A SURVEY OF *CHERRY LEAF ROLL VIRUS* IN INTENSIVELY MANAGED GRAFTED ENGLISH (PERSIAN) WALNUT TREES IN ITALY

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SUMMARY

Blackline disease, caused by *Cherry leaf roll virus* (CLRV), is considered a serious threat limiting English walnut (*Juglans regia*) production in Italy and worldwide if walnut species other than *J. regia*, e.g. 'Paradox' hybrid (*J. regia* × *J. hindsii*), French hybrid (*J. regia* × *J. major* or *J. regia* × *J. nigra*) or northern California black walnut (*J. hindsii*), are used as the rootstock. The virus transmissibility by pollen as well as latent infections can result in the spread of CLRV-contaminated propagative material, which is a major means of the virus dispersal by human activities. In 2014 and 2015, to ascertain the presence and the distribution of blackline symptoms in commercial orchards and to provide a description of the symptomatology, visual inspections and double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) analyses were carried out on 1,684 walnut trees in four different intensively managed grafted English walnut orchards in northeast Italy (Veneto region). Trees with clear blackline symptoms at the scion-rootstock junction, often associated with general decline of the plant, were found only in one commercial orchard in northeast Italy on trees older than ten years of cvs. 'Tulare' and 'Chandler', grafted onto 'Paradox' rootstock. To our knowledge, this is the first report of CLRV (blackline) decline and death in a commercial walnut orchard in Italy.

Keywords: *Juglans regia*, Paradox, tree decline, nuts, CLRV.

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INTRODUCTION

In spring 2014, canopy decline or death of several Persian (English) walnut (*Juglans regia* L.) trees was observed on plants grafted onto 'Paradox' (*J. hindsii* × *J. regia*) grown in a commercial orchard located in the Veneto region of northeastern Italy, an important Italian walnut-producing area. These canopy symptoms were associated with presence of a narrow, black, necrotic strip of cambium and phloem tissues at the rootstock-scion interface (Fig. 1) resembling blackline disease, which is known to be caused by *Cherry leaf roll virus* (CLRV) (Srecko *et al*., 1980; Mircetich and Rowhani, 1985).

Generally, in other species/hosts, CLRV causes foliar symptoms such as chlorotic mosaic, chlorotic or yellow ring patterns or ringspots, yellow vein netting and yellow spotting (Büttner *et al*., 2011). Although CLRV infection does not cause obvious foliar symptoms on English walnut, its presence in a grafted tree with English walnut as scion and a 'Paradox' or northern California black walnut as rootstock induces a hypersensitive reaction, a black necrotic line or canker at the graft union (Srecko *et al*., 1980; Mircetich and Rowhani, 1985).

CLRV is a member of subgroup C, genus *Nepovirus*, family *Secoviridae* (Sanfaçon *et al*., 2012). It was first described in Europe on English walnut in 1933 (Schuster and Miller, 1933). The natural host range of CLRV includes trees and shrubs with global distribution. Some of these are important forest trees (birch, hornbeam, beech, ash, elm) or commercially important crops (walnut, olive, cherry, grapevine) (Ormerod, 1972; Jones and Wood, 1978; Woo *et al*., 2013). Pollen, seed, and graft transmission are reported for this virus (Srecko *et al*., 1980; Büttner *et al*., 2011; EFSA, 2014).

The most frequently used rootstocks belong to the section of "black walnuts" (*Rhysocaryon*) such as northern California black walnut (*J. hindsii*), 'Paradox' hybrids (*J. hindsii* × *J. regia*), and French hybrids (*J. regia* × *J. major* or *J. regia* × *J. nigra*). They are used to increase vigor, productivity and resistance to soil-borne diseases, in particular to *Phytophthora* spp., of English walnut, which is the main economically important walnut species (Leslie and McGranahan, 2014; Browne *et al*., 2015). Using

Fig. 1. Symptoms of blackline disease on a 10 year old walnut tree of *Juglans regia* cv. 'Chandler' grafted on 'Paradox' in a commercial intensively managed walnut orchard.

these rootstocks increases the incidence of blackline disease (Srecko *et al*., 1980; Mircetich and Rowhani, 1985). Though usage of black walnut spp. and hybrid rootstocks is common in the USA, their use has only been recently introduced into intensively managed walnut orchards in Italy (Bortolin *et al*., 2015) as a tentative strategy to avoid severe losses due to infection with *Phytophthora* spp. (Belisario *et al*., 2006, 2016). However, use of English walnut propagated onto black walnut species or hybrids makes such grafted trees susceptible to CLRV. Decline, induced by CLRV ("blackline") in walnut, causes tree symptoms similar to those caused by soil-borne pathogens such as *Phytophthora* spp., nutrient deficiencies, or scion-rootstock incompatibilities. The first symptoms of disease are poor terminal growth, yellowing and drooping leaves, and premature defoliation, particularly in the upper branches. Later, diseased trees show dieback of terminal shoots and decline, often accompanied by profuse suckering from the rootstock. Blackline disease kills tissues at the graft union that transport nutrients and water between rootstock and scion. Necrosis gradually encircles the trunk, completely girdling the tree. The scion is killed in a few years. Trees may become infected at any age, but blackline is most commonly found in trees 15 to 40 years old (http://ipm.ucanr.edu/PMG/r881100711.html). All English walnut cultivars are susceptible to CLRV but remain symptomless unless grafted to a hypersensitive rootstock.

The aims of this work were to ascertain: i) the presence of CLRV in declining and dead walnut trees grafted onto hypersensitive rootstocks [e.g. 'Paradox', *J.* × *intermedia* (*J. nigra* × *J. regia*), *J. nigra*, or *J. major*]; ii) the occurrence of CLRV in Italian commercial orchards; and iii) to provide a description of blackline symptoms in Italian commercial orchards. For this purpose an extensive field survey was carried out in 2014 and 2015 in the Veneto region, where most of the intensively managed walnut orchards are located (Table 1).

MATERIALS AND METHODS

Symptom observations were carried out throughout the growing season, while tissue sampling was done during spring-early summer (from April to June) in both 2014 and 2015. This period is reported as the most conducive to virus isolation (Srecko *et al*., 1980). Plants were visually inspected for blackline symptoms. All plants showing canopy decline or death were carefully examined at the graft union for internal blackline symptoms. Samples from a total of 1,684 walnut trees, belonging to different scion-rootstock combinations from four different intensively managed walnut orchards in northeast Italy, were analyzed by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) in 2014 and 2015 (Table 1). In particular, a thorough 2-year investigation was carried out in the commercial orchard of Agrinord farm where blackline symptoms were initially observed on walnut trees of cv. 'Chandler' and 'Tulare' grafted onto

Table 1. English (Persian) walnut trees sampled and analyzed in 2014 and 2015 in intensively managed orchards located in north-eastern regions of Italy.

Farm/province	Cultivar	Rootstock	Origin	Year of planting	Samples collected in 2014	Samples collected in 2015
Agrinord (Padova)	Chandler	Paradox	U.S.A.	2005	367	213
	Tulare	Paradox	U.S.A.	2005	247	127
	Lara	*Juglans regia*	France	2006	–	62
(Venezia)	Lara	Black walnut*	Europe	2012	144	339
(Venezia)	Lara	Black walnut*	Europe	2012	29	25
(Udine)	Lara	Black walnut*	Europe	2008	51	80
Total					**838**	**846**

* *J. nigra* × *J. regia* or *J. nigra* or *J. major*.

Table 2. Results of visual inspection and DAS-ELISA test carried out in the Agrinord farm in the two year monitoring (2014-2015).

Cultivar	Visual inspection		DAS-ELISA test				
	Inspected plants	Plants showing blackline	Collected samples (2014-2015)	Samples from plants showing blackline	Positive samples/ Tested (2014)	Positive samples/ Tested (2015)	Positives with blackline/ Tot positives (2014-2015)
Chandler	1,420	129	580	48	28/367	19/213	6/47
Tulare	972	38	374	10	4/247	4/127	1/8
Lara	62	0	62	0	-	1/62	0/1
Total	**2,454**	**167**	**1,016**	**58**	**32/614**	**24/402**	**7/56**

'Paradox' imported from the USA. In 2014 a total of 367 trees of cv. 'Chandler' and 247 trees of cv. 'Tulare' were analyzed. In 2015, a total of 340 trees (213 cv. 'Chandler' and 127 cv. 'Tulare') were selected among those in which the virus had not been detected in the previous year. These were retested by DAS-ELISA in order to determine eventual increase of virus incidence and possible direction of virus dispersal. Moreover, in 2015, samples from the same farm were also collected from 62 plants of cv. 'Lara', imported from France, grafted onto *J. regia*, located adjacent to the 'Chandler'/'Paradox' orchard, to verify incidence and dispersal of CLRV and possible relationship to origin of propagative material (Table 1).

In addition to Agrinord, three other commercial walnut orchards in which cv. 'Lara' was grafted on black walnut rootstocks such as *J.* × *intermedia*, *J. nigra* or *J. major* were monitored, totaling 668 samples (224 in 2014 and 444 in 2015) (Table 1).

A total of 838 and 846 samples either from symptomatic or asymptomatic trees (one sampled tree every four plants per row) were analyzed in 2014 and in 2015, respectively.

From each sampled tree, composite leaves were collected in correspondence to the 4 cardinal directions at a height approximately 2 m above ground. A subsample of 8 leaflets was processed for each tree using a commercial DAS-ELISA kit (Bioreba, Reinach, Switzerland) according to manufacturer's instructions. Samples were homogenized in 1:20 extraction phosphate buffered saline (PBS) solution. The antigen-antibody reaction was visible after 30-120 min and/or assessed photometrically at 405 nm. The cut-off value was calculated for each single plate as two times the mean value of negative controls (NC). All values above this cut-off were considered positive.

RESULTS

Visual inspections and DAS-ELISA test revealed the presence of blackline and its causal agent CLRV only on walnut plants from the Agrinord farm. The results of the two year monitoring carried out in this farm are summarized in Table 2.

Visual inspections showed the presence of blackline only on cvs. 'Chandler' and 'Tulare' grafted on 'Paradox'. The overall percentage of symptomatic plants in the two-year investigation was 9.02% (129/1,429) for cv. 'Chandler' and 3.90% (38/972) for cv. 'Tulare'. In turn, no symptomatic plants were found for cv. 'Lara' either grafted on *J. regia* or on black walnut rootstocks.

Symptoms were exactly as those described in the literature and reported above, a general decline in tree vigor and poor terminal growth, yellowing and drooping of leaves, and premature defoliation, particularly at the top of the tree. Blackline-affected plants frequently showed longitudinal bark cracks at the graft union. Removal of bark at the rootstock-scion junction revealed a narrow strip of darkened cambium and phloem tissue (Fig. 1). The necrotic strip varied in length depending on the stage of disease development. Generally, in the initial stages, the black line

was 2-4 cm long. It then progressed to completely girdle the trunk (Fig. 1) resulting in canopy death.

Two-year DAS-ELISA assay results confirmed the presence of CLRV only in walnut trees at the Agrinord farm. An infection percentage of 8.10% (47/580) and 2.13% (8/374) was recorded on cv. 'Chandler' and cv. 'Tulare' respectively, whereas for cv. 'Lara' only one plant out of 62 (1.61%) tested positive for CLRV.

Comparing results obtained in 2014 with those of 2015, an increasing infection rate of 1.41% was recorded on cv. 'Chandler', whereas no increase in infection occurrence was detected in the trees of cv. 'Tulare' in 2015.

Overall, 58 out of 1,016 plants analyzed in Agrinord farm during 2014-2015 exhibited obvious blackline symptoms at the graft union, but only 7 of them (12.07%) resulted CLRV-positive with the DAS-ELISA assay. Conversely, a total of 49 positive samples were found among the 958 symptomless plants (5.11%).

DISCUSSION

In Italy, CLRV has been recorded on several fruit tree species, namely, sweet cherry (Canova, 1976), olive (Savino and Gallitelli, 1981; Faggioli *et al*., 2005), and elderberry (Barba *et al*., 1989). The first record of this virus on English walnut was reported by Savino *et al.* (1977) in Apulia (Southern Italy) and described only leaf symptoms. In this work we describe the first finding of blackline symptoms associated with the presence of the CLRV in an intensively managed commercial orchard located in the Veneto Region.

Blackline disease is considered a serious threat and an important factor limiting walnut production. Transmissibility of CLRV by pollen as well as the possibility of latent infections can easily result in the spread of CLRV-contaminated propagative material, a major factor in virus dispersal by human activities. In addition, virus-infected pollen provides a mechanism for the spread from infected to adjacent healthy orchard trees (Srecko *et al*., 1980).

Trees with clear blackline symptoms at the scion-rootstock junction, often associated with general plant decline, were found only in the Agrinord orchard, on ten-year old English walnut trees (cvs. 'Chandler' and 'Tulare' grafted onto 'Paradox' rootstock) imported from the USA. The presence of CLRV in this orchard was confirmed by DAS-ELISA that detected virus both in symptomatic and asymptomatic plants. Field monitoring and DAS-ELISA tests carried out in other farms in northeast Italy and on diverse rootstock-scion combinations, with material obtained from France, did not detect the presence of CLRV, with the exception of one symptomless plant of cv. 'Lara' grafted onto *J. regia* rootstock, which was adjacent to an infected area. These results suggest a possible relationship between infection and origin of the plant propagation material. This hypothesis could also be supported by the spatial distribution of the infected plants recorded in the field which showed a patchy spread rather than a single outbreak area.

Natural spread of CLRV within the orchard from infected to healthy plants was supported by the data obtained on comparative CLRV occurrence between 2014 and 2015 on cv. 'Chandler'. Nevertheless, the low number of new infected plants recorded in the two-year monitoring suggests a slow spreading of the virus within the orchard. This fact could also explain the low virus incidence recorded on the adjacent cv. 'Lara' (1.61%) trees. However, the differences in the phenological traits between cv. 'Chandler' and cv. 'Lara' may limit pollen transmission of CLRV between these cultivars since bloom of female and male flowers of cv. 'Lara' and cv. 'Chandler' do not overlap completely. DAS-ELISA on leaf tissue was suitable for screening a large number of samples and able to detect the virus also in some symptomless samples. Pollen is reported as more effective than either leaves or catkins for the detection of CLRV by ELISA, but the cost-benefit ratio for general survey of commercial orchards is questionable because for a reliable detection of the virus, a large pollen sample is required and its collection is time and space-consuming (Rowhani *et al*., 1985). At the same time, visual surveys based on the presence of blackline at the graft union can underestimate the percentage of infected trees, as they can remain symptomless for several years due to the slow movement of the virus through the plant. Thus, an ELISA assay from leaves represents a helpful tool for a large-scale disease diagnosis/management in the field, allowing early virus detection.

ELISA tests performed on leaf tissues showed a limited ability to detect virus in symptomatic plants. Similar results have been reported in the literature (Rowhani *et al.,* 1985; Topchiiska, 1990) and can be explained by the variable concentration and distribution of the virus in different plant tissues and by the distance from the blackline area. In particular, ELISA tests carried out by Rowhani *et al.* (1985) on blackline-affected walnut trees showed that the percentage of positive results increased, from about 18% to 100%, when inner bark and cambial tissues sampled 10 cm above the blackline were used instead of leaves. To our knowledge, this is the first report of CLRV (blackline) disease in a commercial walnut orchard in Italy. Molecular analyses are in progress in order to further characterize isolates collected during this study.

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