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Influence of Growth Period on Mycorrhizal Colonization in Roots of *Trifolium Repens* in a Green House Experiment

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Abstract. In natural plant communities are formed fungal-plant associations, associations with the increasing role of the feeding process of both partners, the fungus becoming an extension of the plant's root system, in exchange of carbohydrates synthesized by plants. Mycorrhizal associations are defined by three elements - plant root system, an intraradicular hyphal system and an extraradicular hyphal system.

The study of mycorrhizal symbiosis in controlled environments provided by a green house allows the inplementation of a sampling system in different phase of growth and development of symbiotic fungi. Analisys on samples taken were used to explain the frequency and abundance of different structure in emerging or developed the extraroot by vesicular-arbusculare mushrooms Analizele efectuate asupra probelor recoltate au fost utilizate pentru explicarea frecvenţei şi abundenţei cu care apar diferitele structuri intra- sau extraradiculare dezvolatate de către ciupercile vezicular-arbusculare.

Keywords: vesicular-arbuscular mycorrhiza, trap cultures, stainining solution, colonization parameters, fungal structures

INTRODUCTION

The first researcher who described mycorrhizal symbiosis says that: „It concerns the fact that certain species of trees do not regularly feed in the soil, but that they are everywhere in their whole root system in symbiosis with a fungus mycelium that serves them like a foster-mother and takes charge for the whole feeding of the tree from the soil... The whole body is therefore neither tree root nor fungus alone, but a union of two different creatures forming a homogeneous morphological organ similar to the thallus of a lichen and it may be best termed a fungus root, a mycorrhiza... It (the coat) does not only cover the root tip tightly, but it inserts hyphens through the epidermal cells into the root itself...(though) these hyphens were never traced up to the endodermis... They do never enter the cells lumina.” (B. FRANK, 1885, quoted on www.biologie.unihamburg.de).

It was estimated that in natural communities, about 70% of higher plants are required dependent associations with fungi, 12% are facultative dependent and 18% are colonized. It is believed that vesicular-arbuscular fungi (VA) were disseminated before continental drift, as seen after analysis of higher plant fossils (Remy *et al.* 1994, Taylor *et al.* 2004).

Root - fungi associations are defined by three components: the root system, an intraradicular hyphal system and an extraradicular hyphal system. The two fungal systems provide crucial interfaces involved in the symbiotic uptake of nutrients by partner organisms and the growth and development in different environments. The first interface has a very large variability, influenced by soil heterogeneity, while the second interface is relatively constant and controlled by root homeostasis. Mycelium in the soil is involved in seeking new host

plants and essential nutrients needed both fungus and plant, while the intra-radicular interfaces is dealing with nutrient transfer between symbionts (Smith *et al.*, 2008).

During their growth and development, vesicular-arbuscular fungi arbusculare vesicular differentiate a series of finite structures.

Intraradicular hyphae from a single point of entry, appear to have limited growth, forming an „infection (or colonization) unity", with size adjusted by fungus-host interaction. External hyphae differ morphologically and functionally, ranging from „infection" hyphae, „absorption" hyphae, to „fertile hyphae" (spore-bearing hyphae). Hyphae of infection are those that initiate new colonization points on the same root, other roots of the same plant or roots of nearby plants.

Arbuscules - are hyphae structures from intraradicular hyphae branches, after hyphae branches cross through the cell wall. Arbuscules are formed between cell wall and plasma membrane. Though a short-lived, arbuscule may occur in large numbers in the root, over a long period, as throughout the development of the fungus new ones are produced.

Vesicles - are thin-walled structures and high fat content, products at the junction or ends of hyphae in the root cortex. They usually form ranging from oval to irregular. Vesicles are differentiated very early in development micorizianã certain species, but generally proliferate about the same time with the sporulation phase.

Older mycorrhizae (70-90 days potting) have a small number of arbuscules, but abundant hyphae and vesicles.

MATERIALS AND METHODS

Trap culture establishment

Substrate (soil) necessary for growth and development of trap cultures was harvested from a pasture in the Apuseni Mountains, soil profile is characteristic for *terra rosa* soil. It was prefered to use such a soil because mycorrhizal fungal biomass is great due to low anthropogenic influences on ecosystem. Existing plants in the harvested soil mass were removed by cutting, leaving only their roots in soil. The soil was mixed with coarse sand (*v* 1:1) and transferred to vegetation pots.

Seeding was made with seeds of *Trifolium repens* and cultures were placed in a green house. Temperature was controlled at 20°C during the day, respectively 10°C at night, temperature shift between the two periods was 15°C.

Along the growing season were performed four harvesting, the first being at 8 weeks after plant emergence, following being made every two weeks apart.

Experience located in the green house was composed of four variants, each variant with three repetitions (Fig. 1.). Experimental variants were collected at a distance of 2 weeks apart, variant V_1 was harvested at 8 weeks, V_2 at 10 weeks, V3 and V4 at 12 and 14 weeks after plant emergence. Track parameters were Frequency (F%) of expression, Intensity (M%) of mycorrhizal colonization and arbuscules abundance (A%) in the root system according to plant growth and development.

From each experimental variant were analyzed 30 segments of root, with dimensions of about 1 cm; segments were randomly selected from the mass of roots representing the latest branches in root system.

Fig. 1. Location of experimental variants

Examination method

Vierheilig *et al.* (1998) proposed a simplified method for staining roots in order to analyze the vesicular-arbuscular fungi colonization. The method uses as stain an ink-vinegar solution (5%); solution used to clean the roots is KOH (10%).

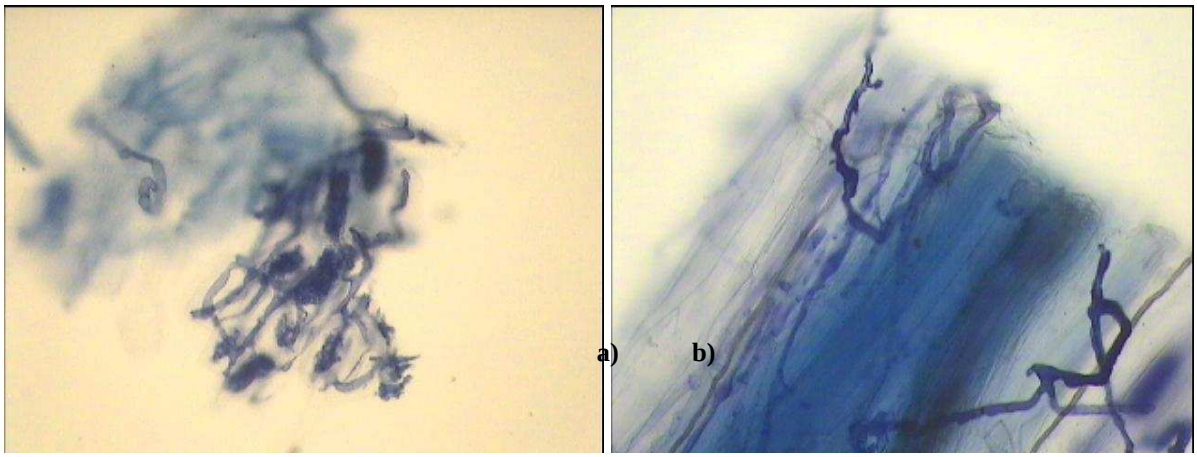

Fig. 2. a) Arbuscules and internal hyphae in root tissue of *Trifolium repens*.
b) penetration point of external hyphae anh internal hyphal development *Trifolium repens* root.

Staining method used for microscopic analysis presented in this paper has suffered a number of changes compared with the original method proposed by Vierheilig. The vinegar used has an acetic alcohol concentration of 9% and the cleaning solution used was NaOH, boiling time for cleaning is one hour. Concentration of these substances was retained as the original method. For staining were used two types of Pelikan ink, blue (Fig. 2) and black (Fig. 3).

Root fragment analysis was done using a microscope, and the results were interpreted using the Mycocalc software (Trouvelot *et al.* 1986, quoted by Przemyslaw *et al.*, 2010).

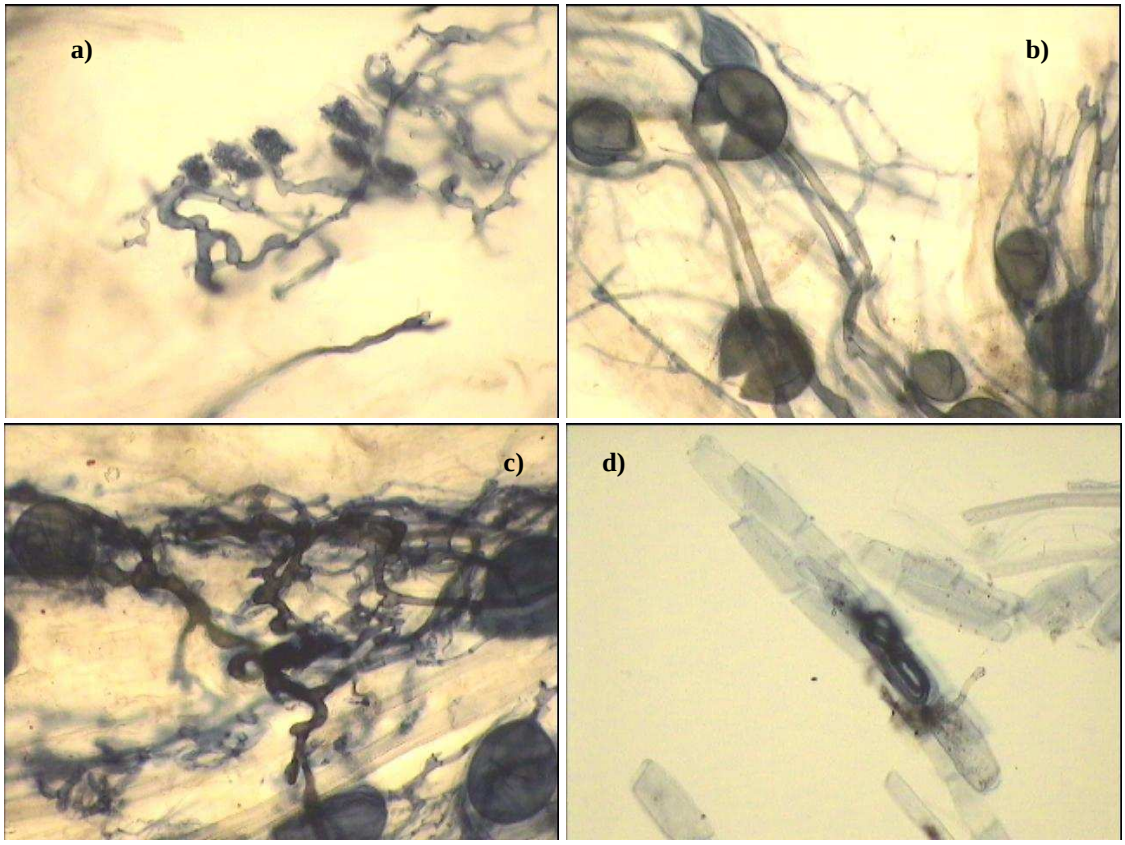

Fig. 3. Mycorrhizal structures on *Trifolium repens*: a) arbuscules and intraradicular hyphae. b), c) hyphae, vesicles and intraradicular spore. d) coiled hyphae.

RESULTS AND DISCUSSIONS

In terms of frequency of mycorrhizal colonization (F), at variant V_1 value is below 85%, for variants V_2, V_3 and V_4 values exceeding 95% (Tab. 1). Increased Frequency of colonization from V_1 to V_3 indicates a more powerfull colonization and stabilization of fungus-plant association with advancing in vegetation of the host plant, the maximum point was reached at 12 weeks after plant emergence; after this point is noticed a slight decrease in the frequency of colonization due to preparation of fungus for extraradicular sporulation phase.

Tab. 1

Frequency of mycorrhizal colonization in root system (F%)

V	R1	R2	R3	Average
1	66.67	96.67	90.00	**84.45**
2	96.67	96.67	100.00	**97.78**
3	100.00	100.00	100.00	**100.00**
4	96.67	100.00	100.00	**98.89**

By analyzing data from microscopic observations, the intensity of mycorrhizal colonization starts at a value of 23% at 8 weeks after emergence of *Trifolium repens* plants, increasing in case of variant V_2 to a value of 40% and reaching a maximum intensity at variant V_3, the value being 48%

(Tab. 2). At stage 14 weeks after emergence, that is assigned to variant V4, colonization intensity decreased to a value of 30%.

Tab. 2

Intensity of mycorrhizal colonization in root system (M%)

V	R1	R2	R3	Average
1	14.57	8.07	46.03	**22.89**
2	50.90	15.63	53.83	**40.12**
3	37.37	33.33	73.10	**47.93**
4	19.47	23.40	45.17	**29.35**

Due to a short live period, arbuscules are the most perishable structures of mycorrhizal fungi. In the the experience described in this paper, arbuscule abundance was the method to quantify the intensity of transfer of nutrients at the interface between the two partners. At variant V_1 arbuscules abundance was at a value of 1.35%, rising at variants V_2 and V_3 from 6% to 10% respectively. After this maximum point, there is a decline in arbuscular abundance , at variant V_4 reaching a value of 3%.

Tab. 3

Arbuscules abundance in root system (A%)

V	R1	R2	R3	Average
1	0.00	1.22	2.82	**1.35**
2	0.32	0.22	17.43	**5.99**
3	3.60	7.55	19.28	**10.14**
4	1.27	1.27	6.27	**2.94**

CONCLUSIONS

Changes proposed to ink-vinegar method showed that after a complete cleaning of root tissue of *Trifolium repens*, the ink and vinegar staining solution highlighted very well internal and external structures of vesicular-arbuscular fungi.

The frequency of colonization in root system for variant V_1 is approximately 85%, at variant V_2 and V_4 the frequency is over 95% and at variant V_3 it was obtained a value of 100%.

In terms of colonization intensity (M%) values increase in proportion to the time of harvest from about 23% at variant V_1, 40% at variant V_2, the maximum being reached at the variant V_3 (48%), compared to V_4 variant where it was observed a decrease in the value of intensity, indicating a decrease in mycorrhizal colonization relative to root development.

Arbuscules abundance (A%) in the root system of *Trifolium repens* reaches the maximum value at variant V_3 (at 12 weeks after emergence), increasing from a low value of 1.35% at variant V_1 and decreasing at variant V_4 to a value of approximately 3%.

By comparing the four experimental variants it can be seen a continuous development of vesicular-arbuscular fungal structures, the maximum values being attained for variant V_3,

the maximum level of transfer between the two partners; while at the variant V_4 was observed a decline in development of the fungalpartner.

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