SHORT COMMUNICATION

A SERIOUS EPIDEMIC OF STOLBUR ON CELERY

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SUMMARY

A 3-year study was carried out in a geographically and ecologically isolated area of north-east Italy with a long tradition in horticulture, where a severe epidemic of stolbur phytoplasma occurred. Among cultivated vegetables, celery was the most susceptible to the disease. In the farms surveyed, *Convolvulus arvensis* a natural host of the phytoplasma and the vector *Hyalesthes obsoletus* were plentiful. Groups of *H. obsoletus* individuals transmitted the pathogen to celery and grapevine under controlled conditions. Other species of insects, shown by PCR to host the phytoplasma, did not transmit it. The surveyed area is a closed ecological niche with high infection pressure and represents a primary example of how an epidemic of phytoplasma can develop among cultivated plants.

Key words: epidemiology, phytoplasma, *Hyalesthes obsoletus*, insect vector, Bois noir.

Phytoplasmas are phloem-limited disease agents of a great number of plant species (McCoy *et al*., 1989) including vegetables, ornamentals, fruit and timber trees, and wild plants. In nature, they are transmittted by sap-sucking insect such as leafhoppers, planthoppers and psyllids (Weintraub and Beanland, 2006). Phytoplasma diseases are considered typically epidemic because, under particularly favourable conditions, they can spread quickly with a high incidence. The success of a phytoplasma disease in a ecosystem depends on several factors, including the contemporary presence of the pathogen, the susceptible host plant(s) and the vector(s). So, in a given ecosystem, a phytoplasma disease can be particularly severe and widespread.

Among phytoplasmas, those belonging to the stolbur group (16SrXII), subgroup A, are present in Europe and cause diseases in different host plants (Lee *et al.*, 2000). Recently, the name '*Candidatus* Phytoplasma solani' was proposed for the reference strain of this '*Candidatus*' species (IRPCM, 2004).

Stolbur was reported several years ago in eastern Europe (Valenta *et al*., 1961), where also the vector *Hyalesthes obsoletus* Signoret was identified (Aleksic *et al*., 1967). After the introduction of molecular-based methods for the diagnosis of phytoplasmas, stolbur has been found in several naturally infected plants species (Marzachì *et al*., 2000) and the list continues to grow (Duduk and Bertaccini, 2006).

Among the known host plants, one of particular importance is the grapevine in which the pathogen causes the disease known as Bois noir. Several studies on Bois noir have shed light on the epidemiology of stolbur phytoplasma regarding the host range (Maixner *et al*., 1995), the vector (Maixner, 1994; Sforza *et al*., 1998), and the presence of other Cixiidae vectors (Gatineau *et al.*, 2001; Trivellone *et al.*, 2005). As a consequence, it is now possible to maintain that the success in nature of *Ca.* P. solani is mainly due to two closely linked factors, i.e. the ability to infect plants of different families and the polyphagy of its main vector *H. obsoletus*. In fact, in the adult stage, the vector feeds on different plant species, infecting them.

The present work describes and analyzes the exceptionally high epidemicity of stolbur phytoplasma on celery in a restricted area of north east Italy, characterised by intensive horticulture. This pathogen, already reported in the region (Osler *et al.*, 1994) had never in the past reached such a high epidemic peaks.

At the end of 2002, our attention was attracted by a severe yellowing of celery in the vicinity of Trieste (north east Italy), suspected to be of phytoplasma origin. So, starting from April 2003, surveys were carried out in three selected farms where the disease had been observed. In these farms, among other vegetables (Table 1), a local celery cultivar is widely grown. Seeds are obtained from mother plants in each farm and seedlings are transplanted fortnightly into open fields from the end of May to the beginning of August.

Cultivated and wild plants showing phytoplasma-like symptoms such as yellowing, stunting, proliferation, virescence and phyllody, and symptomless plants of the

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same species were collected during the surveys.

The presence of possible insect vectors was checked periodically, insects were collected and grouped according to the following criteria: (i) insects common to the three farms; (ii) insects known as vectors of phytoplasmas or, in any case, phloem feeding; (iii) insects available in sufficient numbers for experiments. Sampled plants and insects (in groups of five individuals) were analysed by PCR for the presence of phytoplasmas. Surveys, sampling and PCR analyses continued in 2004 and 2005.

Particular attention was paid to plant and insect species that tested positive for stolbur phytoplasma with analyses of the previous year. Groups of 20-30 individuals/species of insects were used for transmission trials to healthy glasshouse-grown celery plants. The insects, confined in small cages, were maintained on the test plants for two weeks and then sprayed with insecticides; their mortality during the inoculation access period was recorded. Negative controls were healthy celery plants not exposed to the insects. In addition, three one-year-old grapevine plants cv. Chardonnay were exposed to presumably infectious insects. Negative controls consisted of 20 grapevines not exposed to insects.

To test the presence of phytoplasmas in plants and insects, total nucleic acids were extracted from 1g of leaf midribs as decribed by Malisano *et al.* (1996), whereas DNA extraction from insects was according to Doyle and Doyle (1990). Two pairs of universal primers were used for nested PCR assays. First run was done with primer pair P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995) followed by a second run with primer pair R16F2n/R16R2 (Gundersen and Lee, 1996). RFLP analyses on final products was with enzyme *Tru1*I (Fermentas, Vilnius, Lithuania) for identifying phytoplasmas of the 16Sr group associated with plant and insect samples. For routine diagnosis the primer pairs fStol/rStol (Maixner *et al.*, 1995) were also used for nested PCR. A further characterisation of stolbur phytoplasma-positive samples, carried out according to Langer and Maixner (2004), identified three different stolbur types. The extracted DNA was amplified using primer pair fTufAY/rTufAY and the reaction products were digested by *Hpa*II enzyme (Fermentas, Vilnius, Lithuania).

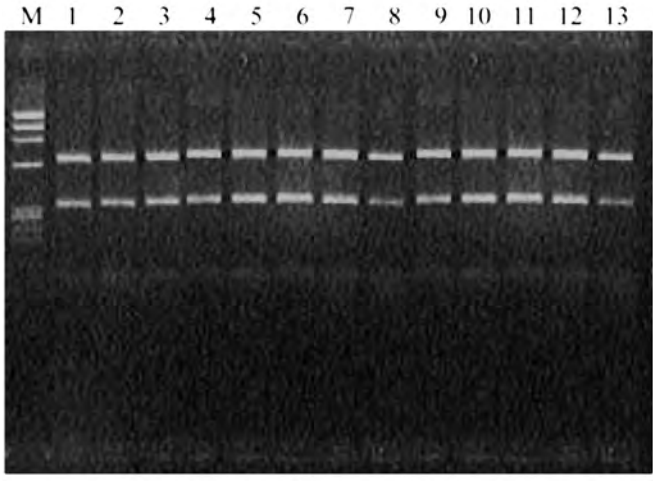

Fig. 1. *Hpa*II restriction profiles of phytoplasma DNA amplified with primer pair fTufAY/rTufAY. Template DNA was from stolbur-infected: 1-3, *Convolvulus arvensis*; 4-6, *Hyalesthes obsoletus*; 7-8 celery from the field (natural infection); 9, celery infected by *H. obsoletus* in greenhouse tests; 10, tomato; 11, carrot; 12, lettuce; 13, periwinkle P-TV reference strain. M, Marker (FX174, New England BioLabs, USA).

DNA was amplified with nested PCR from part of the sampled plants, groups of insects and test plants used for glasshouse transmission trials. After digestion with *Tru1*I, PCR products showed the restriction profile of stolbur phytoplasma (16SrXII-A). PCR-RFLP analyses carried out according to Langer and Maixner (2004) showed the restriction profile of type "b" (=VK-type II) for all stolbur-positive samples (Fig. 1).

Table 1. Main crops and presence of stolbur phytoplasma on cultivated and wild plants in the three farms during the three-year observation period.

Farm	Main crops	Symptomatic plants[a]		Analysed/infected plants No. (%)[b]
		Species	%	
1	Tomato, potato, eggplant, celery, parsley, fennel, carrot, lettuce, chicory, bean, pea, chard, radish, onion, garlic, leek	Celery	10-80	15/15 (100%)
		Tomato	5-7	5/5 (100%)
		C. arvensis	-	12/15 (80%)
2	Tomato, potato, pepper, celery, parsley, lettuce, chicory, onion, garlic, leek	Celery	10-90	14/15 (93%)
		Tomato	5-10	5/5 (100%)
		C. arvensis	-	14/15 (93%)
3	Tomato, potato, eggplant, pepper, celery, parsley, fennel, carrot, lettuce, chicory, bean, chard, radish, onion, garlic, leek	Celery	15-70	14/15 (93%)
		Tomato	5-10	5/5 (100%)
		C. arvensis	-	13/15 (87%)

[a]Minimum and maximum percentage of symptomatic plants, observed during the three-year survey.
[b]Other stolbur-infected plants were: 1 lettuce and 1 carrot (farm 1); 1 carrot, 2 parsley, 2 *Mercurialis annua* (farm 2); 1 lettuce, 1 parsley, 1 wild carrot; 2 *Cirsium* spp. (farm 3).

Among vegetables grown on the farms, celery showed the highest susceptibility and sensitivity to stolbur infection. During the three years of observation the first symptoms, consisting in diffuse yellowing and/or reddening of the leaves and stunting were observed from the second half of July, increasing in severity with time. Symptomatic plants died within one or two months. The percentage of infected plants varied according to the period of transplanting in the open field, i.e. 15 to 20% for those transplanted at the end of May, 80-90% for those transplanted at the end of June/first half of July, and 10% in celeries of the last transplanting (end of July-August).

PCR analysis showed that 43 of 45 symptomatic celery plants were infected by *Ca.* P. solani (16SrXII-A; type "b"). Infection level of 5-8% was detected also in tomatoes that showed symptoms (virescence, proliferations and yellows) in September, after harvesting. Cases of lettuce, carrot and parsley with typical yellows were sporadic (Table 1).

Among wild plants, the presence of the stolbur agent was ascertained in two plants of *Mercurialis annua* with yellows; one plant of *Daucus carota* with reddening; two plants of *Cirsium* spp. with yellows and virescence; 39 plants of *Convolvulus arvensis* with yellows, stunting and proliferation (Table 1). In all farms surveyed symptomatic *C. arvensis* was very abundant at the borders of cultivated plots, over a surface of several square meters. None of the symptomless plants of the weed, tested by PCR, was infected.

Five different species of insects were PCR-positive (Table 2). Captures of adults from *C. arvensis* plants were always abundant in July, but only a low number of insects was collected from celery. In 2004, 23 of 50 (45%) *H. obsoletus* individually tested for the presence of stolbur phytoplasma were positive.

In 2004, a total of 50 healthy celery plants were exposed in the glasshouse to a variable number of insects per plant (Table 3). While the mortality on celery of *H. obsoletus* and *Empoasca decipiens* Paoli was low (20% and 30%, respectively) during the two weeks of exposure, insects of other species died in a few days. About one month after the end of the inoculation access period, all plants exposed to *H. obsoletus* started to show symptoms of yellowing, reddening and severe stunting. All proved to be infected by stolbur phytoplasma type "b", and died within one month. No symptoms were observed in the plants exposed to other insects and in negative controls. PCR tests showed these plants to be healthy.

In 2005 a total of 28 celery plants were exposed to groups of insects. Also in this case, symptoms and positive PCR reactions were obtained only from plants exposed to *H. obsoletus*. Five months after exposure of cv. Chardonnay vines to groups of 30 *H. obsoletus*, one plant showed yellowing and down rolling of the leaves; PCR analyses confirmed it to host the pathogen.

This study, although being carried out in a restricted area, permits some conclusions to be drawn on stolbur and, more generally, on phytoplasma diseases. The surveyed area is clearly an ecological niche, surrounded by the Carso mountains and closed on the other side by the city of Trieste and the sea.

The long tradition of horticulture in this area, probably contributed the three major conditions necessary for a phytoplasma outbreak.

Table 2. Insects collected in the three farms surveyed during the three years of observations. The insects, grouped in batches of five individuals, were analysed for the presence of stolbur phytoplasma.

Species	Month of collection	Groups of insects positive/ tested (No.)
Hyalesthes obsoletus[a]	July	42/50
Empoasca decipiens	July, September	4/20
Austroagallia sinuata	July, August	2/15
Psammotettix alienus	July, August	3/15
Thamnotettix diluitor	June, July	3/20
Cixius spp.	June, July	0/20
Metcalfa pruinosa	June, July, August	0/15
Macrosteles quadripunctulatus	June, July	0/15
Euscelis incisus	July, August, September	0/10
Euscelidius variegatus	July, August	0/10
Cicadella viridis	September, October	0/10
Philaenus spumarius	May, June, July	0/10

[a]Of 50 *H.obsoletus* individually tested in 2004, 23 (45%) were infected by the stolbur phytoplasma

Table 3. Results of transmission trials carried out in 2004 and 2005 by feeding insects collected in the three farms. 20-30 individuals/plant were caged on celery and grapevine plants for a 15-day inoculation access period. The plants were then tested for the presence of stolbur phytoplasma.

Species	Test plant	Positive/tested plants (No.)	
		Year 2004	Year 2005
Hyalesthes obsoletus	Celery	25/25	5/5
Hyalesthes obsoletus	Grapevine cv. Chardonnay	-	1/3
Empoasca decipiens	Celery	0/10	0/8
Austroagallia sinuata	Celery	0/6	0/5
Psammotettix alienus	Celery	0/5	0/5
Thamnotettix diluitor	Celery	0/4	0/5

The first condition is the presence of host plants. In the investigated area, *C. arvensis*, a well known host of *Ca*. P. solani, is abundant inside and around the farms. This is probably because bindweed is a creeping, stolons-producing, perennial plant whose growth is favoured by the periodical mowing done in the surveyed farms. So, the agricultural practices used in the farms facilitated the plentiful presence of *C. arvensis*.

The second condition is the presence of vectors. *H. obsoletus* completes its life cycle on wild plants among which *C. arvensis* is one of the most typical (Sforza *et al.*, 1999; Weber and Maixner, 1998). The registered abundance of *C. arvensis* in the surveyed farms explains the high level of *H. obsoletus* populations which are not affected by aphicide treatments that are usually performed from April to June.

The third condition is the presence of the pathogen. We do not know when and how stolbur phytoplasma was introduced in the investigated area, but we do know that it occurs in the region (Osler *et al.*, 1994) where it elicits Bois noir in grapevines (Refatti *et al.*, 1998). So the pathogen may have been present for years on wild plants in the area but the symptoms it induces in *C. arvensis* may have been overlooked.

It ensues that the contemporary presence of the pathogen, the vector and the natural host, has created a closed ecological niche with a high infection pressure. The profuse occurrence of infected bindweed (thousands of plants), the high infectivity of *H. obsoletus* (45% of the tested individuals) and the presence of the "b" type only of stolbur confirm this likelihood. Thus, celery simply acted as a detector of the endemic phytoplasma.

Celery was by far the most susceptible to infection whereas other alleged *Ca.* P. solani hosts like tomato, potato, and pepper (McCoy *et al*., 1989) were either infected to a low level or were not infected at all (Tab. 1). Furthermore, the highest infection rate was observed on celery plants transplanted immediately before or during July, the period when vectors are most active and infective. This scenario may indicate that the polyphagous *H. obsoletus* adults are preferentially attracted by young, fresh celery plants, a likelihood that seems be confirmed by glasshouse experiments which showed celery to be both readily infected by stolbur phytoplasma (100% of positive transmissions) and a good host for *H. obsoletus* (long survival).

The inability of PCR-positive leafhopper species other that *H. obsoletus* to transmit the phytoplasma further proves that an insect can be considered a vector only after positive transmission of the pathogen to plants or, at least, to an artificial feeding medium (Tanne *et al*., 2001).

Finally, *H. obsoletus* transmission of stolbur phytoplasma to grapevine, further confirms the role of this insect as a vector of Bois noir.

This investigation has shown that in an area under high infection pressure, susceptible plants can totally be compromised when the pathogen occurring in natural reservoirs (wild plants) is transmitted by active vectors from wild to cultivated plants, as previously reported for other phytoplasma diseases like European stone fruit yellows (Carraro *et al.*, 2002).

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REFERENCES

Aleksic Z., Sutic D., Aleksic D., 1967. Transmission intensity of stolbur virus by means of *Hyalesthes obsoletus* Sign. on some host plants. *Zastita bilja* **93-95**: 67-73.

Carraro L., Ferrini F., Ermacora P., Loi N., 2002. Role of wild *Prunus* species in the epidemiology of European stone fruit yellows. *Plant Pathology* **51**: 513-517.

Deng S., Hiruki C., 1991. Amplification of 16SrRNA genes from culturable and non-culturable Mollicutes. *Journal of Microbiological Methods* **14**: 53-61.

Doyle J.J., Doyle J.B., 1990. Isolation of plant DNA from fresh tissue. *Focus* **12**: 13-15.

Duduk B., Bertaccini A., 2006. Corn with symptoms of reddening: new host of stolbur phytoplasma. *Plant Disease* **90**: 1313-1319.

Gatineau F., Larrue J., Clair D., Lorton F., Richard-Molard M., Boudon-Padieu E., 2001. A new natural planthopper vector of stolbur phytoplasma in the genus *Pentastiridius* (Hemiptera: Cixiidae). *European Journal of Plant Pathology* **107**: 263-271.

Gundersen D.E., Lee I.M., 1996. Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs. *Phytopathologia Mediterranea* **35**: 144-151.

IRPCM, 2004. '*Candidatus* Phytoplasma', a taxon for the wall-less, non-helical prokaryotes that colonise plant phloem and insects. *International Journal of Systematic and Evolutionary Microbiology* **54**: 1243-1255.

Langer M., Maixner M., 2004. Molecular characterisation of grapevine yellows associated phytoplasmas of the stolbur-group based on RFLP-analysis of non-ribosomal DNA. *Vitis* **43**: 191-199.

Lee I.M., Davis R.E., Gundersen-Rindal D.E., 2000. Phytoplasma: phytopathogenic Mollicutes. *Annual Review of Microbiology* **54**: 221-255.

Maixner M., 1994. Transmission of German grapevine yellows (Vergilbungskrankheit) by the planthopper *Hyalesthes obsoletus* (Auchenorrhyncha: Cixiidae). *Vitis* **33**: 103-104.

Maixner M., Ahrens U., Seemüller E., 1995. Detection of the German grapevine yellows (Vergilbungskrankheit) MLO in grapevine, alternative hosts and a vector by specific PCR procedure. *European Journal of Plant Pathology* **101**: 241-250.

Malisano G., Firrao G., Locci R., 1996. 16SrDNA-derived oligonucleotide probes for the differential diagnosis of plum leptonecrosis and apple proliferation phytoplasmas. *Bulletin OEPP/EPPO Bullettin* **26**: 421-428.

Marzachì C., Veratti F., d'Aquilio M., Vischi A., Conti M., Boccardo G., 2000. Molecular hybridization and PCR amplification of non-ribosomal DNA to detect and differentiate stolbur phytoplasma isolates from Italy. *Journal of Plant Pathology* **82**: 201-212.

McCoy R.E., Caudwell A., Chang C.J., Chen T.A., Chiykowski L.N., Cousin M.T., Dale J.L., de Leeuw G.T.N., Golino D.A., Hackett K.J., Kirkpatrick B.C., Marwitz R., Petzold H., Sinha R.C., Sugiura M., Whitcomb R.F., Yang I.L., Zhu B.M., Seemüller E., 1989. Plant diseases associated with mycoplasma-like organisms. In: Whitcomb R.F., Tully J.G., (eds). The Mycoplasmas, vol. V, pp. 545-640. Academic Press, San Diego, CA, USA.

Osler R., Firrao G., Carraro L., Loi N., Musetti R., Chen T.A., 1994. Biodiversity of plant MLOs in a well defined ecological area. *IOM Letters* **3**: 284-285.

Refatti E., Carraro L., Osler R., Loi N., Pavan F., 1998. Presenza di differenti tipi di giallume della vite nell'Italia nord-orientale. *Petria* **8**: 85-98.

Schneider B., Seemüller E., Smart C.D., Kirkpatrick B.C., 1995. Phylogenetic classification of plant pathogenic mycoplasmalike organisms or phytoplasmas. In: Razin S., Tully J.G., (eds). Molecular and diagnostic procedures in micoplasmology, vol. II, pp. 369-380. Academic Press, San Diego, CA, USA.

Sforza R., Bourgoin T., Wilson S.W., Boudon-Padieu E., 1999. Field observation, laboratory rearing and descriptions of immatures of the planthopper *Hyalesthes obsoletus* (Hemiptera: Cixiidae). *European Journal of Entomology* **96**: 409-418.

Sforza R., Clair D., Daire X., Larrue J., Boudon-Padieu E., 1998. The role of *Hyalesthes obsoletus* (Hemiptera: Cixiidae) in the occurrence of Bois noir of grapevines in France. *Journal of Phytopathology* **146**: 549-556.

Tanne E., Boudon-Padieu E., Clair D., Davidovich M., Melamed S., Klein M., 2001. Detection of phytoplasma by polymerase chain reaction of insect feeding medium and its use in determining vectoring ability. *Phytopathology* **91**: 741-746.

Trivellone V., Pinzauti F., Bagnoli B., 2005. *Reptalus quinquecostatus* (Dufour) (Auchenorrhyncha Cixiidae) as a possible vector of stolbur phytoplasma in a vineyard in Tuscany. *Redia* **88**: 103-108.

Valenta V., Musil M., Misiga S., 1961. Investigations on European yellows-type viruses. *Phytopathologische Zeitschrift* **42**: 1-38.

Weber A., Maixner M., 1998. Survey of populations of the planthopper *Hyalesthes obsoletus* Sign. (Auchenorrhyncha, Cixiidae) for infection with the phytoplasma causing grapevine yellows in Germany. *Journal of Applied Entomology* **122**: 375-381.

Weintraub P.G., Beanland L.A., 2006. Insect vectors of phytoplasmas. *Annual Review of Entomology* **51**: 91-111.

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