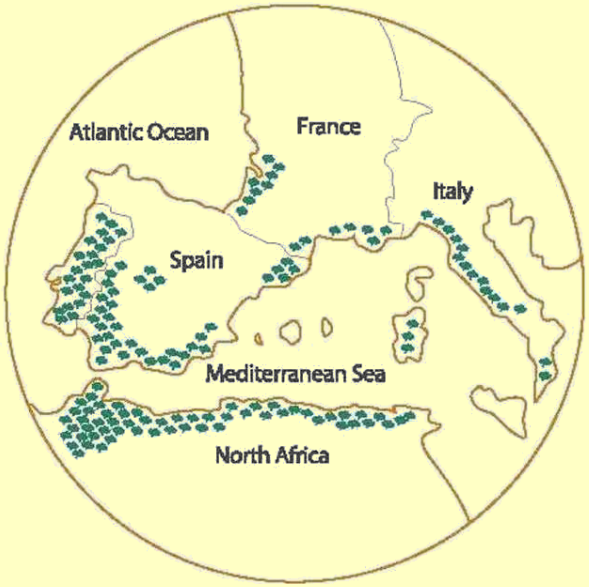
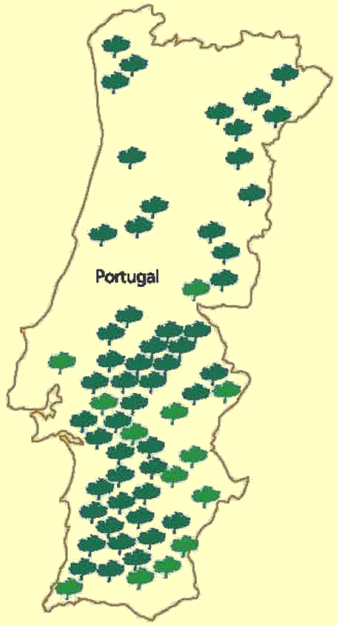
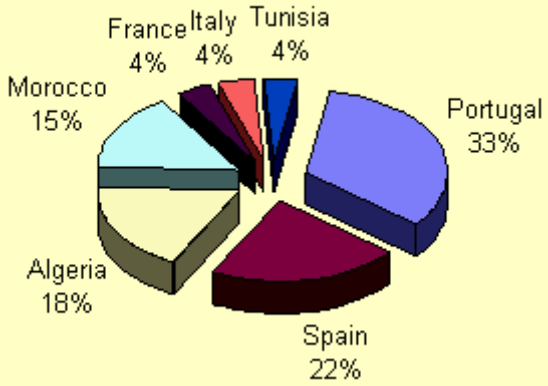
Investigation on virus-like particles associated to decline of *Quercus suber*

Filomena Nóbrega

Cork oak (*Quercus suber*) – Geographic distribution



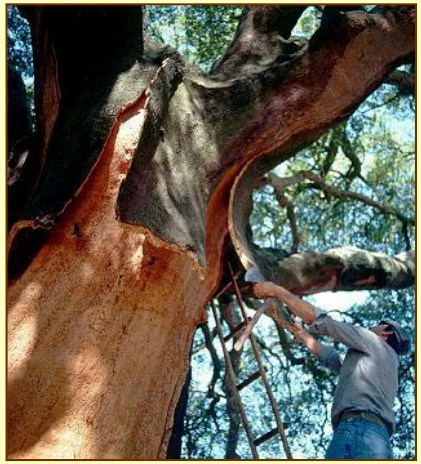




- **730000 ha (21% of the total Portuguese forest area).**
- **175000 tonnes of cork per year, Portugal produces about 60% of the world's output.**

Cork oak forest wide by APCOR

Ecosystems of great socio-economic and ecological importance



The cork oak tree is a unique tree species that is harvested every 9 years for the extraction of cork, once the tree hits the young adult phase – approximately 25 years of age.

Only the third and subsequent harvests produce cork with an even structure good enough to be used for cork bottle stoppers.

The cork oak takes at least 40 years for the bark to become commercially viable.

Cork bark planks after harvesting

The use of cork as wine stoppers is by far the most economically important.

- This represents 70% of the value of all Portuguese cork products and exports making the cork stopper industry one of the most important sectors in the economy.

- Globally around 15 billion cork stoppers are produced each year, 60% of which are made in Portugal.

Quercus suber decline

- The oldest references about cork oak decline in the Iberian Peninsula data back to the end of the nineteenth century.
- In 1980s, cork oak mortality became a matter of concern for the future of these ecosystems.

Contrasting behaviours

Slow decline - gradual fall of the foliage and the presence of branches partially or totally defoliated

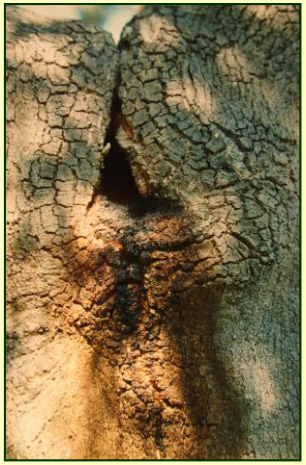
Sudden death - death of twigs and leaves which remain adhered to the tree during some time giving to the canopy a peculiar brownish coloration

General decline symptoms

Gradual deterioration of the crown that starts with leaf chlorosis and dieback of leaf bearing branches



Trunk cankers, wounds, tarry exudates from the bark




Epicormic shooting


Decline symptoms

Mortality occurs in pocket areas with no geographic patterns.

Possible factors linked to cork oak decline

There is no agreement among scientists

- One of the factors which has been more study and presented as a key factor involved in the decline is the presence of *Phytophthora cinnamomi* on the forest soil.

- The decline is caused by unfavorable environmental factors related to:

  - climate (precipitation regimes, drought, extreme temperatures…),
  - soil (lack or excess of nutrients, hydromorphic site conditions,compression of soils…),
  - chemicals (air or soil pollutants…),

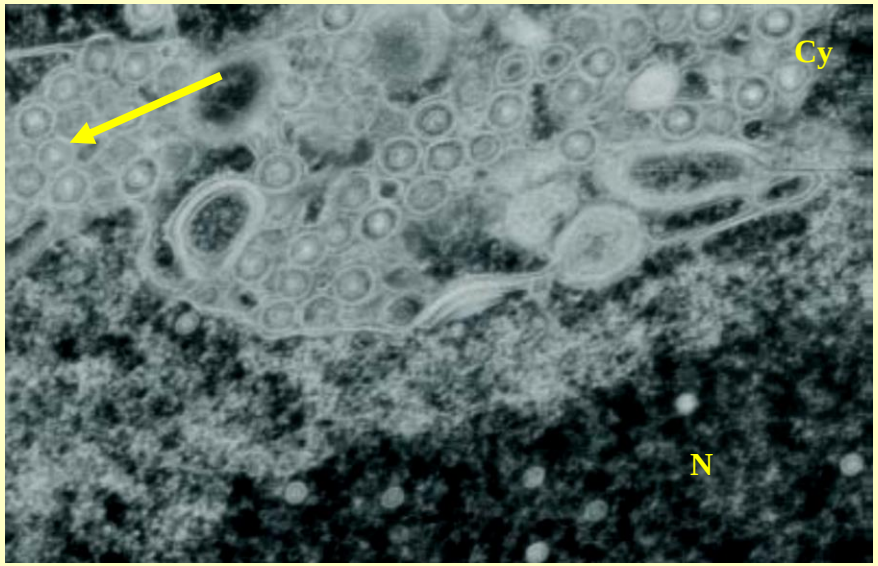
- Silvicultural practices

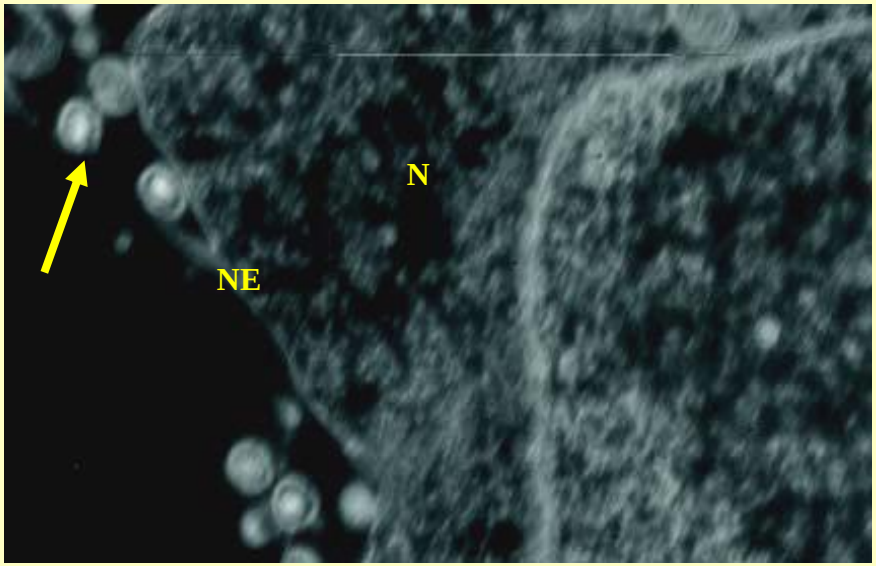
Tat1-like RTn belonging to the Ty3/*gypsy*-type LTR RTns (GenBank accession no. AY428554)

- Mature leaves from several branches on each tree (showing decline symptoms) were collected during December.
- All samples were kept in ice chests for transportation to the laboratory.
- Each tree sample was kept separately in a plastic bag at -80°C until analysis.

Transmission Electron Microscopy (TEM)

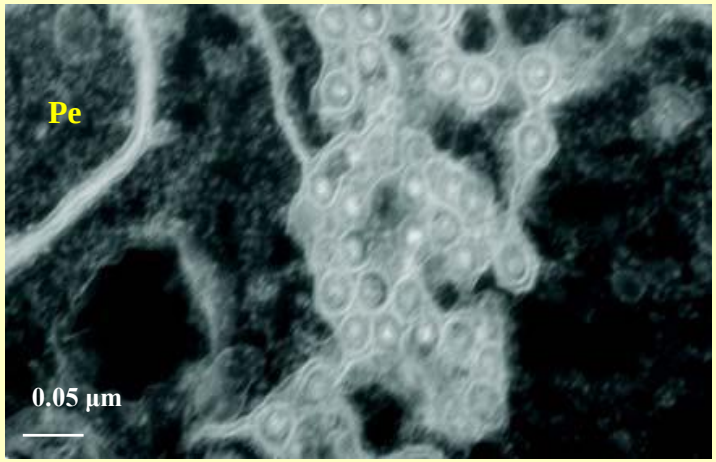
Leaves sterilized by submerging in a 3% sodium hypochlorite were macerated with sterilized water. The resulting suspension was filtrated by using a series of funnels of successive pore sizes (40-100 µm, 16-40 µm and 10-16 µm). The final solution was centrifuged for 15 min at 14000xg and the pellet was then stained by standard TEM techniques.

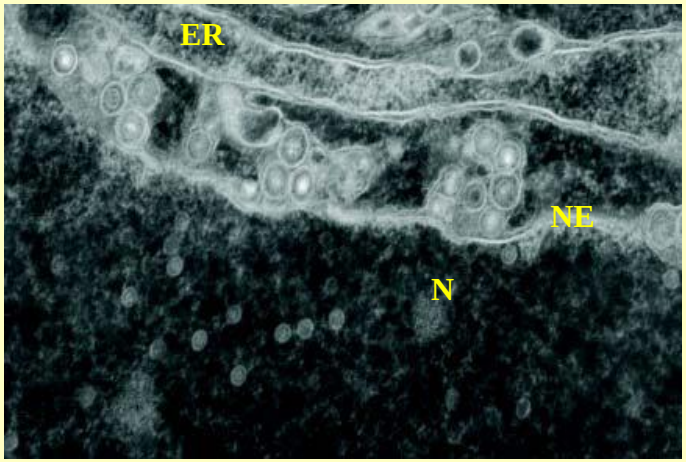


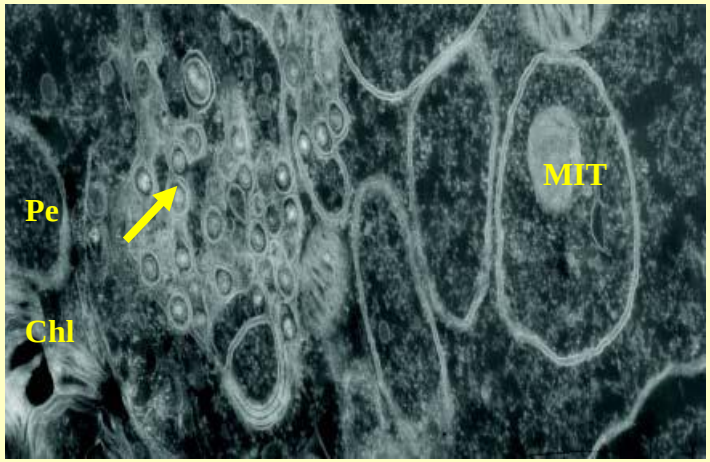


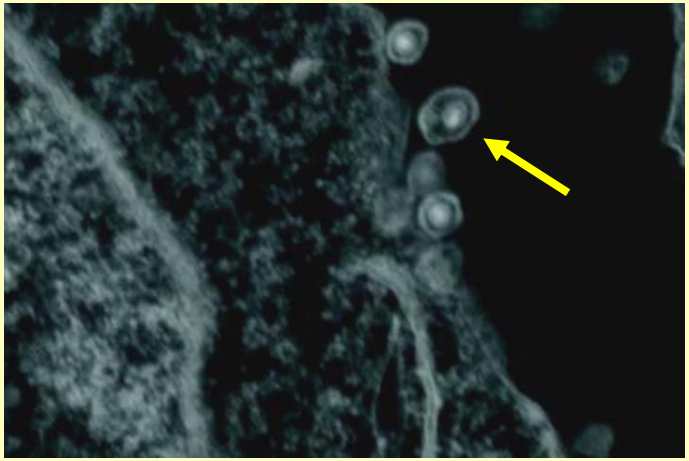
Virus particles can be seen in both cytoplasm (Cy) and near the nuclear envelope (NE)

Transmission Electron Microscopy (TEM)





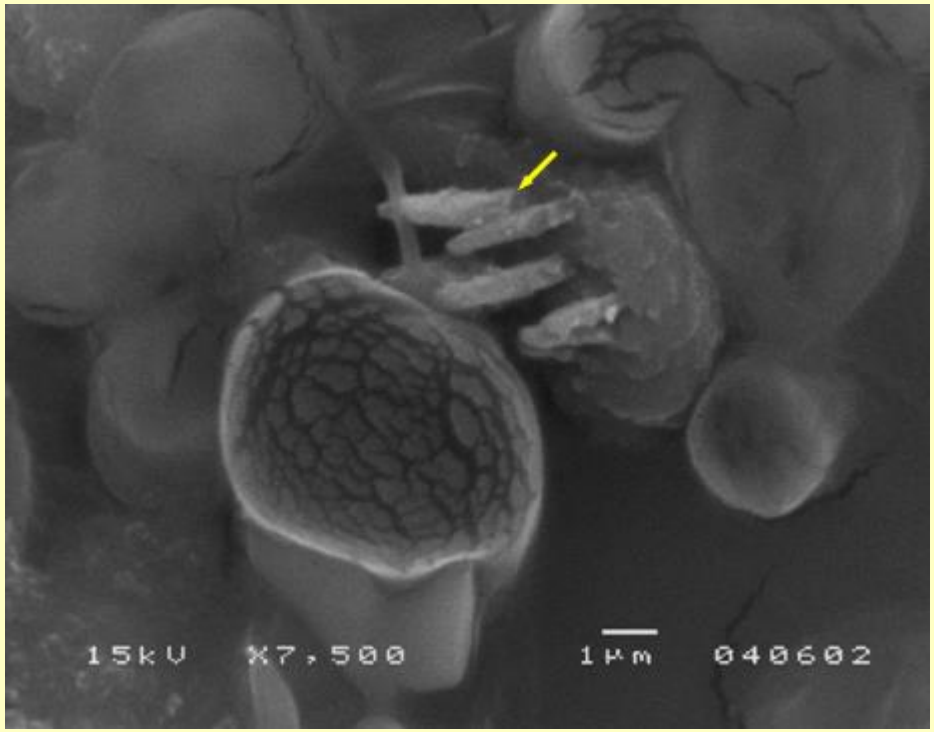




The isometric viral particles detected have an icosahedral structure with a size of approximately 20-30 nm in diameter

Scanning Electron Microscopy (SEM)

Fresh leaves showing disease symptoms were ground in liquid nitrogen and the resulting powder was coated with gold and examined with a JEOL scanning electron microscope.

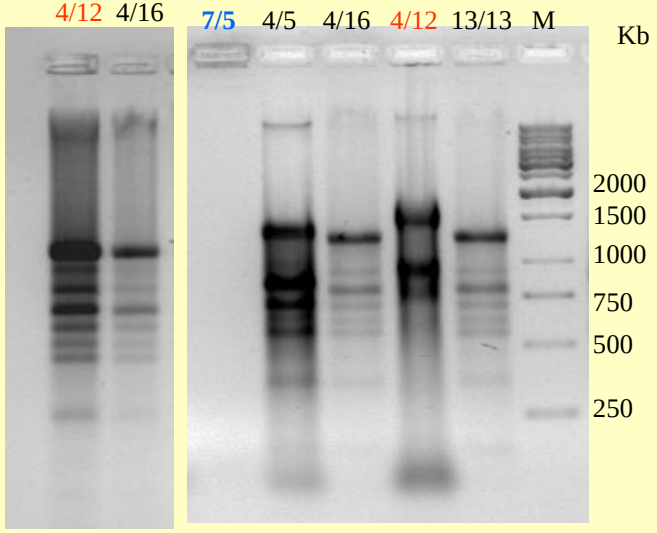


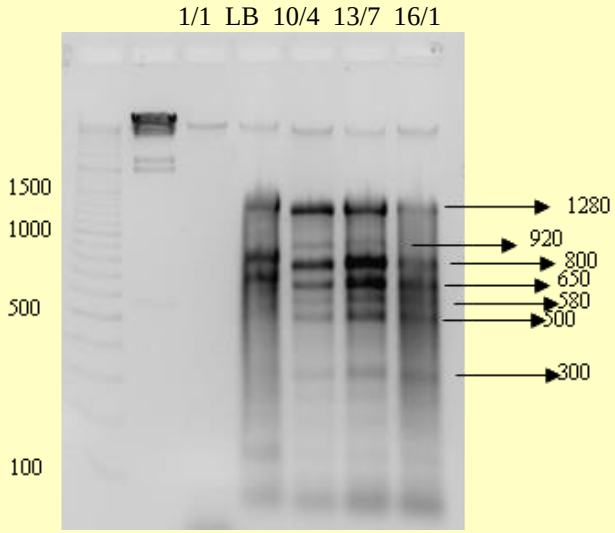
Presence of 5-6 unusual rod-shaped virus particles (???) about 2-3 µm in length, with an end round and the other one flattened, emerging from a broken putative proteinaceous occlusion body (???)

Isolation of virus double-stranded ribonucleic acid (dsRNA)

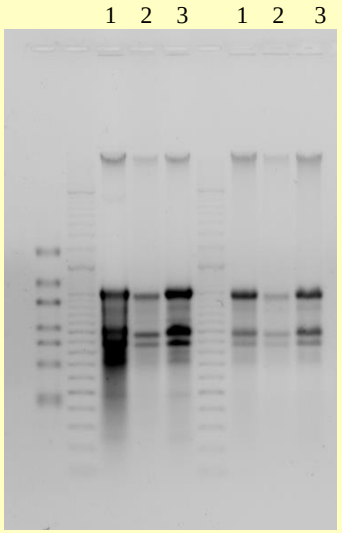
10g leaf samples were ground in liquid nitrogen and suspended in extraction buffer 1x STE, saturated phenol, chloroform:isoamilic acid and cresol.
The dsRNA was purified by binding to cellulose powder CF-11 and was electrophoresed on a 1% agarose gel.

Alentejo region



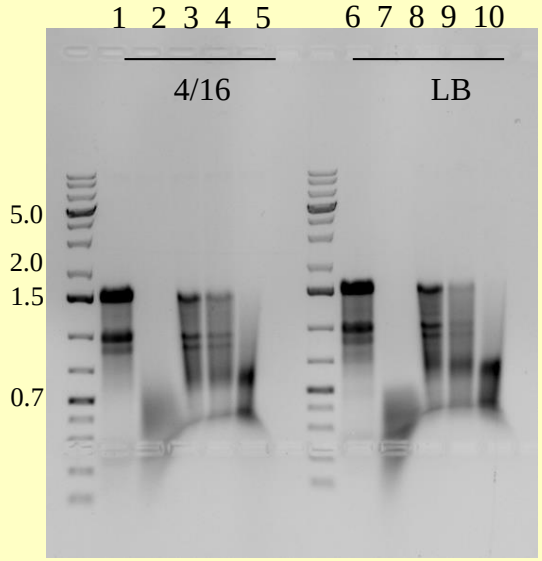


Ribatejo region



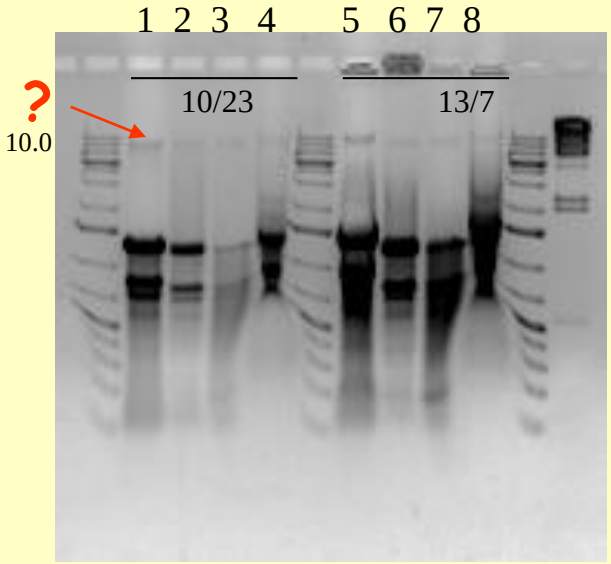
dsRNA profiles with distinct bands were detected.
Different dsRNA profiles from leaves of the same tree (4/12).
No dsRNA bands were detected in healthy tree (7/5)

Treatments with RNase A and DNase I



1,6 – dsRNA (5µL)
2,7 – dsRNA with RNase A (10 ng/mL)
3,8 – dsRNA with RNase A (2,5 ng/mL) + 0,3M NaCl
4,9 – dsRNA with RNase A (5 ng/mL) + 0,3M NaCl
5,10 – dsRNA with RNase A (10 ng/mL) + 0,3M NaCl

- The bands were degraded by RNAse A
- The resistance became strong in the presence of NaCl



1,5 – dsRNA (5µL)
2,6 – dsRNA with Turbo DNAse I (2U) (Ambion)
3,7 – dsRNA with RNase A (10 ng/mL)
4,8 – dsRNA with RNase A (2,5 ng/mL) + 0,3M MgCl2

- The bands were unaffected by DNase I
- RNase treatment without salt was not complete
- The bands were unaffected by RNase with salt

dsRNA?

First- and second-strand cDNA synthesis

RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas)

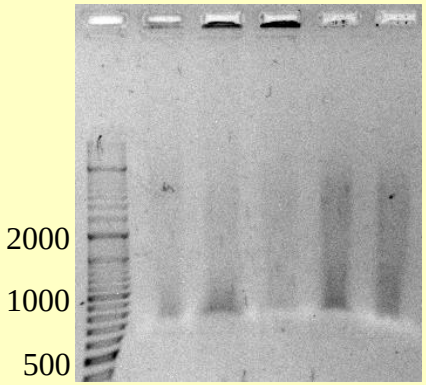
SuperScript III Reverse Transcriptase (Invitrogen)

ImProm-II Reverse Transcription System (Promega)

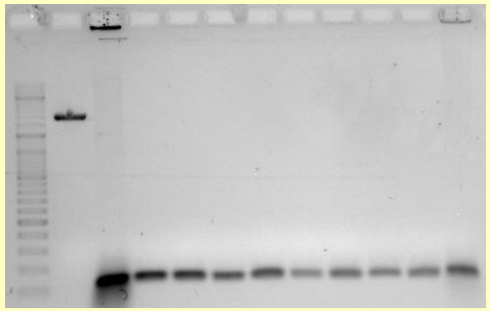
RETROscript™ First Strand Synthesis Kit (Ambion)

DNA polymerase I, *E.coli* and Ribonuclease H, *E.coli*

1. dsDNA (?) → Cloning (CloneJET PCR Cloning Kit and InsTClone PCR Cloning Kit) → Analysis of recombinant clones

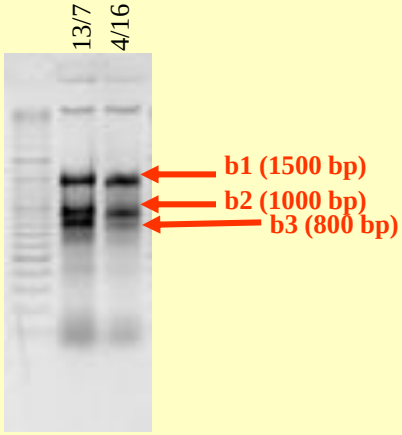


Colonies containing plasmids without inserts



2. General PCR primer sets as well as RT-PCR primer sets for viruses from different genera were tested

First strand cDNA synthesis for RT-PCR



The bands were cut off from the gel, purified with the QIAquick gel extraction kit (Qiagen) and first strand cDNA was synthetised.

Degenerate oligonucleotide primed-PCR (DOP-PCR)

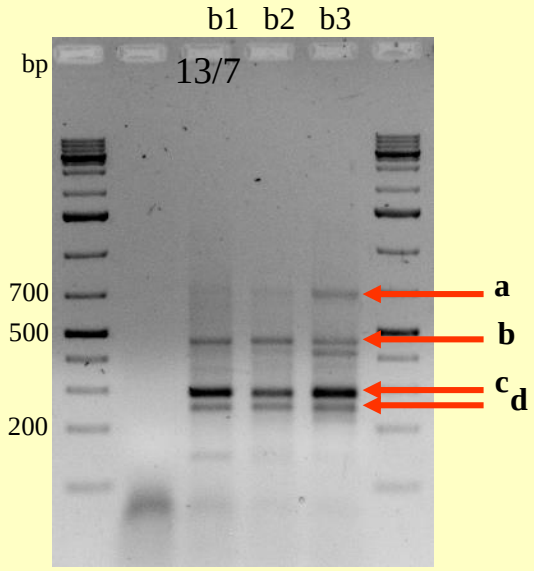
(European Journal of Plant Pathology 107: 411–420, 2001)
DOP1 - 5'CCGACTCGAGNNNNNNATGTGG3'
DOP5 - 5'CCGACTCGAGNNNNNNTTCAGG3'

(Primer designed from the entire genomic *sequence* of the endornavirus
DOPEV1- 5'CGGATGNNNNNNCGCTATTC3'

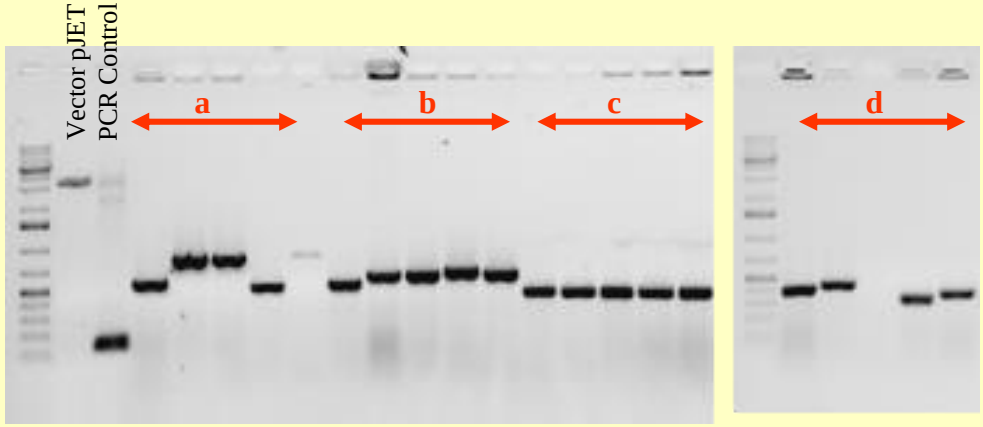
Forest Pathology 37(1): 9-21,2007)
DOPFP – 5'CCGGACTCGAGNNNNNNATGTGG3'

Degenerate oligonucleotide primed-PCR (DOP-PCR)

DOPFP – 5'CCGGACTCGAGNNNNNNATGTGG3'



Analysis of recombinant clones



The bands were cut off from the gel, purified with the QIAquick gel extraction kit (Qiagen) and cloned using CloneJET PCR Cloning kit (Fermentas)

Sequencing

Sequence similarity

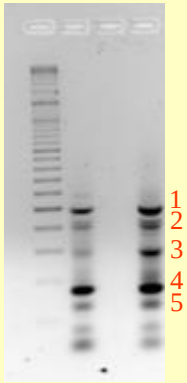
Sequence homology was searched against GenBank using NCBI BLAST (viruses) and against EMBL- EBI using WU-BLAST2 (EMBL Viral) and no similarity was found to plant viruses.

Very low sequence similarity was found to human and animal viruses

Alignment	DB:ID	Source	Length	Score	Identity%	Positives%	E()
1	EM_VI:AF191073	Stealth virus 1 clone 3B43, genomic sequence.	3620	1008	69	69	3.0e-48
2	EM_VI:AB214978	Human picobirnavirus pseudogene for RNA-dependent RNA polymerase.	193	188	69	69	0.26
3	EM_VI:AF065756	Stealth virus 1 clone 3B43 T7.	836	213	67	67	1.8

PCR – Primers for Oak cryptic dsRNA virus

Oak Cryp for (OC1) = 5'-CTCATGTTCATCGAGTCCGTA-3'
Oak Cryp ver (OC2)= 5´-GTCTTAGCTGGATTGAGATACC-3'



5 bands were cut off from the gel, purified, cloned and sequenced.

No similarity was found between sequences of plant viruses

Alignment	DB:ID	Source	Length	Score	Identity%	Positives%	E()
1	EM_VI:DQ149153	Cercopithecine herpesvirus 16 strain X313, complete genome.	156487	214	56	56	1.2
2	EM_PAT:A45258	Sequence 2 from Patent WO9516779.	3957	209	56	56	1.9
3	EM_PAT:DD220300	COMPOSITIONS AND METHODS FOR THE DIAGNOSIS AND TREATMENT OF HERPES SIMPLEX VIRUS INFECTION.	3957	209	56	56	1.9
4	EM_PAT:CS208162	Sequence 110 from Patent WO2005080581.	154746	209	56	56	2.1
5	EM_VI:Z86099	Herpes simplex virus type 2 (strain HG52), complete genome	154746	209	56	56	2.1
6	EM_VI:AY714813	Cercopithecine herpesvirus 2, complete genome.	150715	200	57	57	6.1

Sap containing virus particles (?)

Dead trees are removed

Some trees have holes that descends down inside the tree where the rain water is accumulated

When the trees were cut down, water and sap containing virus particles (?) was collected

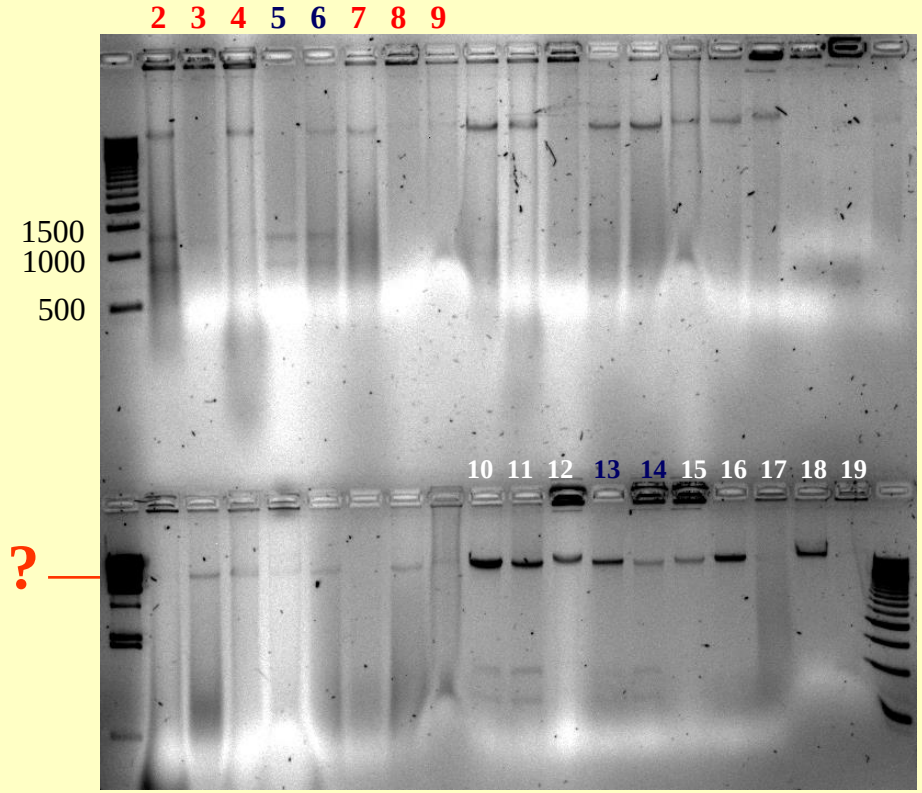
Isolation of RNA and treatment with enzymes

RNAqueous Total RNA Isolation kit (Ambion)

MagMax AI/ND Viral RNA Isolation (Ambion)

Turbo DNAse I (Ambion)

RNAse A (Sigma)



2, 3, 4 -Total RNA

5, 6 - DNAse I

7, 8 – RNAse A

9 – RNAseA + MgCl2

10, 11, 12 -Total RNA

13, 14 - DNAse I

15, 16, 17 – RNAse A

18 – RNAseA + MgCl2

Is it possible to detect virus in this solution?

Return Polyacrylamide Gel Electrophoresis - Viroids

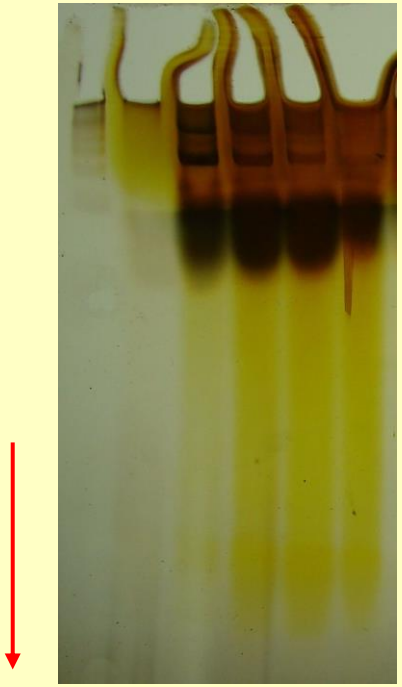
OEPP/EPPO, 2004. *Bulletin OEPP/EPPO Bulletin* 34, 155 -157

The first run was performed under native conditions

1- dsRNA healthy cork oak tree

2,3,4,5, - dsRNA symptomatic cork oak trees

The return run was performed under denaturing conditions (inside an incubator at 70 °C and with reverse the polarity)



The viroid band should appear in the lower two thirds of the gel

Conclusions

- Transmission electron microscopy (TEM) of symptomatic leaves homogenates revealed the presence of isometric virus-like particles with 20-30 nm in diameter.

- Scanning electron microscopy (SEM) of symptomatic leaves revealed the presence of unusual rod-shaped virus particles about 2-3 μm in length with an end round and the other one flattened.

- Analysis by electrophoresis of double stranded RNA (dsRNA) from leaf material showed the presence of a pattern of multiple bands.

- dsRNA was detected in a size range from 0,4 to 10Kb(?)