Indexing *in vitro* for diagnosis of viroids in citrus

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I have been working at the Agricultural Research Institute (ARI) since 1992 mainly on the Programme concerning Sanitation of citrus propagating material. As diagnosis of viroids is the most difficult and time-consuming work of the above Programme, I decided to explore within my MSc project the possibility of the use of an alternative procedure for diagnosis of viroids.

Viroids, the smallest disease-causing agents, cause serious diseases to several crops and are transmitted by mechanical means, such as infested tools. In Cyprus citrus viroids are widespread and the main way to control these disease agents is prevention by planting and grafting viroid-free propagating material. Detection of viroids relies on biological indexing in the glasshouse by the use of the plant indicator Etrog citron.

Technical terms:

Indexing: testing for the presence of a specific pathogenic agent in a plant by the use of an indicator plant

Indicator: a plant which shows characteristic symptoms after its inoculation with a specific pathogenic agent

In vitro means "in glass". Biological or biochemical process performed in the laboratory

In vivo means "in life". Biological or biochemical process occurring within a living organism.

Inoculate: to insert a pathogen into healthy tissue

Aims of the project were the improvement of the citrus sanitation programme in Cyprus and the improvement and speeding up of the process of the biological indexing method for citrus viroids. **Objectives of the project were:** a) the reduction of the time of production of citrus propagating material,

b) the employment of a new faster and relatively simple method for the diagnosis of citrus viroids, c) the employment of a more reliable and environment-friendly method in comparison to the conventional biological indexing in the glasshouse, and d) the reduction of the cost of diagnosis of viroids in the glasshouse.

Research Questions

1. How many days were needed for viroid symptom expression on Etrog citron indicator micro plants *in vitro*?
2. How many days were needed for symptom expression on Etrog citron indicator plants in the glasshouse (*in vivo*)?
3. Which was the best method for the inoculation of citron micro plants? (Graft inoculation with plant bark or inoculation by injection with plant extract)
4. Which were the best indicator citron micro plants? Those propagated from seeds or by cuttings *in vitro*?
5. Which was the best nutrient medium for the micro propagation of citron cuttings *in vitro*?
6. Which was the best nutrient medium for the micro propagation of citron seeds *in vitro*?
7. Which were the symptoms expressed on the infected indicator citron micro plants produced by seeds *in vitro*?
8. What was the symptom expression on the indicator citron micro plants produced by seeds *in vitro*?
9. Which were the symptoms expressed on the infected indicator citron plants in the glasshouse (*in vivo*)?

Methodology

The Research Approach used was the experiment as this tactic was associated with materials and non-human life forms, such as plants, which were the subject of my project. The Experimental approach belongs mostly to the Quantitative Research Family which is concerned with the collection and analysis of data in numeric form.

Data Collection Techniques used were measurements and observations and a combination of both.

Measurements and Observations

1. Each plant used in the experiments was coded with one number and a special label was placed on each glass tube or pot. The date of inoculation, the plant number and the inoculation method were written on the attached label.
2. Two diaries were kept for recording the data:

The first one was used for the plants grown in the greenhouse in pots (*in vivo*) and the other for the plants grown in test tubes (*in vitro*). Data were written in Tables indicating the progress of each plant every week. All data were transferred onto Microsoft Excel Programme for ANOVA statistical analysis.

Data collected were: height of plants, leaf necrosis, severe leaf epinasty, mild leaf epinasty, petiole browning, midvein necrosis and no symptoms.

Records were written in the Tables every week. Time required for detection of viroids by RT-PCR in citron micro plants after inoculation was also recorded.

Scale of research

1st stage: March 2005, 150 Etrog citron plants were planted.
2nd stage: April 2006, 6 source plants were selected.
3rd stage: June 30 - Nov. 30, 2006, "Biological indexing *in vivo"* of 56 citron plants.
4rth stage: May 2 - Nov. 30, 2006, "Biological indexing *in vitro*" of 452 citron plantlets.
5th stage: April - November, 2006, Molecular detection of viroids with 80 tests.

Inside researcher

As an inside researcher I had many **advantages** such as the authority and autonomy to conduct the research work and the responsibility for the organisation and evaluation of the experimental research work. I had also the skills and knowledge required to undertake the project and the resources required to carry out the project. I had the opportunity to use part of the work budget for the needs of the work based learning project and to use the necessary laboratory equipment and facilities and many sources and information available for my research project (Tissue Culture Laboratory, Plant Pathology Laboratory, Greenhouse, Incubator room - controlled environmental conditions - and the Library of the ARI). **Disadvantage** was the lack of time, as I had to undertake at the same time other research tasks too.

Findings:

Indexing *in vivo*

Symptom expression was observed on Etrog citron plants 11 weeks after inoculation on indicators infected by CEVd (severe) and complexes of 3 and 4 viroids (mild)

Indicators infected by a complex of 2 viroids showed symptoms 13 weeks after inoculation and all plants with viroids showed symptoms 14 weeks after inoculation.

Severe symptoms appeared as severe leaf epinasty and leaf necrosis of Etrog citron. Mild symptoms consisted of mild leaf epinasty, petiole browning and midvein browning of citron.

Viroids had negative effect on the height of all infected plants compared to the controls.

Indexing *in vitro*

Etrog seedlings twelve days after inoculation showed symptoms of the disease, such as leaf epinasty. All inoculated Etrog citron plantlets grown *in vitro* and tested by RT-PCR showed the same viroid content present in the source plants. Inoculated Etrog seedlings tested positive to RT-PCR 42 days after inoculation. Etrog cuttings inoculated by grafting tested positive to RT-PCR 50 days after inoculation and Etrog cuttings inoculated by injection tested positive 120 days after inoculation.

Main Conclusions

1. The *in vitro* indexing method for citrus viroids on citron was quicker than the *in vivo* method
2. Indexing *in vitro* of seedlings was quicker (12-20 days) than indexing on cuttings. (Disadvantage was the lack of adequate quantity of seeds and the longer time required for the preparation of micro plants).
3. Indexing on cuttings *in vitro* by grafting was easier and more reliable than indexing either on seedlings or on cuttings by injection.
4. Indexing of cuttings by injection was more time consuming than either one of the other two *in vitro* methods.

Recommendations

1. The *in vitro* indexing method for diagnosis of viroids in citrus could replace the classical *in vivo* indexing method
2. The new method will meet the needs of my target audience through the evaluation of the technique for the research programmes of the Agricultural Research Institute, adaptation of the method and furthermore its use by my colleagues. In addition, I will aim at publications in scientific journals, presentations oral and written in local and/or international Conferences and my ultimate goal would be the evaluation of the technique by other expert scientists.