DIAGNOSIS OF *VERTICILLIUM SP.* FUNGUS FROM SEA BUCKTHORN (*HIPPOPHAE RHAMNOIDES* L.)

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Abstract: *Sea buckthorn is a plant that has few diseases. Among the diseases that can occur in sea buckthorn plantation we mention: verticillium wilt (Verticillium albo - atrum, Verticillium dahliae), fusariosis (Fusarium sp.) and decay caused by fungi from the genera Phytium, Alternaria and Botrytis. Verticillium wilt can occur quite often in sea buckthorn plantations, the pathogen Verticillium sp. being dangerous and able to kill the shrubs during two years. This study was performed on sea buckthorn plantation suspected of being infected with Verticillium sp. from Variaş locality (Timiş County,Romania) belonging to the farm S.C. Classic Agro SRL Variaş. In order to establish an accurate diagnosis there were collected six samples of sea buckthorn (shoots of affected plants, entire plants) from the six cultivated varieties. The most affected variety was Clara (2.5 ha), the samples collected being only entire plants. Sea buckthorn samples were first analyzed visually, followed by microscopic analysis (stereomicroscope and microscope). The visual analysis of the sea buckthorn plants has highlighted the following symptoms: wilting leaves and twigs, yellowing of leaves followed by necrosis, poor developed plants collected and healthy plants, dry shoots, necrotic, primary root cortex soft, putrid, reddish brown in section, 25% of the roots of diseased plants have shown symptoms of decay, in section was observed vascular tissue getting brown, low actynorrhizae colonization, most of the active roots were not affected. The microscope analysis was performed on tissue sections from affected roots and shoots. There were placed tissue samples on different culture media (SAB, DRBC, water agar), which were then incubated for 6 days at 22^0C - 24^0C, obtaining laboratory cultures that have facilitated the correct diagnosis, because the fungal structures weren't visible on diseased plants. On the culture media have appeared fructifications of the fungus Verticillium sp. From the tissues infected with Verticillium sp. have grown mycelia and conidia of Fusarium sp. Both fungi are vascular being particularly dangerous and being able to lead to the plant death. Fusarium sp. has been installed on dead tissue from other causes. In laboratory tests it was confirmed the diagnosis of Verticillium wilt produced by de fungus Verticillium sp. in sea buckthorn shrubs from the cultivar Clara. The fungus was also present in other varieties of sea buckthorn from the plantation. The variety Clara is totally compromised, only 6% from the plants were healthy aspect and the remaining 94% were affected by the pathogen.*

Key words**:** *sea buckthorn, Verticillium wilt, Verticillium sp., diagnosis, infection, analysis.*

INTRODUCTION

In present the sea buckthorn (*Hippophae rhamnoides* L.) has few diseases and pests. From the diseases that can appear in sea buckthorn plantations we are mentioning verticillium wilt (*Verticillium albo atrum* şi *Verticillium dahliae*), fusariose (*Fusarium sp.*) and decays (*Pythium sp., Alternaria sp., Fusarium sp., Botrytis sp*).

The verticillium wilt of the sea buckthorn produced by the soil fungi *Verticillium albo - atrum* and *Verticillium dahliae* appear relatively often in sea buckthorn being a very

dangerous disease. Usually it appears in the sea buckthorn plantations after 5 – 8 years from planting (S. C. THOMAS LI, 2002).

The Verticillium species are common in many soils and they have a great number of host plants with preferences for some certain hosts (tomatoes, peppers, strawberries, sea buckthorn, potato *etc*.) (STEFFEK *et al*., 2006). Sea buckthorn (*Hippophae rhamnoides L*.) is one of its favourite hosts.

This disease can prevent a serious problem on the susceptible hosts from the infested soils because the fungus persists in soil for an undetermined time period, many times on multiple hosts without any symptom. Due to its capacity to produce systemic infections able to kill the plant, verticillium wilt being considered a serious disease. Often it can be confounded with the damages produced by other diseases or by the unfavourable environmental conditions that can have identical symptoms (CYNTHIA L. ASH, 2013).

After S. C. Thomas Li (2002) the symptoms manifested are the progressive yellowing of the leaves, the decolouring of the vascular tissue, the asymmetric growth of the leaves. The attacked fruits are getting dry. During two years an attacked shrub can die. The symptoms caused by *Verticillium sp*. can appear any time during the plants vegetation period, but usually are visible during July and August. In some cases the symptoms can be more severe during or after a cold period of time. The symptoms can be chronic or acute and frequently they are lethal. The chronic symptoms includes yellow foliage with small dimensions, leaves burns (on the leaves edges), slow growing, devitalisation of the offshoots and branches. Often the leaves from one of more many branches can wilt totally. The acute symptoms are including the drying of the leaves or an abnormal red or yellow colouring of some leaves parts comprised between the nervures, partial defoliation, wilting and devitalisation of the leaves. Many times is affected a part from the plant. In its lethal forms *Verticillium* is able to kill the plants (PEGG, G. F., 1974; CYNTHIA L. ASH, 2013).

The fungus survives as saprophyte in soil as mycelium and mycrosclerots. In some plants the fungus can survive in leaves and persists as mycelium or mycrosclerots than when the leaves are falling down on the soil (QIN Q. - M. *et al*., 2008). The mycrosclerots are viable for 10 years and even more (even 14 years) in soil without a host plant. With all these, the warm soils, full of water are leading to the fast death of the mycrosclerots.

The plant multiplication farms set on fields previously cultivated with vegetables or fruit trees are susceptible to a high infection risk. The plants from these multiplication stands can develop the disease after they were transplanted. The mycrosclerots can be formed on the resistant plant roots and not manifests symptoms, but is determining the introduction of the fungus in the non-colonized soils through the infected vegetal material, including seeds, offshoots, plantings, tubercles *etc*. The working machineries can also spread the pathogen.

After JONES and CRILL (1975) that have studied the resistance of the tomatoes to verticillium wilt, the pathogen can be controlled through planting of resistant varieties. With all these, the devitalisation of the infected plants became more severe from a year to another.

The researches from this field are showing that it is impossible to eliminate all the mycrosclerots of *Verticillium* from an infested soil. There isn't any treatment for *Verticillium* sp., but there are many cultural and preventive strategies and that helps to the management of the disease and helps to the management of the disease and helps the infected shrubs to leave with the fungus. There must to be avoided the planting of the susceptible varieties (STEPHANIE PORTER, 2013).Thus, it is recommended the removing of the infected plants and the replanting of tolerant or resistant varieties, avoiding of the stress produced by drought or flooding because these are accelerating the evolution of the disease, the disinfection of the scissors used for the

cuttings, the acquisition of planting material from trusted sources. The sea buckthorn must to not be replanted on that infested field for at least 3-5 years [S. C. THOMAS LI, 2002; 2003.].

The objective of this work is the diagnosis of the fungus *Verticillium sp*. in a sea buckthorn plantation from Timis County (western Romania) suspected to be infected with this pathogen. The plantation is young (third year) and the *Verticillium* symptoms have appeared starting with the first year from the planting, without to be diagnosed by any specialist.

MATERIAL AND METHODS

The sea buckthorn plants collected with roots have been transported to the laboratory and sectioned considering two distinctive zones for the highlighting of the presence of the pathogen: the roots zone and shoots zone with evident disease symptoms, recognized through the existence of the yellow and necrosis leaves, dry shoots, brownish roots, black vessels in section. From roots and shoots were taken pieces of 1-1.5 cm of tissue that have been disinfected by immersion for two minutes in solution of hypochlorite 1:10, the samples were washed twice in sterile distilled water for 5 minutes and then dried on sterile absorbent paper. From these pieces were realised transversal sections of 1-2 mm that were placed on the surface of the culture media in Petri plates (figure 1).

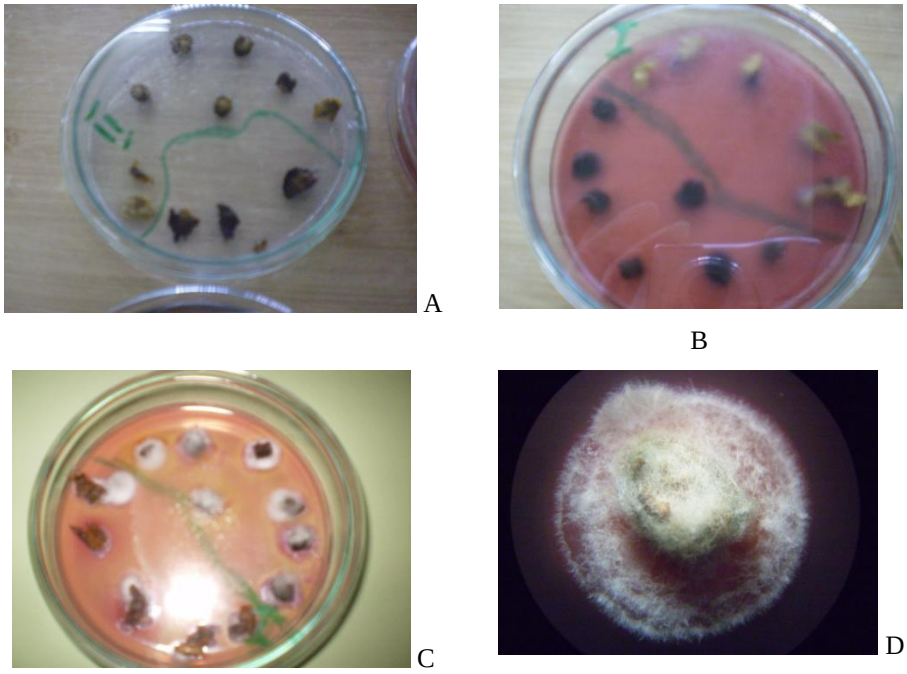


Figure 1. A and B – placing of the plant tissue on the culture media; C and D – growth of the *Verticillium sp*. mycelium (OTILIA COTUNA, RENATA ȘUMĂLAN, 2013)

The variants of the culture media used for the isolation of the pathogen were the following: V1 – water – agar (tap water + 1.5% agar), recommended media for the rapid differentiation of the fructification formations of the fungi and reduced vegetative growth (PAPACOSTEA, 1976), V2 - Sabouraud, (dextrose 4%, peptone 1%, agar 2%) recommended for the growth of the filamentous and membranous fungi saprophytic and parasitic (DOLAN, 1971) and V3 - DRBC

(peptone 0,5%, glucose 1%, potassium dihydrogen phosphate 0,1%, magnesium sulphate 0,05%, dichloran 0,002%, Rose – bengal 0,0025% and agar 1,5%), media recommended for the isolation of the filamentous fungi and of the easts from food products with vegetal origin (PITT, 2009).

For every variant there were done three replicates and in every plate were placed 10 sections of vegetal tissue. The plates were incubated at 22 ± 2^0C, at thermostat in the dark conditions, at least five days. In the sixth days we made microscopic observation for each section of plant stem basis and shoots. Data were analysed according to DOOLOTKELDIEVA (2010), with modification, using the formula:

$$\text{ICS (\%)} = \frac{\text{number of contaminated sections}}{\text{total number of sections on plates}} \times 100$$

The microscopic examining was realised to the stereomicroscope (4x), being completed with microscopic examination on native material (10x).

The ICS (Index of contaminated sections) for each variant were reported as mean ± standard deviation.

RESULTS AND DISCUSSIONS

The sea buckthorn samples consisting on shoots from the affected plants and entire plants were collected from a sea buckthorn plantation suspected to be infected with *Verticillium sp.* from the area of Varias locality (Timis County, Romania) belonging to the farm unit S. C. Clasic Agro S. R. L. Variaș. This plantation was planted four years ago, there being planted six sea buckthorn varieties with different resistance to verticillium wilt. At the moment of planting there wasn't considered the previous crop (tomatoes - that are hosts for this pathogen). The first symptoms have appeared starting with the first year from planting, there being suspicion that the planted material was infected from the plant nurseries. The infection in this plpantation is a particular case because verticillium wilt is installing usually in the older sea buckthorn plantations (after 5-8 days).

There were collected 6 samples from the 6 cultivated varieties. From the variety Clara (2.5 hectares) the most affected the samples were consisting in entire plants (with roots). The variety Clara is compromise almost in totality, only 6% from the plants having a healthy aspect, and the other 94% being affected by the pathogen.

In the first stage the plants were analysed visually both in field and in laboratory. The visual analysis of the sea buckthorn plants has highlighted the following symptoms: the wilting of the leaves and offshoots, the yellowing of the leaves followed by necrosis, the small size of the diseased plants from the variety Clara in comparison with the healthy plants from the same variety; dry and death shoots; the cortex of the primary roots softened, decayed, brown reddish colour in section; 25% from the roots of a diseased plant were presenting decay symptoms; in section were noticed browning of the vascular tissues; the colonisation with actinorrhyzae is reduced, the ones from the affected roots being not active (figure 2).

In the diagnosis phase of the infection in laboratory, the researches have analysed the improvement of the laboratory methodology regarding the isolation of the pathogen on culture media in the case of the *Hippophae rhamnoides* plants that were presenting evident affections of the attack of the *Verticillium sp.* fungus.

The analyses to the binocular magnifier and microscope were realised after the tissue segments from the roots and shoots were placed on different culture media (SAB, DRBC, water - agar) that later were incubated for 6 days at the temperature of 22^0 - 24^0 C. the obtaining

of the fungal cultures in lab has facilitated the setting of the diagnosis because the fungal structures of *Verticillium* weren't visible on the diseased plants.

On the culture media have grown fungi from the genera:*Verticillium sp*. (figure. 3); *Fusarium sp.; Alternaria sp.; Cladosporium* and *Mucor*.

Figure 2. A – dried shoot due to the infection with *Verticillium sp*.; B – the cortex of the root softened and browned; C – section through the diseased root; D – death vascular tissue (OTILIA COTUNA, 2013)

Table 1

Results regarding the fungal contamination of stem roots and shoots sections (%)

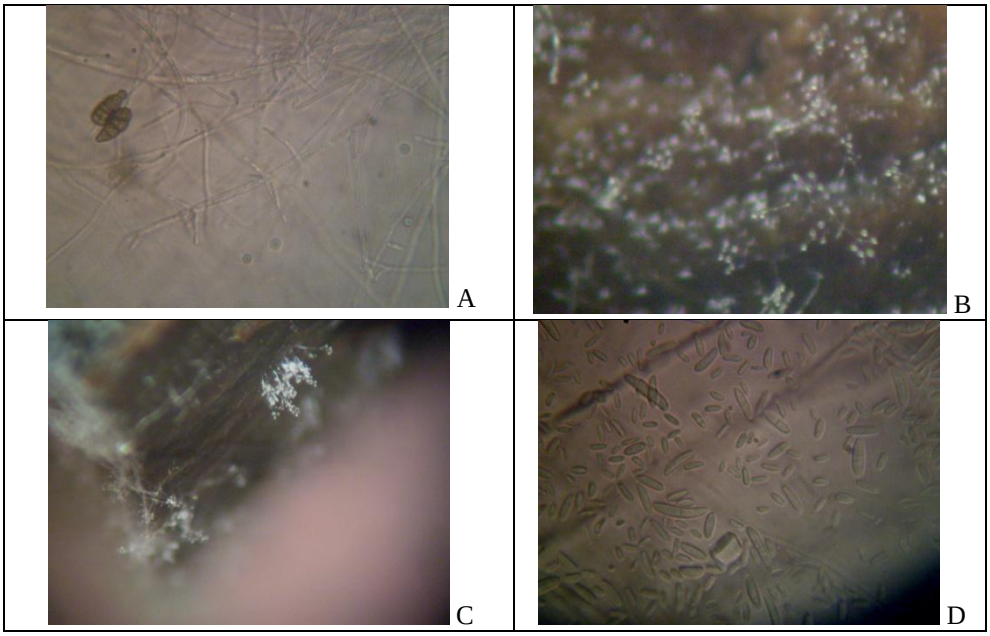
Variants	Control mean group (%)	AW		SAB		DRBC		DL limits
		means ±SD	Diff and signif.	means ±SD	Diff and signif.	means ±SD	Diff and signif	DL 5% DL 1% DL 0,1%
Root sections	32,24	6,73±11,48	-25,5 o	13,33±5,77	18,91- No signif.	76,6±11,54	44,4 *	21,02828 31, 84283 51,15451
Shoot sections	64,44	43,33±11,54	-21,1 o	66,66±11,54	2,22 No signif.	83,33±5,77	18,8 *	13,33611 20,19468 32,44213

Diff<5%=* (significant positive), diff<5%=o (significant negative)

Regarding the percentage of the sections contaminated with fungi there was noticed that on the water-agar was registered the lowest percentage of colonization of the sections, both for the root sections and for the shoots sections. The greatest values of the fungal

contamination index were determined on the DRBC media, the average values being statistically provided (highly significant) (Table 1).

The microscopic examinations were revealed a great fungal diversity on this medium, mainly on the root sections, there being identified mainly species belonging to the genera *Fusarium sp*. (figure 3), *Trichoderma*, *Verticillium* and *Alternaria*.



Figures 3. A –*Verticillium sp*. solitary fialids; B and C – conidiofores growth on vegetal tisues; D – micro and macroconidia of *Fusarium sp*. (OTILIA COTUNA, RENATA ȘUMĂLAN, 2013)

The DRBC mediua succeed to satisfy the nutritive demands of the plant pathogen fungi and can be used for the diagnosis of the *Verticillium sp.* attack, the fungus fructificationd appearing after five days. On water-agar the fungi are growing easy and fructify fungi with ubicuitary spread, without special nutritional needs.

On the shoots sections cultivated on DRBC haven't been registered sections contaminated with *Verticillium sp.* in any replicate. This fact suggests the localisation of the pathogen at the root or crown level much previous to the signalling of the affection at the level of the aerial part, respectively the shoots.

The studies are showing that the *Verticillium sp.* can enter in the root system directly or through the lesions provoked naturally by the root growth and by the organisms from the soil. After the entering in the plant tissues the fungus produces toxins and invades the xylem and then continuates the advancing in the plant through the produced spores. The toxins produced by *Verticillium* can close the plant cells at some distance from those directly invaded. From this reason the fungus cannot be isolated many times from the apex of the diseased shoots or from wilted branches, even the deterioration is very evident.

As response to infection the host plant produces some certain tiloses through that it tries to close the invaded cells, in this way limiting the fungal movement in plant. Practically this closing of the infected vascular tissue reduces the water flow from roots to the aerial part.

In this moment the diminishing of the water flow and the toxins produced by the fungus are leading to the external symptoms (CYNTHIA L. ASH, 2013).

CONCLUSIONS

After the laboratory analyses there is confirmed the verticillium wilt diagnostic in the sea buckthorn plants in the variety Clara. The fungus is present in the other varieties from the plantation. The variety Clara is compromise in totality, 94% being affected by the pathogen.

Near to *Verticillium sp.* on the culture media there have grown *Fusarium sp.* on the tissue sections too. Both fungi are vascular and very dangerous, being able to lead to the plant death. Many times *Fusarium sp.* is installed on death tissue from different reasons.

The greatest values of the fungal contamination index were determined on the DRBC media, the values being statistically provided (highly positive). In contrary, the water-agar media have shown the lowest colonisation rate of the sections, both through the ones from roots and the ones from diseased shoots.

Having in view the obtained results there is recommended in a first stage the clear cut and burning of the diseased plants from all the 2.5 hectares affected by the pathogen (variety Clara), the elimination of the infection sources appeared in the other varieties through the burning of the infected vegetal material, maintenance of a strict cultural hygiene in plantation. The replanting of the affected surface (2.5 ha) will be done with a variety resistant ti verticillium wilt after the previous soil sterilisation. The reccomedation of the specialists in field is to not plat in the *Verticilliuum* affected field for at least 3-5 years.

BIBLIOGRAFY

1. ASH CYNTHIA L., 2013 - *Verticillium wilt of trees and shrubs*, disponibil pe http://www.extension.umn.edu/garden/yard-garden/trees-shrubs/verticillium-wilt/.

2. DOLAN C. T., 1971 - *Appl. Microbiol*. 21. 195 – 197.

3. DOOLOTKELDIEVA T. D., 2010 - *Microbiological control of flour - manufacture: Dissemination of mycotoxins producing fungi in cereal products.* Microbiology Insights 2010, 3:1 - 15.

4. JONES J. P. and CRILL P., 1975 - *Reaction of resistant, tolerant and susceptible tomato varieties to Verticillium wilt.* Plant Dis. *Rep*. 59:3 - 6.

5. PAPACOSTEA PETRE, 1976 - *Biologia solului*, Editura ştiinţifică şi enciclopedică, Bucureşti.

6. PITT J. I., HOCKING A. D., 2009 - *Fungi and food spoilage*, Ed. Springer, New York, 2009:53 - 142.

7. PEGG G. F., 1974 - *Verticillium disease*. Rev. Plant Pathol. 53:157 - 82.

8. PORTER STEPHANIE, 2013 - *Verticillium Wilt Strikes Again*, University of Illinois Extension, Issue 12, july 2013, disponibil pe http://hyg.ipm.illinois.edu/article.php?id=518.

9. STEFFEK ROBERT, ANDREAS SPORNBERGER, JOSEF ALTENBURGER, 2006 - *Detection of Microsclerotia of Verticillium dahliae in Soil Samples and Prospects to Reduce the Inoculum Potential of the Fungus in the Soil*, Agriculturae Conspectus Scientificus, Vol. 71 (2006) No. 4 (145 - 148).

10. THOMAS LI S. C., 2002 – *Product development of sea buckthorn*. In: J. Janick and A. Whipkey (eds), Trends in new crops and news. ASHS Press, Alexandria, VA, p. 393 – 398.

11. THOMAS, S. C. LI, 2003 - *Sea buckthorn (Hippophae rhamnoides L.): Production and Utilization*. Canada: National Research Council of Canada, ISBN 0 – 660 – 19007 - 9.

12. QIN, Q.-M., VALLAD, G. E., AND SUBBARAO, K. V., 2008 - *Characterization of Verticillium dahliae and V. tricorpus isolates from lettuce and artichoke.* Plant Dis. 92:69 - 77.