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Review Article

Xyloporosis: A history of the emergence and eradication of a citrus viroid disease.

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Abstract

The etiology of xyloporosis, a disease that has severe effects on citrus trees grafted onto certain citrus rootstocks, was enigmatic for a long time. Symptoms on test hosts following transmission through grafting suggested that it was synonymous with citrus cachexia, a disease that mainly affects mandarin trees. Recent molecular studies have confirmed that certain Hop stunt viroid (HSVd) isolates induce cachexia and xyloporosis symptoms in disease-sensitive citrus hosts. These HSVd infections are mostly symptomless in numerous Near East and Western Mediterranean fruit trees and grapevines; including plants widely cultivated in those regions for several millennia, long before the emergence of xyloporosis and cachexia as diseases of citrus trees. The present review tracks historical changes in citrus propagation practices and the pathological consequences of those changes that contributed to the emergence of xyloporosis as an economically significant disease of citrus trees grafted onto Palestinian sweet lime rootstocks. The take-home message of these accounts is the need for close cooperation between plant scientists, plant protection scientists, and growers to ensure that changes and proposed improvements in horticultural and plant protection practices are subjected to comprehensive risk-assessment analyses.

Keywords: Hop stunt viroid, phytophthora root rot, inarching, viroid dwarfing, endemic diseases

Introduction

Emerging plant diseases represent a continuous threat to economically important crop plants. Pandemics that affect supplies of grains and other annual subsistence crops are of particular concern (Fletcher et al. 2010). Diseases of perennial fruit trees are often very important for producers, due to the high costs of establishing intensively cultivated orchards and the extended amount of time needed to recover from outbreaks of diseases for which no effective chemical controls are available. For annual crops, a sensitive genotype can be replaced with disease-resistant varieties within a relatively short period of time. Diseases affecting fruit trees and vines may cause considerable losses through the gradual accumulation of newly affected trees over a number of years. This review describes how an important disease emerged, probably not from ingress of a new pathogen, but more likely from changes in cultural practices. It brings together information from a range of literary sources on the historical background of the emergence of xyloporosis and the linkage of this epidemic event with the history of citrus cultivation and with the natural history of Hop stunt viroid (HSVd) and other citrus viroids endemic to the Near East and the Mediterranean region.

Emergence of xyloporosis and how its name was changed to cachexia disease

Reichert and Perlberger (1934) reported on a "new" disease, xyloporosis, which appeared in 1928 among many of the newly planted citrus groves of Shamouti sweet orange trees that had been grafted onto Palestinian sweet lime (PSL), the most commonly used rootstock in the British mandate-ruled area of Palestine. These authors described 3 essential phases of xyloporosis. First, small depressions appear on the stem bark of the rootstock with small conoid pits with interfacing brownish pegs in the inner part of the bark. These symptoms often appear within 1 year of grafting and are most noticeable close to the bud union. In the second stage these symptoms intensify: The wood becomes discolored and, typically, the young tree becomes bent over and its leaves show symptoms typical of trees with root rot. In the third stage, there is blackish discoloration on the bark, the bark splits and leaves are small and yellow. Eventually, the branches wilt and die.

Following intensive observations of a large number of infected trees of different ages in different planting areas, Reichert and Perlberger (1934) concluded that xyloporosis was present in all parts of the country and was not associated with any specific horticultural practice.

Furthermore, although some differences were noticed among trees subject to different edaphic conditions, the disease could not be associated with any specific soil problem. The disease symptoms intensified as the trees aged. Seed source did not affect disease incidence. Pathological tests indicated that the disease was not associated with culturable fungal or bacterial pathogens. These observations led the authors to suggest 2 possible etiological causes of the disease: an unprecedented physiological disorder or an unknown viral pathogen.

A major contribution to xyloporosis research was the observation of transmission by grafting (Childs 1950; 1952) of the cachexia disease affecting mandarins to a number of hosts, including a few that showed xyloporosis-like symptoms (Childs et al. 1965). These results suggested that the names of the 2 diseases, xyloporosis and cachexia, are synonymous and, based on the convention of prioritizing scientific names, the name cachexia was proposed for both diseases. Cachexia was found throughout most or all citrus-growing areas, particularly among Mediterranean varieties in Florida.

Attempts by Norman and Childs (1963) to spread the cachexia disease via 5 different insect species failed and Olson (1965) showed that cachexia is not transmitted through seed. Later studies, reviewed by Bar-Joseph (2003), showed that not only xyloporosis, but also exocortis and 3 other citrus viroids, were not seed-transmitted to citron seedlings. The finding by Calavan and Christiansen (1965) that 'Parsons Special' mandarin showed more distinct symptoms than PSL supported the notion that the disease agents that cause xyloporosis and cachexia on different hosts are closely similar or identical.

The history of citrus production in the land of Israel

Recently, Langgut et al. (2013) reported the finding of typical citron (*Citrus medica*) pollen grains among the extracts from one layer of plaster, deposited to prevent leakage from an ancient irrigation pool belonging to a royal palace garden at present-day Kibbutz Ramat Rachel, near Jerusalem. Archeological evidence dated the construction of the facility to the Persian period (~538 BCE) and provides the first physical evidence for the earliest cultivation of citrus in the province of Judea.

Initially, local growers refrained from grafting citron trees and were not familiar with the grafting technique (Schwartz 1844). The second edition of this book (Schwartz 1862) reported that some growers had started practicing grafting of citron on the PSL rootstock, instead of propagation as seedling plants. Grafting was necessary because of the emergence of the destructive phytophthora gummosis disease, which rapidly decimated non-grafted trees and entire citrus industries throughout the Mediterranean basin (Klotz 1978).

The dependence on grafting to save trees from diseases coincided with the selection of the Shamouti orange, which was obtained from a bud mutation (sport) of the local orange (Spiegel-Roy 1979). Unlike the fruit of its seedy parent, the Shamouti fruit was almost seedless and these trees were propagated by grafting onto rootstocks of an easily rooting citrus species, the PSL.

A seemingly minor horticultural change in the propagation of Palestinian sweet lime rootstock and its grave consequences: The emergence of phytophthora root rot and xyloporosis

Citrus cultivation expanded beyond the coastal plain (Jaffa area), where the Shamouti orange on PSL rootstocks was performing excellently, to new production areas just 10 km east of Jaffa, where all of the trees rapidly succumbed to the phytophthora gummosis disease. Phytophthora damage on PSL was initially blamed on the heavier soils of the new planting areas. However, when local citriculture expanded to sandy soils the root rot problems continued despite the improved edaphic conditions. It took almost 50 years for researchers and growers to realize that PSL rootstocks made from cuttings were different from juvenile PSL seedlings, which were more sensitive to phytophthora root rot, and that the source of this difference was the absence in PSL seedlings of the viroid load commonly present in the rootstocks that were made from cuttings.

The positive effect of citrus viroid infection, the acquired resistance of phytophthora-sensitive rootstocks induced by viroid infection, was first noticed by Rossetti et al. (1980) in Brazil. They noted that trees grafted onto Rangpur lime seedlings were succumbing to gummosis while those infected by the citrus exocortis viroid (CEVd) remained unaffected. Later studies using viroid-free and viroid infested buds of Shamouti grafted on PSL (Ashkenazi and Oren 1977) and also of other citrus stionic combinations (Semancik et al. 2005; Tina et al. 2010) confirmed this observation. Solel et al. (1995) showed that viroid infection also provides citron and Rangpur lime with tolerance to another serious fungal disease, mal secco (*Phoma tracheiphila*). In retrospect, the change in susceptibility to phytophthora root rot could now be associated with the shift from the traditional practice of raising PSL rootstocks from cuttings to producing rootstocks from seed.

The change in nursery practice from the use of cuttings to propagation from seeds was necessitated by the expansion of citrus plantings in the coastal areas of the country. Seedlings were either grafted at the nursery or transplanted into orchards where they were later budded, mainly with Shamouti sweet orange scions. In his chapter on citrus propagation, Karlinsky (2001) wrote:

> Propagation of citrus and especially of the Shamouti variety underwent a considerable change during the years 1890-1939. At the early stage the new growers adopted the traditional practice that consisted of two stages. The first was rootstock propagation, followed with the grafting of the scion variety. The commonly used rootstock was the PSL. The grower would allocate a small plot in his orchard for propagating the rootstock. For

> propagation, he used cuttings that were densely planted and normally maintained in the propagation plot for two years where they developed twigs and root systems and were transplanted into prepared holes at the final planting density. They were then maintained for additional two years before grafting. Grafted plants normally started bearing after additional two years. (p. 120)

Inarching, the savior of trees grafted onto Palestinian sweet lime saplings

One of the final comments of Reichert and Perlberger's 1934 publication stated that "inarching trees on PSL with xyloporosis-tolerant sour orange rootstock offered a potential method for controlling both gummosis and xyloporosis." This was a valuable cultural practice that allowed the continued initial use of the PLS rootstocks. Indeed, despite the immediate danger of infection by xyloporosis, growers did not give up on the practice of grafting their Shamouti groves onto PSL rootstocks. The reasons for this are listed below:

1. Trees grafted onto PSL start bearing fruit 2 to 3 years after planting, at least 2 years sooner than trees grafted onto sour orange rootstocks.
2. Trees grafted onto PSL are moderately dwarfed, which allows for dense planting and, as a result, high levels of productivity can be reached soon after planting.
3. Trees grafted onto PSL, even young trees, produce fruit with excellent quality, including smoother skin and sweeter fruit.
4. Trees grafted onto PSL do not exhibit alternate bearing like trees grafted onto sour orange.

These qualities were especially important since the production of fresh fruit for export was dependent on both high yields and the highest possible fruit quality. In the absence of alternative xyloporosis-resistant rootstocks to produce high-quality Shamouti fruits, which at that time was the country's most important export product, local growers adapted a unique practice of propagating and planting Shamouti on PSL seedling rootstock, and then inarching each tree with 1 or 2 sour saplings 2 or 3 years after planting. This prophylactic practice combined the early production of PSL rootstocks with the xyloporosis and gummosis tolerance of the sour orange inarch. Furthermore, it also incorporated some of the initial advantages of citrus viroid dwarfing practice, including the early productivity of densely planted orange trees.

Xyloporosis-cachexia: The connection to the Hop stunt viroid, a ubiquitous pathogen of fruit trees

Citrus, grapevines, and many other fruit trees which are not closely related to hops share a common pathogen, HSVd (Sano 2003), which is a member of the *Pospiviroidae* family (Flores et al. 2003). The 'dwarf hop' disease was first noticed in Japan around 1940 and its causal agent was identified as a circular single-stranded RNA molecule of 297 nt, which was named HSVd (Takahashi and Takusari 1979; Ohno et al. 1983; Sano 2003). Cucumber pale fruit viroid, originally considered a new viroid species, was found to be caused by a 303 nt HSVd variant that shares 95% sequence identity with the type strain (Sano et al. 1984). Later work detected HSVd variants in symptomless grapevine plants from different geographic regions (Flores et al. 1985; Puchta et al 1988; Amari et. al 2007; Kaponi et al. 2009). HSVd also infects a wide range of fruit trees (Hadidi et al. 1992; Amari 2007; Astruc et al. 1996; Pallas et al. 2003; Kaponi et al. 2009).

A recent GenBank search (September 2014) identified a total of 643 HSVd sequences. Of these, 99 are associated with citrus, 81 with grapevine, 72 with peach, 37 with apricot, 35 with plum, 15 with fig, 14 with pomegranate, 13 with jujube, 8 with mulberry, 6 with apple, 5 with cucumber, and 4 with almond. The fact that HSVd was found among different non-graft-compatible fruit trees in the Mediterranean region and Near East, including wild saplings in remote areas (Kaponi et al. 2009; PE Kyriakopoulou, personal communication), and the apparent absence of any flying insect vector suggested the possibility of mechanical transmission (Garnsey and Jones 1967). One possible mechanical vector over long distances is the goat, as demonstrated by Cohen et al. (2005).

Sano (1988) was the first to report the presence of an HSVd-like molecule in citrus upon examining citron plants from Japan. Puchta et al. (1989) sequenced a citrus viroid from cucurbit plants that had been mechanically inoculated with RNA extracts of a grapefruit tree harboring the GTD #225 dwarfing complex from Israel (Hadas et al. 1989) and found it to be very similar to the HSVd type strain. Top grafting of PSL buds onto GTD #225-inoculated grapefruit trees resulted in xyloporosis symptoms on the PSL stems about 2 years after grafting (M Bar-Joseph, unpublished). The renaissance in the recognition of the role of citrus HSVd isolates in the etiology of cachexia disease had its start with the realization of Semancik, Roistacher, Duran-Vila (1988) and Semancik, Roistacher, Rivera-Bustamante, et al. (1988) that the variation of viroid-induced symptoms on citron is not solely the result of different CEVd isolates.

Developments in biophysical separation of small single-stranded circular RNA molecules allowed Semancik (1986) and Rivera-Bustamente et al. (1986) to demonstrate that old clone citrus trees are often infected with mixtures of citrus viroids (CVd) of different sizes, which they classified on the basis of physical and biological properties into 5 groups (Duran-Vila et al. 1988; Semancik and Duran-Vila 1991) with Group II comprising HSVd-related viroids.

The unique physical nature (heat resistance) of the mechanically transmissible cachexia agent led Roistacher et al. (1983) to suggest that a viroid was probably

involved in the etiology of the disease. This was first demonstrated by Semancik, Roistacher, and Duran-Vila (1988) and Semancik, Roistacher, Rivera-Bustamante, et al. (1988), who showed that cachexia is caused by CVd-IIb, a 299 nt HSVd isolate, and later confirmed by Davino et al. (1991). Cachexia and xyloporosis symptoms were also induced in 'Parson's Special' and PSL by the HSVd isolates CVd-IIc and Ca-903, but not by CVd-IIa. This indicates that not all HSVd isolates cause these diseases in citrus and also confirms the notion that the 2 disease designations reflect the distinct responses of different indexing hosts to similar HSVd isolates, rather than 2 diseases caused by different pathogens (Reanwarakorn and Semancik 1998, 1999).

Another interesting result was the finding that the cachexia-inducing variants are more similar to hop-type HSVd sequences than to the symptomless citrus-type HSVd designated as CVd-IIa. Although we do not know when an HSVd isolate first came into contact with citrus, it seems plausible to assume that isolates like CV-IIa with reduced pathogenicity on cachexia/xyloporosis-sensitive citrus hosts preceded the symptom-inducing isolates that were either maintained in non-sensitive citrus varieties or derived from local wild and/or cultivated alternative host plants.

Molecular and biological comparisons of cachexia-inducing and non-inducing HSVd isolates have revealed the presence of a "cachexia expression motif" of 5 nts within the variable (V) domain that are necessary for disease expression. RNA-folding analyses have suggested probable structural changes within HSVd molecules showing this motif, which are assumed to result in conformational pathogenicity (Palacio-Bielsa et al. 2004). These predictions have been supported by site-directed mutagenesis work, which has shown that even minor changes in the cachexia motif can eliminate or moderate infection (Serra et al. 2008). However, it is interesting to note that analyses of the sequence and secondary structure of the GTD #225 grapefruit HSVd isolate (Puchta et al.1989) conducted by N Duran-Vila (personal communication) suggested that it is a non-cachexia type isolate, based on the presence of 2 additional bases within the V domain (+A in the upper strand; +U in the lower strand) and 3 additional changes.

The GTD# 225 HSVd shows 100% identity with the V regions of cachexia isolate Ca 909 from California and those of several HSVd isolates from grapevine, peach, hops, mulberry, and fig trees; from Germany, China, Tunisia, Pakistan, and Syria (M Bar-Joseph, unpublished). The designation of Ca 909 as a cachexia-inducing isolate (Reanwarakorn and Semancik 1999) has been questioned (Duran-Vila and Semancik 2003; Viladakis et al. 2005). It is possible that the xyloporosis symptoms observed on PSL grafted onto GTD #225-infected plants were produced by typical cachexia HSVd isolates that were not mechanically transmitted to the cucurbit from which the viroid RNA template for GTD #225-HSVd sequencing was derived (Puchta et al. 1989). The reports from Turkey (Onelge et al. 2004) and Egypt (Sofy et al. 2010) on the association between sweet orange gummy bark disease and certain HSVd isolates remain to be substantiated.

The phylogenetic analyses of Sano et al. (2001) suggested the possibility that initial HSVd infections of hop plants in Japan originated from infected grapevines. Similarly, the limits of the long-distance transportation of citrus propagative material (Bar-Joseph 2003) also suggest that citrus most probably reached the Mediterranean area as seed and free of viroids. Yet old clone citrus trees cultivated in the Mediterranean region were found almost universally to be infected with a range of CVds. This could be the result of post-introduction contamination through close cultivation with grapevines that are symptomless carriers of HSVd and CEVd, which were widely cultivated in the area long before the arrival of citrus (Bar-Joseph 2003).

Further evidence for the Near East origin of CVds has been found in the People's Republic of China, where tests conducted in the late 1970s showed that only imported citrus varieties were infected with viroids (e.g., CEVd and HSVd; Wang et al. 2010). This was easy to determine as all varieties were propagated on widely planted trifoliate orange and Satsuma, both of which showed no symptoms of exocortis (Broadbent et al. 1979; P Barkley, personal communication). Import of infected varieties was probably also the cause of dissemination of HSVd among citrus trees in Taiwan (Hsu et al. 1995) and Japan (Ito et al. 2007).

How and why the old and widely spread Hop stunt viroid turned into a serious disease agent of Shamouti trees grafted onto Palestinian sweet lime

What could have caused the emergence of xyloporosis disease among newly planted trees in the 1930s, despite long and extensive use of CVd infected budwood? Alternatively, why had no one noticed severe symptoms of xyloporosis among the Shamouti trees grown on PSL rootstock in this area in previous decades? To answer this question, we need to examine the possible differences in viroid load and viroid effects between Shamouti trees grafted onto PSL rootstock propagated as cuttings and PSL rootstock propagated as seedlings. The PSL cuttings were obtained either from occasionally noticed rootstock suckers or from mature trees grown on PSL rootstocks that were topped to force the production of suckers. In both cases, the cuttings were selected from green, symptomless, and well-developed shoots that were most probably produced on PSL rootstocks that were free of severe xyloporosis-inducing HSVd isolates. Thus, the propagation method commonly adopted in this region prior to the use of the seed bed nursery practice unintentionally selected for mild HSVd isolates.

Sowing seeds did not allow such a selection process, since viroids are not seedborne and the resulting seedlings were free of HSVd. Similarly, the common practice of Shamouti propagation was to select budwood for grafting only from trees on rooted PSL rootstocks that produced typical fruit and were free of any signs of decline. This

selection process unintentionally eliminated severe xyloporosis-inducing HSVd isolates. Thus, despite the wide-scale infection of Shamouti trees with HSVd and other citrus viroids, the traditional growers' experience led them to a practical selection process. Whereas, both the vegetative rootstock and budwood for grafting was only collected from trees that were infected by mild HSVd isolates, or by mixtures of HSVd isolates and other viroids that did not exhibit symptoms of severe xyloporosis or any other graft-transmissible maladies.

Increased demand for trees for planting led growers to use PSL seedlings as rootstock and to collect budwood from the more vigorous trees that were either grafted directly onto sour orange or following inarching. The cost of these improvements was the emergence of xyloporosis affecting the viroid-free PSL rootstocks that became unintentionally infected by the severe HSVd isolates derived from the Shamouti buds grafted onto symptomless sour orange rootstocks.

Concluding remarks

Eighty-five years have passed since the emergence of xyloporosis and the local citrus industry, which used to be a major factor in the local economy, has changed in many ways. Despite its outstanding qualities as a seedless and easy-to-peel sweet orange variety with a perfect combination of taste and aroma, the Shamouti orange, which was once the pride of the local citrus industry, is now less popular in export markets and among Israeli growers. In addition, local citrus nurseries are no longer using PSL as a rootstock. The research of generations of plant pathologists and virologists (listed throughout this review) not only solved the puzzling question of the etiology of xyloporosis/cachexia, but actually managed to almost completely eradicate it from modern citriculture. Nevertheless, this review not only aimed to list these achievements and point out that pests and pathogens often spread readily between crops and are unhindered by growers' fences and international borders, but also to suggest that horticultural developments need to be carefully assessed for hidden dangers that often accompany well-intentioned efforts.

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