Research on the influence of inputs with a biostimulatory role on mycorrhizal dynamics in corn culture

(SUMMARY OF THE Ph.D. THESIS)

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INTRODUCTION

In Romania, agriculture is considered one of the main branches of the national economy. In 2021, Romania ranked first in the European Union in terms of the area cultivated with corn for grains, and the figures are increasing, recording a production of 14,445,000 tons on 2,493,000 hectares cultivated with corn, according to the INS. Sustainable agriculture seeks to increase the productivity of agricultural crops by reducing the amount of chemical fertilizers, pesticides and herbicides used. A favorable alternative for the environment is the application of products with a biostimulatory role, to partially or totally replace synthetic inputs (GOLUBKINA et al., 2020), because their use over a long period can produce an unfavorable effect on soil biodiversity and implicitly human health, an imbalance at the level of the entire agricultural ecosystem (CUI et al., 2010). One of the most sustainable solutions is to increase the use of biological substances on a larger scale, especially those connected to soil diversity and rhizosphere growth (RAYA-HERNÁNDEZ et al., 2020). In biological agriculture, biopreparations based on microorganisms have the role of facilitating the absorption of nutrients from the soil. This direction also includes one of the oldest associations found in nature - symbiosis (LANFRANCO et al., 2018) and one of the forms of symbiotic association is mycorrhiza.

Mycorrhizae are essential actors in organic agriculture due to the many benefits they bring to both crops, soil and the entire ecosystem (AGUILAR-PAREDES et al., 2020, BADRI et al., 2009; FRANCHE et al., 2009). In this association, both partners both the mycorrhizal fungi and the colonized plant are beneficiaries. Plants enjoy increased access to nutrients, especially to inaccessible ones, and fungi receive the carbon produced by plants following the photosynthesis process (BOLDUC, 2011; EVELIN et al., 2009; CHEN et al., 2018).

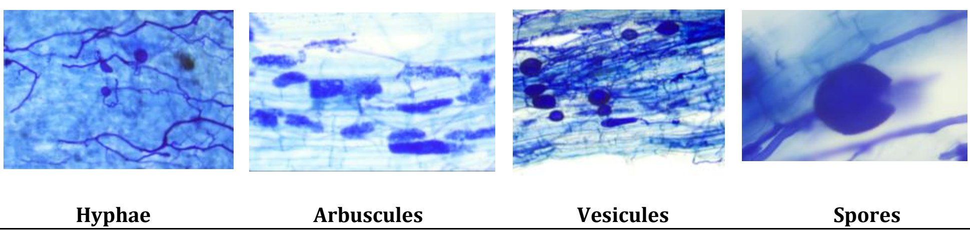

Fig.1. Structurile micoriziene

The fungus grows inside the root cells of the host plant, forming branched structures called (short-lived) arbuscules, where the main exchange of nutrients takes place. The fungus also forms a complex network of extraradicular hyphae that extends beyond the root system of the plant (SAWERS et al., 2008). The fungal structures produced by mycorrhizal fungi are hyphae, arbuscules, vesicles, spores inside the root but are also present outside them (hyphae, vesicles and spores) (KEHRI et al., 2018; KOIDE and MOSSE, 2004) (Figure 1)

1. The structure of the Ph.D. thesis

The doctoral thesis entitled "**Research on the influence of inputs with a biostimulatory role on mycorrhizal dynamics in corn culture**", has 146 pages, 49 figures, 19 tables, and 3 plates, being organized according to the drafting norms of the Doctoral School in two parts.

The first part is entitled: The current state of knowledge, in which the topic was deepened using specialized literature, and the second part includes personal contribution.

Part I of the thesis is structured into 3 chapters:

Chapter 1. *The current state of knowledge regarding mycorrhizae,* represents the part in which information is presented according to the specialized literature about mycorrhizae in terms of definition, mycorrhizal structures present in roots, benefits they bring to crop plants, and representations from agricultural ecosystems.

Chapter 2. *Maize as a mycorrhizal culture plant and test plant in the evaluation of colonization capacity* in which the mycorrhizal performance of corn is presented according to previous studies, the particularities of the root system of corn as well as mineral nutrition and soil requirements.

Chapter *Sustainability of the application of biostimulators in the soil-plant system* this chapter is dedicated to studying the types of biostimulators and their role in sustainable agriculture.

Part II of the thesis includes 7 chapters:

Chapter 4. *The objectives of the research* include the presentation of the purpose of the thesis but also of the specific objectives assumed by the doctoral study.

Chapter 5. *Material and method* are presented: the location and climatic conditions, the biological material studied; the treatment with biostimulators, the organization of the experiments, namely the experimental design related to the two experiments; also mycorrhizal assessment methodologies and techniques, microscopic analysis, laboratory methods, MycoPatt methodology development, and data analysis.

Chapter 6. *Results and discussion- Experiment 1 (2020)* includes 5 subchapters in which the results of the first year of study are presented.

Chapter 7. *Results and discussion -Experiment 2 (2021)* includes 5 sub-chapters in which the results related to 2021 are presented.

Chapter 8. *Results obtained - Mycorrhizal colonization mechanism in maize* includes 3 subchapters in which the most important results regarding the mycorrhizal colonization mechanism in corn are interpreted.

Chapter 9. *The originality and innovative contributions of the thesis* presents the new elements of the study of mycorrhizae in one of the most important cultivated plants.

Chapter 10. *Concluzii și recomandări,* ce cuprinde concluziile aferente studiului doctoral grupate pe cele două experimente (2020-2021).

References: 214 bibliographic titles, namely books, scientific articles, newsletters, and electronic pages.

Annexes: include 3 plates and 2 figures with aspects from both the experimental field and the Microbiology laboratory of the Faculty of Agriculture.

2. Aim and objectives

The purpose of the doctoral thesis entitled "**Research on the influence of inputs with a biostimulatory role on mycorrhizal dynamics in corn culture**", was to evaluate the impact of inputs with a biostimulatory role on the level of mycorrhizal colonization in corn culture in the pedo-climatic conditions of the Transylvanian Plain.
In order to achieve the goal, the research undertaken aimed at a series of specific objectives:

- The influence of pedo-climatic conditions on the mycorrhizal colonization on the corn crop in the Transylvanian Plain;
- The influence of the biostimulator and its concentration on the corn crop;
- Elaboration of the mycorrhization patterns specific to the phenophases and the treatment applied to the corn crop; the influence of the maize crop phenophase in defining clear colonization strategies;
- Synthesis of the complex interaction of all experimental factors on the colonization mechanism and defining how biostimulators influence the level of colonization.

3. Material and method

3.1. Experiment 1 (2020)

Experiment 1 was located in the town of Cojocna, at the Experimental Teaching Station, being located southeast of the city of Cluj-Napoca. The climate is temperate continental, the average annual temperature being +9.6°C, with a minimum in January of −5°C, and a maximum in July/August of +20°C. The soil of Cojocna is of the faeozoic type with a slightly acidic pH, a good supply of nitrogen and phosphorus and a very good supply of potassium. the biological material studied was the maize hybrid MAS 24 C, (experiment 1), which belongs to the FAO 270 group, and adapts to all environmental conditions but also to different densities.

3.2. Experiment 2 (2021)

Experiment 2 was also located in the Transylvanian Plain in the town of Iernut, Mureș county. The relief is characteristic of the Transylvanian Plateau, with a hilly configuration, and extensive flat areas on the left side of the Mureș River. The average temperature of 22-24° C is recorded in July and August, being the hottest months. The soil of the phaeozoum type in the experimental field from Iernut shows an alkaline reaction, against the background of a moderate supply of nitrogen. The biological material studied was the Pioneer P9241 corn hybrid from the FAO 330 group, a hybrid that tolerates very well acidic, eroded soils with low humus content.

3.3 The treatment applied

After emergence, in the plant phenophase of 2-4 leaves formed, the biostimulator AMER 6.3 (Ameropa Company) was applied. The treatment used was identical in both experiments (experiment 1 and 2). It was applied foliarly, in a dose of 1l/ha. In the corn crop, the biostimulator Amer 6.3 is applied in the first phases of vegetation. This product has the role of activating enzymes and enhancing enzyme activities, stimulates plant growth, plant nutrition and increases soil fertility due to the increased content of enzymes and amino acids. The treatment was used to identify potential changes in mycorrhizal colonization in maize roots and possible changes occurring at the level of developmental strategy or mechanism.

3.4. The experimental protocol

Experiment 1 from the locality of Cojocna (2020) and experiment 2 from the locality of Iernut, (2021) were of the bifactorial type, the two factors being: 1 - *Treatmen*t and 2. - *Phenophase of growth*. A control variant (coded A0) is added to the two factors, which shows the native profile of mycorrhization in 2-4 true leaves of the plants (B1) and ovarian comparison for the evolution of colonization during the vegetation period (coded A0-B1).

Factor 1 – Treatment - presents two grades (A1-A2): *untreated A1*, respectively *A2 treated with the AMER 6.3 biostimulator.*

Factor 2 – Phenophase of plant growth with 4 graduations

Within each experimental variant, the most important moments of observation of mycorrhizal colonization were established to capture the periods of plant development (phenophase): B2- phenophase of 6 formed leaves; B3- phenophase of 8-10 formed leaves; B4- cob formation phenophase; B5- phenophase corresponding to physiological maturity. Maize roots were harvested in both the first and second experiments, at each phenophase of plant development.

3.5. Mycorrhiza evaluation methodology

After harvesting the corn roots, they were brought to the Microbiology laboratory and prepared for the determination of mycorrhizal colonization parameters. For the microscopic analysis of the corn roots, their staining was used. Fifteen root segments were analyzed in 15 microscopic fields for each experimental variant (A1 untreated and A2 treated). Within each segment analyzed under the microscope, 15 photographs were taken, resulting in over 6075 photographs within both experimental variants.

The microscopic evaluation methodology was taken from the MycoPatt model (STOIAN et al., 2019), which is a model with an innovative character that shows us the position in a real way of the fungal structures present in the analyzed root and that automatically calculates all the indicators and namely: frequency of colonization, intensity of colonization, fungal structures arbuscules and vesicles, non-colonized areas, ratio between colonized and non-colonized areas, degree of colonization, then the mycorrhiza map will be generated.

4. Results and discussion Experiment 1

4.1. Evaluation of mycorrhizal colonization trend and differences induced by the application of treatments

In the case of colonization frequency, the variations were extremely large between phenophases and between the two variants, untreated (A1), treated with the biostimulator AMER 6.3 (A2). The maximum of this parameter was 50%, reached in the untreated variant at the end of the vegetation period. For the intensity, an increase was recorded simultaneously with the development of the plants, the maximum being in the case of the untreated variant – the physiological maturity phenophase (A1–B5,

20.25%). In the case of arbuscules, the highest value was recorded in the untreated version, 3.67%, in the cob formation phenophase.
Vesicles had approximately the same value of 0.11% in the case of the treated variant in the phenophases of 6 leaves and 8–10 leaves.

4.2. Treatment-induced and growth stage (phenophase) interrelationships between parameters of mycorrhizal colonization

All the correlations are significant which shows us the close connection between the colonization strategy and the developed structures.
The maximum correlation is between frequency and intensity (0.91), which indicates intra-radicular development in almost all cases of root penetration. The expansion observed is very strongly connected with the development of the arbuscules. However, the vesicles appear in a lower weight being very little correlated with the frequency but having a similar weight in the case of the link with the arbuscules and the intensity of the colonization.

4.3. Analysis of interactions between mycorrhizal parameters and prediction of colonization evolution

The analysis of the frequency-intensity interaction of the colonization and the prognosis of the development of the mycorrhizal system in the root cortex was carried out with the help of scatter plots. The evaluation of the intensity-frequency relationship of the colonization indicates a strong variation both at the level of the grouping of experimental factors and relative to the positioning and advance in the root. The regression equations were used due to the large amount of data resulting from experiments based on the MycoPatt methodology. At the graphical level, the most efficient method of presenting regression equations is by superimposing the curve resulting from the application of this equation on a scatter plot type graphic system that presents the parameters two by two.

4.4. Exploratory spatial analysis of mycorrhizal colonization

PCA-type analysis allows both the exploration of observations on mycorrhizal colonization and the projection of colonization parameters related to the synthetic index: degree of colonization. The quality of the microscopic observations is supported by antagonistic intensity gradients and non-colonized areas, with a spatial orientation in the ++ and -- quadrants, respectively.
NMDS analysis is similar to PCA analysis, but this type of ordination is useful for much easier observation of root mycorrhizal colonization as well as for specific parameters of colonization.

4.5. Extraction and evaluation of the specific pattern of colonization induced by the application of treatments

The mycorrhizal model is represented by the mycorrhizal map. This representation makes it possible to observe relatively easily the fungal structures present in the corn root. The experiment resulted in a large database, which was merged into 390 maps with mycorrhizal patterns induced by the treatment applied to the maize crop in different phenophases of the vegetation. The methodology for selecting general patterns associated with treatment and crop phenophase involved heavy filtering of the database. Each stage is associated with a colonization parameter related to the overall value of the parameter for each experimental variant. The first colonization pattern, at the 2–4 leaf stage (A0–B1), showed early colonization, with large areas free of fungal structures. Fungal hyphae had little developmental potential and branching was almost absent. After this point, based on the application of the AMER 6.3 biostimulator treatment, the analysis of the colonization patterns allows the comparison of the two types of symbiosis evolution.

5. Results and discussion Experiment 2

5.1. Evaluation of mycorrhizal colonization trend and differences induced by the application of treatments

Both frequency and intensity show large variations between growth stages for both treated and untreated plants. The frequency of native colonization exceeds 74% native profile – phenophase 2-4 leaves (A0-B1). Intensity of colonization varied greatly between growth stages, with over 40% early in plant growth, followed by a significant reduction at the 6-leaf growth stage. While the first growth stage showed more than 12% of hyphae forming arbuscules, the 6-leaf stage showed a 7% reduction in untreated plants and nearly 10% in treated ones. Vesicles are restricted to less than 1% of colonized roots, with higher values in the last two growth stages for untreated plants compared to the cob formation stage for treated ones.

5.2. Treatment-induced and growth stage (phenophase) interrelationships between parameters of mycorrhizal colonization

Pearson correlations are used to explore the connections between mycorrhizal parameters, their influence, which is related to their simultaneous presence in the same colonized root. The dynamics of the presence and development of mycorrhizal structures in maize roots show a high degree of interconnection. Both untreated and treated profiles show higher correlation coefficients for arbuscules and vesicles compared to the native profile. Differences are greater in arbuscules determined by the presence of the fungal component (frequency) and development (intensity) in the roots.

5.3. Analysis of interactions between mycorrhizal parameters and prediction of colonization evolution

The simultaneous presence of arbuscules and vesicles can be analyzed by plotting them in the same scatterplot. The scatterplot approach allows a visual analysis of the dispersion of the data for each structure and the specific level at which each structure is singular in colonized roots. The native development of these structures is set at over 70% for arbuscules and over 15% for vesicles. The absence of treatment at the untreated 6-leaf stage (A1-B2) leads to a simultaneous presence of both structures with up to 8% for vesicles and 18% for arbuscules. The 10% difference is geared toward improved transfer rather than nutrient storage.

5.4. Exploratory spatial analysis of mycorrhizal colonization

PCA ordinations allow a good visualization of the entire colonization database, with detection of centroids for each growth stage and vector projection for each parameter (untreated) and (treated).

5.5. Extraction and evaluation of the specific pattern of colonization induced by the application of treatments

The entire database consisted of 6075 lines, corresponding to 405 mycorrhizal maps and further divided into 27 sub-databases. The symbiont creates a primary form of hyphal sieve in colonized roots, followed by colonization of adjacent areas and subsequent development of arbuscules. Compared to the development of the fungal structures observed in the native mycorrhizal model, untreated control (A0-B1), a similar development is observed in the treated plants, the growth stage of the treated variant in the phenophase of 8-10 formed leaves (A2-B3), with size and low number of uncolonized areas. Maize plants inoculated with arbuscular mycorrhizae determined a maximum colonization between 86-100%. The mycorrhizal structures were mainly external and internal hyphae, arbuscules and vesicles. The maximum fungal development is recorded in this case within the group of untreated plants.

6. Results obtained Colonization mechanism

Mycorrhizal parameters are very efficient in defining the symbiotic capacity of a soil, the performance of fungi to create a sustainable partnership with crop plants, respectively they allow a realistic comparison of the effect of biostimulators.

The experimental protocol that was the basis of this study is bifactorial, evaluating the level and dynamics of mycorrhizal colonization in the corn crop, under conditions of application of a biostimulator and its effect during the entire vegetation period. The experimental protocol was replicated in two eco-pedological conditions, for the year 2020 in the locality of Cojocna, on a type of faeozoic soil, weakly acid, respectively in the year 2021 in the locality of Iernut, on an alkaline type of faeozoic argilloiluvial soil. Repeating the experiment allows both the identification of the native soil colonization capacity of each location and the in-depth evaluation of the effect of the biostimulators.

6.1. Analysis of variance of multiple comparisons

The variance analysis indicates an amplification of the effect of the biostimulators depending on the soil profile and the specific conditions of each experimental site. In the case of the Cojocna soil, the increases in frequency are significant, and the biostimulator acts to increase the degree of frequency from one vegetation period to another. By comparison, the action of the biostimulators in the Iernut soil conditions, within the native mycorrhizal profile, is high. This aspect allows a variation of the colonization frequency with much wider limits while maintaining a level of the presence of symbionts above 50%.

6.2. Mycorrhizal substitution index (%)

The degree of colonization is an adaptable indicator and can be used as a support in the verification of mycorrhizal substitution, induced by the application of biostimulators and the experimental location. The differences recorded between locations, phenophases and treatments fluctuate within very wide limits. The initial difference in untreated plants is almost 35%, decreasing by half in the six-leaf phenophase, respectively at the end of the vegetation period to below 10%. In the plants to which the biostimulator was applied, the initial difference is only 13%, with an increase up to 19%-23%, respectively a reduction of up to 10% at the end of the vegetation period.

7. The originality and innovative contributions of the thesis

The doctoral thesis brings a series of new elements, both due to the multitude of results obtained and the methodological updates useful in the fields of agronomy and general and applied microbiology.

Also, with this thesis we contribute to the identification and evaluation of the biological mechanism of association of corn with arbuscular mycorrhizae:

1. The first research both nationally and internationally that presents mycorrhizal colonization in maize in an innovative set of procedures: Elaboration of the native mycorrhizal pattern of maize, in 2 locations with different eco-pedological conditions.
2. Realization of specific patterns of corn colonization in different periods of its development under the effect of the application of biostimulators.
3. Implementation of MycoPatt tools for objective assessment of mycorrhizal colonization.
4. Creation of a superdimensional database (over 12,000 microscopic observations) containing observations on fungal structures and colonization parameters.

8. Conclusion and recommendations

1. The application of the biostimulator interferes with the activity of the soil microbiota, thus the biostimulators can have a reaction of amplifying the rate of mycorrhization simultaneously with the growth and development of the plant, as well as prolonging the colonization at high rates.
2. Biostimulators can be used in corn culture because, as it was found in the two experiments, they do not act to inhibit mycorrhizal colonization. It is recommended to use them in corn culture because they offer a good development of the root system as well as the vegetative part, contributing to the fixation of the plant in the soil and a better absorption of nutrients.
3. The general phenomenon recorded in both experimental fields highlights a reduction in the need for abundant colonization in conditions where the plant develops an extensive root system due to the applied inputs and the maintenance of a balanced rate of colonization throughout the entire vegetation period.
4. The mechanism by which the biostimulators act is due to the presence in a high concentration of amino acids, which form interactions with the mineral elements

present in the product recipe, which leads to obtaining a beneficial nutritional complex for both plants and soil microbiota.

5. The location has an extremely important role for the appearance of arbuscules, while for the formation of blisters the treatment has a much higher importance.

6. The pedo-climatic conditions in the Transylvanian Plain had an influence on the mycorrhizal colonization of the corn crop. So:

In the experiment in the Cojocna location, the abundant precipitation in the early stages of plant development and the low temperature caused waterlogging phenomena in the experimental field. The native mycorrhizal profile of the Cojocna soil was reduced to a colonization frequency of only 13.3% and an intensity of 6.08%.

In the experiment in the Iernut location, the different pedo-climatic conditions are visible in the short-term precipitation, with a high intensity, but at the same time with a continuously increasing temperature during the development of the plant. The frequency of mycorrhizal colonization in the native soil profile from Iernut recorded a value of 74.7% with an intensity of 42.6%, both values being 60% and 36% higher than those recorded at Cojocna.

7. The soil profile is decisive in the assembly of a specific microbial community, an aspect that is extremely well evidenced by the punctual abundance and especially the capacity of constant production of arbuscules.

8. Phenophase correlates with root development, which corresponds to the effective size of the colonization surface made available to mycorrhizae by the plant.

9. The use of biostimulators to increase agricultural production while preserving the environment can be considered a forward-looking technological practice without causing disturbances in the mechanisms of interaction between soil microorganisms.

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