Studies on virus-infected cork oak (*Quercus suber* L.)

Filomena Nóbrega[1], Rui Vidal[2], Rita Serrano[2], Raúl Sardinha[3] & Raúl Bruno de Sousa[4]

[1] Estação Florestal Nacional, Av. da República, Quinta do Marquês, 2780-159 Oeiras, Portugal
[2] Faculdade de Farmácia da Universidade de Lisboa, Av. das Forças Armadas, 1600-083 Lisboa, Portugal
[3] Instituto PIAGET, Centro Internacional de Epistemologia e Reflexão Transdisciplinar, Av. João Paulo II, Lote 544 - 2º, 1900-726 Lisboa, Portugal
[4] Instituto Superior de Agronomia, Tapada da Ajuda, 1349-018 Lisboa; Portugal

Introduction

Fig. 1 – *Quercus suber* cork oak trees showing sudden death symptoms

One of the most important ecosystems in Portugal is the typical forest stands of *Quercus suber* ("montados") which has a great social, ecological and economical importance for local people in rural areas. Mainly during the last two decades a gradual reduction of the *Quercus* spp. stands area and density have been observed in Portugal mostly related with general degradation of the ecosystems and the spreading of tree decline phenomena. In the affected areas the decline symptoms in cork trees have been evidenced by the initial deterioration of the crown that started by showing loss of vigor and leaf necrosis followed by sudden death in some trees (Fig. 1).

The health and vitality of the "montados" in Portugal seems to be related with natural causes, anthropogenic influences, inadequate management practices and pests and disease incidence. However, it is difficult to correlate the abiotic or biotic cause with the decline process and the results obtained up to now have not satisfactorily explained the specific causes of the phenomena. This is also the case of the general european oak decline where other factors have been mentioned, such as the involvement of biological agents, mainly fungi, bacteria and viruses. However, about viruses infecting oak trees the available information is still very limited since it is only known, but not yet fully characterized, the ssRNA tobamoviruses isolated from diseased *Q. robur* [1,2] in Germany and the Oak Ringspot Virus infecting *Q. marilandica* and *Q. velutina* in USA [3].

As a consequence, in order to produce a more and consistent knowledge about the cork oak decline process in Portugal, we have initiated a polymerase chain reaction (PCR)-base screening programme for detection of mobile genetic elements on genomic DNA isolated from cork oak leaves. The results have revealed genetic variabilities due to R4-like non-LTR retrotransposons (RTns) insertions on *Q. suber* genome [4] as well as the presence, at least, of three different types of LTR RTns whose partial sequences have been determined and deposited on GenBank database under the following accession numbers: AY099465 (for a Ty1/*copia* RTn named Melmoth), AF512588 (for a Ty3/*gypsy* RTn designated as QSUGY-G1) and AY428554 (for a Tat1-like RTn also belonging to the Ty3/*gypsy*-type LTR RTns). Since the last one seems to be active, and therefore able to be packaged into virus-like particles (VLPs), in order to elucidate this probability we have initiated another programme for detection of virus particles or VLPs, by using scanning electron microscopic (SEM) and transmission electron microscopic (TEM) observations.

Besides confirming our results that have been already reported for the presence of VLPs on the cork oak leaves [5], in the present work we also provide evidence, for the first time to our knowledge, for genomic polymorphisms due to Tat1-like RTn insertions on *Q. suber* genomic DNA and the presence of unusual virus particles on the leaves of diseased cork oak trees.

Methods

Fig. 2 – *Q. suber* leaves showing disease symptoms

1 – Isolation of nucleic acids

Total genomic DNA was extracted from leaves using Qiagen Maxi and Mini DNAeasy kits according to the manufacturer's instructions.

2 – Detection of retrotransposon insertions

PCR amplifications were carried out with a Hybaid Express thermocycler, using Qiagen Taq PCR Master Mix kit with a set of specific gl8 gene primers (primer gl8a58, 5'-AAGAGTGTGGCGCGTGCTATG-3' and primer gl8ain, 5'-CTCAGGAGGTAATGGTAG-3'). For purification and cloning of the PCR products, the following Qiagen kits were used according to the manufacturer's instructions: QIAquick–DNA extraction kit, PCR cloning ligation kit and PCR cloning transformation kit. Both strands of each of some cloned PCR products were sequenced by the sequencing services of "MWG Biotech AG" (Ebersberg, Germany).

3 – TEM and SEM observations

For TEM observations, 5 g of those leaves were macerated with an equal volume of sterilized water and the resulting suspension was filtrated by using a series of funnels (Duran Schott) of successive pore sizes (40-100 µm, 16-40 µm and 10-16 µm). The final fluid was centrifuged for 15 min. at 14000xg and the pellet was then stained by standard TEM techniques and observed. For SEM observations, a few fresh infected *Q. suber* leaves showing disease symptoms (Fig. 2) were stored overnight at -20 ºC and then ground in liquid nitrogen with a pestle and mortar. The resulting powder was coated with gold and examined with a JEOL scanning electron microscope (JSM-5220 LV, 15 kV).

Results and Discussion

1 – *Quercus suber* genetic variabilities due to Tat1-Like insertions

Since the Tat1-like RTn above mentioned seems to be an active genetic element, total genomic DNA was isolated from each one of 17 cork oaks, and directly amplified by PCR using the primer pair gl8. The results showed multiple products on each PCR profile. However, the 249 bp PCR product, corresponding to the Tat1-like RTn partial sequence (GenBank AY428554), was only present on 5 of them (Fig. 3, lanes 1, 2, 4, 5 and 15) although with different yields.

This means that some of the genetic variabilities previously observed on *Q. suber* genomic DNAs could be also due in fact to active Tat1-like RTn insertions because retrotransposition is an irreversible process resulting in insertions of RTns without loss of the parental copies. Otherwise, that is, in the case of an inactive element, it would be either absent or present on all of the investigated DNA templates.

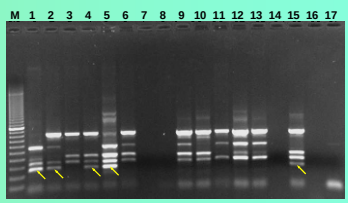

Fig. 3 – 0,8% AGE of PCR products amplified from the genomic DNA templates isolated from 17 cork oaks where the position of the 249 bp PCR product, corresponding to the Tat1-like RTn partial sequence, is indicated by an arrow. Lane M - 100 bp DNA Ladder

2 – TEM and SEM observations

As active RTns propagate via a mRNA intermediate which is then reverse-transcribed and packaged into VLPs [6], TEM observations have been performed as above indicated to confirm or not the putative Tat1-like RTn activity. The results have demonstrated the presence of abundant VLPs approximately isometric, with about 20 nm in diameter, on the infected *Q. suber* leaves, either as aggregates or individual particles (Fig. 4). However, further studies are presently in progress for a definitive validation of these observations in order to know whether any of the nucleic acid components of the observed VLPs corresponds indeed to an active Tat1-like RTn element.

In the meantime, SEM observations have been also carried out on those diseased *Q. suber* leaves. Unexpectedly, the results have shown the presence of 5-6 giant, 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 (Fig. 5).

As those virions bear some resemblances to the viral particles characterised as Baculovirus (a virus often found on cork oaks but not pathogenic for them), total nucleic acid extracts have been obtained from another set of partially purified viral preparations corresponding to the viral particles shown in Figure 5, then digested with DNAse I as well as RNAse A according to standard procedures, and the resulting products fractionated by AGE. The results have apparently revealed that those particles may harbour a supercoiled DNA (cccDNA) since its nucleic acid component was DNAse I sensitive but with a much more slower relative electrophoretic mobility (REM) after the RNAse A digestion (Fig. 6).

Therefore, this REM shift from 9.4-23 kb to more than 100 kb could be due to a single cccDNA genome such as reported for the nucleopolyhedroviruses of the Baculoviridae family which contain a total genome length of 90-165 kb. However, the remaining morphological features of the observed viral particles are not those of the nucleopolyhedroviruses, since possess 2-3 µm in length and one flattened end (a typical characteristic of the cytorhabdoviruses that harbour a negative-sense single-stranded RNA genome).

Consequently, this means that a new type of virus, not yet reported, has been found on *Quercus suber* leaves? Studies are in progress in order to elucidate this situation.

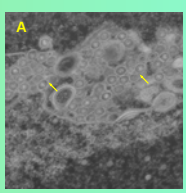

Fig. 4 – TEM observations on the final fluid of macerated *Q. suber* leaves showing aggregates of VLPs (A) and individual VLPs (B)

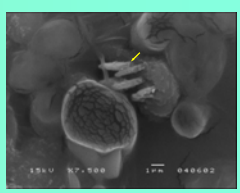

Fig. 5 - SEM observation on diseased *Q. suber* leaves showing 5-6 rod-shaped virus particles with an end flattened (arrow)

Conclusions

From these results, the major conclusions are as follows:

1. Some of the *Quercus suber* genetic variabilities could be due to an active Tat1-like RTn since VLPs have been detected on diseased cork oak leaves;
2. An apparent new type of giant, unusual virus, not yet reported but with some resemblances to nucleo-polyhedroviruses and cytorhabdoviruses, has been also found on diseased cork oak trees;
3. No tobamovirus particles have been detected so far on the Portuguese cork oak leaves, in contrary to the situation reported for diseased *Quercus robur* in Germany.

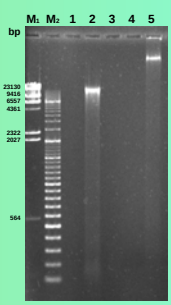

Fig. 6 – 0,8% AGE of the untreated (lane 2), DNAse I digested (lane 4) and RNAse A digested (lane 5) nucleic acid component isolated from partially purified preparations corresponding to the viral particles shown in Fig. 5. Lane M1, λDNA-Hind III digest and lane M2, 3,000 bp DNA Ladder

References

[1] Sabine, H., Bandte, M., Obermeier, C. & Büttner, C., 2003. Investigation on virus-diseased Eurpean oak (*Quercus robur* L.), 8th International Congress of Plant Pathology, Christchurch, New Zealand, 2-7 February 2003.

[2] Steinmöller, S., Bandte, M. & Büttner, C., 2004. Investigations into the pathogen of chlorotic ring spots on oak trees (*Quercus robur*). Gesunde Pflanzen, 56 (1) : 11-16.

[3] Plant Viruses Online: Descriptions and Lists from the VIDE Database. Image.fs.uidaho.edu/vide/refs.htm.

[4] Nóbrega, F., Vidal, R., Sardinha, R. & Bruno de Sousa, R., 2004. Retrotransposons as sources of *Quercus suber* genetic variabilities. XXXI Jornadas Portuguesas de Genética, Oeiras, Portugal, 5-6 February 2004.

[5] Serrano, R., Nóbrega, F. & Vidal, R., 2003. Detection of viral particles on *Quercus suber* L. leaves: preliminary results. Congresso Nacional de Microbiologia, Micro-2003, Tomar, Portugal, 29 November - 2 de December 2003.

[6] Suoniemi, A., Tanskanen, J. & Schulman, A. H. 1998. Gypsy-like retrotransposons are widespread in the plant kingdom. Plant J., 13 (5) : 699-705.

This research was supported by: PIDDAC and AGRO

E-mail: Filomena.Nobrega@efn.com.pt
Estação Florestal Nacional, Avenida da República, Quinta do Marquês
P - 2780-159 OEIRAS (Portugal)
Tel.: (+351) 214463791; Fax: (+351) 214463702