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# Arbuscular Mycorrhizas Traits and Yield of Winter Wheat Profiled by Mineral Fertilization

Roxana Vidican <sup>1</sup>, Florin Păcurar <sup>2</sup>, Sorin Daniel Vâtcă <sup>3,\*</sup> , Anca Pleșa <sup>2</sup> and Vlad Stoian <sup>1,\*</sup> 

<sup>1</sup> Department of Microbiology, Faculty of Agriculture, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Calea Mănăștur 3-5, 400372 Cluj-Napoca, Romania; roxana.vidican@usamvcluj.ro

<sup>2</sup> Department of Grasslands and Forage crops, Faculty of Agriculture, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Calea Mănăștur 3-5, 400372 Cluj-Napoca, Romania; florin.pacurar@usamvcluj.ro (F.P.); anca.plesa@usamvcluj.ro (A.P.)

<sup>3</sup> Department of Plant physiology, Faculty of Agriculture, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Calea Mănăștur 3-5, 400372 Cluj-Napoca, Romania

\* Correspondence: sorin.vatca@usamvcluj.ro (S.D.V.); vlad.stoian@usamvcluj.ro (V.S.)

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**Abstract:** Our aim is to evaluate the changes in arbuscular mycorrhiza characteristics of winter wheat in a three-year experiment. Study results show that fertilizers produce strong variations in arbuscular mycorrhiza extension, with colonization frequency values within 76–98%. The intensity of colonization is only 12% when phosphorus (P) exceeds nitrogen (N) in autumn, but reaches 38% when the N:P ratio is equal. Root colonization shows no consistency from one experimental year to another, with the largest fluctuations recorded in colonization intensity (22–65%) and arbuscules abundance (0–5%). Arbuscules are maintained below 1% by fertilizer with more P than N. Colonization forecasting models indicate P as a factor for the reduction of symbiosis. Each kg of applied P can reduce the colonization frequency by 0.28% and intensity by 0.37%. The maximum of the colonization degree is 61% due to the synergy of equal N and P doses in autumn and ammonium nitrate applied in spring. The application of multiple moderate doses acts as a stimulant for the development of a large root-fungal interface.

**Keywords:** symbiosis; fungal-nutrient interaction; root colonization

## 1. Introduction

Throughout their evolution, most terrestrial plants have developed the ability to form symbiotic associations with mycorrhizal fungi [1]. The role of fungi is to explore soil outside the area accessible to the roots of plants and to relocate the nutrients from fungi to host [2,3]. Their main function is to absorb phosphorus (P) and nitrogen (N), which are major nutrients for plant growth, and receive carbon from host photosynthesis [4–7]. They also act as a biocontrol agent and increase the resistance of plants to stress factors. Fungi develop a large extraradical mycelium that acts as an extension of plant roots in the search for nutrients. The resulting hyphal network can reach areas far from roots and thus inaccessible to plants. General development of arbuscular mycorrhiza in roots takes place both inter- and intracellularly. The arbuscules developed inside the root cells have the role to increase the transfer surface between the two partners [8,9]. Mycorrhizal plants benefit from a higher quantity and variety of nutrients from a larger soil area, thus providing better conditions for growth and development [10,11]. As a response to the presence of symbionts, plants modify their root architecture to ensure the largest area necessary for the installation of fungi [12]. The entire mycorrhiza–host system is defined by a bidirectional nutrient exchange with both partners acting in the regulation of transferred quantity [13,14]. In this system, plants allocate larger amounts of carbohydrates and lipids to the colonized roots where mycorrhiza transfer is more evident.

In agro-ecosystems, the need to ensure a large amount of nutrient resources for crop growth and development is based on the activity of soil microorganisms [15–17]. Fungal symbionts in the rhizosphere of crop plants act as a primary nutrient network. This mechanism ensures the performance of crops in different technologies and provides a positive response to different pressures from agro-ecosystems [18,19]. Current agricultural conditions demand high yields per unit area in order to ensure sufficient food is produced to sustain an increasing population. At the present time, conventional practices are based on high quantities of mineral fertilizers, which partially suppress the effectiveness of the mycorrhiza symbiotic mechanism [20,21]. This can also reduce the level of colonization of the root systems, a phenomenon caused by the abundance of nutrients in the area where plant roots have their maximum potential of absorption. Therefore, it leads to a reduction in root system size and acts as a blocker of the colonization process [22]. Poor mobility of phosphorus in the soil and the absorption process can cause the disappearance of phosphorus from the immediate vicinity of the root. If this coincides with a low level of mycorrhiza colonization, it can induce significant stress on crops [23].

Wheat (*Triticum aestivum* L.) is a crop plant with a global expansion, being present in almost all crop rotations. It has a great plasticity and a good response to mycorrhiza colonization, which produces benefits both for enhanced nutrition and stress protection [24]. Wheat has been tested under different forms of soil tillage, which directly affect the mycorrhiza colonization potential [25–29]. In conventional tillage, the number of infectious propagules decrease and most mycorrhiza activity is moved to the upper soil layer. For minimum and no-tillage, the hyphal network remains undisturbed in the soil, which improves the colonization and the level of nutrient absorption. This mechanism is amplified if the previous crop is an arbuscular mycorrhiza crop, and their fungal symbionts will be more aggressive in colonization. In this context, the analysis of mycorrhiza parameters is a useful technique in the proposal of effective methods for maintaining the productive stability of crops and, at the same time, maintaining their contact with the symbiotic flora of the soil.

Our aim is to assess the impact of mineral fertilization with N and P on intraradical colonization of arbuscular mycorrhiza fungi in winter wheat. In most Romanian farms, P is applied in autumn and supplemented during vegetation time with different amounts of N. Our experiment evaluates two hypotheses regarding the influence of different fertilization practices on the natural mycorrhiza mechanism. As a starting point, we hypothesize that higher amounts of P applied in autumn will affect the degree to which plants will accept arbuscular mycorrhiza symbionts. The second hypothesis was that the coincident application of N and P in an optimum climate will reduce the colonization potential. The main objective was to assess the overall effect of N and P applied in the autumn ( $N_a$ ,  $P_a$ ) and supplemented with N-based fertilizers during the post-winter vegetation period ( $N_v$ ). The second objective was to obtain a general and a specific annual arbuscular mycorrhiza profile. The third objective was to assess the stability of the mycorrhiza mechanism induced by basic fertilization in relation to phased fertilization. The last objective was to assess the forecasting potential of mycorrhiza parameters based on fertilizers, and the yield potential based on the integration of mycorrhiza and fertilizers into models.

## 2. Materials and Methods

Wheat roots were harvested in summer, at the end of the vegetation period, from an experiment with 16 mineral fertilization treatments, in 3 replications (Table 1) over 3 years: 2014–2015 (A), 2015–2016 (B), and 2016–2017 (C). Fertilizers were applied in 3 periods: the first application was in October, before sowing ( $N_a$ ,  $P_a$ ); the second application was at the end of winter, at the beginning of March (S1); and the third application was at the end of April (S2), which corresponds to the boot development stage. Fertilizers were produced by Azomureş, located in Mureş County, Romania [30]. The experimental field was located at an altitude of 400 m, 46°34′57.5538″ latitude and 23°47′15.7158″ longitude in Turda area, Cluj County, Romania on an argic chernozem soil. The previous crop was the common bean (*Phaseolus vulgaris* L.) in all 3 years.

**Table 1.** Experimental design: Type, dose, and phase of fertilizer application.

Treat.	Autumn Fertilizer		Phased Fertilizer		Total N (kg ha <sup>-1</sup> )	Total P (kg ha <sup>-1</sup> )		
	Commercial Formula	Active Ingredient (kg ha <sup>-1</sup> )		Commercial Formula			Active Ingredient (kg ha <sup>-1</sup> )	
		N <sub>a</sub>	P <sub>a</sub>				N <sub>v</sub> , S1	N <sub>v</sub> , S2
V1	N20P20	40	40			40	40	
V2	N20P20	80	80			80	80	
V3	N20P20	120	120			120	120	
V4	N18P46	15	40	AN	25	40	40	
V5	N20P20	80	80	AN	67	147	80	
V6	N20P20	80	80	CAN	67	147	80	
V7	N20P20	80	80	Urea	67	147	80	
V8	N20P20	80	80	AN	33.5	33.5	147	80
V9	N20P20	80	80	CAN	33.5	33.5	147	80
V10	N20P20	80	80	Urea	33.5	33.5	147	80
V11	N18P46	31	80	AN	116		147	80
V12	N18P46	31	80	AN	58	58	147	80
V13	N18P46	31	80	CAN	116		147	80
V14	N18P46	31	80	CAN	58	58	147	80
V15	N20P20	120	120	AN	67		187	120
V16	N20P20	120	120	AN	33.5	33.5	187	120

Note: Treat.—Treatment; N<sub>a</sub>—nitrogen applied in autumn; P<sub>a</sub>—phosphorus applied in autumn; N<sub>v</sub>—nitrogen applied post-winter on vegetation; S1, S2—post-winter number of applications; AN—ammonium nitrate (N—34%); CAN—calcium ammonium nitrate (N—27%); Urea (N—46%).

A total of 30 root segments (1 cm each) were randomly selected from 5 plants in each treatment. Staining was performed with ink based on the method proposed by Vierheilg and modified by Stoian and Florian [31]. All the parameters of colonization are expressed as a percentage (%) and were calculated according to the formulas proposed by Trouvelot et al. [32]: Frequency of colonization (Freq), intensity of colonization (Int) in root system (defined by Trouvelot as M%), and the percentage of arbuscules (Arb). To these parameters, we added a synthetic index called colonization degree (Cdeg), obtained from the product of frequency and intensity, necessary to assess the total volume of the root system explored by arbuscular mycorrhiza hyphae [33].

Data analysis was carried out with the R Studio software [34]. The evaluation of the basic statistics was performed with the “psych” package [35], which allowed the calculation of means and standard errors of parameters. ANOVA and least significant difference (LSD) tests needed to assess the differences between treatments were carried out with the “agricolae” package [36]. An integrated assessment of the arbuscular mycorrhiza profile was performed by assembling the colonization parameters in a cluster analysis, carried out with the “ape” package [37], based on the Euclidean transformation of data with the “stats” package available in R [38]. To highlight the response of mycorrhizas to phased fertilization, we used redundancy analysis (RDA) ordination from the “vegan” package [39], separately by year and basic fertilization. All data analyses were supplemented by the integration of colonization parameters and fertilization into forecast models, based on the Akaike information criterion (AIC) coefficient and the ANOVA analysis of the final results, available in the “MASS” package [40].

### 3. Results

#### 3.1. Global Impact of Fertilization on Arbuscular Mycorrhiza Colonization and Wheat Yield

The colonization frequency in wheat roots is significantly influenced by applied fertilization (Table 2). Equal doses of N and P during autumn, supplemented by 34 kg N in the spring (V9–10), lead to a frequency of colonization more than 98%. The support of plant development with N-based fertilizers applied post-winter in two phases maintains a high level of colonization. The application of

a single dose of 67 kg ha<sup>-1</sup> N from ammonium nitrate (AN) and calcium ammonium nitrate (CAN) (V5, V6) significantly reduces the incidence of colonization to 87%. Applying the same amount of urea (V7) leads to a 7% increase in colonization frequency. Basic fertilization with N18P46 stimulates the frequency of colonization at the phased application of AN (V11, V12) or a single-phase dose of CAN (V13), with values in the range of 93.7–97.4%. Separation of CAN in two doses (V14) produces a significant reduction in colonization frequency by 20%.

**Table 2.** The impact of fertilization on arbuscular mycorrhiza colonization and crop yield of winter wheat.

Treat.	Freq (%)	Int (%)	Arb (%)	Cdeg (%)	Yield (kg ha <sup>-1</sup> )
V1	88.9 ± 1.1 <sup>a-e</sup>	28.4 ± 4.5 <sup>a,b</sup>	0.9 ± 0.5 <sup>b,c</sup>	25.4 ± 4.2 <sup>a-c</sup>	6133.6 ± 72.1 <sup>g</sup>
V2	88.2 ± 3.9 <sup>b-e</sup>	27.1 ± 7.6 <sup>a-c</sup>	0.8 ± 0.3 <sup>b,c</sup>	25.6 ± 7.8 <sup>a-c</sup>	6828.7 ± 73.3 <sup>e,f</sup>
V3	80.7 ± 6.1 <sup>e,f</sup>	16.9 ± 3.5 <sup>b,c</sup>	0.1 ± 0.0 <sup>c</sup>	14.8 ± 3.3 <sup>b,c</sup>	7088.6 ± 83.3 <sup>d,e</sup>
V4	87.4 ± 3.6 <sup>c-e</sup>	21.1 ± 2.2 <sup>b,c</sup>	0.3 ± 0.1 <sup>c</sup>	18.7 ± 2.4 <sup>b,c</sup>	6376.7 ± 60.7 <sup>f,g</sup>
V5	87.4 ± 5.8 <sup>c-e</sup>	24.1 ± 5.4 <sup>a-c</sup>	0.5 ± 0.2 <sup>c</sup>	23.0 ± 5.7 <sup>a-c</sup>	7717.8 ± 74.4 <sup>a</sup>
V6	87.0 ± 3.9 <sup>d,e</sup>	28.2 ± 9.3 <sup>a,b</sup>	0.5 ± 0.2 <sup>c</sup>	26.9 ± 9.5 <sup>a,b</sup>	7176.9 ± 46.0 <sup>b-e</sup>
V7	94.5 ± 2.1 <sup>a-d</sup>	28.5 ± 5.8 <sup>a,b</sup>	0.5 ± 0.2 <sup>c</sup>	27.5 ± 5.9 <sup>a,b</sup>	7170.8 ± 45.7 <sup>b-e</sup>
V8	96.3 ± 1.4 <sup>a-d</sup>	29.5 ± 8.3 <sup>a,b</sup>	0.1 ± 0.0 <sup>c</sup>	28.9 ± 8.3 <sup>a,b</sup>	7575.0 ± 124.4 <sup>a-c</sup>
V9	98.2 ± 0.8 <sup>ab</sup>	38.4 ± 3.6 <sup>a</sup>	1.1 ± 0.4 <sup>a-c</sup>	37.8 ± 3.7 <sup>a</sup>	7458.7 ± 129.8 <sup>a-d</sup>
V10	98.5 ± 0.8 <sup>a</sup>	26.8 ± 2.7 <sup>a-c</sup>	0.7 ± 0.3 <sup>c</sup>	26.3 ± 2.6 <sup>a,b</sup>	7565.6 ± 73.6 <sup>a-c</sup>
V11	94.8 ± 2.2 <sup>a-d</sup>	19.0 ± 2.2 <sup>b,c</sup>	0.3 ± 0.1 <sup>c</sup>	17.8 ± 2.0 <sup>b,c</sup>	7465.9 ± 94.0 <sup>a-d</sup>
V12	97.4 ± 1.2 <sup>a-c</sup>	25.9 ± 3.3 <sup>a-c</sup>	1.9 ± 0.8 <sup>a,b</sup>	25.1 ± 3.1 <sup>a-c</sup>	7756.2 ± 70.0 <sup>a</sup>
V13	93.7 ± 1.6 <sup>a-d</sup>	27.62 ± 7.46 <sup>a-c</sup>	2.1 ± 1.0 <sup>a</sup>	26.8 ± 7.5 <sup>a,b</sup>	7147.0 ± 96.1 <sup>c-e</sup>
V14	76.3 ± 4.2 <sup>f</sup>	12.53 ± 4.13 <sup>c</sup>	0.1 ± 0.0 <sup>c</sup>	10.4 ± 3.7 <sup>c</sup>	7611.4 ± 66.0 <sup>a,b</sup>
V15	88.5 ± 3.0 <sup>a-e</sup>	27.45 ± 6.65 <sup>a-c</sup>	0.4 ± 0.2 <sup>c</sup>	24.7 ± 6.1 <sup>a-c</sup>	6874.3 ± 104.2 <sup>e</sup>
V16	90.7 ± 3.9 <sup>a-e</sup>	24.95 ± 4.47 <sup>a-c</sup>	0.8 ± 0.2 <sup>c</sup>	23.5 ± 4.6 <sup>a-c</sup>	6856.8 ± 164.6 <sup>e</sup>

Note: Values presented in the table are the means of the three experimental years (2014–2015, 2015–2016, 2016–2017) ± standard error. Means followed by different letters indicate differences at  $p < 0.05$  according to LSD test. Freq—Frequency; Int—Intensity; Arb—Arbuscularity; Cdeg—Colonization degree. Total N and P (kg ha<sup>-1</sup>) corresponding to each treatment and fertilizer type: V1–N40P40 (N20P20); V2–N80P80 (N20P20); V3–N120P120 (N20P20); V4–N40P40 (N18P46 + AN); V5–N147P80 (N20P20 + AN); V6–N147P80 (N20P20 + CAN); V7–N147P80 (N20P20 + Urea); V8–N147P80 (N20P20 + AN); V9–N147P80 (N20P20 + CAN); V10–N147P80 (N20P20 + Urea); V11–N147P80 (N18P46 + AN); V12–N147P80 (N18P46 + AN); V13–N147P80 (N18P46 + CAN); V14–N147P80 (N18P46CAN); V15–N187P120 (N20P20 + AN); V16–N187P120 (N20P20 + AN). A full description of treatment recipes is provided in Table 1.

A similar phenomenon occurs following the application of low basic and phased fertilization (V4), with the frequency of colonization decreasing to 87% (Table 2). An interesting case is that of the gradual increase in N20P20, which produces a decrease in frequency (V1–3). Doses of 40 and 80 NP result in a frequency of 88%, while increasing the dose to 120 NP reduces this parameter by 8%.

The application of fertilizers produces a 26% range of variation in the intensity of colonization (Table 2). A divided phased application of AN (V8) and CAN (V9), and the application of one dose of urea (V7) and CAN (V6), lead to the establishment of a colonization intensity within 28–38.35% (V9). A value of 28.37% of this parameter is also obtained when applying a basic fertilization with 40 kg ha<sup>-1</sup> NP (V1). The increase to 120 kg ha<sup>-1</sup> NP (V15) in basic fertilization and the addition of AN, in addition to the 80 kg ha<sup>-1</sup> NP (V2) dose, does not produce significant increases in intensity, with both treatments showing a colonization expansion of 27%. The reduced dose of N18P46 + AN (V4) maintains the intensity at 21%. Compared to this treatment, a double value of basic fertilization and phased AN (V11) leads to a reduction in intensity to 18.96%. The lowest parameter values are recorded at V3 (16.94%) and V14 (12.53%).

Arbuscules abundance is strongly influenced by the type and dose of applied fertilizers, with significant variations between treatments (Table 2). Nine of the experimental treatments recorded values below 0.5% of this parameter. Of these, the treatments with 120 kg ha<sup>-1</sup> NP (V3) and the 31N80P + 58 × 2 CAN (V14) have values less than 0.1%. Treatments with high doses of NP, with (V16) and without phased fertilization (V1–2), and urea in two doses (V10), establish arbuscularity in the

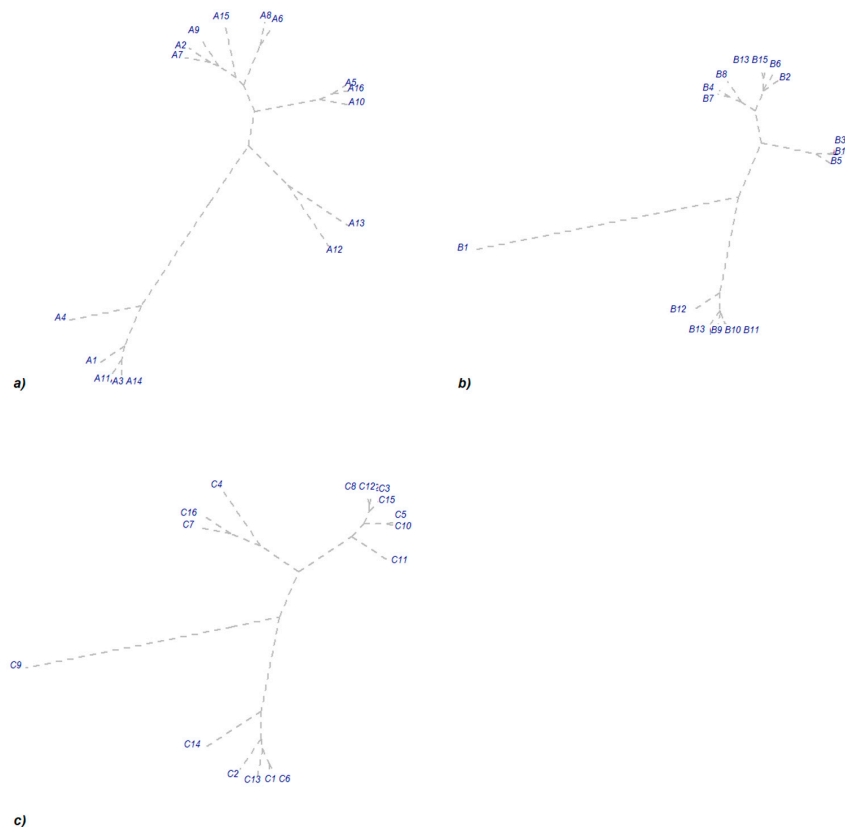
range of 0.73–0.90%. A two-dose application of phased CAN (V9) leads to an increase in intracellular development of arbuscules to a value of 1.14%, with a difference of more than 0.80% from a two-dose AN (V12) and a single-dose CAN (V13), both on N18P46 basic fertilization. This value represents a better development of the arbuscular circuit due to applied treatment and an area where nutrients can be translocated to the host faster.

The degree of colonization expresses the volumetric exploration of roots by mycorrhiza fungi and shows significant variations between treatments (Table 2). The highest AM colonization is 37.1%, obtained by the application of CAN in two doses on a basic fertilization with 80 kg ha<sup>-1</sup> NP (V9). On the same basic fertilization but with the application of CAN in a single dose (V6) and urea (V7), respectively two phased doses of AN (V8) or urea (V10), the level of colonization reached is located in the range of 26.32–28.87%. V13 is the only treatment with N18P46 where the parameter exceeds 26%. The absence of phased fertilization leads to a 25% colonization value in moderate and low-dose NP (V1–2) treatments. The increase in basic fertilization to 120 kg ha<sup>-1</sup> NP (V3) and the absence of phased fertilization decreases the degree of colonization by more than 10%. Treatment V14 has the lowest level of colonization, of only 10.41%.

The yield has significant differences of more than 1600 kg between variants (Table 2). The treatments to which phased AN (V12, V5) was applied recorded yields of more than 7700 kg ha<sup>-1</sup>. Nine treatments had production in the range of 7000–7600 kg ha<sup>-1</sup>, due to either the high doses of NP applied as a base or the supplementation during the growing season with phased fertilizers. An interesting case is the treatments with NP 120 kg ha<sup>-1</sup> base fertilization (V15–16), to which the application of phased AN does not lead to crop yields over 6900 kg ha<sup>-1</sup>.

### 3.2. Inter-Annual Dynamics of Arbuscular Mycorrhiza Colonization

Cluster analysis was performed on all mycorrhiza parameters in order to evaluate the colonization directed by fertilizers. Clusters are presented as groups of treatments formed by the similar value of one or more parameters. Cluster analysis is useful for the selection of fertilizers in order to have a specific arbuscular mycorrhiza pattern. The similarity analysis of the mycorrhiza response to fertilizers highlights different profiles in each experimental year (Figure 1a–c). For 2014–2015, four clusters are distinguished (Figure 1a; Table S1). Cluster 1 consists of two treatments fertilized only with N20P20 (V1, V3) and three treatments with N18P46 (V4, V11, V14) as the basic treatment. For this cluster, the incidence of AM recorded in wheat roots is <80% with the colonization intensity ranging from 22 to 31% (Table S1). The arbuscularity is maintained below 0.5% and the degree of colonization did not exceed 27.89%. The lowest levels of colonization are observed in V4, fertilized with low doses of N and P. The second cluster comprises two treatments with N18P46. Fractional AN (V12) application stimulates colonization of 34.42% and an arbuscules percentage of 5.07%. The application of a single dose of CAN (V13) produces an increase in the degree of colonization to 55.51% and a percentage of arbuscules of 5.87%. The differences between the two treatments indicates a divergence in mycorrhiza development, with a higher number of arbuscules occurring in wheat roots of treatment V12 compared to the total expansion of the symbiotic mycelium. The third cluster is composed of N20P20 treatments, but supplemented with AN (V5), urea (V10), and AN (V16) in two doses. The colonization frequency is 96.67–98.89%, supplemented by a range of 1.33–1.79% for arbuscules. V10 indicates a different trend of sustained colonization frequency and lower values of colonization intensity (36.79%). Cluster 4 is heterogeneous and comprises six treatments. V6 and V8 treatments have values of more than 60% colonization intensity supported by a frequency of 98.89%. Treatment V15 is defined by the colonization intensity of 53.79% and a frequency of 90%. This transitions to a part of the cluster defined by an intensity below 50% and over 2.5% arbuscules, from an intensity of more than 50% but with arbuscules below 2%.



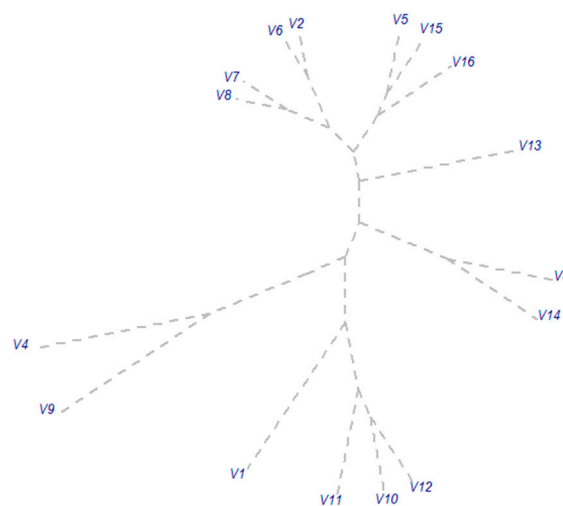
**Figure 1.** Cluster analysis of single-year arbuscular mycorrhiza profile winter wheat due to applied fertilizers: (a) 2014–2015 (A); (b) 2015–2016 (B); (c) 2016–2017 (C). Each point comprises the recorded values of frequency, intensity, arbuscularity, and colonization degree for a specific treatment. Number 1–16, applied treatments. Total N and P ( $\text{kg ha}^{-1}$ ) corresponding to each treatment: 1–N40P40; 2–N80P80; 3–N120P120; 4–N40P40; 5–N147P80; 6–N147P80; 7–N147P80; 8–N147P80; 9–N147P80; 10–N147P80; 11–N147P80; 12–N147P80; 13–N147P80; 14–N147P80; 15–N187P120; 16–N187P120. A full description of treatment recipes is provided in Table 1.

The 2015–2016 season produces a mycorrhiza profile with a clear separation between clusters (Figure 1b; Table S1). The fertilized treatment with  $40 \text{ kg ha}^{-1}$  NP (V1) is defined by a 90% colonization frequency, with a colonization intensity of 42.12% and 2.7% arbuscules. The second cluster (V9–V13) comprises treatments with high doses of N applied as basic or phased fertilizer, with colonization frequencies of more than 93% and intensities in the range of 20.83–26.68%. The third cluster is composed of treatments with an average of 60% colonization frequency and intensities less than 10%, corresponding to treatments with  $120 \text{ kg ha}^{-1}$  NP (V3), NP + AN (V5), and NP + CAN (V14). Cluster 4 incorporates three treatments (V4, V7, V8), at which the frequency is in the range of 85.56–91.11% and with an intraradical expansion of 14.98–10.16%. The fifth cluster (V2, V6, V8, V15, and V16) comprises treatments with degrees of colonization below 10%, due to the poor intraradical development of the arbuscular mycorrhiza mycelium and a reduced frequency of the phenomenon.

In the third experimental year, the cluster analysis groups the mycorrhiza response into four well-separated spatial clusters (Figure 1c; Table S1). The treatment with  $80 \text{ kg ha}^{-1}$  NP and  $116 \text{ kg ha}^{-1}$  N from CAN (V9) is individualized due to the 100% frequency and a colonization intensity of more than 43%. Cluster 2 comprises treatments where the intensity of colonization is less than 15% on a background of 82.22–92.22% frequency. For this cluster, the arbuscularity is almost absent. Clusters 3 and 4 have similar values of colonization frequency and intensity, being differentiated by the presence of 0.27–0.71% arbuscules in Cluster 3 treatments (V4, V7, V16).

For the whole experiment, cluster analysis indicates treatments with the constant potential to maintain mycorrhiza colonization values (Figure 2). There are cases in which colonization is not

consistent and there are large inter-annual fluctuations due to treatments. Fertilization with P exceeding N (V4), both in low doses, produces fluctuations in the colonization frequency, in particular, and keeps the percentage of arbuscules below 1%. A similar case is observed in V9, where the fluctuation of colonization occurs in intensity and the percentage of arbuscules. The interannual assembly of colonization parameters in the treatment with 40 kg ha<sup>-1</sup> NP (V1) highlights a stimulation of the mycorrhiza mechanism in the first two years of experimentation. For this variant, a sharp decrease in colonization intensity in the third year, to 40%, is observed.



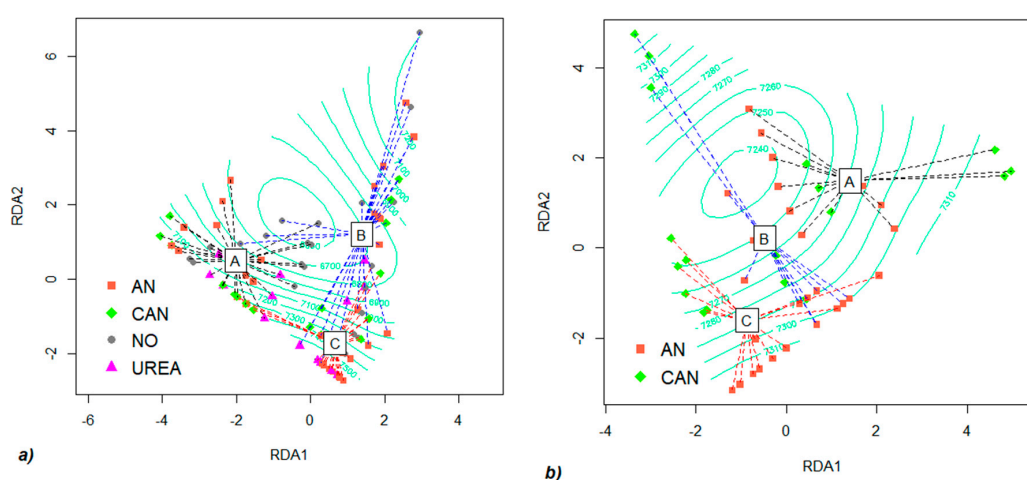
**Figure 2.** Cluster analysis of the inter-annual profile of the arbuscular mycorrhiza colonization winter wheat. Each point comprises the recorded values of frequency, intensity, arbuscularity, and colonization degree for that specific treatment in all three experimental years. Total N and P (kg ha<sup>-1</sup>) corresponding to each treatment and fertilizer type: V1–N40P40 (N20P20); V2–N80P80 (N20P20); V3–N120P120 (N20P20); V4–N40P40 (N18P46 + ammonium nitrate (AN)); V5–N147P80 (N20P20 + AN); V6–N147P80 (N20P20 + calcium ammonium nitrate (CAN)); V7–N147P80 (N20P20 + Urea); V8–N147P80 (N20P20 + AN); V9–N147P80 (N20P20 + CAN); V10–N147P80 (N20P20 + Urea); V11–N147P80 (N18P46 + AN); V12–N147P80 (N18P46 + AN); V13–N147P80 (N18P46 + CAN); V14–N147P80 (N18P46CAN); V15–N187P120 (N20P20 + AN); V16–N187P120 (N20P20 + AN). Full description of treatment recipes is provided in Table 1.

The cluster formed by V10–12 treatments provides an image of a heterogeneous assembly of colonization, integrating cases with parameter compensation and normalizing interannual fluctuations (Figure 2). V3 and V14 form a cluster with similar interannual variations; in both treatments, a reduction in the colonization frequency in the second year of experimentation and similar values of arbuscules can be observed. V13 is a treatment with extreme interannual changes, with an annual reduction of 5% in the colonization frequency. The 35% reduction in intensity from year 1 of experimentation overlaps with the second year, a trend also followed in the third year, with a 15% reduction. Arbuscules in this treatment have a high value only in the first year, falling sharply in the other two years. Three treatments with the same AN value (V5, V15–16) form a group with similar interannual changes in all mycorrhiza parameters. For this group, arbuscules (>1.3%) develop only from an intensity value of more than 40%. Similar frequencies of V2 and V6 are characterized by comparable intensity and arbuscules values, which compresses them into a single cluster. Close to this group are the identified treatments V7 and V8, with high values of colonization intensity in the first experimental year and a similar decreasing trend in the other two years.

### 3.3. Impact of Year and Phased Fertilizer on Colonization Potential

The use of N20P20 and N18P46 as basic fertilization creates a grouped response of the mycorrhiza mechanism related to the year-specific climate (Figure 3a,b). The RDA response assessment explains

81.63% of variations in the case of N20P20 and 89.99% for N18P46 (Table 3). The climate is of much greater importance in the conditions of the N20P20 base ( $F = 130.86$ ); it remains significant in the interaction with phased fertilization and its separate application ( $F = 5.08$ ), but with a reduced influence. For this type of basic fertilization, arbuscular mycorrhiza mechanisms maintain their global dynamics in the conditions of years 1 and 3, but have a high fluctuation in the second year of experimentation. The phased application of CAN maintains stable yields based on N20P20, but only in the first year of experimentation. The dynamics of colonization and crop yield are heterogeneous in the ordination plan at the application of AN and urea. Under the condition of N18P46 as basic fertilizer, the climate is of much lower singular importance ( $F = 54.77$ ), but acts more strongly to ensure multiple interactions with the phased fertilization and separation of this in doses on vegetation ( $F = 20.48$ ). For this type of base, the type of phased fertilization and the mode of application are much more restrictive ( $F = 64.10$ ), especially under the conditions of year 3 of experimentation. A clear separation of the colonization is observed due to the crop yield potential and fertilization with AN or CAN.



**Figure 3.** Redundancy analysis (RDA) of arbuscular mycorrhiza colonization in winter wheat, separated by year, basic fertilization, and yield potential: (a) N20P20; (b) N18P46. (A) 2014–2015; (B) 2015–2016; (C) 2016–2017. AN, ammonium nitrate (N—34%); CAN, calcium ammonium nitrate (N—27%); urea (N—46%).

**Table 3.** The importance of experimental factors in arbuscular mycorrhiza development in winter wheat.

Basic Type	N20P20		N18P46	
	RDA 1	RDA 2	RDA 1	RDA 2
Variance explained (%)	73.50	8.13	74.27	15.72
Interaction	F	<i>p</i> -value	F	<i>p</i> -value
year	130.86	0.005	54.77	0.005
phase	6.61	0.005	13.79	0.005
split	6.66	0.015	6.41	0.015
year × phase	3.61	0.005	39.13	0.005
year × split	6.22	0.005	1.81	0.205
phase × split	2.42	0.095	64.10	0.005
year × phase × split	5.08	0.010	20.48	0.005

Note: Significance levels:  $p < 0.001$ ;  $p < 0.01$ ;  $p < 0.05$ . Year—the influence of the three experimental years (2014–2015, 2015–2016, 2016–2017); Phase—the influence of the application period (autumn or post-winter); Split—the influence of the post-winter applications (at the end of winter or at the end of winter + boot development phase).

The assessment of arbuscular mycorrhiza colonization in autumn wheat highlights the perturbation potential of nutrients from treatment recipes (Table 4). The forecast of the colonization frequency in the roots is 93.51%, plus a value of 0.19% for each kg of N applied in autumn, and 0.12% in the case of the phased application of N. Phosphorus leads to a reduction in the colonization almost equal to the total N



applied. The trend is also maintained in the case of colonization intensity, but with a much higher value of the P reduction influence ( $0.37\% \text{ kg}^{-1} \text{ P}$  applied) and with a maximum for the colonization degree. Arbuscularity is a phenomenon present with a lower proportion in mineral fertilized winter wheat, and, for this parameter, P and N applied on vegetation are responsible for its growth. For the forecast of the potential yield, five different models were created, based either on the unique effect of fertilizers, colonization parameters, or their integration. Three models were obtained with an average yield estimate, accompanied by one model for high- and one for low-yield estimates. Model 2 integrates the fertilization recipe and the main parameters of colonization, highlighting the role of the phased application of N and the frequency of colonization as positive factors in determining the yield. In the same model, the colonization intensity and the arbuscules development are negatively involved in the yield forecast. The model that starts from a high production value ( $7214.4 \text{ kg ha}^{-1}$ ) is based to a large extent on the arbuscules value, and a reduction occurs due to the way the hyphae expand into the cortex observed through the colonization degree. For the model based on the interaction between frequency, intensity, and arbuscules, high yields can be obtained in the context of a high colonization frequency, but with the development of a high number of arbuscules. The integration of fertilizers with arbuscularity and degree of colonization indicates the antagonism between the structures developed during colonization ( $\text{Arb} = -22.96$ ,  $\text{Cdeg} = -1.50$ ) and the minerally applied elements. At the same time, it can be observed that the role of N in the formation of the crop yield, and of P in its reduction, are related to the partial blocking of the mycorrhiza absorption mechanism.

**Table 4.** Forecasting potential of fertilizers over arbuscular mycorrhiza parameters and crop yield in winter wheat.

Mycorrhizas (%)	Inter	N <sub>a</sub>	P <sub>a</sub>	N <sub>v</sub>				
Freq (%)	93.51	0.19	-0.28	0.12				
Int (%)	31.55	0.27	-0.37	0.09				
Arb (%)	0.68	-0.005	0.003	0.003				
Cdeg (%)	29.76	0.29	-0.39	0.11				
Yield ( $\text{kg ha}^{-1}$ )	Inter	N <sub>a</sub>	P <sub>a</sub>	N <sub>v</sub>	Freq	Int	Arb	Cdeg
Model 1	6439.88	6.80	-4.72	10.26				
Model 2	5858.45	6.81	-4.53	9.83	8.20	-5.76	-4.85	
Model 3	6500.10	7.11	-5.25	10.48			-22.96	-1.50
Model 4	6024.77				15.08	-9.34	32.01	
Model 5	7214.41						6.18	-1.82

Note: Inter.—Intercept; N<sub>a</sub>, P<sub>a</sub>—nitrogen and phosphorus applied in autumn; N<sub>v</sub>—nitrogen applied as a phasal fertilizer; Freq—Frequency; Int—Intensity; Arb—Arbuscularity; Cdeg—Colonization degree.

#### 4. Discussion

The three experimental years showed a number of differences during the growing period of the winter wheat [41–44], which act as a supplementary source of variation in AM colonization potential. This allowed the monitoring of the arbuscular mycorrhiza profile both in response to applied fertilization and in response to the specific climate of each year. Year one of experimentation was a normally warm year, but with rainfall only in the autumn period. This aspect induces stress for plants and a need to enter into symbiosis with mycorrhizas for the provision of water, in particular [45,46]. The second experimental year was characterized by a dry period in winter, but with an excess of precipitation from the beginning of spring until harvest, and a normally warm thermal regime. In this climatic context, plants have a normal development and act toward partially blocking the installation of mycorrhizas in the root cortex. The third year was characterized by an alternation of dry and rainy periods and cold and warm periods. Arbuscular mycorrhiza colonization is restricted by this type of climate, being more present in the roots only during dry periods to supplement the nutrients necessary for plant development [47,48].

Fertilizers produced fluctuations in the AM colonization of wheat from one experimental year to another. There were three treatments that maintained the differences of all colonization parameters below 15%. The lowest doses of N18P46 + AN (V4) resulted in the lowest colonization in the first year. For this treatment, the increase in colonization frequency was 9% from year one to year two, and 14% from year two to year three. Intensity of colonization decreased by 7% in the second year, followed by an increase of 11% in year three. This recipe produced a decrease of just 4% in colonization degree in the first two years, followed by an increase of 13% in the third year. N20P20 with two doses of urea (V10) maintained a stable colonization frequency, above 96% in all experimental years. For this variant, the largest decrease, of 14%, was in the intensity of colonization from year one to year two, which was also visible in colonization degree, which fell 13%. For this treatment, arbuscularity decreased slightly between years. A similar trend was visible for treatment with N18P46 + AN (V12), with just 10 to 12% changes in intensity of colonization and 8–12% in colonization degree. Arbuscules presented a high variation within colonization parameters, with values over 5% in only two treatments with N18P46 (V12–V13) and one year. Mycorrhiza colonization showed a lack of consistency between years, which sustains the perspective of choosing fertilizers based on both local climatic conditions and the desired level of colonization.

Arbuscular mycorrhiza networks work to balance the transfer of nutrients between symbiotic partners and prevent losses from ecosystems [49–51]. The presence in the root area of easily accessible nutrients makes the plant restrict the access of symbiotic fungi [52–54]. In this context, the application of high doses of fertilizers gives plants an immediate surplus of nutrients and reduces their ability to form symbiosis. On the other hand, moderate doses of fertilizers work both to stimulate plant and root development and to provide a high area of installation of a much stronger partnership between fungi and plants. In this case, fungi compensate for the lack of nutrients directly accessible to the plant that normally come from fertilization [55]. However, the development of arbuscules occurs only following a massive root colonization; under moderate fertilization conditions, this parameter is maintained at low levels.

The presence of arbuscular mycorrhiza fungi in the rhizosphere of wheat plants leads to a stronger accumulation of P by plants [56]. The solubility of fertilizers strongly influences the development of symbiotic fungi through the current level of nutrients present in the rhizosphere. The high availability of fertilizers leads from a reduction to a blockage of colonization during the period when the plant has ensured the source of nutrients [57]. The application of fertilizers with high concentrations of P reduces the potential for colonization, which leads to a lower response and resistance of plants to the appearance of stress.

The current context of agriculture promotes the reduction in mineral inputs and the identification of alternative solutions to ensure the nutrients necessary for the maintenance of high yields in wheat [58–60]. Arbuscular mycorrhiza develops a hyphal network in host roots with a role in supplementing the nutrient transfer from soil to their partner [61]. The level of nutrient uptake and transfer to the host is related to the intensity of colonization and the overall extension of hyphae in the roots. The intensity of colonization represents the entire transport network that will provide the potential quantities of nutrients available to the plant. When root cells are penetrated by hyphae, they branch and form arbuscules, which improve the nutrient exchange between partners [62]. The arbuscular circuit provides the benefit of enhanced transfer of nutrients to the host. Assessing mycorrhiza dynamics and linking it to applied fertilization levels and yield is a useful tool in determining the optimal doses in terms of the symbiotic potential of the soil. The response of plants to arbuscular mycorrhiza colonization is dependent on the wheat variety [63], which can be further analyzed as colonization patterns related to the structures developed by fungi in roots. Limiting chemical sources of nutrients stimulates plants in the development of a highly permissive root system toward symbionts and the more efficient exploitation of soil resources. Regular evaluation of the connections of wheat plants with mycorrhiza networks is essential for adapting fertilization systems to specific climate and soil

conditions. Based on the mycorrhiza response, an efficient strategy can be established for the selection of an appropriate fertilizer formula and application phase.

## 5. Conclusions

Results of this study show that the frequency of colonization is above 98% at the base application of N20P20, and two doses of phased urea and CAN. The application of CAN to basic fertilization with N20P20 leads to an average colonization intensity of 38.35% and an arbuscules percentage of 1.14%. For periods with water stress at the end of winter (e.g., 2014–2015), the application of AN as a phased fertilizer maintains the intensity of colonization at over 40%. For years of excess rainfall during the growing season (e.g., 2015–2016), it is recommended to use fertilizers with a high percentage of P compared to N and to apply AN to maintain a degree of colonization of more than 22%. In precipitation and temperature alternations (e.g., 2016–2017), the most favorable treatment is CAN-based, with a phased application in two doses, which maintains the degree of colonization at over 43%. The arbuscular mycorrhiza colonization forecast models indicate P as a symbiosis reduction factor, with a value of 0.39% for each kg of fertilizer applied. The lack of water in the autumn period leads to strong changes in the potential for colonization.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/2073-4395/10/6/846/s1>, Table S1: Impact of the experimental factors on arbuscular mycorrhiza colonization in winter wheat.

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