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

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Abstract: The typical forest stand of *Quercus suber* (montados) is one of the most important ecosystems in Portugal and is of great social, ecological and economical importance for rural populations; it contributes extensively to Portuguese PNB through cork production and transformation. However, over the last few decades, ecophysiological disturbances of the montados were observed. In affected areas, the decline symptoms of cork oak trees evolved by the deterioration of the crown which, starting with leaf necrosis, led some trees to sudden death. Virus particles on cork tree leaves were detected by SEM observation and partial dsDNA sequences of three different reverse transcribing-like viruses from *Q. suber* leaves were already determined. Our study aims to increase the knowledge about the infectious agents in order to better understand the cork oak decline and define suitable measures to overcome them.

Key words: virus, cork oak decline, *Quercus suber*

Introduction

One of the most important ecosystems in Portugal are the typical *Quercus suber* stands ("montados") which have great social, ecological and economical importance for rural populations. A gradual reduction of the *Quercus* spp. area and density was observed in Portugal, mainly during the last two decades, related to the general degradation of the ecosystems and the spreading of tree decline phenomena. In the affected areas, the decline symptoms in cork trees was evidenced by crown deterioration which started by vigour loss and leaf necrosis inducing the sudden death of some trees.

The "montados" health loss in Portugal seems to depend on natural causes, anthropogenic influences, inadequate management practices and pests and disease incidence. However, correlating the abiotic or biotic factors with decline process is difficult and research results have not yet satisfactorily explained the specific causes of the phenomena. The same general decline phenomena was observed in European oak; the inducing factors involved are mainly biological agents such as fungi, bacteria or viruses. However, concerning virus-infecting oak trees, the available information is still very limited, because they are known, but not yet fully characterized; the ssRNA tobamoviruses were isolated from diseased *Quercus robur* (Sabine et al., 2003; Steinmöller et al., 2004) in Germany and the oak ringspot virus from *Quercus marilandica* and *Quercus velutina* in USA (Plant Viruses Online).

Therefore, in order to obtain more consistent knowledge about the cork oak decline process in Portugal, we initiated a polymerase chain reaction (PCR)-base screening program to detect mobile genetic elements on genomic DNA isolated from cork oak leaves. The results revealed a genetic variability due to R4-like non-LTR retrotransposons (RTns) insertions on

Quercus suber genome (Nóbrega et al., 2004) as well as the presence of at least three different types of LTR RTns, whose partial sequences were determined. They were 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). The last one seems to be active, and therefore able to be packaged into virus-like particles (VLPs). Aiming at elucidating this probability, we initiated another detection program of virus particles or VLPs, using scanning electron microscopic (SEM) and transmission electron microscopic (TEM) observations.

Besides confirming our already reported results concerning the presence of VLPs in cork oak leaves (Serrano et al., 2003), the present work also provides evidence, for the first time, of genomic polymorphisms due to Tat1-like RTn insertions on *Quercus suber* genomic DNA as well as the presence of unusual virus particles on leaves of diseased cork oak trees.

Methods

Isolation of nucleic acids

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

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 several cloned PCR products were sequenced by the sequencing services of "MWG Biotech AG" (Ebersberg, Germany).

TEM and SEM observations

For TEM observations, 5 g of infected 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 mn at 14000xg and the pellet then stained by standard TEM techniques before observation. For SEM observations, a few fresh infected *Q. suber* leaves showing disease symptoms were stored overnight at -20 °C 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

Q. suber genetic variability 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 g18. 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. 1, lanes 1, 2, 4, 5 and 15) although with different yields.

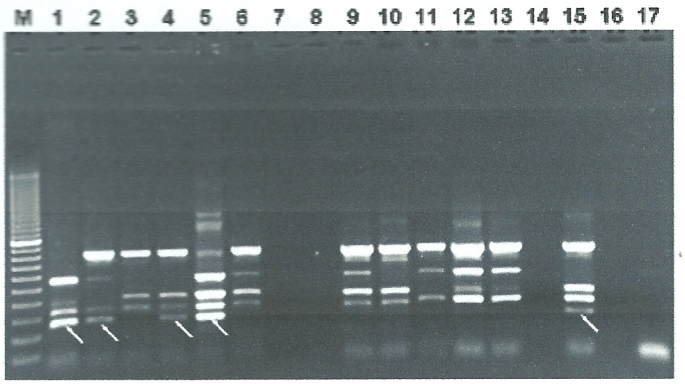

Figure 1. 0,8% AGE of PCR products amplified from the genomic DNA templates, isolated from 17 cork oaks. The position of the 249 bp PCR product, corresponding to the Tat1-like RTn partial sequence, is indicated by an arrow. Lane M – 100 bp DNA Ladder

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

TEM and SEM observations

As active RTns propagates via a mRNA intermediate which is then reverse-transcribed and packaged into VLPs (Suoniemi et al., 1998), TEM observations were performed as indicated, to confirm or nullify the putative Tat1-like RTn activity. The results demonstrated the presence of abundant VLPs approximately isometric, about 20 nm in diameter, on the infected *Q. suber* leaves, either as aggregated or individual particles (Fig. 2). 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 to an active Tat1-like RTn element.

Meanwhile, SEM observations were also carried out on diseased *Q. suber* leaves. Unexpectedly, the results showed the presence of 5-6 giant, unusual rod-shaped virus particles about 2-3 µm in length, with a rounded end and the other flattened, emerging from a broken, putative proteinaceous occlusion body (Fig. 3).

As those virions had some resemblance to the viral particles characterised as Baculovirus (a virus often found on cork oak but not pathogenic for the tree), total nucleic acid extracts were obtained from another set of partially purified viral preparations corresponding to the viral particles shown in figure 3. These particles were digested with DNAse I as well as RNAse A according to standard procedures, and the resulting products splited by AGE. The results apparently revealed that these particles may harbour a supercoiled DNA (cccDNA) since its nucleic acid component was DNAse I sensitive, but they showed a much slower relative electrophoretic mobility (REM) after the RNAse A digestion.

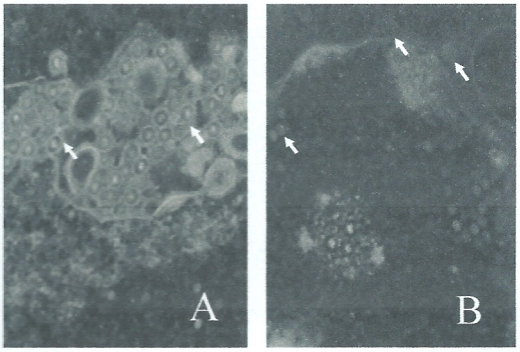

Figure 2. TEM observations on the final fluid of macerated *Q. suber* leaves showing aggregates of VLPs (A) and individual VLPs (B)

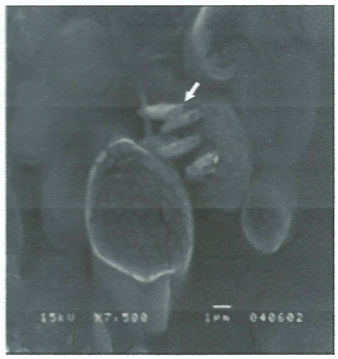

Figure 3. SEM observation on diseased *Q. suber* leaves showing 5-6 rod-shaped virus particles with a flattened end (arrow)

Therefore, this REM, shifted 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 they 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, does this mean that a new type of virus, not yet reported, was found on *Q. suber* leaves? Studies are in progress in order to elucidate this situation.

Conclusions

Based on these results, the major conclusions are as follows:

1. Some of the *Quercus suber* genetic variability could be due to the active Tat1-like RTn because of VLPs 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, was also found on diseased cork oak trees;
3. To date, No tobamovirus particles were detected on the Portuguese cork oak leaves, contrary to the situation reported for diseased *Quercus robur* in Germany.

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