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In Vitro Cultures in Agricultural Research: A Means to Contain Phytoviruses and Genetically Modified Plants (GMPs)

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Agricultural research into developing new pathogen-resistant plant varieties often involves the use of biological agents and/or organisms that require special bio-containment, which can be severely limiting especially when research funds are scarce. In this editorial, the *Plum pox virus* case study is presented and the possibility of using *in vitro* cultures to contain both phytoviruses and genetically modified plants (GMPs) is discussed.

Plum pox virus (PPV), a member of the genus *Potyvirus,* is the causal agent of sharka, the most detrimental viral disease affecting stone fruits (*Prunus* spp.) including plum, peach, apricot and cherry. PPV causes severe symptoms in the fruit and in some cases premature drop, resulting in reduced fruit quality and yield. The losses, in susceptible cultivars, may affect total fruit yields, thus making sharka a significant agricultural, economic and social constraint. PPV spreads locally in orchards by aphids, when infected plants are present and over long distances by the use of infected propagative plant materials. Sharka was first reported in Bulgaria and, in about a century it has spread through Europe, around the Mediterranean basin and in most stone fruit producing countries worldwide except South Africa, Australia and New Zealand [1,2].

In many countries PPV thus has quarantine status and considerable efforts are being made to eradicate or contain it (e.g. EPPO http://www.eppo.org/QUARANTINE/listA2.htm and NAPPO http://www.pestalert.org). Since PPV, like all phytoviruses, cannot be directly controlled by chemical application on infected plants, and the reduction of aphid vectors by insecticides is not effective against non-persistent viruses such as PPV, current strategies to contain its spread are based on the use of certified PPV-free plant materials, periodic surveys of orchards and the eradication of diseased trees, together with the development of PPV-resistant stone fruit cultivars.

To date, classical breeding has not achieved the goals expected, mainly due to there being only a few valuable sources of resistance to PPV in stone fruits [2,3]. On the other hand, a PPV-resistant plum variety, genetically modified and registered as Honey Sweet [4], has been obtained in USA, and more recently, GM plum clones were also obtained in our laboratories in Italy using RNAi [5].

In whatever way the plants are produced, by classical breeding or genetic modification, they need extensive analysis in order to verify their ability to resist PPV infection. In addition, given that an effective disease-control strategy should protect the host from the most economically important and geographically widespread variants of the pathogen, it is critical to analyse the spectrum of resistance of the plants to different isolates of the virus [6]. This is especially true in the case of resistance based on the sequence-specific RNA silencing mechanism, as the above GM plums are [7,8]. All the infection test procedures, in most countries, should be performed in special authorized quarantine greenhouses/screenhouses [9] and, if the tested plants are transgenic, the greenhouse should adhere to containment conditions to prevent not only insect movement but also pollen escape [10]. The evaluation of PPV in *Prunus* species is further constrained by the need to alternate cycles in greenhouses and cold chambers and the need to move the plants grown in pots. Furthermore, PPV can only be artificially transmitted during a limited period of the year. Its distribution and expression throughout the plant can be irregular and influenced by environmental conditions, and lastly its evaluation is very time-consuming-at least two vegetative periods are needed [2].

Therefore, there has been increased focus on alternative methods that would minimize viruses and GM escapes. In this context, *in vitro* culture represents a good tool.

In vitro culture techniques, in particular micropropagation, refer to the multiplications of plants in jars, in aseptic conditions and on artificial growth medium. These techniques have been developed to rapidly propagate clonal plants, by shortening the time necessary to introduce new varieties, and to preserve germplasm. They have been applied in cases where plant cloning is difficult or even impossible by current methods, such as cutting, budding and grafting. Although one of the main prerogatives of *in vitro* tissue culture is to obtain and maintain pathogen-free plants, some researchers have considered this technique as a tool in virology research. The micropropagation of virus-infected plants, particularly interesting for plant trees, could be used as a continuous source of viral inoculums. Thus various strategies have been tested for PPV infected stone fruits, such as the micropropagation of previously *in vivo* PPV-infected GF305 (an indicator for virus indexing), or *in vitro* GF305 inoculation by shoot grafting, dipping, injection, scarification or aphid [11].

Although initially the durability of the infected culture and the maintenance of the pathogenicity level were considered uncertain, the possibility of maintaining *in vitro* cultured shoots of PPV infected GF305 [12], also for several years [5] has recently been demonstrated. This could be of particular practical interest for molecular and biological studies. In those countries where PPV is not endemic or some PPV strains are not present, it is possible to maintain and use a collection of PPV isolates in the laboratory, thus avoiding the risk of virus release into the environment.

For example, we have developed an *in vitro* evaluation method for PPV resistance [5]. Micropropagated shoots of the germplasm (which was also transgenic) were micrografted *in vitro* onto PPV infected

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GF305 rootstock. Successful grafted scions were thus ready for analysis in order to detect PPV. This procedure means that dozens of plants can be analysed rapidly in a laboratory, avoiding the restrictive and expensive control measures that are necessary for *in vivo* manipulation of quarantine viruses and GMPs. Although an *in vitro* test cannot be compared to an *in vivo* test, a high degree of correspondence between *in vivo* and *in vitro* evaluations for PPV resistance was observed [13] making the *in vitro* procedure useful as a preliminary screening.

Although *in vivo* studies are needed, compliance with biosafety measures is mandatory: the use of *in vitro* cultures means that precautions can be taken while at the same time advancing our knowledge of issues of fundamental agricultural importance.

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