

Efficacy of fungal Rust Disease on Willow Plant in Egypt

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Abstract: Limited literature exists on the subject of disease control in woody plants in Egypt. Uredinio spores cycle of *Melampsora* sp repeatedly and reinvest willow (*Salix tetrasperma*) during the vegetation period. Different treatment were applied before and after the appearance of rust infection. The application of proline, gibberellin and methionine increased the severity of infection while sodium silicate decreased severity of infection where, the percentage of infected leaves was 51.53, 47.0, 46.44 and 22.88 respectively. On the other hand, application of *Trichoderma harzianum* and *Saccharomyces cerevisiae* gave reasonable control of leaf rust severity with infected leaves percentages of 10.69 and 19.18 % respectively compared with control (Plant inoculated with pathogen uredinio spores without treatment). The chlorophyll content a(a & b) were decreased in infected plant than in the healthy. The GC/MS analysis in this study revealed that great differences in the chemical structure and molecular weight of detected compounds in healthy and infected plant, although 12 and 8 compound were detected in healthy and infected plant respectively, two compounds only were similar in chemical structure Methyl 5-nitro-2,12-dioxocyclodecane- 1 - carboxylate and Methyl 5-nitro-2, 11 -dioxo-cycloundecane- 1-carboxylate were detected in healthy and infected plant respectively. Ultrastructures of healthy leaves confirmed the regular form of host cells and equal distances of the intracellular spaces with defenatly cell wall, mitochondria and large number of plastides. On the other hand distortion in cellular component and lyses of host cell wall were observed as a result of infection. The ultrastructure of intercellular hyphae and dikaryotic haustoria of *Melampsora* sp, and the host response to haustorial invasion was investigated with multishaped haustorial lobes were present inter- and intracellular in host cells

Key words: Rust disease, *Salix tetrasperma*, Control, Ultrastructures

INTRODUCTION

With more than 7000 species, rust fungi (Basidiomycota, Uredinales) are the largest group of obligate plant pathogens known to date (Aime, 2006). Among them, there are disease agents that severely affect field crops, mono- and dicotyledonous angiosperms, ferns, vegetables (s, ornamentals, fruit, and forest trees (Alexopoulos *et al.*, 1996 ; Agrios, 1997). Willows (*Salix* spp.) are grown as a major crop in short rotation coppice (SRC) willow plantations for renewable energy because of their yield potential and coppicing ability. Compared with annual crops, SRC is better suited for biocontrol because of the carry-over effect on biological control agents, assuming a 3 to 5 year harvest interval, *Salix* spp (willows) may also be used for phytoremediation. Lindbergh and Greger (1996); Giachetti and Sebastiani (2007) reported that Willow plants are capable of accumulating many different heavy metals. In Northern countries, *Salix viminalis* L. and *S. dasyclados* Wimm have been found to be suitable for use as bioenergy sources to provide an alternative to fossil fuels (Johansson & Alström, 2000). Besides energy production, willows can be used as a vegetation filter in the process of bioremediation of wastewater or contaminated land, and for increasing the content of organic material in soil (McCracken & Dawson, 1998; Nejad, 2005).

Monocultures of energy crops are endangered by various diseases and pests. The most widespread and frequent disease in willow plantations is leaf rust, caused by *Melampsora* spp. Mostly *Melampsora larici-epitea*, with many pathotypes, parasitizing on different willow clones, is found to cause the greatest damage in energy forest plantations (Pei *et al.*, 1993, 2002; Ramstedt, 1999; Ramstedt *et al.* 2002; Hurtado and Ramstedt, 2003). Leaf rust (*Melampsora larici-epitea* Kleb.) is considered a major limiting factor for growth of short rotation coppice (SRC) willow (*Salix* spp.), a bioenergy crop grown in Northern Europe (Larsson 1998; McCracken and Dawson 2003). It can cause premature defoliation, poor cold acclimation, and

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production losses (Rnnberg-Wstljung and Thorsén 1988). Biotrophic rust fungi in the genus *Melampsora* are common parasites of arctic willows (*Salix* spp.) with at least 12 willow species reported as hosts in arctic Canada alone (Parmelee 1989). Many species of *Salix* in the European and Asian arctic also have been reported to be infected with *Melampsora* rust (Beerling 1998). It is likely that none of the 400 or 500 species of *Salix* of the world are entirely free of rust infection. In temperate regions, *Melampsora* spp. are macrocyclic and heteroecious with *Tsuga*, *Abies*, *Larix* and *Ribes* spp. being described as aecial hosts (Sinclair 1987; Pei *et al.* 1993). The taxonomic status of *Melamposora* rusts and their associations with willow hosts are often unclear (Pei *et al.* 1993 and Fritz *et al.* 1996) In England, *M. epitea* infects several willow species, while other *Melamposora* species were found only to infect one species of *Salix* (McCracken and Dawson, 1992; Pei *et al.* 1993). Although Pei *et al.* (1999) stated that the association between willow (*Salix*) and rust (*Melampsora*) is highly specific.

Inducible resistance mechanisms such as systemic acquired resistance (SAR) are broad spectrum plant defense responses that can be induced biologically by challenging a plant with a weaker strain of a specific pathogen or exposing a plant to natural and/or synthetic chemical compounds (Elliston *et al.* 1977). Applications of low concentrations of jasmonic acid (JA) to plants induce proteinase inhibitors, proline-rich cell wall protein, and a range of enzymes involved in plant defense reactions (Sticher *et al.* 1997). Other chemical or plant extract agents with proposed SAR-inducing activity include various inorganic salts, compost and compost water extracts, low molecular weight proteins (elicitors) silicon, oxalate, phosphate, 2-thiouracil, polyacrylic acid, nucleic acids, unsaturated fatty acids and N-trimethyl-L-lysine (Kessmann *et al.* 1994). Because of economic, technical, and environmental considerations, control of leaf rust by fungicides is not favored. Alternative methods, including biological control, are thought to be a better choice (Pei *et al.*, 1996 & 2003). Antagonistic fungi are a major component of the microbial community on the plant rhizosphere and phyllosphere, and play a major role in regulating many interactions between plants and parasitic microorganisms (Jeffries, 1997). The fungus *Darluca filum* which behaves as a hyper-parasite on uredosori of the fungus *Melampsora epitea*, was also identified for the first time (Markovic *et al.* 2007). Fungi in the genus *Trichoderma* (Harman, 2004) has evolved multiple mechanisms that result in improvements in plant resistance to disease and plant growth and productivity. Strain T22 of *T. harzianum* generally increases plant growth and development and controls diseases in both commercial use and controlled experimental settings (Harman, 2000). *Sphaerellopsis filum* is a fungal hyperparasite that attacks a wide range of rust fungi, including willow *Melampsora* spp (Yuan *et al.*, 1999). The aim of this study was to (1) assess rust damage on willow in Egypt, (2) reveal the abundance of rust uredinia during the growing season, (3) estimate the impact of fertilization on leaf rust damage.(4) biological and metabolic control of rust disease (5) effect of rust on metabolic activity and ultrastructures of willow.

MATERIALS AND METHODS

Plant Cultivation:

Cuttings (18–20 cm length) of *Salix tetrasperma* stems were raised in 15 cm diameter plastic pots containing 1.5 kg autoclaved soil and irrigated with water in a greenhouse during the first days of February month.

Urediniospores Inoculation and Treatments Preparation:

Proline, methionine and gibberillic acid were used in treatments at concentration 50 ppm, Sodium silicate was used at 0.5 %, Suspension of *Trichoderma harzianum* and *Saccharomyces cerevisiae* was prepared from 7 and 3 days old cultures respectively by flooding cultures plates with 1-2 ml sterile water, collect spores with a pipette, and adjust the spore and vegetative cells of *T. harzianum* and *S. cerevisiae* respectively concentration to / ml in 0.0 1% Tween 20 in distilled water as a wetting agent and the spore suspension was applied (0.5 ml per plant) with a hand-held garden sprayer. After inoculation with urediniospores suspension according to (Onfroy *et al.* 1999) (Urediniospores were collected from old infected leaves of *Salix tetrasperma* and was adjusted at concentration spores / ml), each tray was covered with a clear plastic cover to maintain 100% relative humidity and plants were regularly sprayed with distilled water. Spray control plants with 0.0 1% Tween20 (Mould *et al.* 1991).

Treatments Application:

The plants were sprayed with chemical and bio-agents (*Trichoderma harzianum* and *Saccharomyces cerevisiae*) treatments at three different times, when they reached the three- to four-leaf stage, followed with

3 days was inoculated with urediospores and sprayed with treatments, then followed with 6 days (after appearance of rust pustules) were inoculated with urediniospores and retreated. The appeared symptoms were examined daily and number of pustules / leaf was detected. Three replicates were used for each treatments. Infection % = number of infected leaves / number of healthy leaves x 100

Quantitative Determination of Chlorophylls:

Chlorophylls content was determined according to Vernon and Seely (1966) using the following equations:

mg chlorophyll a / gm tissue = 11.63 (A665) -2.39 (A649).

mg Chlorophyll b / gm tissue = 2.11 (A649) -5.18 (A665).

mg chlorophyll a + b / gm tissue = 6.45 (A665)+17.72 (A649).

Where (A), denotes the reading of the optical density

Light Microscopy:

Samples of healthy and infected leaves of *Salix tetrasperma* were fixed in 6% glutaraldehyde buffered with 0.1 M phosphate, pH 7.4 for 48 h at 4 °C, dehydrated in ethanol and embedded in Epon 812 resin. Semi-thin sectioning (thickness 20 nm) cut with ultramicrotome were stained with bromophenol blue and examined under image analysis system microscope.

Electron Microscopy:

Electron microscopy studies were carried out at the Regional Center for Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt. Tissues of tissues healthy and infected *Salix tetrasperma* leaves were cut into small pieces. Tissue samples were fixed in a solution of 1% paraformaldehyde, 0.025% glutaraldehyde and 0.01 M phosphatebuffered saline (PBS, pH 7.2) for 10 h at 10°C, and then washed with the same buffer for 5 h at 10°C. Then the buffer was removed and the samples covered with an aqueous solution of 1% osmium tetroxide for 2 h. After this the osmium solution was removed and the samples dehydrated by passage through a series of ethanol concentration ranging from 50% to 96%. Embedding. The absolute alcohol was removed and propylene oxide was added to the sample for 1 h. The samples were put in propylene oxide and Epon 812 resin (2:1) then in pure resin for overnight, and placed in an oven at 60°C for 48 h. Small blocks were sectioned (50 nm) using ultra microtome. The sections were stained by uranyl acetate-lead citrate 500A and subsequently examined with the transmission electron microscope (C Joel Jem-1200 EX II. Acc. Voltage 120 KV. MAG- medium).

Gas Chromatography/mass Spectrometry (GC/MS) Analysis:

For a comparative study of the chemical components in healthy and infected plants, 5 g of fresh leaves were grinded in 10 ml chloroform and then filtrated and concentrated into 1 ml. The concentrated extract was placed in GC autosampler vials until they were analyzed. A Varian Star 3400 Cx Ion Trap GC/MS Shimadzu GCMS-QP 5050 A. software class 5000. Searched library: Wiley 229 LIB. Column: DBI, 30m, 053 mm ID; 1.5 um film. Carrier gas: Helium (flow rate 1 ml/min.). Ionization mode: EI (70 ev). Temperature program: 70 (static for 2 min) then gradually increasing (at a rate of 2 /min) up to 220 (static for 5 min). Detector temperature 250 injector temperature 250 The chromatographs were compared and individual peaks were identified by comparing mass spectra to the library references. at the Regional Center for Mycology and Biotechnology AL-Azhar University).

RESULTS AND DISCUSSION

Results:

Plant growth and fungal rust disease were studied under applications of different treatments, where the application of sulfur containing amino acid methionine and proline and chemical compound sodium silicate decreased the plant growth including length and width of leaves and length of lateral branches. On the other hand, the application of biocides *T. harzianum* and *S. cerevisiae* increased plant growth. The plant growth was clear at application of gibberellin, where the length of lateral branches was 30.67 cm, while the total number of leaves were decreased with comparison with other treatments (Table 1 & Fig. 1)

Table 1: Effect of different treatments on growth and severity of infection

Treatments with	Leaf					
	Length (cm)	Width (cm)	Length of lateral branches (cm)	Total number of leaves	Number of Infected leaves	Infected leaves %
Control (1)	9.33±1.53	2.33±0.15	12.9±0.17	48.66±1.53	0.0	0.0
Control (2)	9.33±1.15	2.26±0.21	12.66±0.58	60.33±1.53	23.66±1.53	39.21
Methionine	7±0.5	1.87±0.23	10.77±1.96	42.33±2.08	19.66±1.53	46.44
Proline	6.73±0.49	1.73±0.15	9.6±0.36	54.33±2.08	28±1.0	51.53
Gibberellin	8.63±1.27	2.07±0.15	30.67±1.15	34±1.0	16±1.0	47.05
Na silicate	7.17±0.15	1.83±0.25	10.47±0.92	51±1.0	11.67±2.31	22.88
<i>S. cerevisiae</i>	9.73±0.38	2.57±0.15	16.8±1.59	57.33±1.53	11±2.0	19.18
<i>T. harzianum</i>	8.33±0.47	2.3±0.10	12.8±0.35	53±2.0	5.67±1.53	10.69

Control(1) uninoculated and untreated plant

Control(2) Inoculated and Untreated Plants



Fig. 1: Growth of willow plant at different treatments

The application of these treatments was evaluated to increase the efficacy of immune system of plant against pathogen or reduce leaf rust severity of willow plant. The data in Table (1) and Figs.(2 &3), generally showed that *Salix tetrasperma* treatments with amino acids and gibberellin were very less effective than *Salix tetrasperma* treatments with *T. harzianum*, *S. cerevisiae* and sodium silicate. *T. harzianum* was found to be the best bioagent and significantly reduced leaf rust on willow. *T. harzianum*, *S. cerevisiae*, and sodium silicate exhibited significant reduction in leaves rust of willow with percentage 10.69, 19.18 and 22.88 % respectively. On the other hand, proline increased the sensitivity of willow to rust infection, where the percentage of leaves infection was 51.53% compared with control (inoculated with urediniospores and untreated) 39.2 1%.

After repeating the inoculation of urediniospores and treatment application the number of infected leaves increased at all treatments except sodium silicate and biocides (*T. harzianum* and *S. cerevisiae*) beside, the number of developed uredinia become unlimited on infected leaves in proline, methionine, gibberellin and control (inoculated & untreated plant) (Figs. 2 &3B, C, D & G).

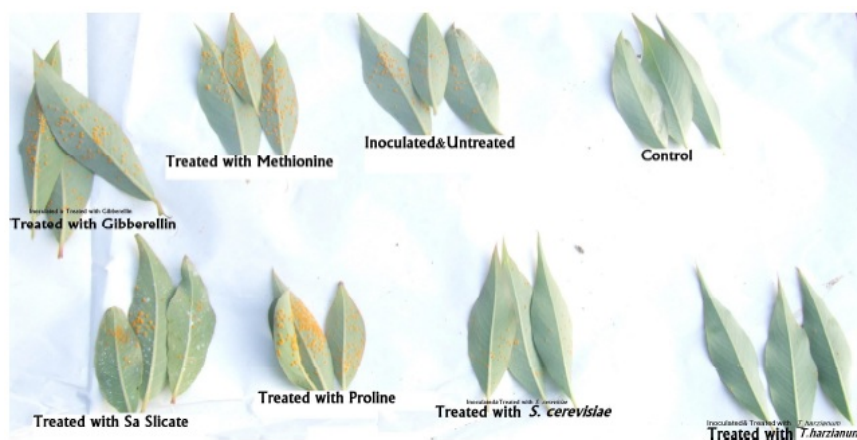


Fig. 2: Willow leaves infected with fungal rust disease under at different treatments



Fig. 3: Severity of infection on willow plant at different treatments (A) control without inoculation and untreated, (B) inoculated and untreated, (C) inoculated and treated with proline (D) inoculated and treated with methionine, (E) inoculated and treated with gibberellin, (F) inoculated and treated with sodium silicate, (G) inoculated and treated with *S. cerevisiae*, (H) inoculated and treated with *T. harzianum*.

The first appearance of uredinia on treated and untreated plant was after 6 days of uredinio spores inoculation on leaves surface, proline and then methionine were less effect on the pathogen than other treatments. After 6 days of inoculation the number of infected leaves was 8, 7,2,2,1, and 1 for proline,methionine, sodium silicate, *T.harzianum*, *S. cerevisiae* and control (inoculated & untreated plant) respectively. Each infected leaf in all treatments containing one uredinum at 6 days but with increasing time the number of infected leaves increased, also the number of uredinia on each infected leaf increased (Table 2), where at 15 days of inoculation the infected leaves and number of appeared uredinia respectively were 9 and 15 in control (inoculated& untreated plant), 15 and 21 in proline treatment, 5 and 8 in *T. harzianum* treatment. Additionally, the size of developed uredinum increased with increasing time after inoculation.

Table 2: Number of uredosours and infected leaves at different times after inoculation

Treatments with	Time (day)							
	6		9		12		15	
	NIL	NU	NIL	NU	NIL	NU	NIL	NU
Control (1)	0	0	0	0	0	0	0	0
Control (2)	1	1	2	2	6	10	9	15
Methionine	7	7	9	14	12	19	15	21
Proline	8	8	9	16	13	22	14	23
Gibberellin	2	2	4	4	15	20	15	22
Na silicate	2	2	3	3	8	8	8	16
<i>S. cerevisiae</i>	1	1	4	4	5	7	7	11
<i>T. harzianum</i>	2	2	3	4	3	5	5	8

NIL, number of infected leaves; NU, number of uredosours

The chlorophyll contents in infected plant was more than in healthy plant (Table 3) particularly chlorophyll a. However, no significant change in chlorophyll b content in infected or healthy plants. The presence of pathogen (*Melampsora* sp) on willow plant might affect the metabolic biosynthesis of metabolites in plant. Generally, the detected metabolites in the presence of the pathogen were less than detected in healthy plant (8 in the infected and 12 in healthy).The pathogen induced the biosynthesis of certain metabolites such as 1 -Phenyl- 1,2,3,4,-Tetrahydrophosphinoline, 1 0-Methoxy-Akuammilan- 1 7-ol, 1 0-Methoxy-Akuammilan- 1 7-ol, 3 -n-Hexyl-Delta -9 -tetrahydrocan-nabinol, 2,4,6- Triphenoxy-1,3,5-Triazine

(Table 3 & Fig. 4). On the other hand, compounds containing benzyne such as 1,2-Benzenedicarboxylic acid, diethyl ester, 1,2-Benzenedicarboxylic acid, diethyl ester, 1,2-Benzenedicarboxylic acid, diethyl ester not synthesized in infected plant. Two compounds similar in the molecular weight and molecular formula were detected in both healthy and infected plant Methyl 5-nitro-2,1-dioxocyclodecane-1-carboxylate (molecular formula $C_{14}H_{21}NO_6$ & molecular weight 299) and Methyl 5-nitro-2,1-dioxo-cycloundecane-1-carboxylate (molecular formula $C_{13}H_{19}NO_6$ & molecular weight 285) respectively.

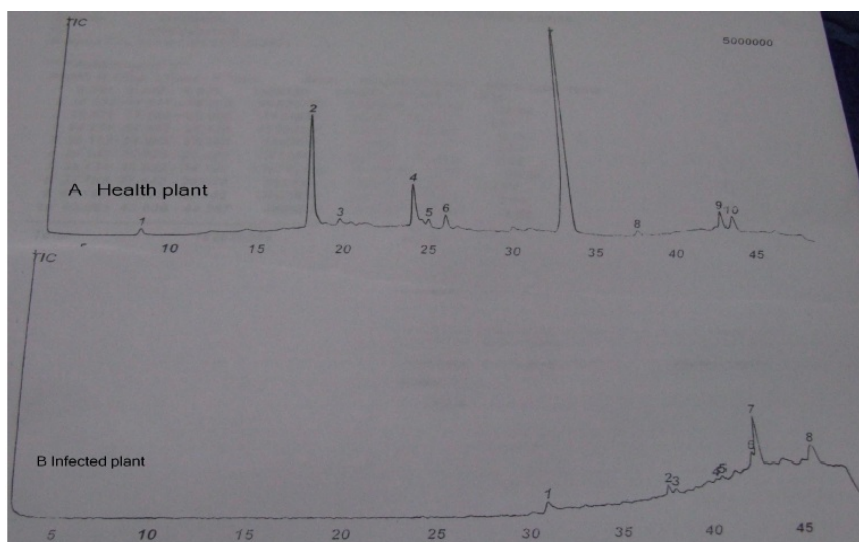


Fig. 4: Gas chromatography/mass spectrometry (GC/MS) chromatograms showing the peak of separated compounds in healthy (A) and infected (B) plant.

Table 3: Chlorophyll contents of healthy and infected willow plant

Chlorophyll Type	Chlorophyll content (mg/ g fresh weight) of	
	Healthy plant	Infected plant
a	15.25±0.43	11.13±0.26
b	3.99±0.74	3.06±0.16
a + b	19.24±0.38	14.28±0.03

Effect of rust infection of *S. tetrasperma* leaves by *Melampsora* sp was studied by light, and transmission electron microscopy. Germ tubes developed from urediniospores (Fig.5 Ur) via pre-formed germpores and extended enteroblastically. Germ tubes closely followed leaf surface features and occasionally penetrated epidermal cells directly. Such germ tubes were not seen to establish infection. Germ tubes generally entered leaves via stomata which appeared in the lower epiderms (Fig 5A). The epidermis of healthy leaves composed of two layer of cells (Fig 5 B.) while the infected leaves composed of one layer of cells (Fig 5C). On the other hand, the cells of mesophyll tissue were approximately regular in shape and size in healthy leaves (Fig.6A) while its irregular in infected leaves (Fig 7A). Ultrastructures of healthy leaves confirmed the regular form of host cells and equal distances of the intracellular spaces with irregular cell wall, mitochondria and large number of plastids (Fig.6B&C).

Later hyphae extended from cells subtending the primary haustorial mother cell and made contact and established infection in host cells lining the substomatal cavity. Although, the effect of pathogen on host cells was clear, the host cells become irregular in shape, the intracellular spaces and vacuoles increased in size (Fig.7A&B) Distortion in cellular component and lyses of host cell wall were observed (Fig.7E). The ultrastructure of intercellular hyphae and dikaryotic haustoria of *Melampsora* sp, and the host response to haustorial invasion was investigated. The intercellular hyphae share common characteristics with those of other uredinial stages of rust fungi. Clear septa were recognized inside the intercellular hypha (Fig. 7D). The formation of haustoria was observed in the pathogen which penetrate the host cells and large aggregations of host cell cytoplasm were present near haustoria in infected host cells (Fig7D) Multi shaped haustorial lobes were present inter- and intracellular of host cells (Fig. 7 C).

Table 4: GC/MS analysis of compounds in health and infected plant

Chemical compounds of					
Health plant			Infected plant		
Compound name	Molecular formula	Molecular weight	Compound name	Molecular formula	Molecular weight
Citroconic anhydride	C ₅ H ₄ O ₃	112	1-Phenyl-1,2,3,4,-Tetrahydrophosphinoline	C ₁₅ H ₁₅ P	226
1,2 - benzenedicarboxylic acid,diethyl ester	C ₁₂ H ₁₄ O ₄	222	10-Methoxy-Akuammilan-17-ol	C ₂₀ H ₂₄ N ₂ O ₂	324
Methyl 5-nitro-2,12-dioxocyclodecane-1- carboxylate	C ₁₄ H ₂₁ NO ₆	299	3-n-Hexyl-Delta - 9 -tetrahydrocannabinol	C ₂₂ H ₃₂ O ₂	328
3,4- dihydro-2-(morpholin-4-yl)-5, 7- dinitrospiro (cyclopentane-1, 3- quinazoline)	C ₁₆ H ₁₉ N ₅ O ₅	361	2,4,6-Triphenoxy-1,3,5-Triazine	C ₂₁ H ₁₅ N ₃ O ₃	357
Citronellyl acetate	C ₁₂ H ₂₂ O ₂	198	Methyl 5-nitro-2,11-dioxocycloundecane -1-carboxylate	C ₁₃ H ₁₉ NO ₆	285
Stearaldehyde	C ₁₈ H ₃₆ O	268	Palmitaldehyde	C ₁₆ H ₃₂ O	240
9-Octadecanoic acid	C ₁₈ H ₃₄ O ₂	282	2 -methyl-heptadecane	C ₁₈ H ₃₈	254
1,2 - Benzenedicarboxylic acid,diebutyl ester	C ₁₆ H ₂₂ O ₄	278	Unknown	-	-
1,2 - Benzenedicarboxylic acid,dioctyl ester	C ₂₄ H ₃₈ O ₄	390			
Thiocyanic acid,5,α-cholestan-3-β-y-lester	C ₂₈ H ₄₇ NS	429			
Hexadecane	C ₁₆ H ₃₄	226			
DL-Lauryl thio-DLPropionate	C ₃₀ H ₅₆ O ₇ S	514			

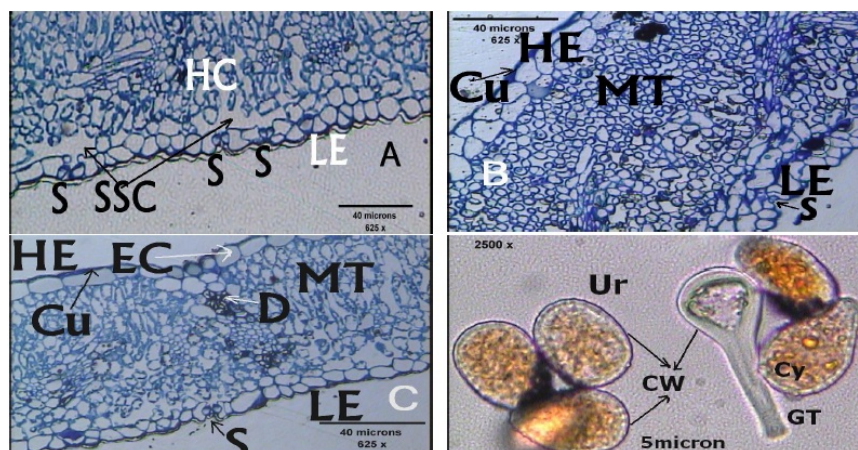


Fig. 5: Light micrographs of willow leaf showed (A) healthy leaf with normal host cells (HC), lower epidermis (LE) with stoma (S) and sub-stomatal chamber (SSC); (B) Healthy leaf with higher epidermis (HE) containing multilayer cells covered with cuticle (Cu) and normal mesophyll tissue (MT); (C) Infected leaf containing higher epidermis with one layer of epidermal cells (EC) and irregular cells of mesophyll tissue containing crystals druses (D); Urediniospores (Ur) of *Melampsora* sp containing clear cell wall (Cw) and cytoplasm, growing Urediniospore with germ tube (GT).

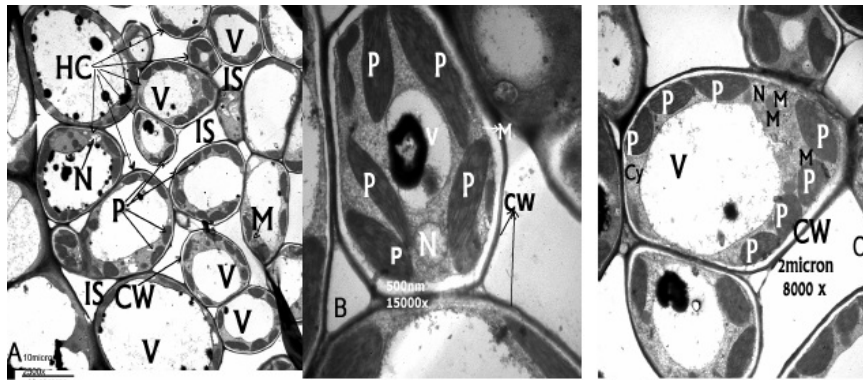


Fig. 6: Electron micrographs of healthy leaf of willow A, host cell (HC) containing cells with plastids (P), cell wall (CW), nucleus with regular intracellular spaces (IS); B enlarged part showing clear plastids, nucleus and vacuole (V); C host cell containing numerous plastids, mitochondria (M) and large vacuole in cytoplasm (Cy).

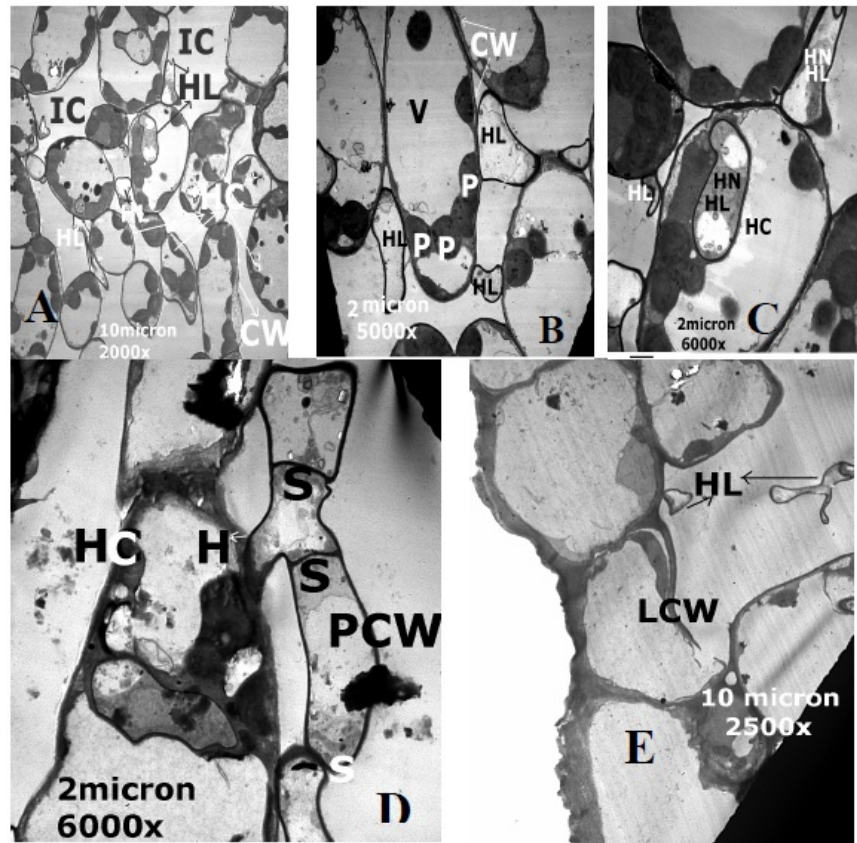


Fig. 7: Electron micrographs of infected leaf with *Melampsora* sp (A) irregular host cells containing haustorial lobes (HL) of pathogen with presence of large size of intracellular space (IC); (B) host cell containing thin cell wall (WC) with large vacuole (V) and limited number of plastids (P); (C) intracellular and intercellular haustorial lobes containing clear nucleus (HN) and mitochondria (HM); (D) hypha containing septum (S) and cell wall (PCW) present in the intercellular space penetrate the host cell (HC) wall with haustorium (H); (E) lysis of host cell wall (LCW)

Discussion:

Limited literature found concerning the control of disease in woody plants in Egypt. From our observation on rust of *Salix tetrasperma* in Egypt, it appeared in the late of February month. After inoculation of urediniospores of *Melampsora* sp on leaves of *Salix tetrasperma*, the disease symptoms appeared after 6 days on lower surface of leaves. This may be due to the ability of urediniospores to repeatedly part of their life cycle. While the appearance of orange pustules of urediniospores on lower surface of leaves may due to large number of stomata on lower surface which helped the urediniospores to penetrate the host leaves. Spiers & Hopcroft, (1988) stated that the penetration of plant leaves by the fungus is accomplished by germ tubes which are formed by the urediniospores. These germ tubes follow the leaf surface and enter the leaf through the stomata. This data was confirmed by examination the ultrastructures of infected and health plant. Also, the size of first appearance of orange pustules was very small and then increased with increasing vegetation time. It has been observed in many studies that first infection with leaf rusts occurs usually in late spring when the aeciospores produced on larch spread to willows (Pei *et al.*, 1995; Alexopoulos *et al.*, 1996). Six or seven days after spore germination, the first orange pustules containing urediniospores appear on the lower leaf surface.

Urediniospores cycle repeatedly and reinvest willows during the vegetation period, until late autumn when they form teliospores to over winter on fallen leaves (Pei *et al.*, 1999).

The obtained results showed that, there is no relation shape between increasing in plant growth and their sensitivity to their pathogen where *S. cerevisiae* and *T. harzianum* stimulated the plant growth and decreased the severity of infection. On the other hand, proline and methionine decreased plant growth but increased severity of infection. This phenomenon could be expected as a result of utilization of applied amino acids as a source of nutrient and energy or interference with plant metabolic pathways to prevent the biosyntheses of defense compounds. Leaves on fertilized plants were generally more damaged than on unfertilized plants. This indicates that plants with a good nutrient supply are more infected by rust fungus (*Melampsora pinitorqua*) than in poor habitats (Desprez- Loustau & Wagner, 1997). Plants invest less in leaf protecting chemicals in better nutrient and water conditions, causing higher susceptibility to plant pathogens (Bennett & Wallsgrave, 1994). Toome *et al.* (2006 and 2008) studied the willows rust by *Melampsora epitea* and found that infection increased significantly during the growing season, and the leaves collected from fertilized plants had more and larger rust pustules than those from unfertilized plants.

The data also showed that application of *T. harzianum* and *S. cerevisiae* gave the best control respectively among other using treatments in rust of *Salix* sp. The inhibition of rust severity by the antagonistic species when they were applied to leaves may be due to inhibitory substances produced by these bioagents or to competition to nutrients and space or induced the plant to produced defense chemical compounds against their pathogen. These results are in a good agreement with those obtained by Biles and Hill (1988), who found that *T. harzianum* was effective in reducing sporulation capacity of fungus *Cochliobolus sativus* on excised wheat seedling leaves. Also, Cook and Baker (1983); Mausam (2007) indicated that several *Trichoderma* spp have proved to be effective mycoparasites. Papavizas and Lumsden (1980) demonstrated that *T. harzianum* is known for parasitizing the mycelium of several important plant pathogens. Recently, Hamdy *et al.* (2001) stated that under greenhouse condition of plant guard (*Trichoderma harzianum* and yeast *Saccharomyces cerevisiae* gave reasonable control of leaf rust severity with disease reduction percentages of 64.29 and 19.14 % respectively. From our results sodium silicate plays an important role in the controlling of rust disease on *Salix tetrasperma* where the severity of infection was decreased than control plant (inoculated without treatment). Similar results were obtained by other researches (Fauteux *et al.* 2005; Bi *et al.* 2006) using this compound or other their salts. *In vitro* tests Guo *et al.* (2007) showed that sodium silicate, when added to potato dextrose agar, was effective in suppressing the radial growth of the pathogen *Trichothecium roseum* on the medium.

Several studies have suggested that Si activates plant defense mechanisms. Results by Chérif *et al.* (1992) suggest that a relationship exists between Si treatment, resistance to *Puccinia ultimum* attack, and expression of plant defense mechanisms. Although Datnoff *et al.* (1997); Belanger *et al.* (1995) stated that Si inhibits fungal disease by physically inhibiting fungal germ tube penetration of the epidermis. Recently Savvas (2009) found that the nutrient solution of silicon is capable of enhancing resistance to powdery mildew disease. Results obtained from the effect of rust disease on *Salix tetrasperma* showed that chlorophyll contents

(a&b) was decreased in infected plant than in the healthy. Reduction in photosynthesis was explained by McGrath and Pennypacker (1990) McCracken & Dawson, (1998); Pei *et al.*, (2003). They stated that rust severity was associated with decrease apparent in photosynthetic rates per unit of leaf area. However, Keutgen and Roeb (1996) found that, declined of photosynthesis might be due to the formation of appressoria and the growth of infection tubes into the stomata, leading to a reduction in gas exchange In the case of heavy detriment. The infection appeared on older leaves in *Salix* sp while young leaves were more resistant to infection for certain time and then infected. The young leaves may be containing more defense chemical compounds. This explanation agreed with Jon and Yong (2005) they shown that defense compounds are present in immature poplar leaves that likely contribute to *Melampsora medusae* rust immunity.

The GC/MS analysis in this study revealed that presence of waxes in healthy leaves such as Stearaldehyde, Hexadecane and Octadecanoic acid which interfere with leaves cutical, and therefore it may be play a protected role against pathogen. Hietala *et al.* (1997) found that epicuticular waxes contained *n*-alkanes, *n*-alcohols, *n*- aldehyde, wax esters and free fatty acids. On the other hand, most of the detected compounds in leaves in infected plant were not detected in the healthy plant, this may be duo to disorders in plant metabolic pathways as a result of presence of pathogen in host cells. Although, the pathogen induced the synthesis of important compound such as 3-*n*-Hexyl-Delta -9 -tetrahydrocannabinol. Pate (1994) stated that Cannabinoids are divided into three groups. The first are naturally occurring 21- carbon terpenophenolic compounds found to date solely in plants of the *Cannabis* genus, currently termed phytocannabinoids, The best known analgesic of these is - tetrahydrocannabinol. Also, the detected compound 1-Phenyl-1,2,3,4,-Tetrahydrophosphinoline in infected plant indicated for the relationship between the biosynthesis of salicylic compounds which plays an important role willow plant and resistant to their pathogen. According to Ruuhola and Julkunen-Tiitto (2000) Salicylates, the main phenolic glucosides of willow (*Salix* spp.), Salicylates are synthesized from phenylalanine (Phe) via the shikimate pathway. 2-Aminoindan-2- phosphonic acid (AIP), a strong inhibitor of Phe ammonia-lyase, was used to block the biosynthesis of salicylates. The results of ultrastructures revealed distortion of host cells as result of *Melampsora* sp infection and aggregations of cytoplasmic components near haustoria in infected host cells. Similar results were obtained on rust fungus *Uromyces euphorbiae* (Baka, 2002) and on rust fungus *Puccinia hemerocallidis* Mims *et al.* (2002).

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