Improvement of biological indexing technique for citrus viruses detection

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ABSTRACT

Psorosis and concave are citrus diseases of undemonstrated etiology that can be diagnosed by biological indexing. Its presumed causal agent is Citrus psorosis virus (CPsV) type species of the genus Ophiovirus. In this study improvement of a biological indexing assay for the detection of Citrus psorosis virus in citrus trees using rooted inoculated cuttings of indicator plants (Madam vinous, Dweettangor and Naveilina Orange) was achieved. Compared the results of detection of CPsV by traditional indexing using indicator seedlings and rooted inoculated cuttings of indictor plants were confirmed by (DAS-ELISA) and correlated with observation of symptoms thought to be specific, in field trees or in graft-inoculated indicator plants. The results showed that, improved technique of biological indexing using rooted inoculated cuttings of indicator plants was faster than the traditional method in addition sensitive and more consistent than ELISA test. RT-PCR technique was used to recognize Egyptian isolate of CPsV and would be adaptable for large scale application. GA3, ABA and IBA content in cuttings is remarkably higher than seedlings, while IAA content in leaves of cuttings was considerably lower than in the seedlings. The detection protocol described in this study could be used in citrus certification programs and to test trees in nurseries and commercial orchards for CPsV infection.

Keywords: Psorosis CPsV, detection, Biological indexing, ELISA, Rooted inoculated cuttings.

INTRODUCTION

Citrus psorosis is a damaging disease caused by Citrus psorosis virus (CPsV). It induces typical bark scaling lesions on sweet orange, mandarin and grapefruit trunks and limbs, and occasionally ringspot symptoms on their leaves and fruit. Wood staining often accompanies bark scaling on infected branches and trunks. Sour orange, lemon, pummelo and rough lemon usually show no external bark symptoms **(Roistacher, 1991).** Based on symptom expression, two types of psorosis were proposed by **Fawcett and Klotz (1939),** psorosis-A and psorosis-B**. Wallace (1957)** showed that psorosis-A protects against a challenge from the more severe bark lesions produced by psorosis-B. The disease is widespread in many parts of the world, including South America and the Mediterranean areas **(Roistacher, 1993.** Field diagnosis of the disease was practiced and trees with bark lesion symptoms were eliminated. This probably contributed to the limited spread of the disease in Tucumán **(Fawcett and Klotz, 1939).** The causal agent of the disease is Citrus psorosis virus (CPsV), a type member of Ophiovirus genus with a genome of three single-stranded RNAs of negative polarity **(Milne *et al.*, 2000).** Several methods are available for CPsV detection (**Martín *et al.*, 2004).** Biological indexing is undertaken by graft-inoculating citrus indicator plants, and then testing for cross protection with a severe isolate **(Roistacher & Calavan, 1965 and Roistacher, 1991 and 1993**). DAS-ELISA **(García *et al.*, 1997),**

TAS-ELISA-AP (AP, alkaline phosphatase) **(Alioto *et al.*,1999)** and TAS-ELISA-HP (HP, horseradish peroxidase) are methods developed and applied for diagnosis in field trees. Several primers have been designed for CPsV detection by RT-PCR, thus providing alternative methods for diagnosis. Although Concave Gum induces young leaf symptoms in the same indicator plants, this disease cannot be grouped with psorosis-A **(Roistacher, 1981).** This disease produce oak leaf patterns in leaves of both field trees and indicator plants, but they rarely induce shock symptoms in indicator plants. The concave gum pathogen will not protect against a challenge from psorosis -B lesion inoculum **(Roistacher and Calavan, 1965)** and does not produce scaly bark, but induces other trunk or fruit symptoms distinct from those of psorosis. In addition, concave gum, impietratura, and cristacortis isolates do not contain a 48 kd protein commonly associated with psorosis. Moreover, ringspot isolates **(da Graça *et al.*, 1991, 1993; Roistacher, 1993**) and tissue of concave gum infected trees do not react with psorosis-A antiserum **(D'Onghia *et al.*, 1998).** A new technique of biological indexing used of indicator cuttings instead of seedlings was developed for the detection of the main citrus virus and viroids **(ElBacki *et al.*, 2005; El Sayed, 2005 and D'Onghia *et al.*, 2009).**

The objective of this study is improvement of a biological indexing assay for the detection of CITRUS psorosis virus in citrus trees using rooted inoculated cuttings of indicator plants (Madam vinous, Dweettangor and Naveilina Orange).

MATERIALS AND METHODS

Psorosis and concave gum sources were collected from symptomatic field trees of citrus orchards in Qualubia governorate (Moshtohor) (fig.1). The collected samples were biologically indexed as described by **Roistacher (Roistacher, 1991 and Roistacher *et al.*, 2000)** on indicator seedlings of Madam Vinous, Dweettangor and Navelina Orange budded on Rough lemon and kept under greenhouse conditions

Figure 1. Bark scaling.

Figure 2. Bark scaling and gum

Figure 3. Gum in a transversal trunk section.

Figure 4. Atypical bark scaling.

1- Rooted cutting

About 50 fresh, semi-hard wood and hard wood cuttings/each indicator with 4-6 nodes were inoculated by chip budding using the bark tissue collected from the CPSV and Concave Gum source (Figure1). During grafting cuttings were always maintained with the basal part in the water. Grafts were sealed with parafilm and the inoculated cuttings were, dipped in the IBA solution (500 ppm) and placed in the peat moss and perlite media inside a plastic bag. A negative control was also included in the trial using ten cuttings/indicator which were chip budded with healthy tissue. Inoculated cuttings were grown in the indexing greenhouse at warm temperatures (34-36°C). Four days after inoculation, the plastic bag was opened from the top and 10 days later completely removed for acclimatization. Inoculated cuttings were examined after 10 days for grafts viability and after 2 weeks for rooting. Symptom observations were routinely carried out since shoots flushing. Plants to be further investigated (i.e. bark crokcing symptoms) were maintained longer (more than 3 months) in the same pots. After the 1st month of growth, organic amendant for macro and microelements was weekly foliar sprayed every 2 weeks. Symptoms were observed after 3 weeks for CPSV and Concave Gum. Nevertheless, most of the symptoms developed after 25-30 days.

2- Indicator seedling

Seedlings of Madam vinous, Navelina sweet orange, Dweettangor and budded on rough lemon were used as indicator plants for characterization. For each tested isolate, four indicator plants were inoculated by grafting three blind buds. Non-inoculated plants were the negative controls. All grafted plants were kept under cool temperatures (24ºC to 27ºC maximum during the daytime and 18ºC to 21ºC minimum in the night).

3- DAS-ELISA assay

DAS-ELISA assay was used to confirm the results of biological indexing, a commercial kit were used for the detection of CPsV and Concave gum (Agritest SRL. Italy). Plates were coated with antibodies in coating buffer, diluted 1:1000 and incubated for 2 h. at 37°C, then washed 3 times using PBS Tween at 3 min. intervals and dried by blotting on paper. Samples were extracted at 1/10 concentration with extraction buffer, using cortical scraping, 200 µl of the extract was added per each well of the plate, then incubated overnight at 4°C. Plates were washed 3 times, and then incubated 2 h. at 37°C with labeled antibodies with alkaline phosphatase. Washing was repeated, then a substrate (P-nitrophenyl phosphate) in a substrate buffer freshly prepared (1 mg/ml) in a substrate buffer freshly prepared were added and incubated at room temperature, till yellow color developed. Absorbance was read 1 h. and 2 h. using an automatic plate reader at 405 nm with a reader **(Djelouah*et al.*, 2000).**

4- RT-PCR

RT-PCR was used on all indexed bud chips from the first flushes of indicator trees. Total RNA was extracted from 100 mg of symptomatic or healthy citrus leaf tissue using Qiagen mini plant RNeasy kit according to manufacturer's instructions (Qiagen). DNA complementary to specific region (434 bp) from nt 766 to 1200 of CP gene of CPsV was amplified using primers Cons1(5_ACAAAGAAATTCCCTGCAA GGG-3_) and Cons2 (5_AAGTTTCTATCATTCTGA-AACCC-3_) which were designed based on nucleotide sequences of CP gene of Florida isolate CPsV-6. For preparing cDNA standard method was followed using forward Cons1 and reverse Cons2 primers in 20 µl of a reaction mixture (RNeasy kits). The cDNA (1 µl) was added to 24 µl of a PCR mixture containing 1× PCR buffer, 3 mMMgCI2

200 µMdNTPs, 1 µl of each primer (10 µM) and 1 Uof Taq polymerase. Amplification was performed at 94 °C for 1 min followed by 40 cycles of: 94 °C for 15 s,54 °C for 25 s, 72 °C for 1 min, with a final extension of 72°C for 5 min. PCR products were run on 1.5 % agarose gels, stained with ethidium bromide and viewed over a UV light source.

1- Determination of plant hormones

Plant hormones were determined quantitatively by using the HPLC "high – performance liquid chromatography" according to the method of **Koshioka, *et al.*, (1983),** at central lab. Of *Horticulture Research Institute* (HRI), Agricultural Research Centre (*ARC*), *Egypt*.

RESULTS

Symptoms expression on rooted inoculated cuttings of the indicator plants and traditional indexing on the indicator seedlings used in this experiment recorded in the tables (1&2) including Chock, leaf drops and Oak leaf patterns. All rootstock citrus plants (Dweettangor, Madam Vinous and Navelina) which grafted on Rough lemon as rootstock were indexed by graft inoculation with two blind buds from CPsV-and Concave Gum infected citrus plants. The rootstock tested were differed in response to CPsV-and Concave gum isolate where Dweettangor appeared low response whereas gave oak leaf pattern symptoms while Madam Vinous appeared hyper sensitivity whereas gave shock symptoms while Navelina appeared higher response whereas gave oak leaf pattern and shock symptoms. Control inoculations with tissue no showing symptoms gave negative results, none of the tested plants showed systemic symptoms. These tested plants gave positive results were tested serologically by DAS-ELISA against specific CPsV and Concave gum antiserum.

Table 1.Symptoms and incubation period of CPsV on inoculated on rooted cuttings and seedlings.

Inoculated cuttings	Rooting		Seedlings		DAS-ELISA
	Symptoms	Incubation period (days)	Symptoms	Incubation period (days)	
Madam Vinous	**Chock**	**28**	**Chock**	**35**	+
Dweet tangor	**Oak leaf (OLP)**	**25**	**Oak leaf**	**32**	+
Navelina orange	**Drops leaf**	**25 days**	**Drops leaf**	**35**	+

Table 2. Symptoms and incubation period of Concave Gum virus on rooted inoculated cuttings and seedlings.

Inoculated cuttings	Rooting		Seedlings		DAS-ELISA
	Symptoms	Incubation period (days)	Symptoms	Incubation period (days)	
Madam Vinous	**OLP**	**22**	**OLP**	**35**	+
Dweettangor	**OLP**	**20**	**OLP**	**33**	+
Navelina orange	**OLP**	**18**	**OLP**	**35**	+

Figure 5. CPsV chock symptoms in Madam vinous and Navelina orange

Figure 6.CGV symptoms Oak leaf patterns in Dweet tangor and Navelina orange

Figure 7. Oak leaf pattern in Navelina Orange.

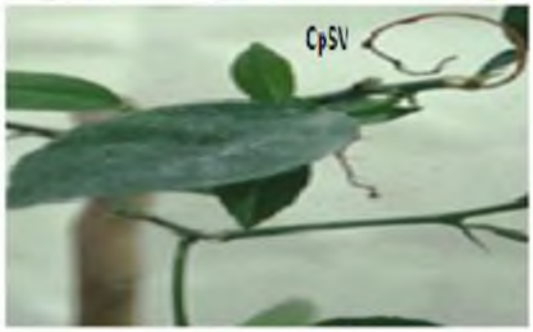

Figure 8. Shock reaction in Madam vinous.

Results obtained verify prior findings. The importance of temperature for symptom expressions especially for shock reaction. Cool temperatures preferred the appearance of shock symptoms in young emerging shoots (Rooted inoculated cuttings). Psorosis induced shock reaction in Madam Vinous, Navelina sweet orange grafted on Rough lemon under cool conditions, whereas concave gum induced none of these symptoms, but induced Oak leaf patterns

RT-PCR appears to recognize Egyptian isolate of CPSV and would be adaptable for large scale application. This procedure used in present study is very simple, quick and reliable for the detection of citrus psorosis virus infection.

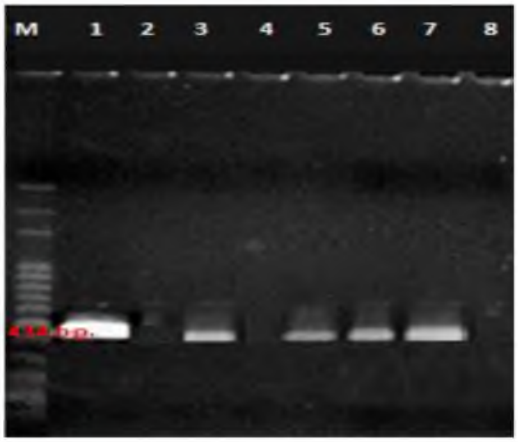

Fig. (9). 1.5% Agarose gel electrophoresis analysis of RT-PCR showing the amplified fragment of (CpSV) isolate from infected citrus trees. The amplified of the coat protein coding region. The arrow indicated a specific CpSVcDNA band 434 bp by using specific CpSV primers. Lanes 1, 3, 5, 6 and 7 gave positive results. While lanes 2, 4 and 8 gave negative results and Lane M is the v c 100bp. plus DNA size marker. The size of the cDNA fragment is indicated on the presence of CpSV at 434 bp.

Plant hormones: (IAA, ABA, GA3) contents in the leaves of rooted inoculated cuttings and seedlings were measured to elucidate the relationship between endogenous plant hormones and buds differentiation. GA3, ABA and IBA content in cuttings is remarkably higher than seedlings, while IAA content in leaves of cuttings was considerably lower than in the seedlings. In the experiment of, sprouting of seedling buds was delayed by IAA, while, GA3, ABA, and IBA enhanced it in cuttings. In summary $GA_{1/3}$ content in the leaves cuttings was higher than in seedlings. These data suggested that, $GA_{1/3}$ increase of leafless inflorescence and enhancement of the ABA content and of the IAA content ringing suggested that endogenous IAA and ABA influence the pattern of inflorescence. These hormones might affect bud's development.

Table 3. Plant hormones contents in the leaves of rooted inoculated cuttings and seedlings.

Hormone	Rooted cuttings	Seedlings
GA3	24.57µg/ 1g	22.40µg/ 1g
Abscisic (sa) 4	3.84µg/ 1g	0.254µg/ 1g
3Indol acetic acid	0.10µg/ 1g	2.40µg/ 1g
3Indol butyric acid	0.09µg/ 1g	0.08µg/ 1g

DISCUSSION

Results of biological indexing by cuttings were confirmed by ELISA using plant tissue from symptomatic and symptomless indicator plants. All inoculated indicator stem cuttings were CpSV and CGV ELISA-positive; these results were agreement with **(ElBacki *et al.*, 2005; El Sayed, 2005 and D'Onghia *et al.*, 2009)** they found that, A new system of biological indexing based on the use of indicator cuttings instead of seedlings was developed for the detection of the main citrus virus and viroids.

Considering the important factors for rooting ability of stem cuttings as the juvenility stage of the plant source and the seasonal timing of cutting harvesting **(Bhusal *et al.*, 2001).**

After 5 weeks of growth from inoculation clear symptoms were observed on the new emerging shoots of indicators. Symptoms observed were shock, leaf crinkle, and mottle, oak leaf pattern (OLP). Results of biological indexing were confirmed by ELISA, these results were agreement with **(D'Onghia and *et al.*, 2009).** Cool temperatures preferred the appearance of shock symptoms in young emerging shoots (Rooted inoculated cuttings). Psorosis induced shock reaction in Madam Vinous, Navelina sweet orange grafted on Rough lemon under cool conditions, whereas concave gum induced none of these symptoms, but induced Oak leaf patterns these results were in harmony to those obtained by **(Figueroa *et al.*, 2009).** GA3, ABA and IBA content in cuttings is remarkably higher than seedlings, while IAA content in leaves of cuttings was considerably lower than in the seedlings, this results supported by **Pennazio and Roggero (1996)** they indicated that, auxin activity was reduced in diseased viruses plants.

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