SILVA FENNICA

Silva Fennica 41(4) research notes

www.metla.fi/silvafennica · ISSN 0037-5330
The Finnish Society of Forest Science · The Finnish Forest Research Institute

Cherry leaf roll virus Abundant on *Betula pubescens* in Finland

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Jalkanen, R., Büttner, C. & von Bargen, S. 2007. *Cherry leaf roll virus* abundant on *Betula pubescens* in Finland. Silva Fennica 41(4): 755–762.

Virus-related symptoms such as vein banding, leaf roll, chlorosis and subsequent necrosis on birch leaves were increasingly recorded throughout Finland since 2002. They are widespread in this country and have also been detected in northern Norway and Sweden. Symptomatic foliage has so far been found on *Betula pendula*, *B. pubescens*, *B. pubescens* subsp. *czerepanovii*, and *B. nana*.

A *Cherry leaf roll virus* (CLRV) specific IC-RT-PCR was applied to young leaves, buds and catkins of symptomatic shoots of nineteen pubescent and one silver birch trees grown in the centre of Rovaniemi, Finland. CLRV was found in seventeen *B. pubescens* trees. This is the first time that *B. pubescens* has been confirmed to be a host species for CLRV in Finland. Nor has CLRV been recorded earlier in northern Finland.

Keywords *Betula pendula*, *Betula nana*, *Betula pubescens* subsp. *czerepanovii*, virology
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Received 4 April 2007 **Revised** 6 September 2007 **Accepted** 17 September 2007
Available at http://www.metla.fi/silvafennica/full/sf41/sf414755.pdf

1 Background

Since the summer of 2002, increasing numbers of birch trees (*Betula* spp.) of various sizes and ages with foliar disorders of unknown origin were discovered throughout Fennoscandia. Symptomatic birches showing vein banding, leaf roll and chlorosis with subsequent necrosis were found from the southern coast of Finland to the conifer tree-line area in northern Finland, in the sub arctic zone of northern Norway as well as in Sweden (Fig. 1). Affected birch species were silver (*Betula pendula* Roth), pubescent (*B. pubescens* Ehrh.), mountain (*B. pubescens* subsp. *czerepanovii* (Orlova) Hämet-Ahti) and dwarf birch (*B. nana* L.). Diseased birch trees were either growing alone or in groups. The phenomenon occurred in trees and bushes on mineral sites and peatland, in

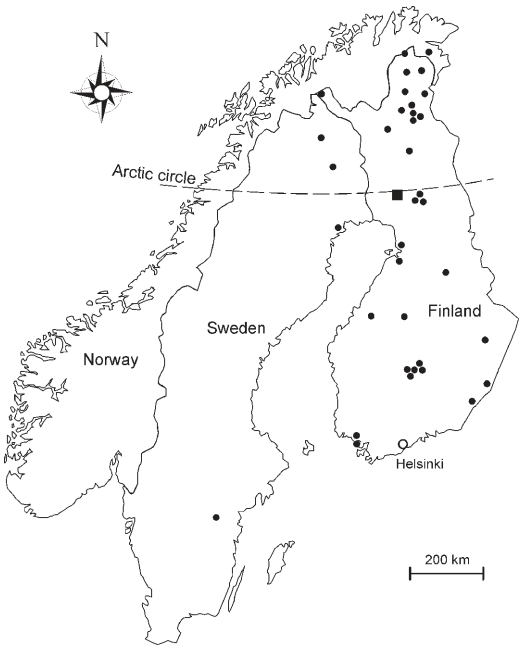


Fig. 1. Locations, where *Cherry leaf roll virus* -type symptoms have been recorded on *Betula* spp. in Fennoscandia. The black square represents 25 records in Rovaniemi, N Finland.

inhabited areas such parks, and forests, and in valleys as well as exactly at the birch alpine tree line. Leaf symptoms appeared either in a small part, even a branch tip, or in most parts of the green canopy (Fig. 2). Tops of taller trees seemed to be least affected. Leaf symptoms of this type have neither been found ever to this extent in Finland nor confirmed for the causal agent on *B. pubescens* or other birch species except *B. pendula*.

2 Symptom Description

First symptoms on *B. pubescens* appeared soon after full expansion of first leaves and after the primary shoots at tips of first-order branches had grown a few centimetres at the end of May (Rovaniemi 2006). At this time, chlorotic vein banding, lighter than the pale green early summer foliage colour, developed and affected leaves started to show leaf roll, leading to a convex appearance (Fig. 3a). In some cases chlorotic line patterns were more irregular and concentrated on the leaf tips. First symptoms appeared in the primary leaves, but not every leaf was affected. While symptom-free leaves expanded to regular size with a normal shape and green colour, the final size of the symptomatic leaves remained noticeably smaller.

In further development all leaves at the tip part of the new shoot exhibited symptoms. From July onwards the general colour of the symptomatic foliage increasingly changed from a lighter green to yellow and, in most cases, exhibited strong leaf roll symptoms. Chlorotic bands were clearly visible either between the main veins or on both sides of a vein, forming either diamond structures or chlorotic areas widening towards the leaf edge. Sometimes the whole interveinal space was chlorotic (Fig. 3c), while veins and adjacent tissues remained green most of the season. Ring spots were absent but patterns of the mosaic type occurred in some leaves of normal size (Fig. 3b). Throughout the summer strongest symptoms were present at the leaf tips, where chlorotic lesions gradually turned necrotic (Fig. 2d). Leaf roll symptoms increased in time as well (Fig. 3d, also 3a and 3c). Additionally, in the very dry summer of 2006, symptomatic leaves were drooping, thus the affected parts of the canopy resembled to suffer from drought (Fig. 2b). Further, distorted foliage, either due to hypertrophic growth of interveinal tissue or reduced vein extension was common (Fig. 2e, 3c).

In late summer the symptomatic parts of the canopies could easily be distinguished from symptom-free parts or the neighbouring crowns due to their pale colour (Fig. 4). Normally, a lot of parasitic fungi inhabited birch leaves at the end of the growing season so that fungal infections partly hid symptoms caused by other diseases. In this respect fungal pathogens infected birch foliage exceptionally scanty in the summer of 2006, this allowed the studied phenomenon to appear well. – Shedding time of symptomatic leaves in respect to healthy ones seemed to vary depending on site, tree and even location within the canopy. Once a tree exhibited the above-described symptoms, they clearly appeared to have spread the following year. In fact, the entire canopy could become symptomatic in a few years.

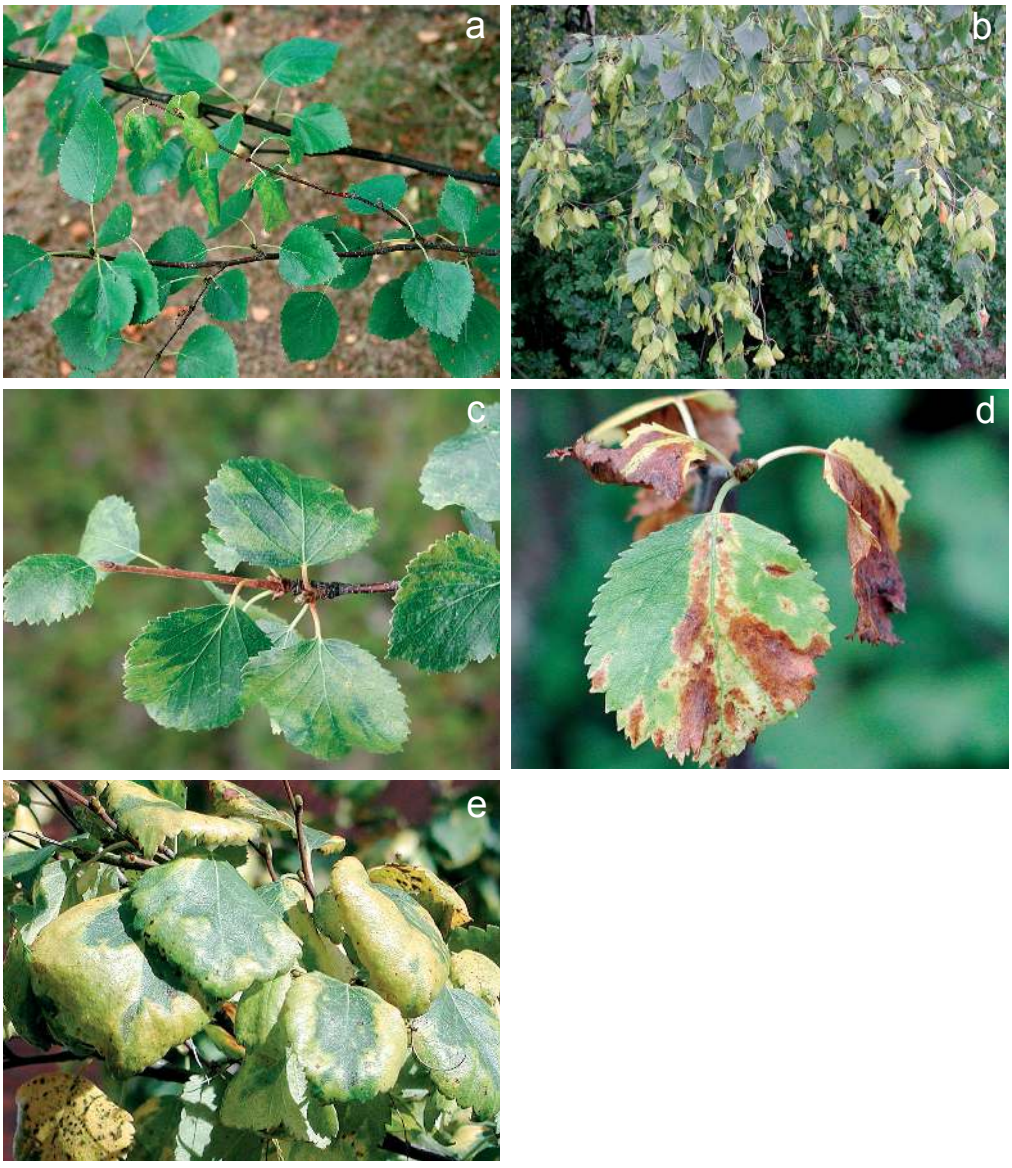


Fig. 2. Virus-like symptoms found throughout Fennoscandia. **a)** On a shoot tip of *Betula pubescens* (sample tree no. 10) in the town Rovaniemi, N Finland, 19.8.2006; **b)** in the lower canopy of *B. pendula* with healthy and infected leaves/shoots in Jyväskylä, central Finland, 1.9.2006; **c)** in a shoot of *B. pubescens* var. *czerepanovii* in Neiden, NE Norway, 19.7.2006; **d)** necrotic lesions on *B. pubescens* leaves, Inari village, Inari, N Finland 22.8.2006; and **e)** hypertrophic leaf growth of *B. pubescens* tree, in the town Luleå, N Sweden, 14.9.2004.

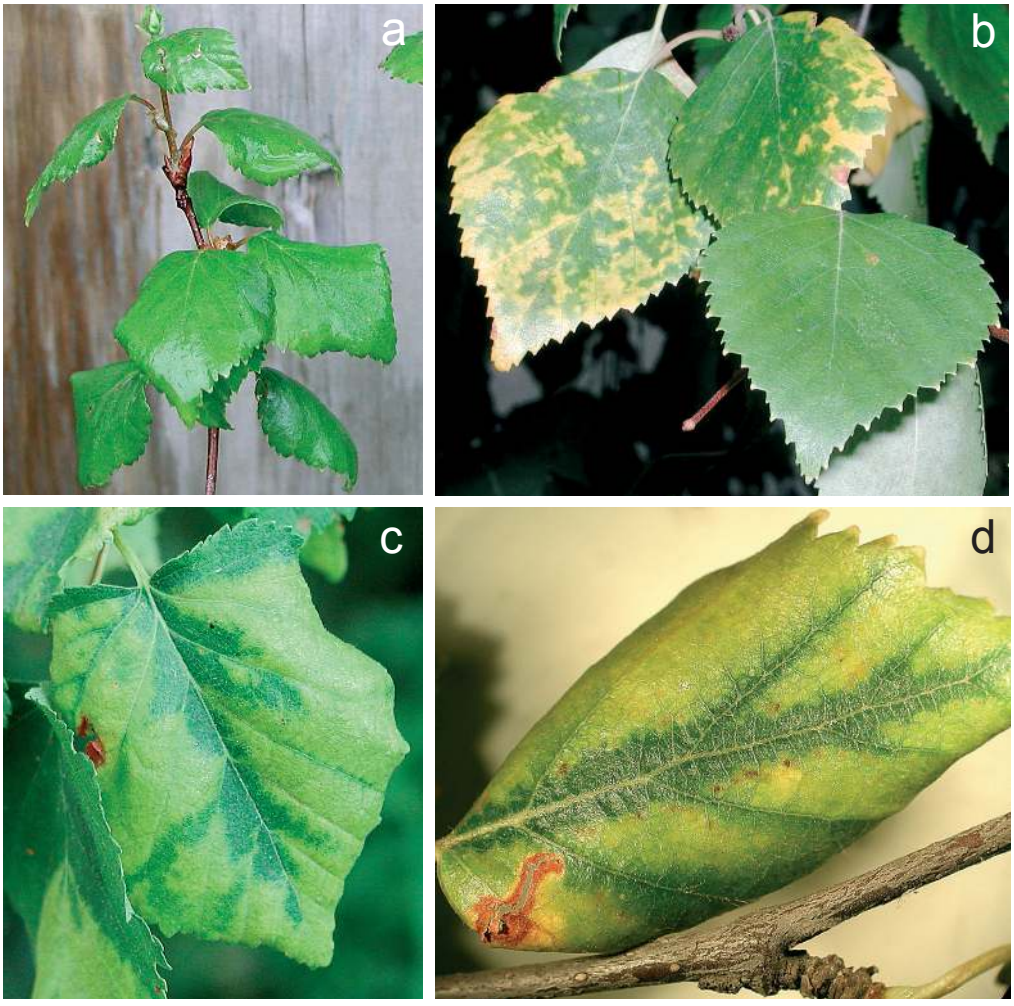


Fig. 3. Symptoms of *Cherry leaf roll virus*, CLRV, infected *Betula pubescens*, town Rovaniemi, N Finland **a)** Leaf-roll symptoms and beginning chlorosis soon after the shoot flush, sample tree no. 8, 1.6.2006; **b)** mosaic pattern, sample tree no. 14, 21.9.2006; **c)** typical chlorotic vein banding, line pattern and leaf roll, sample tree no. 2, 14.7.2006; and **d)** leaf-roll symptoms together with line pattern, sample tree no. 2, 4.8.2006.

The syndrome of birch trees described here led to the hypothesis of a viral etiology. Although, several viruses like *Tobacco rattle virus* (TRV, Schmelzer 1972) and *Apple mosaic virus* (ApMV, Grüntzig et al. 1996) have been reported to infect *Betula* species, symptoms resembled those described for *Cherry leaf roll virus*, CLRV (Schmelzer 1972, Cooper and Atkinson 1975), known to have a wide geographic distribution (Rebenstorf et al. 2006). To confirm our suspicion, we aimed to apply the IC-RT-PCR technique to test the presence of CLRV in northern Finnish birch trees.

3 Material and Methods

Nineteen pubescent birches and one silver birch, which exhibited the above-mentioned symptoms,



Fig. 4. Birch habitus with typical *Cherry leaf roll virus*, CLRV, type symptoms in the entire canopy in Rovaniemi, N Finland, 4.8.2006.

were numbered (1 to 20) and sampled in the centre of the Rovaniemi town in northern Finland (66°29´…66°31´N) in September 21, 2006. At the time of sampling only one symptomatic silver birch in Rovaniemi was known. The sampled trees were 3 to 14 m tall, and their green canopy exhibited slight, moderate or strong symptoms. In the slightest case, symptoms appeared as a rule only in the lower parts of the green canopy. One to two twigs with leaves, buds and, if possible, catkins were sampled from symptomatic areas of the lower canopy. In case of trees nos. 18–20 symptoms were different or became already diffuse due to the autumn colouration of leaves. Sampled material was stored wrapped in moist paper towels in plastic bags at +5 °C.

The samples were analysed within one week from sampling by application of a modified CLRV specific immuno capture reverse transcriptase polymerase chain reaction (IC-RT-PCR) developed by Werner et al. (1997). One symptomatic leaf, two to three buds and one catkin, if available, per tree were tested. To apply for routine testing, the protocol was shortened by omitting denaturation steps and carried out as a single tube reaction assay as well as reducing required chemicals and enzymes, in short: One 0.2 ml reaction tube per sample was coated with 10 µl antibody solution (1:200 dilution of IgG in carbonate buffer, pH 9.6) for 2 h at 37 °C. The polyclonal antibody was raised in rabbit against a CLRV isolate E603 from a black elderberry, *Sambucus nigra* L. (in Rebenstorf et al. 2006). Immunisation and purification of IgG were done using standard protocols (Harlow and Lane 1988). After washing the tubes three times with 150 µl phosphate buffered saline containing 0.05% (v/v) Tween (PBS-Tween), 10 µl homogenised plant material (1:10 dilution in PBS-Tween supplemented with 2% (w/v) polyvinylpyrolidone 40) was applied and incubated over night at 4 °C. Unbound material was washed away as described above and 10 µl of reverse transcriptase mix, which consists of 50 mM Tris-HCl pH 8.3, 50 mM KCl, 4 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM each dNTP, 50 pmol first strand primer (RW1, 5´ GTCGGAAAGATTACGTAAAAGG) and 200 units M-MuLV-RTase (Fermentas), was added to reaction tubes and incubated for at least 1 h at 37 °C. Afterwards 40 µl of PCR mix – 12.5 mM Tris, 62.5 mM KCl, 0.125% (v/v) TritonX100, 0.25 mM each dNTP, 2.5 mM $MgCl_2$, 25 pmol each first and second strand primer (RW2, 5` TGGCGACCGTGTAACGGCA) and 5 units Taq-Polymerase (Promega) – was added directly

to the cDNA reaction and subjected to PCR in a robocycler (Stratagene) using the following program: 1× 120 s 94 °C; 30× 60 s 94 °C, 45 s 55 °C, 60 s 72 °C; 1× 5 min 72 °C. PCR-products were analysed by separation in 1% agarose gels and visualised after staining with ethidumbromide under uv-light.

In parallel, samples of six trees (nos. 2, 8, 14, 15, 17 and 20) were checked for virus particles stained with 2% (w/v) uranylacetate by transmission electron microscopy (TEM).

4 Results and Discussion

CLRV was detected in leaves, buds and/or catkins in 85% of the sampled trees (Table 1). IC-RT-PCR specifically amplified a single fragment of the expected size (412 bp), from sampled tissues. This was in accordance with amplification products received from CLRV infected woody control plants (*Sambucus nigra* and *B. pendula*), while no fragments were obtained from the water control. Only in three birches IC-RT-PCR results were negative. Symptoms of the two CLRV negative *B. pubescens* trees differed from the patterns described above and referenced in the literature for CLRV. The observed leaf-roll symptoms might be due to the very severe drought in the sampling area in 2006 (Monthly weather report 2006), together with the appearance of autumn colours. The third tree with a negative result was the only sampled *B. pendula*, but with similar symptoms as the trees, which resulted in CLRV infected. The failure to detect CLRV in this sample might be due to improper sample material, because only very few shoots were symptomatic. Although, CLRV detection is usually possible in leaves from woody hosts showing chlorotic spots and line pattern, virus concentration is often low in these plants and the virus is irregularly distributed within the canopy (Bandte and Büttner 2001). Leaves of the tested silver birch tree were already necrotising at the end of the vegetation period, which also might be a cause for lack of detectability of CLRV. Secondary plant compounds in sampled material such as phenols and polysaccharides, which are known inhibitors of PCR (Demeke and Adams 1992, MacKenzie et al. 1997, Osman and Rowhani 2006), may be highly concentrated in senescent tissue.

Table 1. Presence of *Cherry leaf roll virus*, CLRV, in *Betula* spp. growing along the streets in the centre of Rovaniemi, Finland.

Tree no.	Species	Symptom appearance [a]	CLRV detection [b] Leaves	Buds	Catkins
1	*B. pubescens*	C, T, o	+	-	-
2	*B. pubescens*	C, T, p	+	-	na
3	*B. pubescens*	C, T, o	+	+	+
4	*B. pubescens*	C, T, p	+	+	+
5	*B. pubescens*	C, T, p	+	-	+
6	*B. pubescens*	C, T, d	-	+	+
7	*B. pubescens*	C, T, p	-	+	na
8	*B. pubescens*	C, T, p	+	+	na
9	*B. pubescens*	C, T, p	-	+	+
10	*B. pubescens*	o	+	+	na
11	*B. pubescens*	L	-	+	+
12	*B. pubescens*	C, T, p	+	+	na
13	*B. pubescens*	C, T, p	-	+	+
14	*B. pubescens*	C, T, p	+	+	na
15	*B. pubescens*	C, T, p	+	+	na
16	*B. pubescens*	C, T, o	-	+	na
17	*B. pubescens*	C, T, p	+	+	na
18	*B. pendula*	C, T, p	-	-	na
19	*B. pubescens*	atypical, p	-	-	na
20	*B. pubescens*	atypical, p	-	-	na

[a] C = clear; T = typical; L = leaves partly fallen; p = symptoms present at time of sampling; d = symptoms decreasing at time of sampling; o = symptoms over at time of sampling
[b] + = specific fragment amplification in IC-RT-PCR, - = no amplification, na = sample not available

Electron microscopy revealed isometric virus particles with an estimated diameter of 28–30 nm in leaf samples, originating from *B. pubescens* tree nos. 15 and 17. This is in accordance with the reported morphology of *Cherry leaf roll virus* (Jones 1985), although other viruses as for instance ApMV are known to infect *Betula* species (Grüntzig et al. 1996) exhibiting similar isometric particle sizes. On the contrary no other virus-like structures with different morphologies were visible, so at least filamentous viruses like TRV are unlikely to participate as causal agents of the disease.

This is the first time that *Cherry leaf roll virus* has been confirmed by molecular methods to occur in several *B. pubescens* trees in Rovaniemi and based on symptoms in many birch populations all over Finland. In a previous report on

viruses and mycoplasmas in forest trees in Finland, Bremer et al. (1991) suspected two silver birches (*B. pendula*) at two separate locations in southern Finland to have been CLRV positive by symptom evaluation. In the first case, leaves of the silver birch had pale green lesions and ring spots, which did not appear in recent samples on any birch species. According to Bremer et al. (1991), it was possible to transmit the agent and cause similar symptoms by grafting an infested shoot to a healthy seed-borne silver birch sapling. In the other case, another silver birch with similar symptoms was analysed serologically by J.I. Cooper, who, by personal communication, confirmed that the birch exhibited CLRV (Bremer et al. 1991).

Although Hamacher et al. (1987) has mentioned CLRV-infected *B. pubescens* occurring in Europe before, our positive findings are the first ones on this *Betula* species in Finland. Referring to the visual observation, it seems that most of the trees with CLRV-type symptoms in Finland have been *B. pubescens*, at least so far.

In our countrywide records during the summer of 2006, symptoms on birch appeared very distinct. Although trees were sampled in September, we were able to confirm CLRV infection in birches in Rovaniemi, assuming that this virus is widely spread in Fennoscandia. CLRV is transmitted via pollen and seed in nature (Massalski and Cooper 1984) allowing effective intra-specific dispersal of the virus. While vertical transmission of CLRV by pollen and seed is effective, horizontal transmission by pollen originating from infected birch trees seems to be limited (Cooper et al. 1984). It was also reported by Cooper (1976) that germination rates of birch seedlings from infected mother plants were reduced in comparison with healthy trees, which suggests that vertical transmission is not alone responsible of natural CLRV dissemination and other routes of virus dispersal with epidemiological relevance may exist. The rate at which symptoms have spread in the last few years indicates CLRV to be able to become a serious problem in northern birch forests and for the local forest industry. Therefore rapid efforts are needed to conduct detailed studies on distribution, ways of spread as well as biological and economic importance of CLRV among birch species and possible other hosts. It has to be revealed, why CLRV is spreading so fast and widely and whether increasing summer temperatures may be responsible for a higher susceptibility to CLRV. The means of viral spread is of special interest because CLRV-typical symptoms were found in a few years time in a large geographical area, in several birch species and on very distinct site types far from each other.

Acknowledgements

The work was supported by Deutsche Forschungsgemeinschaft (DFG, grant no. Bu 890/8-1).

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Total of 17 references