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Reliable Analytic Strategy to Correlate the Morphological and Cytological Parameters on *Lupinus termis* L. against *Fusarium oxysporum* Infection

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Abstract: Many analytic strategies have emerged to estimate plant responses to Fusarium wilt. The demand for fast and reliable method (diagnosis, prediction) to determine isolate strength accurately is not established yet. Early determination of pathogen strength helps in plant medication. The aim of this study was to develop a faster strategy and method for early determination of fungal isolates strength in correlation to plant response. Till now, the scientists have no consensus on the most correlated parameters that could express wilt precisely. In this study, 30 isolates of *Fusarium oxysporum* isolated from *Lupinus termis* L. were used to provide an explicit image about the real strength of *Fusarium* isolates and its impact on the plant. Wilting percentage ranged from 26.67% to 93.33% of the infected plants depending on isolate virulence. Some of cellular, morphological and physical measurements were conducted on 8 out of 30 isolates, including root (length, fresh weight (FW) and dry weight (DW)), nodules (water content (WC), FW, DW), stem (height, WC, FW, DW), total leaves/plant (WC, FW, DW) and the fourth leaf (WC, FW, DW, leaf area, epidermal cell area, epidermal cell number, succulence). Hierarchical clustering was used to determine the variance between the isolates. Detrended correspondence analysis (DCA) and canonical correspondence analysis (CCA) were used to determine the most important growth parameters that could express wilting accurately. The CCA results showed that most of the measured parameters on the fourth leaf, except for leaf epidermal cell number, were highly and positively correlated to wilt. That makes these specific parameters valuable and sensitive for any changes in isolates strength. Accordingly, a mathematical model was created to be helpful in the quick determination of isolate strength and precise medication.

Key words: *F. oxysporum*, *L. termis* L., leaf area, epidermal cell area, water content, linear and exponential decay models.

1. Introduction

Fusarium is one of the most world-wide fungal pathogens that known to cause wilt disease to many crop plants. It infects mainly the root system, causing damping-off, root rot and wilt because of the distribution of the pathogen in the soil [1] and leading to great decrease in seed yield [2-4]. Besides, the chlamydospores may persist for long periods in soil and this is the main mode of survival in unfavorable conditions [5, 6]. *L. termis* is one of the crops that occupied a large area of search against *Fusarium* infection. It was considered as an old crop and widely used not only as a protein source and a fodder but also for soil improvement [7, 8]. In Egypt, specifically, the cultivated lupine area is about 1,200 ha and produces 2,400 ton lupine, with productivity of 20,000 kg/ha [9].

Infected plants undergo changes in many vital physiological processes, specifically, disturbance in water balance [10] and hormonal imbalance [11].

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Disruption of water movement from roots to leaves is explained by different theories, such as plugging theory [12] and systematic toxin theory [13]. The plugging theory mentions that the vessels of the infected plants are plugged with the fungal hyphae or by other plant materials, such as callose and tyloses, and this limits water transport in the xylem. Whilst, the systematic toxin theory indicates that the fungal toxins disturb the metabolism of the infected plants, leading to leaf wilt and many other symptoms on the plant in general.

Leaf is an important plant organ, which reflects the effect of biotic-like fungal infection and any abiotic stress factor, like salinity, temperature, humidity and others. Visually, any of these factors causes a decrease in leaf area and color which is mainly depending on change in photosynthesis and cellular processes "cell expansion and cell division" [14]. Therefore, leaf area measurements are required in most physiological studies involving plant growth [15]. Besides, any change in leaf area is highly related to changes in leaf epidermal cell area and epidermal cell number [14]. Furthermore, measuring leaf water content (WC), fresh weight (FW) and dry weight (DW) against *Fusarium* infection has been addressed in some studies [16] without precisely estimating their correlation to Fusarium wilt. On the level of the plant, additional growth parameters were studied, such as plant height, the number of leaves, FW and DW of the shoot to determine the changes occurring on them under fungal infection [16].

Most authors focused on measuring some specific parameters under fungal infection. These plant parameters (responses) were correlated to individual isolate virulence. More worthy, it is imperative to take into account possible correlations between those parameters and different fungal isolates (different wilt (0-100%) on specific plant cultivar. This requires an appropriate analysis of data (which is by nature multivariate) in order to identify which parameters could express a greater correlation to Fusarium wilt disease (wilt%).

This study focused on testing the pathogenicity of 30 *Fusarium* isolates on *L. termis* L. plant. Only eight isolates were selected for further 21 morphological and cytological measurements, with the following questions addressed:

(1) To what extent could be the effectiveness of analyzing a package of parameters (morphological and cytological) on estimating fungal virulence (wilt%) at the early stages of plant infection?

(2) What would be the rank correlation between the measured growth parameters and wilt?

(3) Which parameters have the greatest and strongest correlation to wilt to express it precisely?

(4) Could model some specific plant parameters to express wilt percent immediately on the onset of infection to decide for the appropriate therapy before losing the whole crop?

2. Materials and Methods

2.1 Source of the Pathogen

F. oxysporum isolates were isolated from infected lupine plants from different areas, specifically the root to the basal part of the stem. Then, the collected pieces of the plants (1 cm) were surface sterilized by immersing in 2% sodium hypochlorite solution for 2 min, washed with sterile distilled water and dried by sterile filter papers. The dried parts were laid on PDA medium for fungal growth enhancement, then sub-cultured into another sterile plate and kept in pure culture.

2.2 Pathogenicity Test

Pathogenicity test was carried out in an open-roof greenhouse using 30 isolates of *F. oxysporum* isolated from lupine plants under natural growth conditions. Specifically, the daily day/night temperature was 25/13 °C, average relative humidity was 61% and light intensity at the top of the plants was around 350 ± 30 μmol/m^2/s photosynthetic photon flux density (PPFD). For inoculum preparation, the isolates were

firstly inoculated on 500 mL barley medium (75 g barley, 25 g clean sand, 2 g sucrose, 0.1 g yeast extract and 100 mL distilled water) and incubated at 28 °C for 15 d [17]. The pots of 30 cm diameter containing 4 kg sterilized soil were inoculated with the prepared inoculum of *F. oxysporum* (5% by weight). The soil was mixed thoroughly with inocula, watered and left for one week [5, 18]. Lupine seeds Giza 1 were sterilized in sodium hypochlorite (0.1%) for 2 min, then washed with sterile water and planted in the pots (5 seeds/pot and 3 pots/isolate). Wilt percentage in the infected plants was calculated after 30 d of seed sowing. Pots without inocula were used as a control.

2.3 Measurements of Growth Parameters of Root, Nodules, Stem and Total Leaves

WC, FW and DW data of roots and nodules were collected after washing the roots with tape water. Afterwards, the roots and nodules were separated to calculate their FW (W_1/g). Then, both were dried overnight in an oven at 100 °C and reweighed once again to calculate their DW (W_2/g). WC (g) was calculated by subtracting FW and DW [19], as Eq. (1):

$$WC\ (g) = FW - DW \quad (1)$$

Besides, root length of the control and infected plants was estimated by using a ruler. The previously mentioned parameters (WC, FW and DW) were measured on the other plant organs (stem and total leaves).

2.4 Morphological Parameters of Leaf Area, WC, FW and DW of the Fourth Leaf

The 4th leaf was tagged and selected for measuring leaf area, WC, FW and DW. This leaf was selected after considering so many aspects. First of all, it is a true leaf and is fully expanded after 30 d of fungal attack; this considers an opportunity to estimate fungal virulence at the early stage of plant infection. Subsequently, this gives a chance to decide for an appropriate remediation before losing the whole crop. Second, selecting a leaf in a lower rank, such as the 1st, 2nd or 3rd leaf are slightly affected by fungal infection, might be not accurate to estimate an effective dose of any treatment used for remediation. Third, selecting a leaf in a higher rank, such as the 5th, 6th or 7th leaves show more visual symptoms of fungal effects, but it weakens the chance of plant recover by any treatment used for remediation. To sum, early diagnosis of the disease helps to gain more time to select the desired treatment to save crop quality and quantity. A picture was captured for the selected leaf of control and infected plants by using a digital camera (HP Photosmart M447 digital camera). To keep accurate measurements, the distance between the camera and the measured leaf was fixed by a special stand designed in the lab [19]. Besides, magnification was also fixed to 20×. A photo of reference area was captured for calibration. To calculate leaf area, ImageJ 1.47 software was used to analyze the captured pictures. WC, FW and DW were measured by the explained methodology in roots, stems and nodules.

2.5 Cytological Parameters

Cytological parameters were conducted on the selected leaf (the 4th leaf) and included measuring the epidermal cell area and epidermal cell number. Epidermal cell area was measured by sampling discs (5 mm diameter) of control and treated plants from the middle part of the mid leaflet of the 4th leaf after 30 d. The discs were mounted on a glass plate and painted carefully with transparent nail varnish (Rossmann, Germany), and then left to be dried at room temperature. After 40 min, a replica of the epidermal cells was peeled out from the disc and kept on lactic acid to be protected from being wrinkled when they are used after a while. Later on, the replica was mounted onto a glass slide, and an image was captured to it by using a microscope connected to a camera (Zeiss microscope (Axiostar), 200×). ImageJ 1.47 software was used to calculate the exact area of

five cells from each replica in μm^2. Epidermal cell number per leaflet was calculated by dividing the 4th leaf area on the mean epidermal cell area [19]. Leaf succulence, defined as water content per unit area, was determined by (FW-DW)/leaf area [19].

2.6 Statistical Analysis

2.6.1 ANOVA

One way ANOVA was carried out using SPSS software 19.0 (SPSS Inc., Chicago, IL, USA). Differences with *P* values less than 0.05 were significant.

2.6.2 Clustering, Detrended Correspondence Analysis (DCA) and Canonical Correspondence Analysis (CCA)

Hierarchical clustering was carried out by using Free Statistics Software (v1.0.3) to all the tested isolates to determine the variance between them [20].

Multivariate ordination techniques were applied to analyze the plant parameters data measured as responses to eight isolate of *F. oxysporum*. This was to investigate the dominant parameters characterizing the fungal isolates effects on the lupine plant. A unimodal response of the selected growth parameters to the isolates was expected, but it was not supported by DCA, as the gradient analysis was less than 4 [21]. Consequently, it was decided to use DCA as an indirect gradient analysis to derive the isolates gradient from the variance between their effects on the measured parameters. Besides, CCA, a direct gradient analysis was chosen to relate the variance in the selected growth parameters to wilt disease. From previous analysis, it was apparent that the study data including the isolates and the measured parameters from the infected plants required both direct and indirect ordinations to provide deep and clear insight to this experiment. All analysis was conducted with Canoco 4.5 [21].

Standard CCA was used to determine the impact of isolates severity "pathogenicity" on wilt. Isolates were then used as a covariable in all further analysis. Eigenvalues in particular analysis were, therefore, related to the residual variance after removing the effect of isolates. Further, CCA was used to determine which of the measured parameters have a distinct correlation with wilt. Comparison of DCA and CCA eigenvalues (the first and the second axes) identified the proportion of variability between the eight isolates explained by specific growth parameters; if the selected parameters explained most of the variation between the isolates (explored by DCA), eigenvalues of correspondent axes would be nearly identical [22, 23]. Results of DCA and CCA analysis were visualized using CanoDraw ordination diagrams.

2.6.3 Modeling (Fitting)

Modeling of the parameters was done for highly correlated parameters using SigmaPlot (v8.0).

3. Results

3.1 Pathogenicity Test

Pathogenicity test was carried out in a greenhouse using 30 isolates of *F. oxysporum* collected from infected *L. termis* from different areas. Wilt percentage was calculated after 30 d. Table 1 shows only wilt percentage for eight selected isolates. A cluster analysis was carried out for the 30 isolates depending on their wilt percentage and displayed a high variation between the isolates (around 250 variance; Fig. 1). In general, wilt percentage in all 30 isolates lied within 23.0%-93.3%. Consequently, the selection of eight isolates was mainly depending on wilt percentage. Specifically, among the eight selected isolates, AUMC 9272, 9290 and 9291 showed high ability to cause wilt on lupine by 93.3%, 80% and 73.3%, respectively. Besides, three isolates with similar visual wilt symptoms about 60% were also chosen (AUMC 9264, 9274 and 9292) to see the difference between them on the further measured parameters. Besides, the isolate AUMC 9291 selected among four showed 53% wilt percentage. Furthermore,

Table 1 Plant parameters measured for different plant parts against different *Fusarium* isolates causing wilt with different virulence.

Plant organs	Code	Parameters	Control	AUMC isolates (wilt%)							
				9264 (60%)	9272 (93%)	9274 (60%)	9283 (60%)	9288 (33%)	9290 (80%)	9291 (73%)	9292 (53%)
Root	1	WC (g)	1.34 ± 0.03	1.25 ± 0.06	1.06 ± 0.03*	3.12 ± 0.07*	1.69 ± 0.06*	3.22 ± 0.13*	1.96 ± 0.01*	1.83 ± 0.03*	3.02 ± 0.04*
	2	Length (cm)	31.00 ± 2.08	15.00 ± 1.15*	17.67 ± 1.20*	29.33 ± 2.33	17.67 ± 1.20*	25.33 ± 2.60	19.67 ± 1.76*	16.00 ± 0.58*	15.67 ± 1.20*
	3	FW (g)	1.49 ± 0.03	1.40 ± 0.04	1.17 ± 0.03*	3.46 ± 0.08*	1.81 ± 0.06*	3.57 ± 0.12*	2.15 ± 0.01*	2.00 ± 0.03*	3.34 ± 0.04*
	4	DW(g)	0.15 ± 0.01	0.16 ± 0.02	0.11 ± 0.00*	0.34 ± 0.01*	0.12 ± 0.00*	0.34 ± 0.02*	0.19 ± 0.01*	0.16 ± 0.00	0.32 ± 0.00*
Nodule	5	WC (g)	0.46 ± 0.05	0.03 ± 0.003*	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*	0.022 ± 0.022	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*
	6	FW (g)	0.50 ± 0.06	0.03 ± 0.03*	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*	0.03 ± 0.03*	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*
	7	DW (g)	0.04 ± 0.00	0.006 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*	0.007 ± 0.007*	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*
	8	Stem height (cm)	23.00 ± 0.58	12.67 ± 1.20*	21.33 ± 1.20	20.00 ± 1.15	17.33 ± 1.45*	19.67 ± 1.20	16.33 ± 1.45*	14.00 ± 1.35*	13.33 ± 0.88*
Stem	9	WC (g)	2.01 ± 0.03	2.24 ± 0.04*	2.06 ± 0.02	2.83 ± 0.07*	2.42 ± 0.05*	3.05 ± 0.04*	2.05 ± 0.08	1.95 ± 0.06	2.66 ± 0.01*
	10	FW (g)	2.32 ± 0.04	2.44 ± 0.04*	2.29 ± 0.02	3.15 ± 0.06*	2.61 ± 0.05*	3.46 ± 0.04*	2.23 ± 0.08	2.17 ± 0.04	2.89 ± 0.01*
	11	DW (g)	0.31 ± 0.01	0.21 ± 0.01*	0.23 ± 0.00*	0.32 ± 0.01	0.20 ± 0.01*	0.42 ± 0.00*	0.18 ± 0.00*	0.22 ± 0.02*	0.23 ± 0.00*
Total leaves	12	WC (g)	2.06 ± 0.16	1.87 ± 0.07*	1.10 ± 0.07*	2.61 ± 0.07*	2.02 ± 0.08	2.75 ± 0.09*	1.62 ± 0.04*	1.70 ± 0.05*	2.36 ± 0.04*
	13	FW (g)	2.33 ± 0.16	2.06 ± 0.07	1.19 ± 0;07*	2.90 ± 0.07*	2.21 ± 0.09	3.04 ± 0.09*	1.82 ± 0.05*	1.93 ± 0.05*	2.62 ± 0.05*
	14	DW (g)	0.27 ± 0.01	0.19 ± 0.01*	0.09 ± 0.00*	0.30 ± 0.02	0.19 ± 0.00*	0.29 ± 0.01	0.20 ± 0.01*	0.22 ± 0.00*	0.27 ± 0.01
The 4th leaf	15	WC (g)	0.24 ± 0.02	0.10 ± 0.05*	0.13 ± 0.03*	0.20 ± 0.02	0.21 ± 0.01	0.23 ± 0.03	0.12 ± 0.05*	0.15 ± 0.02*	0.10 ± 0.02*
	16	FW (g)	0.28 ± 0.01	0.11 ± 0.05*	0.15 ± 0.03*	0.23 ± 0.02	0.24 ± 0.02	0.26 ± 0.02	0.16 ± 0.05	0.17 ± 0.02	0.12 ± 0.02*
	17	DW (g)	0.04 ± 0.00	0.02 ± 0.00*	0.02 ± 0.00*	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.01	0.02 ± 0.00*	0.02 ± 0.00*
	18	Leaf area (cm^2)	8.03 ± 1.74	6.16 ± 0.14*	6.49 ± 0.91	6.69 ± 0.98*	6.32 ± 1.03	7.26 ± 1.06	4.75 ± 1.74*	5.97 ± 0.74*	4.56 ± 0.93*
	19	Epidermal cell area (μm^2)	381.6 ± 131.5*	292.51 ± 44.59*	300.07 ± 16.43	288.02 ± 38.00*	389.55 ± 118.23	180.86 ± 33.35*	193.21 ± 25.20*	156.81 ± 55.45*	84.15 ± 23.21*
	20	Epidermal cell number (cell/leaf)	210 ± 14.02	224 ± 61.31	250 ± 18.75*	301 ± 33.98*	204 ± 31.16	405 ± 24.12*	246 ± 52.41*	381 ± 84.39*	542 ± 170.91*
	21	Leaf succulence (mg/cm^2)	30 ± 0.01	20 ± 0.01*	20 ± 0.00*	20 ± 0.00*	30 ± 0.01	30 ± 0.00	30 ± 0.01	30 ± 0.00	20 ± 0.00*

* Significant at 5%.

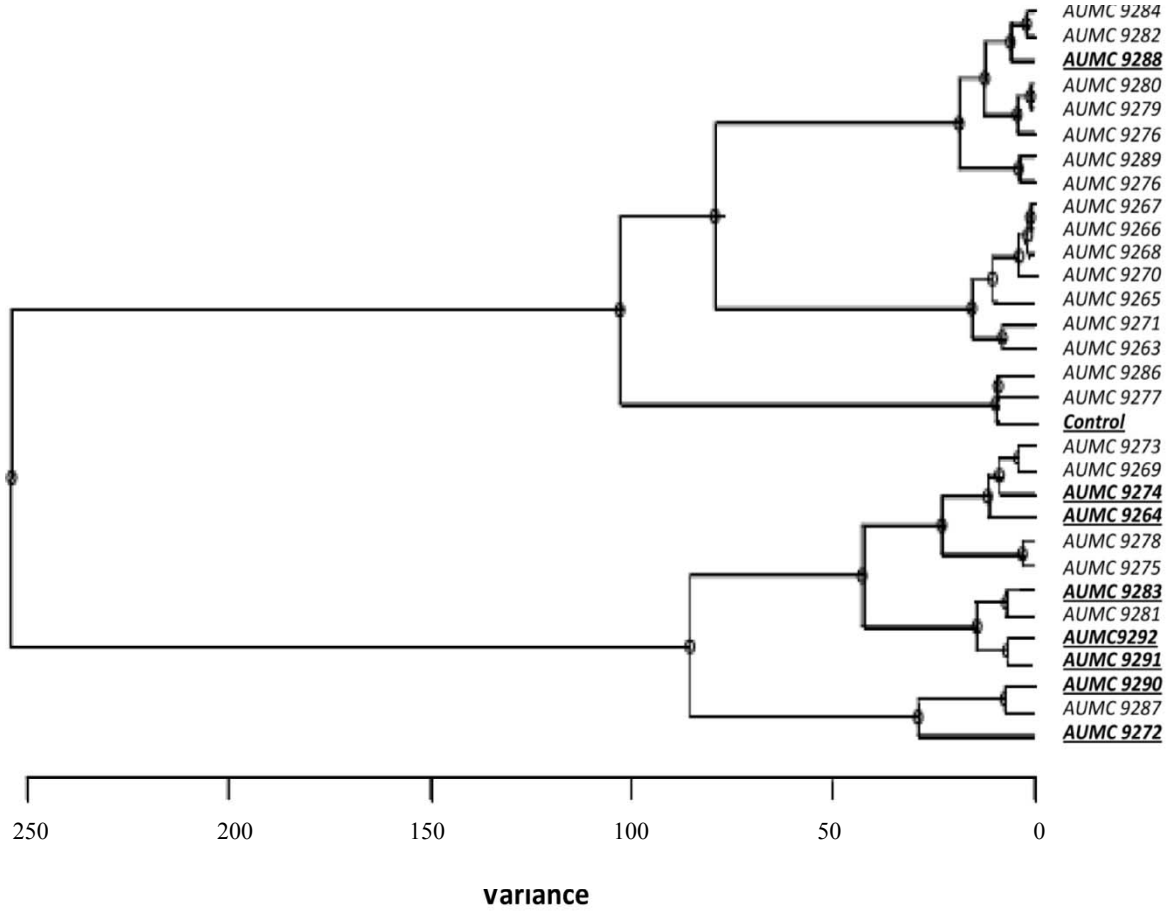

Fig. 1 Dendrogram from cluster analysis of the pooled data of 30 isolates (*F. oxysporum*, wilt%) of wilted lupine in addition to the control plant.

the isolate AUMC 9288 was selected among 10 isolates with wilt percentage of 33.3%. All the remaining isolates displayed wilt percentage within 47%-23%. Then, the response of the lupine plant to the eight chosen isolates was recorded in different plant parts, i.e., nodules, root, stem total leaves and the 4th leaf as recorded in Table 1.

3.2 Root and Nodules

All the measured parameters in root and nodules exhibited specific responses to the tested isolates. Regarding WC of the root, it was reduced significantly by only two isolates AUMC 9264 and 9272 of *F. oxysporum* in comparison with all the other isolates (Table 1). On the contrary, the remaining six isolates increased the WC of root significantly. Generally, root length decreased in all tested isolates (Table 1). This decrease was significant in only six isolates AUMC 9264, 9272, 9283, 9290, 9291 and 9292. Regarding FW of the root, isolates AUMC 9274, 9283, 9288, 9290, 9291 and 9292 increased significantly from 20% to 100%. Contrarily, AUMC 9272 displayed a significant decrease 20% in root FW. The DW of root also increased as a response to some isolates (AUMC 9274, 9288, 9290 and 9292) with a range from 26% to 70%; while the remaining isolates showed insignificant results (Table 1). Regarding the results of FW and DW of nodules, six isolates (out of eight) of *F. oxysporum* caused complete inhibition of nodulation. The two other isolates (AUMC 9264 and 9288) caused nodulation with a significant reduction in FW and DW in comparison with the control (Table 1). Namely, most of the infected plants possessed small and few nodules. The density of the root system was visually poor in the infected plant and that was

accompanied by reduced nodulation.

3.3 Stem and Total Leaves

In general, all tested isolates decreased stem height of lupine (Table 1). The reduction of stem height was hardly affected as a response to the isolates AUMC 9264, 9288, 9290, 9291 and 9292 of *F. oxysporum*. Nevertheless, the other three isolates (AUMC 9272, 9274 and 9283) showed a slight and insignificant reduction in stem height in comparison with the control plant. The WC of stem significantly was increased by the following five isolates of *F. oxysporum* AUMC 9274, 9288, 9264, 9283 and 9292 in a range from 11% to 49%. In spite of that, the isolates AUMC 9272, 9290 and 9291 showed an insignificant reduction in stem WC in comparison with the control plant (Table 1). The FW of stem results was consistent to WC of the stem as a response to the tested isolates. Precisely, WC displayed a significant increase in the same five isolates (mentioned above) and insignificant decrease in the other remaining isolates (Table 1). Generally, DW of the stem was significantly reduced (22% to 40%) by most of the isolates, except for AUMC 9283, which showed an increase of almost 30% (Table 1). The measured parameters of total leaves (WC, FW and DW) showed a high similarity with the same parameters of the stem. Specifically, they were decreased by five isolates (AUMC 9264, 9272, 9288, 9290 and 9291) and increased significantly by three isolates (AUMC 9274, 9283 and 9292) with a percentage of reduction in FW and DW of total leaves within 7.14%-50% (Table 1).

3.4 The Fourth Leaf Growth Parameters

In general, all the growth parameters of the 4th leaf, except for the epidermal cell number, were reduced in comparison with the control. Regarding WC and FW of the selected leaf, theses parameters decreased significantly in five isolates (AUMC 9264, 9272, 9290, 9291 and 9292). Precisely, the percentage of reduction in WC and FW ranged from 46% to 58% in comparison with the control plant. The other three isolates (AUMC 9274, 9288 and 9283) reduced WC and leaf FW insignificantly and showed nearly no change as a response to the last mentioned isolate (Table 1). The DW of the 4th leaf showed a significant reduction in four isolates (AUMC 9264, 9272, 9291, and 9292) with a reduction around 50% in comparison with the control plants. The other four isolated (AUMC 9274, 9283, 9288 and 9290) reduced DW of the 4th leaf with only 25% (Table 1). Leaf area of the 4th leaf was significantly reduced by the isolates AUMC 9264, 9290, 9291 and 9292 with a reduction around 45%. The other three isolates (AUMC 9272, 9283 and 9288) reduced leaf area by around 10%. Surprisingly, the isolate AUMC 9274 exhibited an insignificant increased in the 4th leaf around 5%. On the whole, the epidermal cell area was significantly reduced due to infection of lupine by six isolates of *F. oxysporum* (out of eight), although the two remained isolates AUMC 9272 and 9288 showed an insignificant reduction. However, it was very remarkable that the isolate AUMC 9292 (Table 1) caused a highly significant reduction in the epidermal cell area about 75% of the control plant. The epidermal cell number was the only parameter that showed a negative response to the tested isolates. Namely, the epidermal cell number was increased significantly as a response to six isolates, which are AUMC 9272, 9274, 9283, 9290, 9291 and 9292. As well as, the remaining two isolates (AUMC 9264 and 9288) increased the epidermal cell area but insignificantly. This exhibited the adverse behavior of the epidermal cell area (decreased) and the epidermal cell number (increased) as a response to fungal infection. That might be related to a direct or indirect effect on cell division and cell expansion. Finally, leaf succulence showed a visible significant reduction by the isolates AUMC 9264, 9272, 9274 and 9292. Additionally, the isolates AUMC 9283, 9288, 9290 and 9291 showed no effect on leaf succulence of the 4th leaf.

3.5 Cluster Analysis

All of the collected data of the selected eight isolates and their 21 measured parameters were pooled in cluster analysis to determine the variance between these isolates in the case of including all the measured parameters. These measurements were described in Table 1, involved root (WC, length, FW, DW), nodules (WC, FW, DW), stem (height, WC, FW, DW), total leaves/plant (WC, FW, DW) and the 4th leaf (WC, FW, DW, leaf area, epidermal cell area, epidermal cell number, succulence). The outcome of this step showed a hierarchical clustering with two main clads (Fig. 2), which classified the isolates into two groups (A and B) at about 800 variances. Group A included the control beside the isolates AUMC 9288, 9290, 9274, 9272 and 9264. The other group B included three isolates AUMC 9292, 9283 and 9291. It was noticeable that the isolates on the same clad were also highly varied.

3.6 DCA and CCA

DCA was conducted to depict the degree of differences between the eight selected fungal isolates in case of including the 21 growth parameters (Fig. 3). In DCA, total inertia (the total amount of variance) was 0.097. Besides, the eigenvalues of the first and second axes were 0.09 and 0.002, with an accumulative variance of isolates 93.3% and 94.9%, respectively. It was confirmed by DCA that the isolate AUMC 9288 was close to the control plant in comparison with the other isolates. The other isolates displayed gradual differences with the control. Prominently, the isolate AUMC 9292 showed a clear and great variance from the control (Fig. 3). Additionally, isolate AUMC 9274 displayed specific variation than the tested isolates and the control plant.

CCA was carried out to determine which growth parameters were highly correlated to wilt in all of the tested isolates (Fig. 4). The summary of the performed CCA diagram included total inertia equal to 0.035. However, the gradient length of axes 1 and 2 were 0.428 and 0.000, respectively. This cleared that only the first axis was significant and explained 100% of the isolates variance. Besides, the eigenvalues of the first axis and second axes are 0.003 and 0.022, respectively.

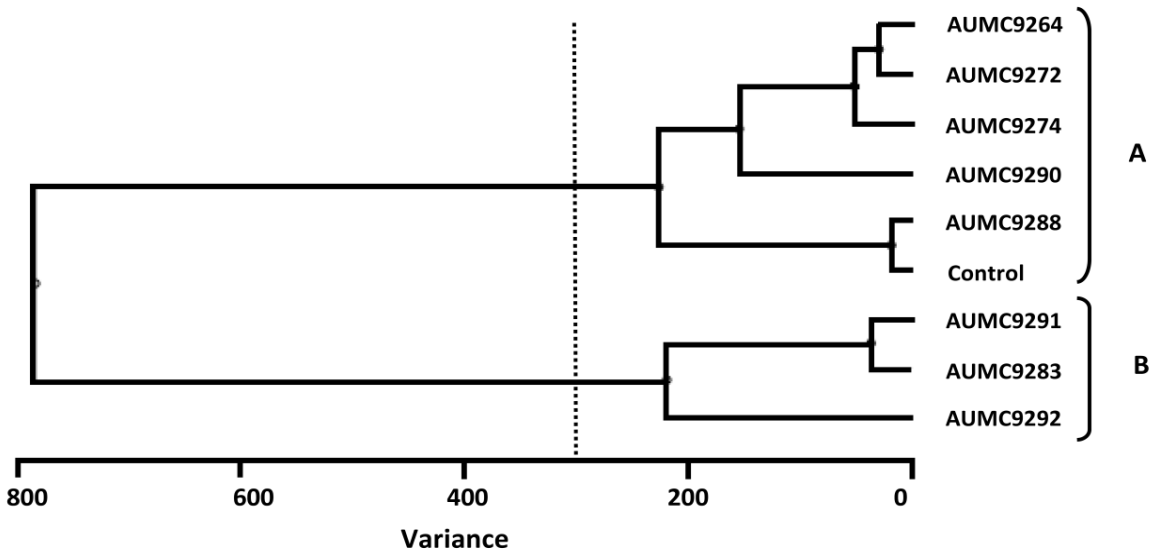

Fig. 2 Dendrogram from cluster analysis of the pooled data of 21 parameters (morphological and cytological plant responses) of the wilted lupine plants in response to eight *F. oxysporum* isolates.

Root (WC = 1, length = 2, FW = 3, DW = 4); nodules (WC = 5, FW = 6, DW = 7); stem (height = 8, WC = 9, FW = 10, DW = 11); total leaves/plant (WC = 12, FW = 13, DW = 14); the 4th leaf (WC = 15, FW = 16, DW = 17, leaf area = 18, epidermal cell area = 19, epidermal cell number = 20, leaf succulence = 21).

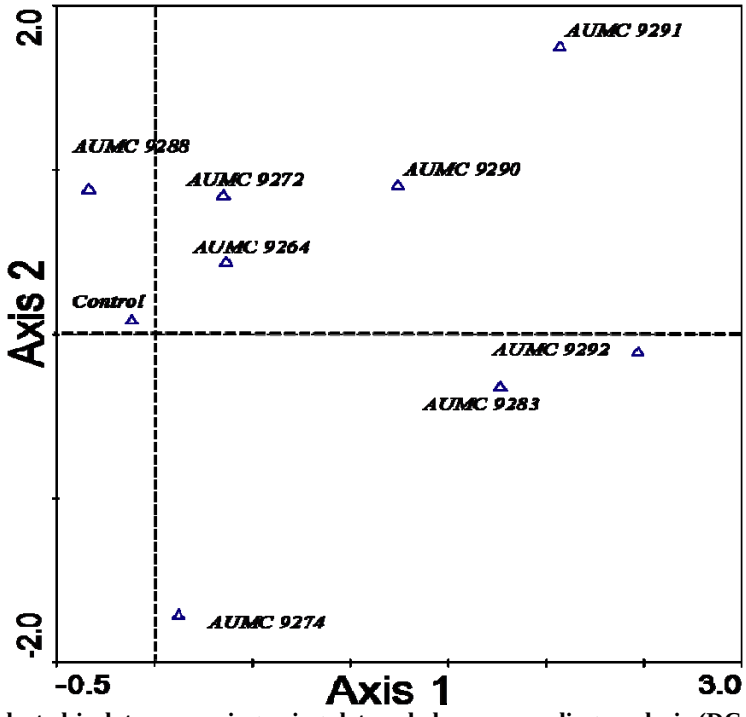

Fig. 3 Ordination for selected isolates screening using detrended corresponding analysis (DCA) to show the differences between them.

The obtained result divided the growth parameters into three main groups according to its correlation to wilt disease. The first group showed a strong positive correlation to wilt disease and included most of the measured parameters on the 4th leaf except for the epidermal cell number (Fig. 4). Specifically, the FW, DW, WC and leaf area of the selected leaf occupied a privileged position of the wilting arrow. Although the epidermal cell area and leaf succulence showed the same trend as the previously mentioned parameters, the strength of their correlation was slightly lower. Additionally, stem height and root FW were also positively correlated to wilt. The second group was located in the opposite direction of wilt and included all the remained parameters, i.e., root (WC, length and DW), total leaves, epidermal cell number of the 4th leaf. This clarified their negative correlation with wilt under fungal infection. Surprisingly, the nodules showed an independent group (group 3) and uncorrelated to wilt (Fig. 4). The previous CCA diagram led us to re-cluster the selected isolates once again with considering that only the parameters of the 4th leaf showed the highest positive correlation to wilt (FW, DW, WC and leaf area from group 1). The obtained cluster analysis showed less variance between the eight isolates (one-tenth of the previous clustering variance in Fig. 2) and displayed three main clads (at variance 30), forming three groups (A, B and C) with new distribution for the selected isolates (Fig. 5). Again, the isolate AUMC 9288 was grouped with the control (this is the isolate with low wilt percent). All the remained isolates were deposited on the other clads and configured two smaller groups (B and C).

3.7 Linear and Exponential Decay Models

On the basis of the results obtained from DCA and CCA, particularly the strongly correlated parameters of the 4th leaf (FW, DW, WC and leaf area) to wilt lead us to depict a mathematical relation between them and wilt. Accordingly, a mathematical model was created to predict the isolates strength by considering the selected parameters of the 4th leaf individually. A linear model represented leaf WC and leaf FW (Fig. 6) as Eq. (2):

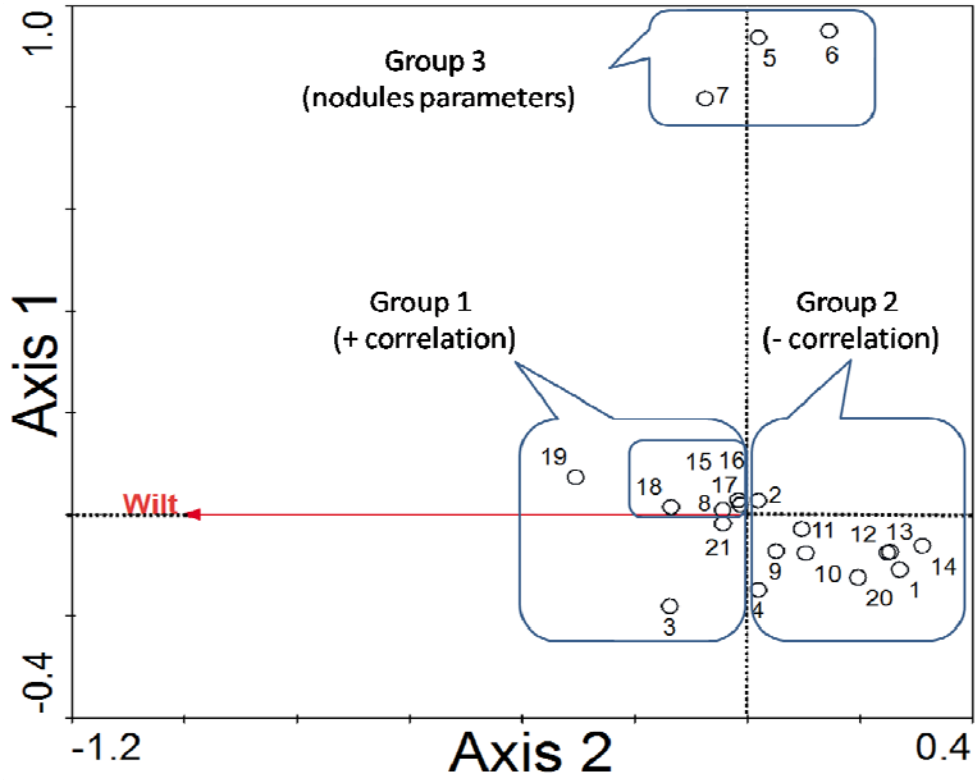

Fig. 4 Canonical correspondence analysis (CCA) of all measured parameters and its correlation to wilting factors.
Root (WC = 1, length = 2, FW = 3, DW = 4); nodules (WC = 5, FW = 6, DW = 7); stem (height = 8, WC = 9, FW = 10, DW = 11); total leaves/ plant (WC = 12, FW = 13, DW = 14), the 4th leaf (WC = 15, FW = 16, DW = 17, leaf area = 18, epidermal cell area = 19, epidermal cell number = 20, leaf succulence = 21).
CCA shows three groups of parameters, each group has a distinct correlation to wilt: group 1, positive correlation; group 2, negative correlation and group 3 is uncorrelated to wilt. Each group is surrounded by a rounded rectangular callout. Some of the 4th leaf parameters which show the strongest correlation to wilt are surrounded by a rounded rectangular in group 1.

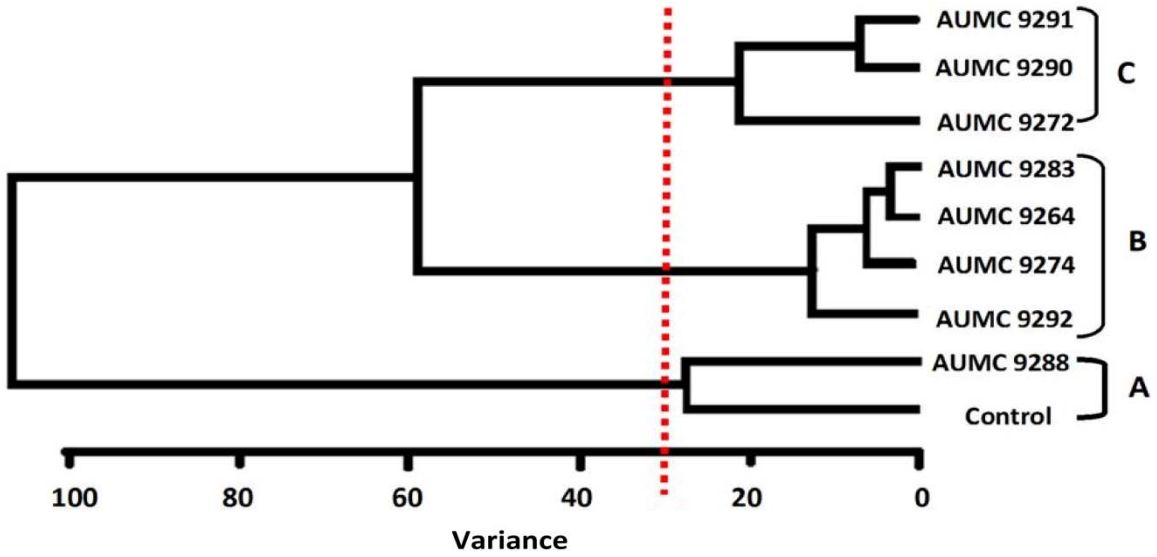

Fig. 5 Dendrogram from cluster analysis of isolates depending on the strongest correlated parameters of the 4th leaf (WC, FW, DW, leaf area) to wilt.
It shows a decrease in the variance between the isolates to one-tenth of the variance of the 21 parameters in Fig. 2.

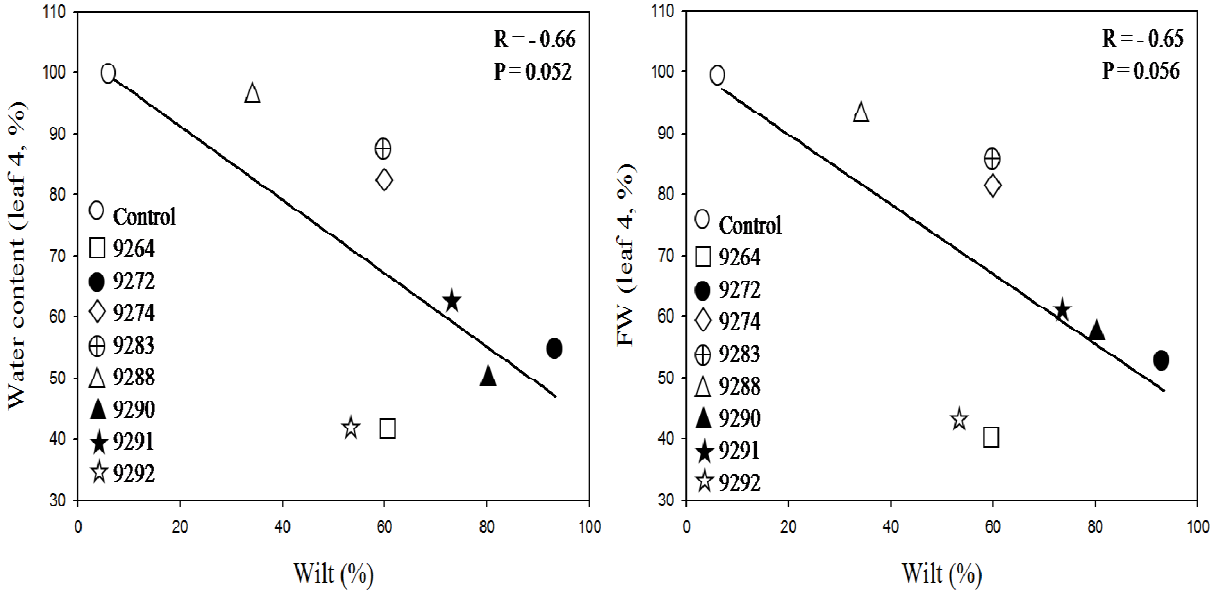

Fig. 6 Linear model for WC% (a) and FW% (b) parameters of the 4th leaf.

Regression coefficient (R) for both equations was more than 0.66 and 0.65, respectively; P values for both equations were 0.052 and 0.056, respectively.

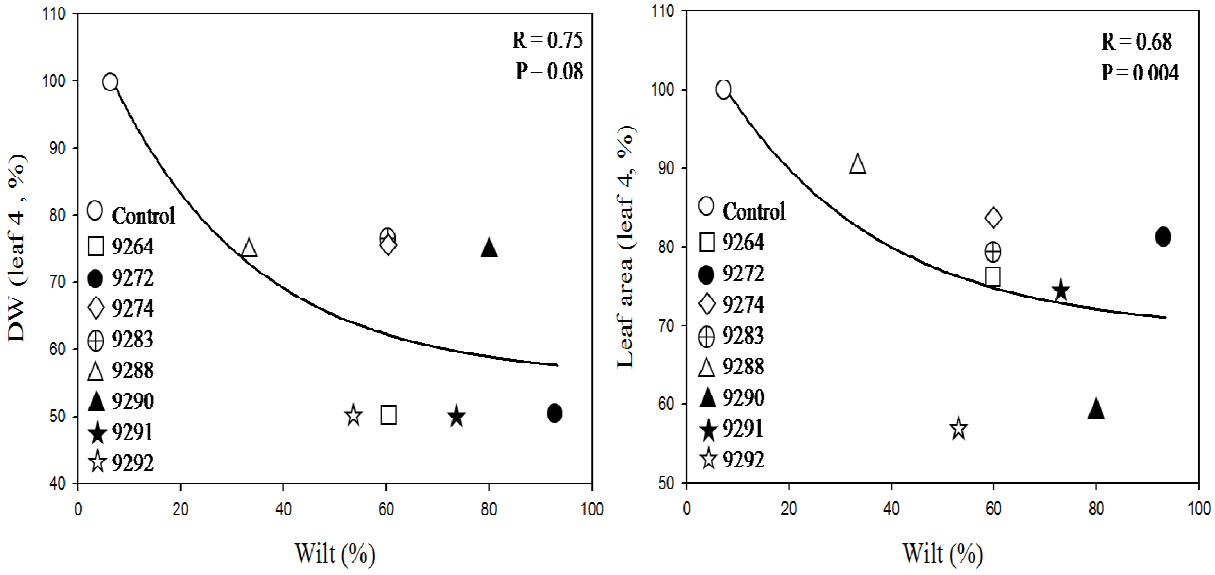

Fig. 7 Exponential decay model for the 4th leaf DW% (a) and leaf area% (b).

The regression coefficient for both variables against wilt was 0.75 and 0.68, respectively; P value was 0.08 and 0.004, respectively.

$$F = y_0 + ax \quad (2)$$

where, F is a linear equation (expressed as WC and FW), y_0 (reciprocal) equals to 105.1 and 102.8, a (slope) equals to 0.59 and 0.56 for WC and FW, respectively.

While, exponential decay model with three parameters expressed the relationship between leaf DW and leaf area to wilt percent (Fig. 7) as Eq. (3):

$$F = y_0 + a \cdot e^{-bx} \quad (3)$$

where, F is an exponential decay equation (expressed as DW and leaf area), y_0 (reciprocal) equals to 55.7 and 73.8, a (slope) equals to 56.4 and 32.3 for DW and leaf area, respectively; b is a constant and equals to 0.035 and 0.021 for DW and leaf area, respectively.

4. Discussion

In the recent years, many studies were dedicated to determine the exact plant responses to Fusarium wilt. The work on this point required considering different plant growth parameters under fungal infection. From these parameters, many theories emerged mainly to illustrate the underlying mechanisms of plant growth in the case of fungal infection. The most important parameters that showed consensus from scientists as a response to wilt included water status, root system, plant height, leaf area, plant FW and DW [10, 16]. In this study, the authors considered measuring 21 growth parameters (morphological and cytological parameters) on the lupine plant as a response to eight isolates of Fusarium wilt. Covering a large scale of measurements revealed an important finding of the parameters that could express wilt accurately under infection conditions. In the current study, screening of 30 isolates of *F. oxysporum* on the basis of their ability to cause wilt showed different wilt percentages from 13.3% to 93%. The eight tested pathogenic isolates (AUMC 9264, 9272, 9274, 9283, 9288, 9290, 9291 and 9292) were selected according to their wilt percentage. Surprisingly, they displayed a high level of variance in the case of considering 21 further growth parameters than in considering their wilt percentage (Figs. 1 and 2). This might be related to genetic variation between them, as it was reported by Bentley et al. [24], who emphasized the presence of genetic variation among worldwide collected isolates of *F. oxysporum*. Regarding the underground parts of the plants, including root and nodules, in most infected plants, the density of the root system was visually poor and had no or small nodules. The results from these plant parts in this study were consistent with many studies, except for FW of the root. Specifically, FW of root was increased by all the isolates, except for AUMC 9272. The specific effect of the isolate AUMC 9272 on root FW agreed with Mennan et al. [25], who recorded that *F. oxysporum* caused reduction in FW of roots on *Heterodera cruciferae*. These contrasting results might be depending on plant age at the time of measuring FW of the root. In this study, the authors measured the parameter at the seedling stage of 30-day-old seedling. Even though the WC and DW of root did not show the same response to fungal infection, they were reduced. Additionally, root length was decreased, and that corresponds with a research conducted on pea as a response to *F. oxysporum* and *F. solani* [26]. Also, Hwang et al. [27] reported that shoot and root DW of lentil decreased with increasing inoculum density of Fusarium wilt. Regarding nodule formation, FW and DW of nodule were reduced and this is supported by the results of John et al. [28], who reported a reduction in nodulation in soybean plant under fungal infection. Furthermore, as the FW of nodules was highly reduced, WC was diminished significantly in all infected plants.

In this study, it was remarkable that stem height was reduced in all the tested isolates of *Fusarium* (Table 1). This reduction was reported in some studies carried on tomato as a response to Fusarium wilt [16, 25, 29]. Surprisingly, WC of stem showed a striking result, in which five isolates (out of eight) displayed a significant increase in WC in this specific organ. Besides, the other three isolates displayed an insignificant increase in WC. Actually, in the literature, there are two conceptions regarding WC of the stem in case of infection with *F. oxysporum.* Some studies reported that WC is reduced significantly in this specific organ [25]. However, others recorded only 2% reduction in stem WC [16] in age of 30-day-old seedling and they did not provide an explanation for this reduction since it was insignificant. Although, the result obtained in this study is in agreement with the last data reported by Nogués et al. [16], which established a clear vision for water use strategy during the early stages of the plant growth under fungal infection. This strategy was highly confirmed and supported by measuring WC in different plant organs (root, stem and leaf) under

infection with different isolates of *F. oxysporum*. The authors proposed that the increase in WC of stem occurs at the early stage of fungal infection (around 30-35 days old seedlings) before the vascular tissues are blocked with fungal cells and the formed gummy substances and tyloses. At this stage, the plant deals with a dual challenge: first to meet its requirements of this rapid growth stage and second to keep the vital physiological processes for a longer time. If this happens, the water has only a possibility to be stored in the parenchymatous cells of the stem, as they make up the bulk of the cortex in the stem. In later growth stages, when the water flows from the vascular tissues of roots becomes very slow, the water would be defused from the parenchymatous cell of the stem to the mesophyll cells in the leaves. This assumption is highly supported by the recent study, which confirmed a state of water loses through non-stomatal pathway as a consequence of leaf cell membrane injury under fungal infection [10]. Specifically, Wang et al. [10] reported that leaf cell membrane injury occurs under fungal infection induces water movement out of the vascular tissues and creates a chance of uncontrolled water loss from damaged cells. Interestingly, this strategy of plant water status under fungal infection is similar to a large extent to the hydraulic segmentation hypothesis (HSH). This hypothesis, generally, proposes that stem and leaves operate separately to adjust water balance in a performance, like a body of a bottle (stem) and bottleneck (leaves) in shrubs and woody plants [30]. In this study, exclusively this hypothesis is emphasized for the first time on crop plant lupine at the early growth stage of Fusarium infection. In all the tested isolates, WC increased in the stem and decreased in the leaves, specifically on the photosynthetic area. This boosts this assumption and opens a new point of research to investigate the underlying mechanism behind this finding.

Generally, the tested isolates reduced leaf area significantly in the range of 53%-60%, but only one isolate (AUMC 9264) shows a positive and significant effect on leaf area than the other isolates. Some studies reported a clear and significant reduce in plant height and leaf area by *F. oxysporum* on sweet basil [31] an tomato [16]. This also was accompanied by reduction in WC, FW and DW. This might be explained by some studies that reported that leaf area is reduced when the specific weight, chlorophyll content, net photosynthetic rate, stomatal conductance and transpiration rate decreased under infection with *F. oxysporum* [15]. In accordance, FW and DW of sweet lemon basil plant cv. Citriodorum were reduced due to *F. oxysporum* infection [31]. Many studies had a consensus on the changes of different influences processes, such as water uptake, root pressure, stomatal conductance, photosynthesis, enzymatic activity, roots and shoots growth and mineral nutrition in case of infection by *F. oxysporum* [32-34].

In the current study, it was exclusively measured the epidermal cell area and epidermal cell number as a response to *F. oxysporum* infection. It was very remarkable that epidermal cell number increased in all the infected plants with a notable percentage, in some plants up to 98% (AUMC 9274, 9293, 9292 and 9292). However, this increase in the epidermal cell number was accompanied by a significant reduction in the epidermal cell area. This led to a final reduced leaf area. Actually, this finding was confirmed in most of the isolates and that may be due to the hormonal imbalance, specifically, auxins that control cells expansion [11]. This result supports the presence of an inverse relationship between the epidermal cell area and the epidermal cell number under the fungal infection. Exceptionally, leaf succulence displays more or less no change as a response to the tested isolates. However, it was expected to get a reduction in leaf succulence as a result of the reduced WC, but the reduction in leaf area affected mainly on the obtained results. A study by Waisel et al. [35] confirmed that some plants may develop succulence to various levels in order to maintain a suitable leaf water potential and solute potential.

Clustering, DCA and CCA used to describe the variance between the isolates and to determine the most important growth parameters that could express wilt precisely and the degree of their correlation to wilt. This discriminated analysis clustered 30 studied isolates into different groups depending on wilt percentage (Fig. 1). This step was followed by selecting only eight isolates depending on their wilt percentage (different isolates) to compare them on the morphological and cellular level. This cluster analysis showed that pooling 21 growth parameters for different isolates revealed a big variance between of them more than 800 variance level (Fig. 2). DCA determined accurately the variance between the selected isolates in comparison with the control plant (Fig. 3) and confirmed the data obtained from the previous cluster analysis. Most importantly, CCA analysis was very efficient, because it determined not only the correlation of the 21 tested parameters to wilt, but also the degree of their correlation. This diagram revealed crucial finding of some growth parameters measured in the 4th leaf. In this step, it was confirmed that leaf area, WC, FW and DW of the leaf are the parameters, which we are looking for to feasibly and precisely express Fusarium wilt severity (Fig. 4). Hierarchal clustering for the eight selected isolates once again by considering only the four defined parameters reduced the variance between them to the one-tenth in comparison to considering all the measured parameters (Figs. 2 and 5). The previous data provided an opportunity to create a simple model to determine wilt percent for any Fusarium isolate by measuring only the previous mentioned parameters. This model is used exclusively in this study and very specific to the measured parameters. Namely, for leaf area and WC, a linear model could express wilt percent precisely (Fig. 6). However, for FW and DW, an exponential decay model with three parameters is used to achieve the same purpose (Fig. 7). This model was applied to some data excluded from previous studies [16], from leaf area, where they estimated the WC and wilt disease severity on the tomato plant. Following this model, it was also possible to get nearly the same results as they reported.

5. Conclusions

In conclusion, the tested isolates of *F. oxysporum* infected lupine plants with different severities predispose the plant to suffer from wilting disorders. Moreover, a decrease in root WC, leaf area, leaf succulence, root length, DW and FW of the root, leaves and nodules is consequent to fungal infection. Interestingly, increased WC of stem revealed an important fact about water conservation strategy at the early stages of plant growth under fungal infection. Furthermore, epidermal cell area and number were conversely affected by *F. oxysporum* infection. Clustering the tested isolates on the base of considering 21 growth parameters clarifies the exact variance level rather than only considering wilt percentage. This study is different in its strategy because it represents an integrated investigation of a wide range of morphological and cytological parameters to find out the most reliable plant parameters responding to the wilt disease. DCA and CCA analysis were efficient in determining the variance between the selected isolates and in specifying the most important growth parameters that could express wilt precisely. Ultimately, this study uses linear and exponential decay models to correlate the determined growth parameters (leaf area, WC, DW and FW) to wilt and suggests using them as a nucleus for estimating wilting and isolates strength in later studies.

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