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Research Article

Identification and Detection of *Citrus* Psorosis Virus (CPsV) In *Citrus* Varieties And Rootstocks Cultivated In Mitidja Region, Algeria

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Abstract
Psorosis is one of major graft-transmissible diseases of *Citrus*; the virus was reported to occur in different *Citrus* cultivated areas and constitutes a serious threat to *Citrus* establishment and development. This study focuses on the identification of *Citrus* Psorosis Virus (CPsV) by tow diagnostic methods; biological indexing in indicator seedling and serological test (DAS-ELISA). Thereby, different varieties of sweet orange (*Citrus sinensis* (L.) *Osbeck*), mandarin (*Citrus* × *Deliciosa*), lemon (*Citrus* × *limon*), Grapefruits (*Citrus* × *Paradisi*), clementine (*Citrus* × *Clementine*) and tow rootstocks sour orange (*Citrus* × *aurantium* L.) and trifoliate orange (*Poncirus trifoliata)* that exhibit typical symptoms of Psorosis disease have been detected and collected from five different orchards located in the regions of Mitidja (Algeria). All collected samples were subjected to biological testing and double antibody sandwich ELISA (DAS-ELISA) as diagnostic tests. The serological method, specifically DAS-ELISA, yielded positive results on varieties from the prospect station, indicating the presence of psorosis in Algeria. The typical symptoms of psorosis were observed through the indexed seedlings and grafts.

Keywords: *Citrus*, CPsV, *Ophiovirus*, indexing, DAS-ELISA.

Introduction

Psorosis is a widespread graft-transmissible viral disease of *Citrus* [1]. The disease was the first to be diagnosed by the use seedling index [2]. Psorosis was reported in Florida in 1896 and distribution documented [3]. In fact, the disease presence has been reported in the Americas, Africa (including the Mediterranean basin) and probably in Asia [4, 5]. Moreover, disease seems disseminated around the world through the distribution of infected *Citrus* species and varieties [1, 6, 7].

Citrus Psorosis Virus (CPsV), the causal agent of psorosis disease [8], which belongs to the genus *Ophiovirus* [9] within the family *Aspiviridae* [10], is a virus tripartite and its genome is consisting of three single-stranded RNA segments of negative sense [11]. Based on differences in their biological behavior, several strains of this virus have been identified [1]. The disease infects most economically important *Citrus* varieties, including sweet orange, mandarin and grapefruits. Usually, the symptoms appear on 10-15 years-old trees, which include bark scaling of the trunk, chlorotic

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flecks and spots on young leaves. Gum may accumulate below the bark scales and may impregnate the xylem producing wood staining and vessel occlusion. All these symptoms are used for field diagnosis of psorosis [4, 12]. Typically, infected trees are not killed by the virus but induces slow decline that may result in unproductiveness [4]. The potential economic losses caused by CPsV to *Citrus* producers have been assessed in different *Citrus*-producing regions. In Argentina and Uruguay, psorosis is a serious disease causing annual losses of about 5% of trees [13] and the disease is still present as recently reported by Zanek et *al.* [14]. Another earlier investigation was conducted in a Valencia orange orchard located in California. It has been shown that the estimated reduction in yield, on producing trees, is 35% [15].

Many authors such as D'Onghia and *al.* [16], Potere et *al.* [17], Alioto and *al.* [18], and Djelouah et *al.* [19] detected CPsV using Enzyme-linked Immunosorbent assay (ELISA) method. This technique of detection showed that results were positively correlated with data from biological indexing for a selection of CPsV isolates worldwide. Until recently, the only effective method for diagnosis was biological indexing on differential indicators and is still considered quick and inexpensive method [4].

In Algeria *Citrus* are crops of major importance of agriculture economy were the Mitidja plain represents the largest surface area with 16970 ha or 30% or the Algerian *Citrus* growing area with a yield of 23.4 tonne/ha [20]. However, these yields have been subject to various phytosanitary constraints for many years due to destructive diseases caused by viruses, which have accelerated the decline of orchards both quantitatively and qualitatively. Amongst plant viruses, those transmitted by grafting cause damages of economic importance to *Citrus* industry [21].

The *Citrus* psorosis was first reported in Algeria by different investigations such as; Brichet [22], Lamour [23], Blonde [24], Frezal [25], Chapot [26] and Bové [27]. These authors indicated that the diseased trees were generally sweet orange trees and clementine trees over 15 years old and showing serious symptoms of peeling bark in the varieties Valencia late, Thomson navel and Clementine.

The main objectives of this investigation were to study the effect of the rootstocks on *Citrus* psorosis virus using serological detection (DAS-ELISA) and bio indexing and to determine the occurrence of the disease in different *Citrus* varieties.

Materials and methods

Samples collection

Thirty varieties of citrus and two rootstocks (Sour orange (*Citrus aurantium*) and Trifoliate orange (*Poncirus trifoliata*) distributed across six sites in the Mitija region were included in this survey (Figure 1). A total of 467 trees were sampled between leaves and ovary *Citrus* tissue. Samples were randomly collected from symptomatic trees; along the periphery of square areas [28]. The collected samples were stored in sterile labeled bags and transmitted to laboratory within 24 hours.

Serological test (DAS-ELISA)

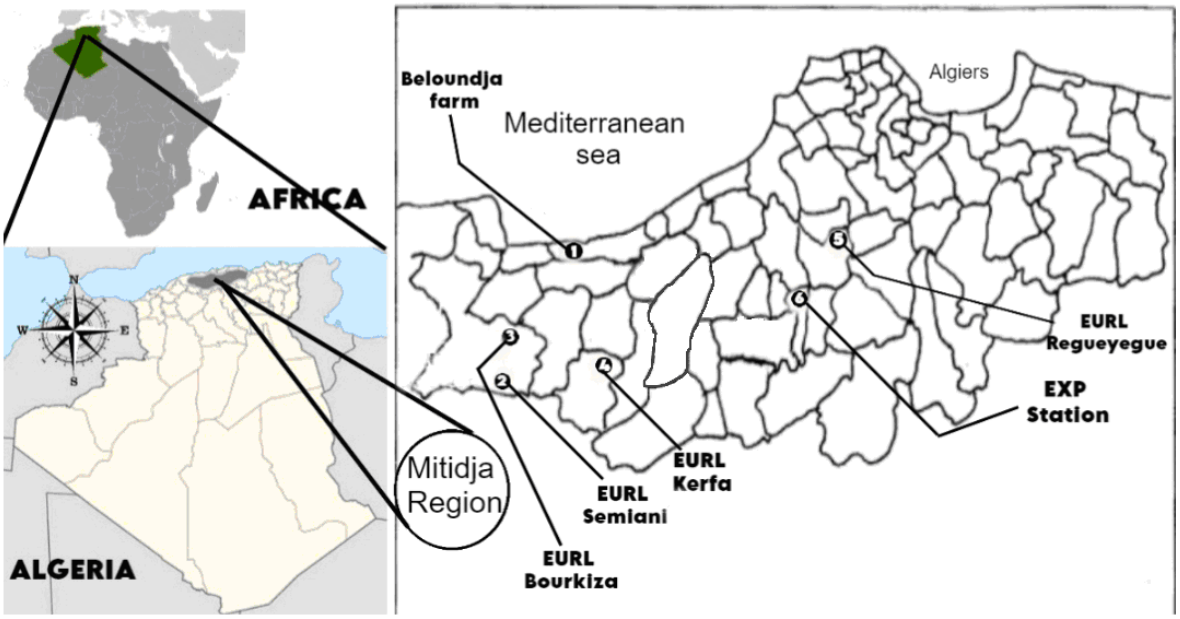
For the serological detection an antiserum produced at the laboratory (AGRITEST SRL, Valenzano, Italy) was employed, based on double antibody sandwich (DAS) methode [29] using monoclonal antibody alkaline phosphatase conjugate (hybridoma line Ps 29, DPPMA University of Bari, Italy [17,19].

ELISA Plate wells were coated with specific antibody diluted (100 µL/10 mL) in coating buffer and incubated for 2 h at 37°C. In the second step, the young leaves and ovary *Citrus* tissue samples were grinded and diluted (1/10, w/v) in an extraction buffer containing PBS + Tween 0.05% + PVP 2% (polyvinylpyrrolidone) + BSA 2%; then dispense at 200 µL per well and incubated overnight at 4°C. In the third step, specific antibody conjugate alkaline phosphatase diluted (100 µL/10mL) in conjugate buffer was added and incubated for 2 h at 37°C. The diluted substrate of paranitrophenyl phosphate (2 pellets of 5 mg of PNPP in 10 mL of substrate buffer) was then added and incubate at room temperature. The reading of results by the ELISA reader at 405 nm was made from 60 minute to 120 minute after adding the substrate and optical density were measured at the end.

Biological indexing

120 bud sticks were randomly collected from separate area of thirty *Citrus* varieties and two *Citrus* rootstocks without considering the presence or absence of symptoms and were used for indexing by the method of Roistacher [1]. Indexing of psorosis was done at indicator seedlings of Hamlin [30, 31]. A total of eighty tow indicator seedlings were graft-inoculated from each tree. Tow bark patch from each bud stick were inoculated on each of three to five 0.5 cm diameter indicator seedlings. Grafts were wrapped with plastic tapes for two week following inoculation. Seedlings were immediately cut back to 20 to 30 cm above the graft in order to force new growth. All new growth flushes were

examined for Symptoms (flecking, spots, shock and death) from 6 month post-inoculation. Two plants were inoculated with positive control from provided from the virology laboratory of Algerian institute for fruits (ITAFV Boufarik). Watering was carried out 2 to 3 times/week and regularly fertilized by an Agrispon deficiency corrector (1mL/L) during the indexing period to maintain vigorous growth and avoid possible problems of deficiency. Plants were grown in a greenhouse at 18-25°C and inspected for symptoms, after incubation period, the plants were serologically tested [19].



Site	Varieties	Surface area(Ha)
1	Hamlin	5
2	Lemon	3
	Double fine	6.2
	Washington navel	2.28
	Valencia late	3.5
	Clementine	5.4
	Sour orange (*Citrus aurantium*)	Planted isolated from the orchard
	Trifoliate orange (*Poncirus trifoliata)*	Planted isolated from the orchard
3	Blood orange	6
	Portugaise	4
	Double fine	5.8
	Sour orange (*Citrus aurantium*)	planted isolated from the orchard
4	Thomson	4.2
5	Tarocco	5
	Satsuma	1
	Grapefruit	3
	Salustiana	2,5
6	Thomson	1,25
	Lemon	0.7

Figure 1. Context map showing the survey locations in of Algeria

Results and discussion

DAS-ELISA

Serological tests realized on the leaves samples (Table 1) revealed that CPsV occurrence varied among locations between 16% to 100%. CPsV where detected in 61.64% of tested trees. The highest infection in trees grafted with sour orange was detected in Double fine, Salustiana and Valencia late (100%), followed by thomson 76.92% and Hamlin 74.19%. Among sweet orange cultivars: Washington navel, Portugaise, blood orange and Tarocco showed 72.22%,

56.52%, 52.94% and 44.83% of infection, respectively. However we have register 70% for the cultivar grapefruit, 63.64% in lemon and obtained the lower infection in Satsuma with 40%
For trees grafted with *Poncirus trifoliate*, within the intervarietal ranking, sweet orange cultivar Valencia late was the most frequently infected (90%) followed by double fine 68.18%, Thomson 55.56%, and lemon 33.33%. The lowest infection occurrence was recorded for Clementine with 16.13%.
On the other hand in trees sour orange the proportion of infected plants were much higher (70%) in rootstocks trifoliate orange compared with sour orange trees 20%, 45%, respectively.

Table1. Results of ELISA test for the detection of CPsV at leaves of different *Citrus* varieties

***Citrus* species**	**Rootstocks-cultivar**	**Origine**	**Trees**			**Symptom Type***
			Tested	**Infected**	**%**	
Sweet orange	sour orange-Hamlin	EURL Beloundja	31	23	74.19	1, 2, 3
Sweet orange	Sour orange-Valencia late	EURL Semiani	17	17	100.00	1, 2
Sweet orange	Sour orange-Double fine	EURL Semiani	9	9	100.00	3
Lemon	Sour orange-Lemon	EURL Semiani	11	7	63.64	1
Sweet orange	Sour orange-Washington navel	EURL Semiani	18	13	72.22	3
Mandarin	Sour orange-Satsuma	EURL Regueyegue	5	2	40.00	1, 6, 7
Sweet orange	Sour orange-Salustiana	EURL Regueyegue	10	10	100.00	1, 5
Sweet orange	Sour orange-Tarocco	EURL Regueyegue	29	13	44.83	5, 7
Grapefruits	Sour orange-Grapefruit	EURL Regueyegue	10	7	70.00	1, 5
Sweet orange	Sour orange-Portuguaise	EURL Bourkiza	23	13	56.52	1, 3, 5
Sweet orange	Sour orange-Blood orange	EURL Bourkiza	34	18	52.94	1, 3, 5
Sweet orange	Sour orange-Double fine	EURL Bourkiza	27	27	100.00	1, 3
Sweet orange	Sour orange-Thomson	EURL Kerfa	13	10	76.92	1, 2, 5, 7
Celementine	Trifoliate orange-clementine	EURL Semiani	31	5	16.13	1
Sweet orange	Trifoliate orange-Valencia late	EURL Semiani	10	9	90.00	1
Sweet orange	Trifoliate orange-Double fine	EURL Semiani	22	15	68.18	1
Sweet orange	Trifoliate orange-Lemon	Exp station	6	2	33.33	1
Sweet orange	Trifoliate orange-Thomson	Exp station	9	5	55.56	1, 5
Sour orange (*Citrus aurantium)*	Sour orange	EURL Bourkiza	20	4	20.00	2, 5
Sour orange (*Citrus aurantium*)	Sour orange	EURL Semiani	20	9	45.00	2
Trifoliate orange (*Poncirus trifoliata*)	Trifoliate orange	EURL Semiani	10	7	70.00	2
Total			**365**	**225**	**61.64**	

*Symptom type: 1, flecks and spots on young leaves; 2, embossing on old leaves; 3, interlineal flecking; 4, narrowing of the leaf surface; 5, bark scaling of the trunk and accumulated gum; 6, green spot in fruits 7: oak leaf pattern.

The serological test carried on *Citrus* flowers ovary samples collected in different prospect sites (Table 2) revealed the presence of CPsV in the studies variety. From 102 tested samples 55 were positively at DAS-ELISA (occurrence of 53.92%).
The presence of symptoms of CPsV at the different prospect fields of *Citrus* in Mitidja were confirmed by DAS-ELISA. The results confirms the presence of the virus in the tested leaves samples of different *Citrus* varieties and rootstocks (sour orange and trifoliate orange); probably due to cultural practices and use of infected propagating materiel (grafts or rootstocks) [32]. The lack of prophylactic procedures during grafting constitutes a major concern in the multiplication of *Citrus* varieties and contributes to the spread of different disease, particularly *Citrus* psorosis virus. Our findings also demonstrates that the varieties affected by CPsV have propagated by infected rootstocks (sour

orange and trifoliate orange). Our results agree with those obtained by Bové [33] and Bové and *al.* [34] where positives responses were registered in the diagnosis of the disease of psorosis in the Mediterranean basin. The results obtained with DAS-ELISA on flower samples confirmed the presence of CPsV in flowers of different varieties, highlighting the systemic infection process of psorosis. These results are consistent with the findings of Cambra [35] and Garcia *et al.* [36].

Table 2. Results of ELISA test for the detection of CPsV at flowers ovary different *Citrus* varieties

***Citrus* species**	**Rootstocks-cultivars**	**Origine**	**Trees**		
			Tested	**Infected**	%
Sweet orange	sour orange-Portuguaise	EURL Bourkiza	10	5	50
Sweet orange	sour orange-Double fine	EURL Bourkiza	5	2	40
Sweet orange	sour orange-Double fine	EURL Semiani	5	2	40
Sweet orange	sour orange-Valencia late	EURL Semiani	6	3	50
Sweet orange	sour orange-Thomson	EURL Kerfa	51	30	58.82
Clementine	Poncirus Trifoliata-Clementine	EURL Semiani	5	3	60
Sweet orange	Poncirus Trifoliata-Double fine	EURL Semiani	9	7	77.78
Sweet orange	Poncirus Trifoliata-Thomson	Exp station	11	3	27.27
Total			**102**	**55**	**53.92**

Biological indexing

After six month of inoculation, observation of potential symptoms on the leaves of the emerging young flushes as growth indicator seedlings, revealed a typical psorosis symptoms on almost all tested cultivars (Table 3). The symptoms were flecks and spots on young leaves and narrowing of the leaf surface, on the hamlin cultivar with interlineal flecking only between secondary leaf vein and leaf chlorosis (Figure 2).

On satsuma plants the appearance of leaf yellowing followed with narrowing of the leaf surface were observed. The symptoms of leaf chlorosis and slight leaf embossing were registered on the seedling inoculated with tarocco and salustiana cultivar. However, the symptoms observed on double fine were severe; these symptoms are discoloration between leaf vein also in principal leaf and narrowing of the leaf surface. Important symptoms of slightly rounded spot were noticed in seedlings Inoculate with candidate washington navel and other varieties (portugaise, lemon, sour orange clementine, blood orange, valencia late, grapefruit and trifoliate orange) (Figure 3). The symptoms revealed were small light spots and leaf narrowing of the leaf surface. The plants inoculated with positive control were externalizing a typical psorosis such as flecks and spots on young leaves and narrowing of the leaf surface.

The serological test carried out on inoculated seedlings revealed the presence of CPsV on 42 samples of *Citrus* leaves corresponding to an occurrence of 52.5% (Table 3). The percentage values varied from 20% to 100%, all the trees that indexed positive were also ELISA positive. From the 82 *Citrus* indicators indexed 34 (41.46%) induced symptoms. Cultivar grafted with sour orange, which are lemon, satsuma, washington navel, salustiana, tarocco, grapefruit, portugaise and blood orange that indexed negative, gave a positive ELISA reaction. However in seedling grafted with *Poncirus trifoliate*, we registered one of indicator that indexed negative, exhibited a positive DAS-ELISA for each variety namely Clementine and Thomson and for sour orange and trifoliate orange species.

In the indexing on indicator plant (hamlin) we found that the disease has developed on the large number of plants indexed. Indeed, the plants expressed characteristic symptoms of *Citrus* psorosis virus. Our results agree with those obtained by Roistacher [1]. This development of symptoms indicates the good receptivity of these plants to the virus resulting from the installation of the pathogen, its development and the expression of pathogenesis. All the symptoms observed on the indexed plants are typical and characteristic of psorosis disease and are very similar to those observed on varieties and rootstocks of prospected sites. The very high correlation of positive DAS-ELISA results with indexing demonstrates the feasibility of using serological assays for low-cost diagnosis of psorosis disease. Moreover, since ELISA-positive samples may indexed negative on indicators, suggest that

ELISA can be more sensitive than indexing for detecting CPsV.

Table 3. Comparative result of CPsV detection using DAS ELISA and bioindexing

Citrus species	Rootstocks-cultivars	Origine	Diagnostic tests				
			Bioindixing	symptom type*	DAS - ELISA	positif result	%
Sweet orange	sour orange-Hamlin	EURL Beloundja	5	3	5	5	100
Sweet orange	sour orange-Double fine	EURL Bourkiza	2	3, 4	5	2	40
Lemon	sour orange-Lemon	Exp station	1	1, 4	5	2	40
Sweet orange	sour orange-Washington navel	EURL Semiani	3	1	5	4	80
Satsuma	sour orange-Satsuma	EURL Regueyegue	1	4	5	2	40
Sweet orange	sour orange-Salustiana	EURL Regueyegue	4	1, 2	5	5	100
Sweet orange	sour orange-Tarocco	EURL Regueyegue	3	2, 1	5	4	80
Grapefruits	sour orange-Grapefruit	EURL Regueyegue	2	1	5	3	60
Sweet orange	sour orange-Portuguaise	EURL Bourkiza	1	1, 4	5	2	40
Sweet orange	sour orange-Blood orange	EURL Bourkiza	1	1, 4	5	2	40
Clementine	Trifoliate orange-Clementine	EURL Semiani	1	1, 3, 4	5	1	20
Sweet orange	Trifoliate orange-Valencia late	EURL Semiani	2	1, 4	5	2	40
Sweet orange	Trifoliate orange-Double fine	EURL Semiani	2	1, 4	5	2	40
Sweet orange	Trifoliate orange-Thomson	EURL kerfa	1	1, 4	5	2	40
Sour orange	Sour orange	EURL Semiani	2	1, 2, 4	5	2	40
Trofoliate orange	Trifoliate orange	EURL Semiani	1	1, 4	5	2	40
Total			**32**		**80**	**42**	**52.5**

*Symptom type; 1, flecks and spots on young leaves; 2, embossing on old leaves; 3, interlineal flecking; 4, narrowing of the leaf surface.

a **b**

Figure 2. Healthy leaf (a); interlineal flecking between secondary leaf vein (inoculated by hamlin) (b)

a b c

Figure 3. Healthy leaf (a); flecks and spots on young leaves and narrowing of the leaf surface on plant inoculated with sour orange (b & c),

Our study permitted to report the presence of *Citrus* psorosis virus disease on thomson, valencia late and clementine varieties in Algeria as previously demonstrated [22-27]. Interestingly, the CPsV were also identified on other varieties namely; lemon, washington navel, double fine, portugaise, hamlin, satsuma, salustiana, blood orange, grapefruit, tarocco, and tow rootstocks; sour orange (*Citrus* × *aurantium*) and trifoliate orange (*Poncirus trifoliata*).

Conclusions

This work is part of an ongoing program in Algeria investigating citrus pathogens, including *Citrus exocortis* viroid (CEVd) [37] and Citrus psorosis virus (CPsV). This study confirms the presence of CPsV in various citrus varieties (e.g., Hamlin, Washington Navel, Salustiana, Valencia Late, Thomson Clementine) and rootstocks (sour orange, trifoliate orange). Psorosis is a globally devastating disease of *Citrus* caused by an infectious filamentous *Ophiovirus*, *Citrus* psorosis virus (CPsV), which causes annual losses and a progressive decline of trees by affecting the conductive tissues.

The Control of sanitary procedures in propagating materiel is crucial since psorosis dispersal in most *Citrus* areas occurs only by the use of infected bud wood. The simplest way to control this disease is the deployment of *in vitro* shoot-tip grafting in combination with thermotherapy or somatic embryogenesis from stigma and style cultures, which already has proved effective for eliminating CPsV from plant propagating material. *Citrus* culture in Algeria faces many constraints, especially a lack of studies on *Citrus* graft-transmissible disease and their distribution in the country.

Ours results underline the importance of a launching bud wood certification and rigorous indexing programs at nursery level and establishing a quarantine system for the introduction of new varieties in Algerian *Citrus* orchard. In fact, the use of psorosis-free certified buds in new plantings would need supplementary measures like vector control once the vector species will be unequivocally identified, or in the long term, the use of resistant varieties.

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