PERIODICO di MINERALOGIA Volume 89, No. 2, 2020

Biochemical Investigations for Studying the Effect of Mycorrhizas on Mulberry Vitroplants (*Morus* sp.)

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Abstract

This study presents the biochemical investigations performed for pointing out the effect of the fungi of vesicular-arbuscular mycorrhizas on vitroplants of Morus sp. (Calafat and Ichinose genotypes). For this purpose inorganic P concentration assay through Fiske – Subbarow method was performed and the concentration of the proteins was determined by Bradford method. At the same time, electrophoretic analyses were carried out of the proteins extracted from the colonized roots.

Keywords: mulberry, root, fungi of vesicular-arbuscular mycorrhizas, symbiosis, phosphorous, protein.

Introduction

Morus sp. is an important and acknowledged source of natural products that may be used for pharmaceutical, cosmetic, food, sericicultural purposes, etc. Its multiple utilizations make of this species one with economic implications that recommend it for the culture in the system of sustainable agriculture.

According to the principles of sustainable agriculture, the biofertilizers of the mycorrhizal type count among the approved fertilization methods. In nature, the mycorrhizal fungi are indissolubly linked to the processes of growth and development of the plant, especially in microbe- rich and nutrient – poor environments [1].

The mycorrhizas increase the natural resistance to plants to abiotic and biotic stresses, they make it possible the more efficient exploitation of the soil resources, they facilitate the obtaining of higher quality plants in conditions of low chemical input, they improve the transportation and mobility of the ions in the soil, etc. [1;2].

The mycorrhizal type of association is considered as a mutualistic symbiosis, because the host plant receives the mineral elements via mycelium and the heterotrophic fungus benefits by the compounds obtained through the photosynthesis carried out by the host [3].

The aim of our researches was to point out the effect of the fungi of vesicular-arbuscular mycorrhizas on vitroplants of *Morus* sp. (Calafat and Ichinose genotypes), determined through inorganic P and protein assay, as well as by the analysis of the electrophoretic spectrum of the proteins.

This study points out for the first time in Romania, at biochemical level, the benefic effect of the mycorrhizal symbiotic associations between the mulberry vitroplants and the fungi of vesicular-arbuscular mycorrhizas (VAM).

Materials and Methods

The Biological Plant Material

The mulberry vitroplants (Calafat and Ichinose genotypes) were obtained through micropropagation, using the technique of *in vitro* culture of axillary meristems prelevated from annual young shoots grown on mature mulberry trees from the germplasm collection of S.C. SERICAROM S.A. The culture medium used for micropropagation consisted in the basic medium Murashige and Skoog [4], supplemented with 2 mg/l BAP (6-benzylaminopurine), 1 mg/l KIN (kinetin), 30 g/l sucrose and 7 g/l agar [5]. The rooting medium consisted in the same basic medium, but supplemented with 1mg/l IAA (indole-3-acetic acid) and 30 g/l sucrose. The culture vessels were incubated at temperatures of 22±2ºC during the photoperiod (16 h), and respectively 18±2ºC during the period of darkness (8 h).

The Biologic Fungal Material

The fungal material consisted of a *Glomus* inoculum under the form of the commercial product "endorize SOL" (10 propagule/g), produced by the "BIORIZE" Company under INRA license.

The Experimental Colonization of the Root System

Experimental inoculation of the root system was carried out in the moment of vitroplant transfer on peat type substrate (autoclaved peat). The quantity of inoculation used for each vitroplant was of 0.5 respectively 1 g of commercial product "endorize SOL"/pot. No VAM fungi inoculation was used in the case of control in which the substrate consisted in the autoclaved peat. The vitroplants were subsequently maintained in the greenhouse, being watered periodically.

Obtaining of Total Protein Extract

The roots were mortared with quartz sand up to the homogenization. The extraction of proteins was carried out in Tris-HCl 20 mM buffer with pH 8, containing 10 mM NaHCO3, 10 mM MgCl2, 0.1 mM Na_2EDTA, 10 mM β-mercaptoethanol, 10% sucrose, 0.1% Triton X-100 at 4ºC for 24 hours, in order to optimize the extraction method. After centrifugation at 18.000 rpm, for 20 minutes, the supernatant was used for assay of the total proteins and P.

Assay of the Phosphorus from the Soil using Fiske-Subbarow Method [6]

Orthophosphate was spectrophotometrically determined assaying by the reaction with the ammonium molybdate in acid medium. Phosphomolybdate was reduced in low acid medium to "molybdenum blue" that was spectrophotometrically assayed. The reducing agent was 1 amino-2 naphtol-4 sulphonic acid.

Proteins Assay by Bradford Method

Bradford method [7] is based on the binding of the Coomassie Brilliant Blue G-250 a staining reagent used for proteins and the determination of absorption of the complex formed at 595 nm. The dye presents the red color that turns to blue when the dye formed a complex with the protein.

Total Migrated Proteins

After the electrophoresis in the denatured system SDS-PAGE discontinuous buffer the gels were incubated 30 minutes in a solution of 40% methanol-10% trichloracetic acid and then they were stained with Coomassie blue R250, G250 prepared in ethanol-methanol and acetic acid 10%. Gels were stand in an 7% acetic acid solution.

Determination of the Molecular Weights (Mw) of the Proteins

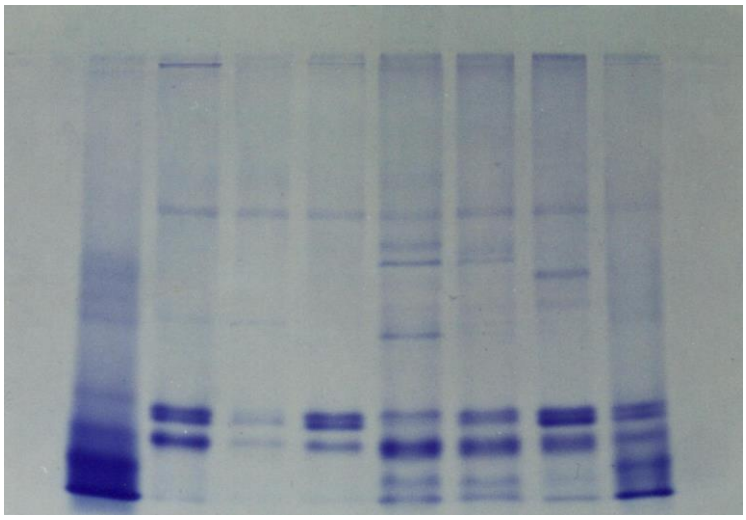
Determination of the molecular weights of the proteins from the analyzed samples was carried out using markers that contains proteins with known molecular weights (marker of molecular weight – kit Sigma with proteins that have Mw between 14,200-66,000 Da). The Mw estimation was carried out by determination of the retention factor (Rf) that is

proportional to lg molecular weights of the proteins (Mw) from the marker. The molecular weights of the proteins (Da) from samples is determined by the extrapolation of Rf values that characterize these proteins, on the graphic Rf=f (Mw).

Results and Discussions

Our previous researches aimed at pointing out the bio-fertilizing, bioregulating and bioprotecting effects of the VAM fungi on the vitroplants of *Morus* sp. through morphometric, volumetric, and gravimetric determinations. These researches were confirmed by qualitative (the electrophoretic spectrum of the proteins) and quantitative biochemical analyses (dosing of inorganic P and of total proteins).

The analysis of electrophoretic spectrum of the proteins extracted from root tissues proves the existence of a variable number of protein bands in case of plants that established mycorrhizal symbiosis compared to the control (Figure 1).



E C1 C0.5 CM I 1 I0.5 IM E

Fig. 1. The electrophoretic spectrum of the proteins extracted from mulberry (*Morus* sp.) roots, colonized with mycorrhizas (E – marker of molecular weight; C1 – Calafat genotype inoculated with 1g of "endorize SOL"; C0.5 – Calafat genotype inoculated with 0.5 g of "endorize SOL"; CM – control for the Calafat genotype; I1 – Ichinose genotype inoculated with 1g of "endorize SOL"; I0.5 – Ichinose genotype inoculated with 0.5g of "endorize SOL"; IM – Ichinose genotype non-inoculated with "endorize SOL").

In the case of Ichinose genotype the number of electrophoretic bands is higher with a unit (11 against 10 bands) at the samples I1 and I0,5 compared to the control. This electrophoretic band appeared in the case of mycorrhizal roots is due to the effect of mycorrhizal colonization. The number of electrophoretic bands in case of the Calafat genotype is smaller with a unit (4 against 5 bands) for the C1 sample compared to control and C0.5, so the relation between the number of protein bands and the concentration of the inoculum is not maintained. These observations lead to the idea that the number of protein bands varies depending on the concentration of the inoculation used at this genotype.

The molecular weights of the proteins that were determined electrophoretically are presented in Figure 2 and Table 1.

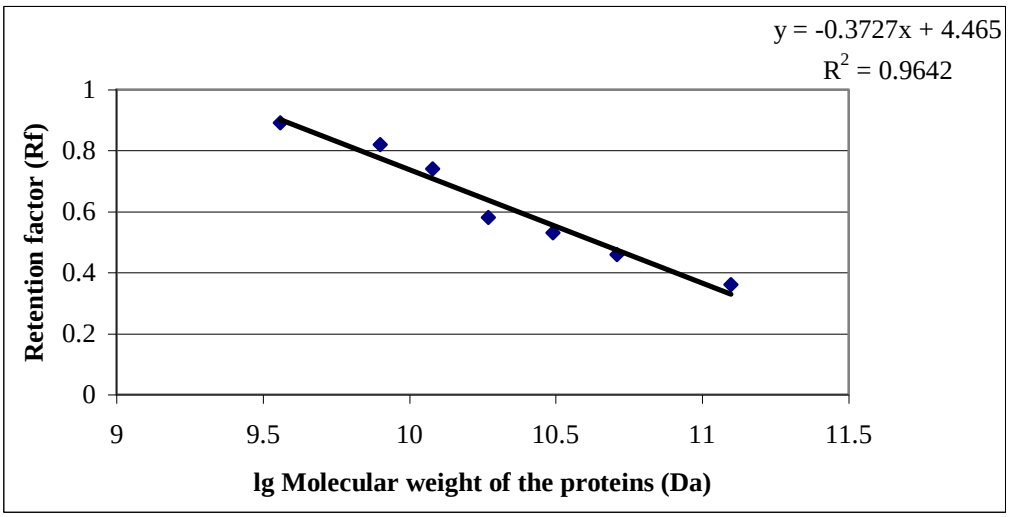


Fig. 2. Variation of the molecular weight of proteins (Mw) with the retention factor (Rf).

Table 1. Molecular weight of the electrophoretically pointed out proteins

Sample MC Mw (Da)	Sample C0.5 Mw (Da)	Sample C1 Mw (Da)	Sample MI Mw (Da)	Sample I0.5 Mw (Da)	Sample I1 Mw (Da)
11.04105	11.04105	11.04105	11.2557	11.01422	11.3362
10.3971	10.3971	9.860478	11.20204	10.79957	11.01422
9.860478	9.860478	9.806815	11.01422	10.77274	10.63858
9.806815	9.806815	9.672659	10.77274	10.50443	10.50443
9.672659	9.672659		10.34344	10.3971	10.3971
			9.860478	10.34344	10.34344
			9.806815	9.860478	9.860478
			9.672759	9.806815	9.806815
			9.618997	9.672759	9.672759
			9.458009	9.618997	9.618997
				9.458009	9.458009

The possibility of identifying certain compounds of protein nature is opened, which may prove to be useful to complete the vast range of the biosynthesized protein compounds of mulberry plants, that are recognized for the abundance of active principles used in human and veterinary medicine, in cosmetics, food, etc.

Our experiments went on with the determination of P variation in mulberry roots colonized with VAM fungi. The P concentrations in the analyzed samples were determined on the basis of the standard curve presented in Figure 3.

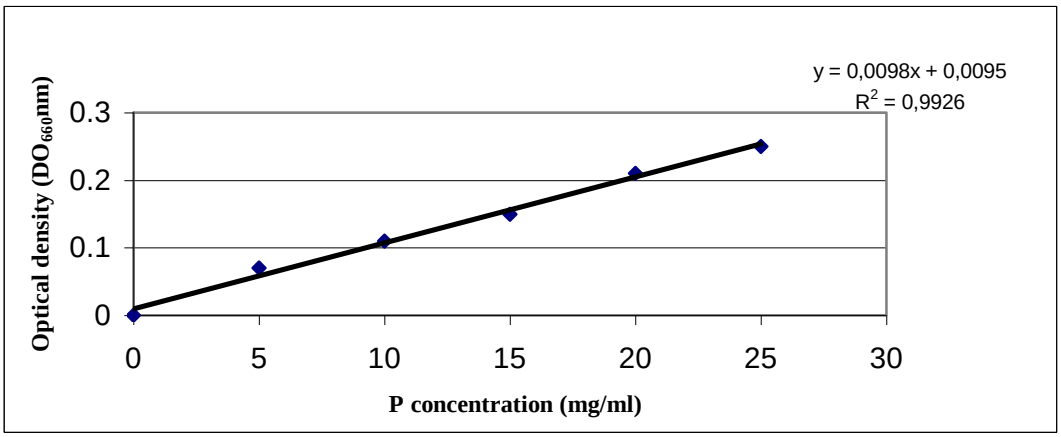


Fig. 3. Variation of P concentration in mulberry roots colonized with mycorrhiza fungi depending on the optical density at 660 nm.

Table 2. P concentrations in mulberry roots colonized with different quantities of fungal inoculum

Samples	CM	C0.5	C1	IM	I0.5	I1
P concentrations (mg/ml)	13.97959	14.08163	11.78571	14.54082	15.66327	16.88776

On the basis of the P concentration presented in Table 2 it may be seen that, in some cases, there is a direct relation between the genotype, the presence of VAM inoculum and their effect over P concentration in the roots. For the Calafat genotype, it is ascertained that in the C1 variant the P concentration is lower than in the C0.5 variant and even smaller than in the CM variant. On the contrary, for the Ichinose genotype a constant increase of the P concentration is ascertained, increase that confirms the bio-fertilizer effect that the mycorrhizas have on the plant roots.

The studies regarding the beneficial effect of the mycorrhizal fungi was continued with performing quantitative analysis of the protein content in the roots of mulberry vitroplants.

The concentration of proteins was determined on the basis of the standard curve represented in Figure 4. The use of different quantities of VAM inocula determined a variation of the concentration of proteins depending on the genotype (Table 3).

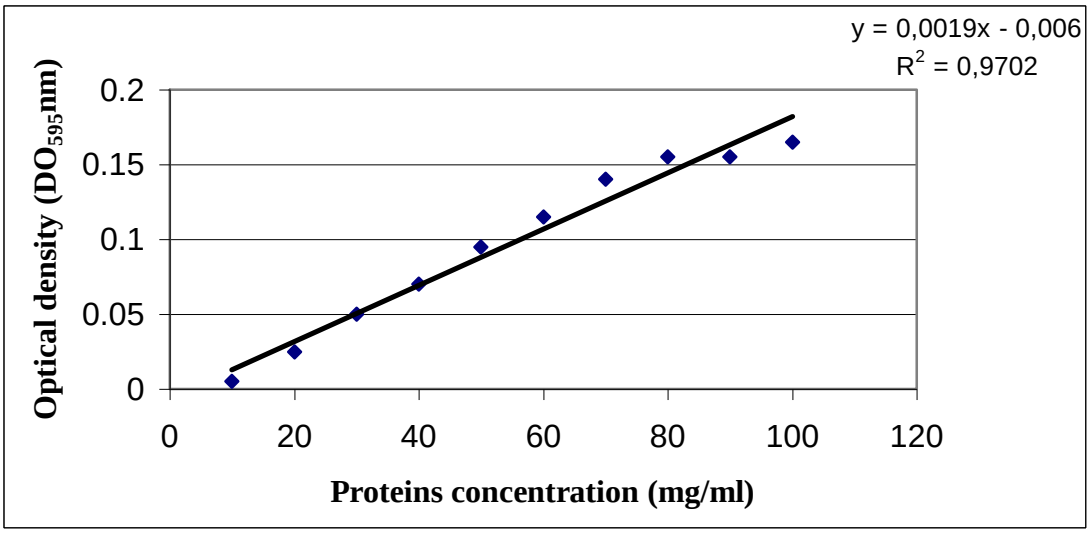


Fig. 4. The variation of the protein concentration from the mulberry roots colonized with different quantities of VAM inocula depending on the optical density at 595 nm.

Table 3. The protein concentration of the mulberry roots colonized with different quantities of VAM inocula.

Samples	CM	C0.5	C1	IM	I0.5	I1
Protein concentration (mg/ml)	3973.684	3184.211	3184.211	2263.158	2526.316	2657.895

In the case of Ichinose genotype, it is observed that in the variant in which 1g of inoculum was applied, the total protein concentration is higher comparatively to the one with a 0.5 g inoculum. In the case of Calafat genotype, it is ascertained the maintenance of the total protein concentration at a constant level no matter what the quantity of the used inoculum was.

The reduction in the protein concentration in the case of the Calafat genotype colonized with VAM fungi could be explained by an declined stage in the evolution of the symbiosis, or by an degrading effect performed by the fungal proteases. In the case of Ichinose genotype it is evident an increase of the protein concentrations in both variants. Thus can be explained the benefic role of VAM fungi on the biosynthetic processes of the proteins, and generally on plant growth and development, as there is a direct correlation between the protein concentration and the quantity of the inoculum, in the case of Ichinose genotype.

Conclusions

Our experiments proved the biofertilizing effect of the mycorrhizal fungi in the case of studied mulberry genotypes. Their positive role was revealed by performing biochemical analyses which aimed to evaluate the protein and P concentrations, as well as the electrophoretic spectrum of the proteins.

1. The protein concentration was higher in the case of the roots colonized with VAM fungi compared to the control variant, for the Ichinose genotype;
2. The P concentration in the roots colonized with VAM fungi is superior to the control variant, thus explaining their biofertilizing effect in the case of the Ichinose genotype. In this genotype a direct correlation between the protein - P concentrations and the quantity of the applied inoculum was revealed;
3. The electrophoretic spectrum revealed an increase of the number of the protein bands in the case of the roots colonized with VAM fungi in the Ichinose genotype, thus proving an amplification of the protein biosynthes after the installing of the symbiosis;
4. The higher concentrations of the phosphorus and of the proteins in the root tissues previously colonized with VAM fungi point out their effect as bio-fertilizers, according to the concept of sustainable agriculture.

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