Epiphytic Population Dynamics of Olive Knot Pathogenic Bacterium, *Pseudomonas savastanoi pv. Savastanoi*

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ABSTRACT

The dynamics of an epiphytic population of *Pseudomonas savastanoi* pv. *savastanoi*, the causal agent of olive knot disease was studied during 2015/2016 growing season at Al-Neir in Wadi Al-Sir in Jordan. The pathogen was recovered from washing leaves collected from symptomless as well as sick trees. The recovered population was found to change with fluctuations in air temperature and relative humidity. The higher epiphytic population was recovered during spring and fall seasons, while the lower one was recovered during summer and winter seasons. The highest recovered population, which was obtained during the first week of June, coincided with an average temperature of 21.5^{o}C and relative humidity of more than 52%. During the field inspection, newly formed knots appeared late in June, following the highest epiphytic population. In Jordan, the occurrence of this pathogen as epiphytic population and their fluctuations in relation to temperature and RH on olive leaves could bring to light a new dimension in the epidemiology of disease as well as a source of primary disease inoculum.

Keywords: Disease epidemiology, inoculum, plant-pathogenic bacteria, Olive Tuberculosis.

INTRODUCTION

Olive knot disease or olive tuberculosis (Gardan, *et al*., 1992) on olive *(Olea europaea* L.), which is caused by the bacterium *Pseudomonas savastanoi* pv. *savastanoi* (*Psv*) (Vivian and Mansfield, 1993) is characterized by tumor formation on the trunk, branches, and less frequently on the leaves of olive trees. Tumor formation on olive branches is related to the production of the phytohormones; indole-3-acetic acid (IAA) and cytokinins by the pathogenic bacterium (Smidt and Kosuge, 1978; Comai and Kosuge, 1980; Surico *et al*., 1985). It has been reported that olive knot disease is also *hrp/hrc* gene dependent (Sisto *et al*., 2004). The disease is widespread in most of the Mediterranean countries, including Jordan where olive groves have been grown for centuries. In Jordan, the disease occurs in most of the olive growing areas and their incidence varies with location and cultivar (Khlaif, 2006).

The endophytic phase of *Psv* in olive stems and the structural and ultra-structural histogenesis of olive knots had been studied in detail (Rodrı´guez-Moreno *et al*., 2009). *Pseudomonas savastanoi* pv. *savastanoi* can multiply in its saprophytic phase on olive tree phyllosphere (Ercolani, 1978; Quesada *et al*., 2007) and spread at short distances by splashing rain, wind blown aerosols, insects, and cultural practices. Infection occurs through wounds caused during harvesting, pruning, as well as by hail, frost and leaf scars (Wilson, 1935). The pathogen was isolated from symptomless olive plants (Hijazin and Khlaif, 2005), it could also move systemically with plant sap and develop secondary tumours in new wounds as has been suggested in oleander (*Nerium oleander)* (Wilson and Magie, 1964) and in olive

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(Penyalver *et al*., 2006; Marchi *et al*., 2009). The pathogen could survive inside knots from one season to another and if the humidity and their populations are high enough, exudates containing the bacteria might be produced, providing the source of inoculum (Wilson, 1935).

Epiphytic populations were studied by many researchers, especially those who deal with plant-pathogenic bacteria, ice-nucleation active bacteria, and plant growth promoting leaf-associated bacteria (Lindow *et al*., 1978; Hirano *et al*., 1982; Colin and Mc- Carter, 1983; Zagory *et al*., 1983; Knudsen *et al*., 1988; Lindow *et al*., 1988; Beattie, 2002). This phenomenon is very important in the case of plant-pathogenic bacteria. These epiphytic populations could play a role as a source of primary inoculum able to do penetration, infection, and colonization in order to promote the occurrence and development of the disease under its favorable environmental conditions.

Pseudomonas savastanoi pv. *savastanoi* can survive in the olive tree phyllosphere as epiphyte (Ercolani, 1978) and as endophytes, migrating to a certain extent along the xylem vessels (Marchi *et al*., 2009). The epiphytic *Psv* population shows seasonal fluctuations; higher populations were detected in warm and rainy months (Quesada *et al*., 2007).

In addition to knots, *Psv* has been isolated from leaves and from the surfaces of stems and olive fruits (Ercolani, 1978; Lavermicocca and Surico, 1987). However, several studies were conducted on the epiphytic population distribution and fluctuation of *Psv* on the phyllosphere (the total above-ground portions of plants as habitat for microorganisms) of olive trees (Ercolani 1978, 1985, 1991). *Psv* had a resident phase colonizing the phyllosphere of symptomless leaves, where it dominated bacterial communities throughout most of the year (Ercolani, 1978). A seasonal distribution of *Psv* populations has also been shown, reaching high levels on symptomless leaves during spring and fall (Ercolani, 1978). Recently, Lamichhane and Varvaro (2014) reported that there was no presence of olive knot pathogen on the phylloplane of 28 Italian olive cultivars introduced to Nepal although a consistent number of bacterial species were found in association with leaf.

There is a lack of information about the epiphytic population fluctuation of olive knot pathogen as affected by the ambient air temperature and RH% throughout the growing season. Therefore, this study was conducted in order to do possible isolation and quantification of *Psv* on leaves of naturally infected and symptomless olive plants throughout 2015/2016 olive growing season, and to find the relationships between *Psv* populations and seasonal climatic fluctuations.

MATERIALS AND METHODS

Experimental orchard and sampling

Olive leaf samples were collected at different intervals during the study period (Table 1) from symptomless and naturally infected olive trees of the same orchard located at Al-Neir in Wadi Al-Sir in Jordan throughout the growing seasons in 2015/2016. Naturally infected olive trees and symptomless trees were randomly selected in the orchard for sampling. Nabali Mohassan was the common olive cultivar. Fifty samples were collected fortnightly. Samples were collected from the experimental orchard; 25 samples were collected from symptomless leaves of naturally infected olive trees, 25 samples were collected from leaves of symptomless trees, and 5 samples per tree were collected from five labeled branches. These 5 samples were mixed to form composite sample for each tree, then the composite samples for the trees were mixed, forming a bulk sample for the orchard. Collectively, two bulk samples for the orchard were collected. The bulk samples were kept in plastic bags in an icebox and processed 24h afterwards.

Isolation of bacterium

Ten grams of leaves (200 to 300 leaves) from each sample were processed at each sampling date (Table 1). Leaves of each sample were placed separately in 100ml

Erlenmeyer flasks, containing 90ml sterile distilled water (SDW) and shaken for 90min at 200rpm at room temperature. The resulting suspensions were 10-fold diluted in SDW in 3 steps to 10^{-3}, and an aliquot of each dilution (0.1ml) was spread using sterilized L-shape glass rod onto the surface of BCBRVB medium plate (Autoclaved 950ml of King's B (KB) medium (containing g/L: (10 gm protease peptone, 1.5 gm K_2HPO_4. $3H_2O$, 1.5 gm $MgSO_4$. 7 H_2O, 15 gm Agar, 15 ml glycerol dissolved in 1l SDW), cooled to 45°C, then a mixture of the following antibiotics was added in 10ml 70% ethanol diluted with 40ml SDW: 10mg Bacitracin, 6mg Vancomycin, 0.5mg Rifampicin, 75mg Cycloheximide, 0.25mg Benomyl) (Schaad *et al*., 2001), then 5 plates were inoculated and incubated at 25°C. Plates were checked daily and putative *Psv* colonies were streaked on new KB plate, and then each bacterial isolate was identified and its identity was confirmed through biochemical LOPAT tests and pathogenicity tests. The resulted bacterial colonies of *Psv* were counted and expressed as colony forming units (CFU) per ml of leaf suspension, and then the average of CFU/ml for the plates was taken.

Identification of bacterium

Putative *Psv* colonies were subjected to LOPAT tests as recommended by Schaad *et al*. (2001), including: Levan production, oxidase, potato soft rot, arginine dihydrolase, hypersensitivity reaction to white burley tobacco leaves, and pathogenicity test. These tests were also carried out against the reference culture of *Psv* (CFBP1670) obtained from M Lopez IVIA Lab Valencia, Spain for comparison purposes.

Pathogenicity test

Four different twigs on two different two-year old seedlings of cv. Nabali Mohassan, i.e. two twigs/seedling, were inoculated with a bacterial mass of 24 hours old culture of the different obtained and identified bacterial isolates. There were three inoculation sites on each twig, and then the inoculated sites were wrapped with parafilm. The inoculated seedlings were kept under a mist chamber (100% RH) for 48hrs, then transferred to a greenhouse bench at 25 ± 2ºC and checked periodically for knots development.

Climatic variables

Climatic variables, namely, temperature, RH, and rainfall were recorded from an automatic weather station (Jordan meteorology, Amman, Jordan): cumulative rainfall, min, max and average of temperature, and RH were calculated from a given sampling date to the next one (Table 1). Averages of *Psv* populations per leaf suspension from each bulk sample were estimated by dilution-plating as described above.

RESULTS

Isolation of the bacterium

Pseudomonas savastanoi pv. *savastanoi* was isolated from both olive leaf suspensions of infected and symptomless olive trees. Raised, circular, smooth, glistening, and fluorescent colonies were obtained on BCBRVB medium plates, when the surface of these plates was inoculated with the suspension of leaves collected from naturally infected and symptomless plants after subjecting them to long wave UV light (367nm).

Identification of the bacterium

The putative *Psv* colonies subjected to LOPAT tests were Oxidase-negative, Levan-negative, Pectinolytic activity- negative, Arginine dihydrolase-negative, and tobacco white burley Hypersensitivity-positive. The obtained results were similar to those tests run against the reference culture of *Psv* (CFBP1670)**.**

Pathogenicity test

When a bacterial mass from a 24-hrs-old culture of *Psv* of each tested isolates was applied at the wounded sites on the twigs of two-year old olive seedlings of cv. Nabli Mohassan, typical knots developed four weeks after inoculation. The knot started at the inoculation site as a swelling of fleshy tissues. These knots were similar to those induced by the reference culture of *Psv* (CFBP1670).

***Pseudomonas savastanoi* pv. *savastanoi* population dynamics**

Epiphytic populations of *Psv* recovered during the study from symptomless leaves were significantly lower from symptomless than infected trees although the obtained populations of both suspensions were coincided with RH, temperature, and rainfall (Table 1 and Fig. 1).

The results of the recovered epiphytic populations (REP) of *Psv* are listed in Table 1. However, the obtained results showed that the epiphytic population of *Psv* was recovered from both symptomless and sick olive trees. In addition, the recovered population was found to change with the fluctuation of temperature and RH (Fig. 1).

The REP of *Psv* in October 2015 was $3.8x10^4$ and $1.5x10^3$ CFU/ml from sick and symptomless trees, respectively, then this population was found to decrease during winter season until it reached $1.2x10^3$ and $5.2x10^2$ CFU/ml in sick and symptomless trees, respectively by February 17th. This coincided with a gradual decrease in average temperature during winter (October – February period) which ranged from 21.3–6.9ºC, an average RH of 51-88 %, and 36.5mm rainfall on February 17th (Table 1 and Fig. 2 b).

Following that, the REP increased gradually during spring to reach$1.9x10^5$ and $1.9x10^5$ CFU/ml of sick and symptomless trees, respectively. This coincided with a gradual increase in average temperature during spring (March – June period), ranging from 9.4–24.8ºC, RH average of 41–55 %, and 20–108 mm rainfall (Table 1 and Fig. 2b). Furthermore, the REP increased until it reached its peak during the period that extended from March until the first week of June, where the REP reached $9.6x10^5$and $2.5x10^4$ CFU/ml of sick and symptomless trees respectively. This coincided with an average temperature of 21.5 ºC and RH that ranged from 46-52% (Table 2, Fig. 1 and 2).

Moreover, the 2nd peak of REP appeared during autumn where the population reached $3.5x10^4$ and $1.2x10^3$ CFU/ml for sick and symptomless trees respectively, coinciding with an average temperature of 23.4–25 ºC and RH of 33–60 % (Table 2 and Fig. 2).

Throughout the study period, the highest REP of Psv obtained was during spring, where the mean *Psv* populations ranged from $1.1x10^5$ to $9.6x10^5$CFU/ml on leaves obtained from infected trees and $0.75x10^4$ to $6.3x10^4$CFU/ ml from leaves of symptomless trees (Table 1). During this period, the temperature averages ranged from 13.3– 21.5ºC, and RH averages ranged from 52–56 % (Table 1, Fig. 1 and 2).

During summer months, it was found that the lowest obtained *Psv* populations from leaves of infected and symptomless trees over the study period were $2x10^3$–$9x10^3$ and $4x10^3$–$1.6x10^2$ CFU/ml, respectively (Table 1). This population coincided with average temperatures which ranged from 25-27.6ºC and 41-49 % RH (Table 1and Fig. 2). Furthermore, there was a higher decline in the recovered populations during December and January, and it coincided with an average temperature that ranged from 7.2–13ºC, and then increased during November to reach 14.2–15.5 ºC (Table 1, Fig. 1).

DISCUSSION

Olive knot is considered one of the most important diseases of olive trees in the Mediterranean region (Vivian and Mansfield, 1993). The disease was recorded to occur in most of olive growing areas in Jordan, causing economic losses. It drastically affected olive trees both qualitatively and quantitatively (Khlaif, 2006).

The results of our study revealed the occurrence of epiphytic population of *Psv* on olive leaves as reported by Ercolani (1978, 1985, and 1991), Lavermicocca and Surico (1987), and Quesada *et al*., (2010a). However, they disagreed with Lamichhane and Varvaro (2014). Nevertheless, this epiphytic population dynamically changes with the fluctuations in temperature and RH under orchard conditions, in which higher population density occurs during spring and fall seasons. This is in general agreement with Quesada *et al*. (2007), where

suitable temperature and RH can encourage *Psv* epiphytic population to build up.

The highest population of *Psv* found during the first week of June where the temperature was 21.3ºC and there was 52% of RH could be favorable for disease development.

Furthermore, the results showed that the rainfall increased the recovered populations of *Psv* from both leaf suspensions collected from infected and symptomless trees, providing suitable conditions for epiphytic population to increase through increasing RH% and providing the leaves with a film of water necessary for bacterial growth and reproduction, and consequently the infection.

Moreover, the tendency of the pathogen to remain on olive leaves constitute a source of disease inoculum, especially if it coincides with the availability of wounds which resulted from fruit harvesting practices that allow the bacteria to penetrate olive tissues and cause knot formation.

A delay until late June in the onset of the disease, through the appearance of a fresh knot on olive branches was observed in the experimental field (Al-Neir in Wadi Al-Sir in Jordan) during 2015 and 2016, either on infected trees from a previous season or on symptomless trees. Soon after that, the knots in the same orchard were observed developing. More knots also developed until the end of the season. These observations are in general agreement with Quesada *et al*. (2010a), who reported that the higher populations were detected in warm and rainy months.

These results could be explained if we take into account that the pathogen build up their epiphytic populations after winter time during spring season to reach the highest population at the first week of June (Table 1). Thus, the pathogen will be ready to cause infection after indirect penetration. In Jordan, it is confirmed that the epiphytic population constitutes a new dimension as a primary source of inoculums, which so far could be in agreement with the observations of Ciccarone (1950), Quesada *et al*. (2007), and Quesada *et al*. (2010 b).

In conclusion, this study provides information about the dynamics of epiphytic population of olive knot pathogen as affected by temperature and RH throughout the year, and where the population is high enough to be a serious problem causing the disease. Hence, the investigation of the epiphytic population under field condition leads the farmer for the best disease management practices, helping him to lower the incidence possibility of olive knot disease.

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Table 1: Averages of the recovered epiphytic population of *Pseudomonas savastanoi* pv. *savastanoi* from olive leaves of cv. Nabali Mohassan in relation to temperature, relative humidity, and rainfall in Al-Neir in Wadi Al-Sir in Jordan in Jordan during 2015/2016 olive growing season.

Date	Population number CFU/ml		Temperature (°C)			Relative Humidity (%)			Rainfall (mm)
	Diseased	Symptom-less	Max	Min	Mean	max	Min	mean	
Oct. 30	3.8×10^4	1.5×10^3	25	15.3	21.3	96	37	51	0
Nov. 15	5.3×10^4	1.9×10^3	20	11	15.5	74	57	66	0
Nov. 30	4.2×10^4	1.6×10^3	14.2	9.4	14.3	76	51	64	0
Dec. 14	2.1×10^4	1.1×10^3	17	9	13	75	56	66	0
Dec. 30	6.5×10^3	5.3×10^2	11.2	6.9	9	79	74	76	0
Jan. 14	4.1×10^3	2.2×10^2	9.3	5.1	7.2	89	80	88	0
Jan. 28	3×10^3	1.1×10^2	11.6	6.3	8.9	85	69	77	0
Feb. 7	2.9×10^3	8.3×10^2	15.4	8.5	11.9	76	60	68	0
Feb. 17	1.2×10^3	5.2×10^2	9.3	4.5	6.9	85	74	80	36.5
Mar. 2	1.7×10^4	9.3×10^2	12.6	7.2	9.4	75	58	67	20
Mar. 21	1.5×10^4	1.4×10^2	14	7.8	10.9	69	55	62	88
Apr. 11	1.1×10^5	6.3×10^4	17.3	9.3	13.3	62	50	56	28
May. 7	1.9×10^5	0.75×10^4	25	15.9	20.4	53	50	51	108
May. 16	1.5×10^5	1×10^4	23.8	15.7	19.8	58	41	50	0
Jun. 2	9.6×10^5	2.5×10^4	27	15.4	21.5	61	42	52	0
Jun. 20	3.1×10^4	4×10^3	29.9	19.6	24.8	51	36	44	0
Jul. 13	9×10^3	2×10^3	30	21.1	25	54	38	46	0
Jul. 27	7.5×10^3	2.5×10^2	32.8	22.4	27.6	52	37	46	0
Aug. 16	2×10^3	1.6×10^2	34.6	24	24.7	50	37	49	0

Date	Population number CFU/ml		Temperature (°C)			Relative Humidity (%)			Rainfall (mm)
	Diseased	Symptom-less	Max	Min	Mean	max	Min	mean	
Sep. 5	2.8 x 10^3	2 x 10^2	32.7	20.1	24	49	33	41	0
Sep. 19	3 x 10^3	1.8 x 10^2	33.1	20.4	26.7	51	33	42	0
Oct. 17	3.5 x 10^4	1.2 x 10^3	28.1	18.5	23.4	60	40	50	0

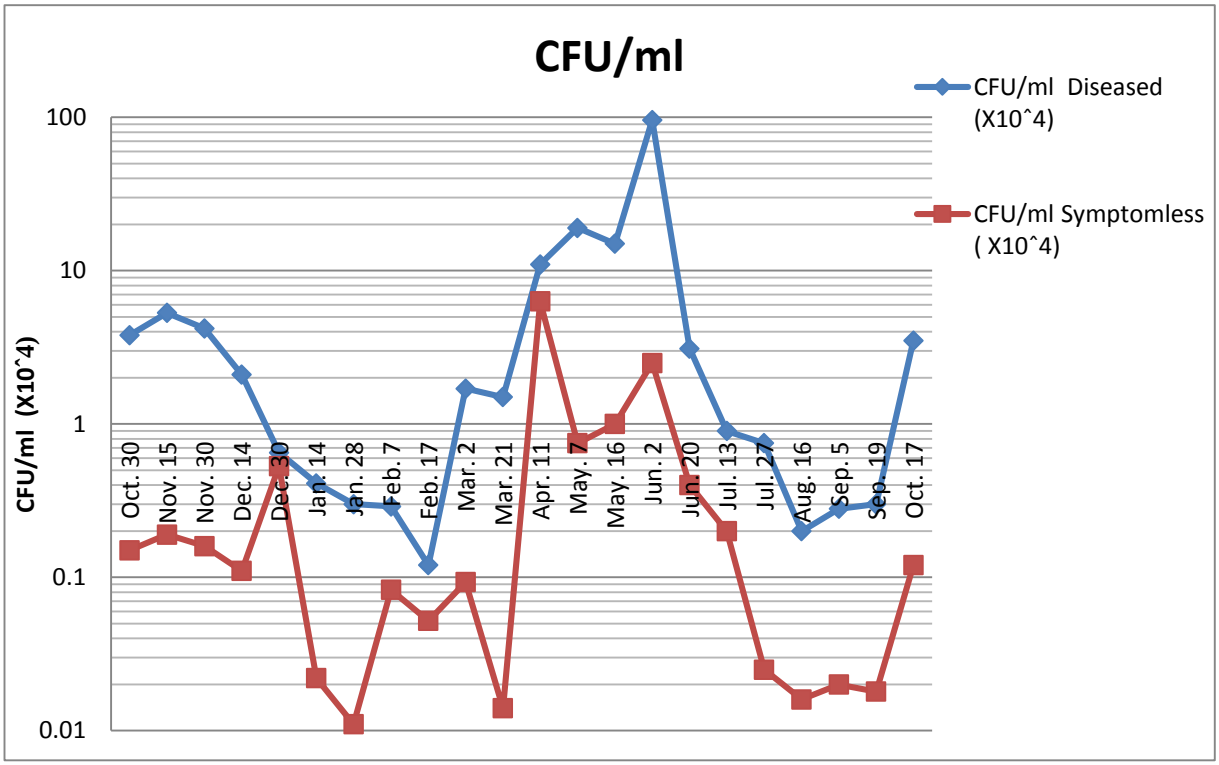

Figure 1: Mean of *Pseudomonas savastanoi* pv. *savastanoi* populations recovered from symptomless leaf of naturally infected and symptomless olive trees cv. Nabali Mohassan. Each point consists of 25 samples of olive leaves that are mixed to form bulk sample.

Table 2: Seasonal fluctuations of *Pseudomonas savastanoi* pv. *savastanoi* population on olive leaves of cv. Nabali Mohassan collected from symptomless and diseased trees.

Season	Duration	Average population number CFU/ml	
		Diseased	Symptomless
Fall	Sep. 19 – Nov.14	3.42 x 10^4	0.128 x 10^4
Winter	Dec.14 – Feb.17	0.645 x 10^4	0.055 x 10^4
Spring	Mar.2 – Jun.20	21.2 x 10^4	15.8 x 10^4
Summer	Jul.13 – Sep. 5	0.532 x 10^4	0.065 x 10^4

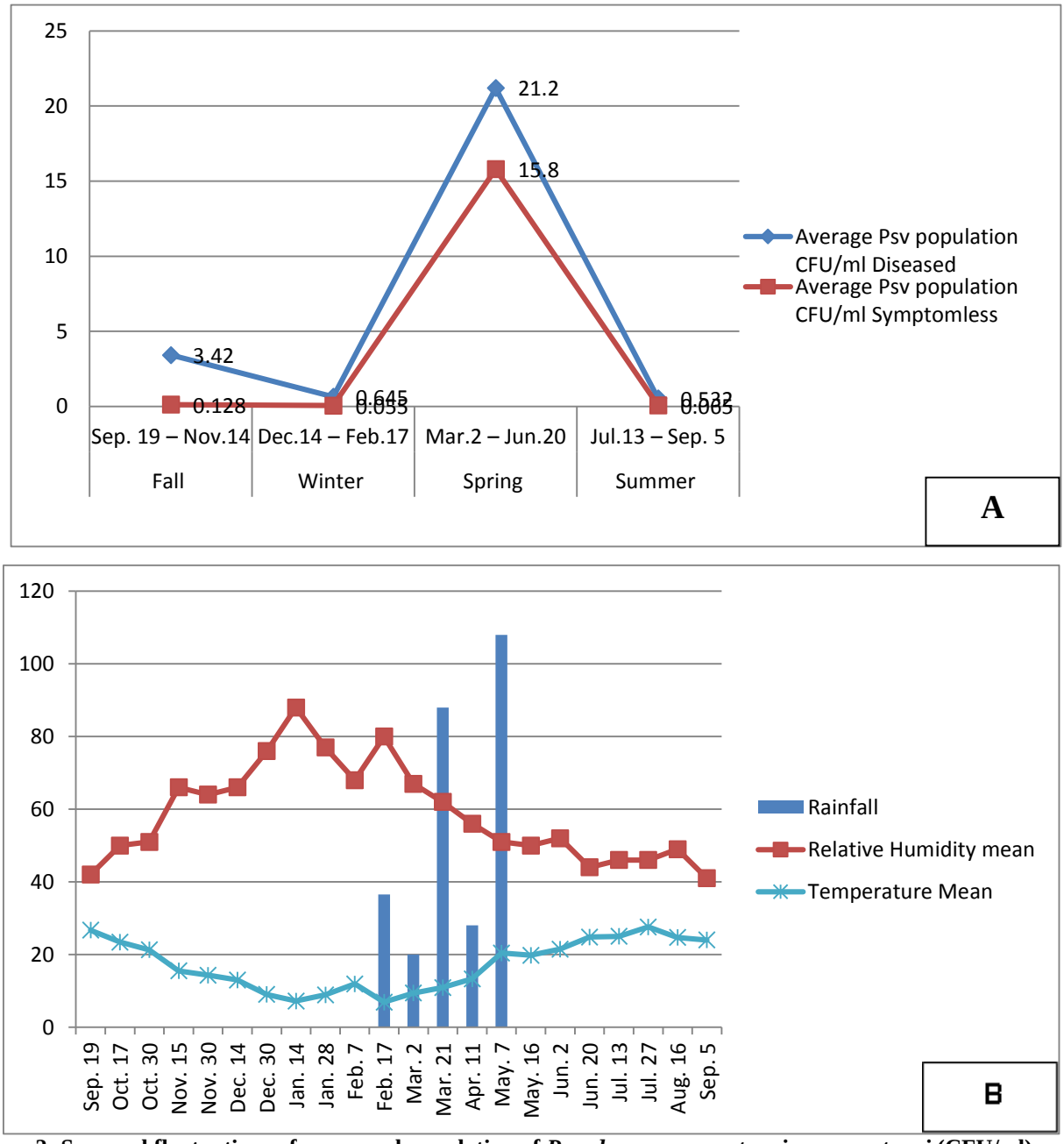

Figure 2: Seasonal fluctuations of recovered population of *Pseudomonas savastanoi* pv. *savastanoi* (CFU/ml) on leaf surfaces of naturally infected and symptomless olive trees all around the year (A), and average temperature, RH and cumulative rainfall (B).

التذبذب في الاعداد السطحية لمسبب مرض تعقد الزيتون البكتيري *Pseudomonas savastanoi* pv. *savastanoi*

بلال ابراهيم اوريكات[1]، حامد مصطفى اخليف[2]

ملخص

تم دراسة التذبذب (التغيرات) في الأعداد السطحية لبكتيريا تعقد الزيتون على أوراق الزيتون على مدار الموسم الزراعي 2016/2015م في بستان زيتون في منطقة وادي السير – الأردن. تم عزل المسبب المرضي من معلق أوراق الزيتون التي جمعت من أشجار مصابة وسليمة، حيث وجد أن أعداد المسبب المرضي تتغير تبعاً للتغيرات في الحرارة والرطوبة النسبية. وقد وجد أن أعلى الأعداد التي تم الحصول عليها كان خلال الربيع والخريف بينما أقل الأعداد كان خلال الصيف والشتاء، وكانت أعلى أعداد للمسبب المرضي قد ظهرت خلال الأسبوع الأول من حزيران وكان ذلك متزامناً مع معدل درجات حرارة 21.5 ᵒم ومعدل رطوبة نسبية 52%. كما أظهر المسح الحقلي للحقل الذي أجريت فيه التجربة خلال 2015–2016م، أن هنالك تأخراً في ظهور المرض، حيث ظهرت عقد خشبية تشكلت حديثاً في أواخر حزيران والتي تلت اعلى أعداد للبكتيريا. في الاردن، قد يجلب وجود المسبب المرضي سطحياً على أوراق الزيتون بعداً جديداً في تحديد وبائية المرض، حيث يعتبر مصدراً أساسياً للعدوى بالمرض.

الكلمات الدالة: وبائية الأمراض، مادة اللقاح، البكتيريا الممرضة للنبات، سل الزيتون.

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