 from Amiad and Modiin, respectively, belong to the same strain while transmissibility by those vectors is not the same, then the cause must lie in the difference between C. tenellus A and C. tenellus P.

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Appendix A

S. citri isolates and geographical regions in Israel from where they originated.

Isolate	Common name	Citrus species and hybrids	Region
Sc 25	grapefruit	*Citrus paradisi*	Amiad
Sc 30	"	" "	"
Sc 136	"	" "	Jordan Valley
Sc 20	orange	*Citrus aurantium*	Bet-Dagan
Sc 50	pummelite	*C.paradisi* x *C.grandis*	Modiin
Sc 83	"	" "	"
Sc 84	"	" "	"
Sc 200	red grapefruit	*Citrus paradisi*	Hadera
Sc 102	pummelo	*Citrus grandis*	Arava

Sp Jer - *Spiroplasma mellifera* (provided by Hadassah Medical School Jerusalem)