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The Effect of Mycorrhizal Inoculum and Phosphorus Treatment on Growth and Flowering of Ajania (*Ajania pacifica* (Nakai) Bremer et Humphries) Plant

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Abstract: The influence of mycorrhizal inoculum in combination with different phosphorus treatments on growth and flowering parameters of Ajania (*Ajania pacifica* (Nakai) Bremer et Humphries) plants was investigated in two growing seasons (2015 and 2016). Plants of the cultivar 'Silver and Gold' were transplanted into pots either with added mycorrhizal inoculum or without inoculum and assigned to four phosphorus treatments. Mycorrhizal colonization was assessed by evaluating the frequency of colonization, intensity of colonization and density of fungal structures (arbuscules, vesicles, coils and microsclerotia) in the roots. During the growing season, the content of plant available phosphorus in the soil was analyzed, and shoot length, number of shoots, number of inflorescences, number of flowers and flowering time were evaluated. Inoculated Ajania plants were successfully colonized with arbuscular mycorrhizal fungi and dark septate endophytic fungi. In the root segments, hyphae were mainly observed, as well as vesicles, coils, arbuscules and microsclerotia, but in lower density. The density of fungal structures did not differ among phosphorus treatments, but did differ between years, with a higher density of fungal structures in 2016. Mycorrhizal plants developed higher number of shoots in 2016, higher number of inflorescences, higher number of flowers, and they flowered longer compared to uninoculated plants.

Keywords: arbuscular mycorrhizal fungi; dark septate endophytic fungi; phosphorus; fertilization; growth; flowering; fungal colonization

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1. Introduction

Arbuscular mycorrhiza (AM) is the most common form of endomycorrhiza. It is a symbiotic relationship between soil fungi from the phylum Glomeromycota [1] and the roots of higher plants in which there is a characteristic intracellular and intercellular proliferation of hyphae in the parenchymal and epidermal cells of the primary cortex of the root. With the help of AM, the metabolism between the two partners is improved and the area of water uptake with minerals is up to forty times larger, to which extracellular hyphae contribute [2]. Symbiosis with AM fungi has many beneficial effects on plants. An important effect of this symbiosis may be an increase in the uptake of mineral nutrients from the soil, especially relatively immobile ones such as phosphate [3]. The fungus provides mineral nutrients, mainly phosphorus (in the form of phosphate) [4], hormones and water to the plant via the fungal mycelium, and the plant fungus provides energy (in the form of sugars) [5]. AM root colonization is an important factor affecting seedling development and competition with other species. The degree of colonization depends, among other factors, on the nutrient conditions in the substrate, especially the availability of nitrogen and phosphorus, which are considered the most restrictive nutrients for plant growth [6]. Mycorrhizae enhance the uptake of nutrients by the plant by increasing the

absorptive surface of the receiving system. Mycorrhizal plants are able to obtain more nutrients from nutrient-poor soils than non-mycorrhizal plants because the hyphae of mycorrhizal plants use a larger soil volume [7]. It has been reported that mycorrhizae play little or no role in substrates where there is high availability and supply of nutrients, especially phosphorus [6,8]. Plants colonized with AM fungi are often colonized with dark septate endophytic (DSE) fungi [9], which belong to the class Ascomycetes [10] and contain structures called microsclerotia. The effect of DSEs on plant growth and mineral nutrition can be positive or negative depending on the plant species and environment [11]. In general, DSEs improve plant growth and development, enhance plant tolerance to various environmental stressors, play a role in phytohormone production, plant nutrient supply, degradation of complex carbohydrates and plant simple sugars, increase photosynthetic activity, and regulate the concentration of plant metabolites [12–15].

Ajania (*Ajania pacifica* (Nakai) Bremer et Humphries) is a perennial ornamental plant in the Asteraceae family. It is a relative of chrysanthemum and also a short-day plant [16,17], flowering in a short day, so the production technology is very similar to that of chrysanthemum. It is mostly used as a pot plant, for garden beds, as attractive single plants, but there are also varieties used for cut flowers. It could be an interesting alternative to the well-known chrysanthemum in the future [16]. The cultivar 'Silver and Gold' is probably the most popular cultivar of the genus Ajania, mainly because of the ornamental value of the leaves and the flower [16].

The positive influence of AM fungi on the growth and development of herbaceous perennials in pot experiments and in the greenhouse is cited by many authors in their studies. McGraw and Schenck [18] and Sohn et al. [19] in an experiment on chrysanthemum (*Chrysanthemum morifolium* Ramat.) reported better rooting of cuttings, longer roots, more lateral roots, greater shoot growth, faster flowering and more and larger inflorescences in plants to which inoculum was added. Gaur et al. [20] found higher dry matter, larger plants, higher number of flowers, and earlier flowering in an experiment with petunia (*Petunia hybrida* Vilm.) with added inoculum. Hemla Naik et al. [21] found higher shoot dry matter, improved phosphorus supply, higher flower yield and fertilizer saving (25% less phosphorus) in inoculated *Callistephus chinensis* (L.) Nees plants. Asrar et al. [22], in an experiment on flowering Kalanchoe (*Kalanchoe blossfeldiana* Poelln.) plants found that inoculated plants had higher number of flowers and shoot dry matter compared to uninoculated plants. Moreover, some authors reported improved response of inoculated *Antirrhinum majus* L. [23], *Dianthus caryophyllus* L. [24], *Imaptiens balsamina* L. [25], *Kalanchoe blossfeldiana* Poelln. plants [22] to different stressors.

To our knowledge, there are no studies in the literature on the effect of mycorrhizal fungi on the growth and flowering of Ajania plants. The aim of our study was to investigate (i) the degree of colonization of the root system of Ajania by mycorrhizal fungi, (ii) how the addition of phosphorus affects the colonization of plants by mycorrhizal fungi, (iii) the effect of mycorrhizal fungi on shoot length, number of shoots and flowering time of Ajania plants.

2. Materials and Methods

2.1. Experimental Design

A two-year pot experiment with Ajania (*Ajania pacifica* (Nakai) Bremer et Humphries) plants, cultivar 'Silver and Gold' was conducted in the greenhouse at the Biotechnical Faculty of the University of Ljubljana, Slovenia (46°2′ N, 14°28′ E, 297 m a.s.l.). The growing conditions (temperature, humidity, lighting, etc.) in the greenhouse were in accordance with the recommendations for growing chrysanthemums [26].

In the experiment, 40 annual seedlings cut to 4–5 cm height were planted on 26 May 2015 in Humko substrate consisting of 40% white peat (50% fractionated, 50% std), 20% black peat, 20% lava, 15% pumice, 5% zeolite, with pH 6.05 and 43.9 mg P_2O_5 L^{-1} substrate in pots of 1 L volume. At the time of planting, 40 mL of AM fungal inoculum was added to half of the plants in the root zone (inoculated plants). Commercially available inoculum

Symbivit (Symbiom Ltd., Sázava, Czech Republic) with six AM fungal species (*Glomus etunicatum* WN Becker and Gerd., *Glomus microaggregatum* Koske, Gemma and PD Olexia, *Glomus intraradices* NC Schenck and GS Sm., *Glomus claroideum* NC Schenck and GS Sm., *Glomus mosseae* (TH Nicolson and Gerd.) Gerd. and Trappe and *Glomus geosporum* (TH Nicolson and Gerd. C.) Walker) was used. The inoculum contained no other ingredients that could have a direct positive or negative effect on plant performance (www.symbiom.cz (accessed on 25 February 2021)). Other plants were planted without added AM fungal inoculum (uninoculated plants).

Two factors, mycorrhizal inoculation and phosphorus treatment, were studied in our experiment. The phosphorus treatment included four levels of substrate fertilization (Control, P1, P2, and P3) that differed in the amount of phosphorus added (Table 1). The control treatment did not receive phosphorus through superphosphate fertilizer. Forty pots were randomly arranged and placed in a greenhouse. Each combination of two inoculum-phosphorus factors (8 treatments) was carried out in five replicates. Later, in early June, 8 g of meat bone meal per liter of substrate was added to all plants by sprinkling. Meat bone meal (K3 Koto, Koto, Ljubljana, Slovenia) was added in the form of powder (particles up to 2 mm in size) containing 6–8% P_2O_5. Thus, each plant received 0.5 g of P_2O_5 L^{-1} substrate.

Table 1. Fertilization norms according to different fertilization treatments (in mg L^{-1} substrate).

Fertilization Treatment	Superphosphate Fertilizer	P_2O_5	0-0-60 Fertilizer	K_2O	KAN-Kutina	Calcium Ammonium Nitrate
Control	0	0	20	12	150	40.5
P1	20	5.2	20	12	150	40.5
P2	40	10.4	20	12	150	40.5
P3	80	20.8	20	12	150	40.5

In accordance with the experimental design, only plants in treatments P1, P2 and P3 were fertilized with superphosphate (26% P_2O_5, Fertoz Co., Melbourne, Australia) for the first time in mid-June and then at regular weekly fertilizations. At the same time, all plants were fertilized with potassium and nitrogen fertilizers. For the potassium treatments, commercial fertilizer 0-0-60 (The Andersons, Inc., Maumee, OH, USA) was used. For the nitrogen treatments, KAN-Kutina (Calcium Ammonium Nitrate Fertilizer, Petrokemija, Kutina, Croatia) was used, which contains equal amounts of nitrogen in nitrate and in ammonium forms (soluble mineral forms) (Table 1).

Twice during the growing season in 2015 (3 July and 30 September) and in 2016 (27 July and 11 October), the same amount of substrate (10 g) was taken from the root zone per pot to analyze the content of plant-available phosphorus according to the Egner–Riehm–Domingo AL method [27].

2.2. Measurements of Plant Growth and Flowering Parameters

During the growing season, growth parameters (total shoot length and total number of shoots) per plant were monitored four times in 2015 (on 19 June, 4 July, 4 August and 30 September) and five times in 2016 (17 April, 15 May, 28 June, 4 August and 13 September). In addition, flowering parameters (total number of flowers, total number of inflorescences, and total flowering time per plant) were monitored during the growing seasons. Due to excessive elongation of shoots, some of them also broke off at the end of the growing season.

2.3. Assessment of Root Mycorrhizal Colonization

Fine lateral roots (3 g), 1 cm long, smaller than 3 mm in diameter, were collected from inoculated and uninoculated plants during flowering to assess mycorrhizal colonization. The substrate was manually cleaned from the roots and washed under running and distilled water. The cleaned roots were kept in tubes and stored in distilled water in a refrigerator at 4 °C until further work in the laboratory.

To estimate of mycorrhizal colonization, roots were prepared according to the modified method of Phillips and Hayman [28]. The roots were soaked in 10% KOH for 30 min at 90 °C, then washed under running water and stained with trypan blue. Subsequently, the stained roots were dried at 90 °C for 10–15 min. Each root sample consisted of 30 fragments of 1 cm length.

Mycorrhizal colonization was assessed according to the method of Trouvelot et al. [29], as previously described in Alarcón and Cuenca [30]. The degree of mycorrhizal colonization was evaluated microscopically in lactoglycerol using Mycocalc software (Wuhan, Hubei, China). This method allows simultaneous evaluation of the frequency of colonization (F%), the intensity of colonization (M%), the proportion of AM fungal structures (arbuscules (A%), vesicles (V%) and coils (C%)) and the proportion of DSE fungal structures (microsclerotia (MS%)) in the roots.

2.4. Statistical Analysis

The experiment was set up as a factorial design. The analysis was performed using the software version 3.6.1. of the R statistical environment [31]. The plant available phosphorus and mycorrhizal colonization data were subjected to two-factor analysis of variance (ANOVA) (factors phosphorus treatment and time), while data regarding flowering were subjected to three-factor ANOVA (factors: mycorrhizal inoculation, phosphorus treatment and time). The phosphorus treatment factor had four levels (Control, P1, P2 and P3) and the mycorrhizal inoculation factor had two levels (inoculated and uninoculated). In the analysis of plant available phosphorus data, the time factor had 4 levels (3 July 2015, 30 September 2015, 27 July 2016 and 11 October 2016), while in the analysis of mycorrhizal colonization and flowering data it had 2 levels (2015 and 2016). Duncan's multiple range test was then performed at a significance level of $p < 0.05$ to detect significant differences between means, where required. Growth parameter data (total shoot length and total number of shoots) were subjected to a linear mixed model (*lme* function in the *nlme* package) where the dependence of measurements on the same pot over time was considered, including the random effect of pot. The model included three factors (time with 4 levels in 2015 and 5 levels in 2016; factor phosphorus treatment with 4 levels and factor mycorrhizal inoculation with two levels). When the analysis of variance showed statistically significant results (see Supplementary Tables S1 and S2 for growth parameters in 2015 and 2016, respectively), a contrast analysis was performed using a generalized hypothesis testing procedure, considering simultaneous hypothesis tests. If the *p*-value for differences between means was less than 0.05, the difference was considered statistically significant.

3. Results

3.1. Phosphorus Available to Plants

In our experiment, the content of plant available phosphorus in the soil increased over all sampling times in both years according to the experimental design (Table 2). The control treatment had the lowest content of plant available phosphorus in the soil, followed by P1, while P2 and P3 had the highest values of phosphorus available to plants. In 2015, the content of plant-available phosphorus in the soil was higher than in 2016, with the sampling date of 11 October 2016 showing the highest values. With the additional fertilization during the growing season and the addition of meat bone meal, phosphorus levels in the substrate on average increased from double (Control) to almost four times (P3) the recommended levels for growing chrysanthemum by the end of the experiment.

Table 2. The average content ± standard error (n = 20) of phosphorus available to plants (mg P_2O_5 L^{-1} substrate) regarding phosphorus treatment and time of sampling.

Main Effect	Phosphorus Available to Plants
Phosphorus Treatment	
Control	1367 ± 126 c
P1	1630 ± 135 b
P2	1852 ± 133 a
P3	1938 ± 130 a
Time	
3 July 2015	1732 ± 114 b
30 September 2015	2434 ± 93 a
27 July 2016	1459 ± 67 c
11 October 2016	1163 ± 79 d
ANOVA	
Phosphorus treatment	***
Time	***
Phosphorus treatment × Time	NS

Means followed by different letters in a column indicate statistically significant differences (Duncan test, $p < 0.05$). ***, statistically significant differences at $p < 0.001$; NS, not significant.

3.2. Mycorrhizal Colonization

No mycorrhizal colonization with AM fungi or DSE fungi was observed in the control (uninoculated) roots, whereas in all observed root segments of the inoculated plants, mycorrhizal abundance (F%) averaged 100% (±0%) in both years, indicating that certain fungal structures were present. Arbuscules, vesicles and coils were observed among AM fungal structures, while microsclerotia among DSE fungal structures. The intensity of arbuscular mycorrhizal colonization and the frequency of observed fungal structures did not differ statistically significantly among phosphorus treatments, but did differ between years, except for the frequency of arbuscules. The results showed that arbuscules were not present in 2015, while they were present in 2016, but their density was not significantly different between years. The density of vesicles, coils and microsclerotia was significantly higher in 2016, compared to 2015 (Table 3).

Table 3. Frequency (F%) and intensity (M%) of arbuscular mycorrhizal colonization, with frequencies of arbuscules (A%), vesicles (V%), coils (C%) and microsclerotia (MS%) in roots of inoculated Ajania plants in 2015 and 2016. Data are presented as means ± standard errors (n = 20).

Main Effect	F%	M%	A%	V%	C%	MS%
Time						
2015	100 ± 0.0	22.9 ± 3.1 b	0.0 ± 0.0	0.1 ± 0.0 b	0.01 ± 0.0 b	0.7 ± 0.2 b
2016	100 ± 0.0	38.1 ± 3.4 a	0.1 ± 0.0	1.0 ± 0.3 a	2.7 ± 0.4 a	2.9 ± 0.4 a
ANOVA						
Phosphorus treatment	NS	NS	NS	NS	NS	NS
Time	NS	**	NS	*	***	***
Phosphorus treatment × Time	NS	NS	NS	NS	NS	NS

Means followed by different letters in a column indicate statistically significant differences (Duncan test, $p < 0.05$). *, statistically significant differences at $p < 0.05$; **, statistically significant differences at $p < 0.01$; ***, statistically significant differences at $p < 0.001$; NS, not significant.

3.3. Growth and Flowering

According to the model, time affected the total shoot length of the plants in 2015 and 2016 (Tables S1 and S2; Figures 1 and 2). Ajania grew more in 2016 than in 2015 (Figures 1 and 2).

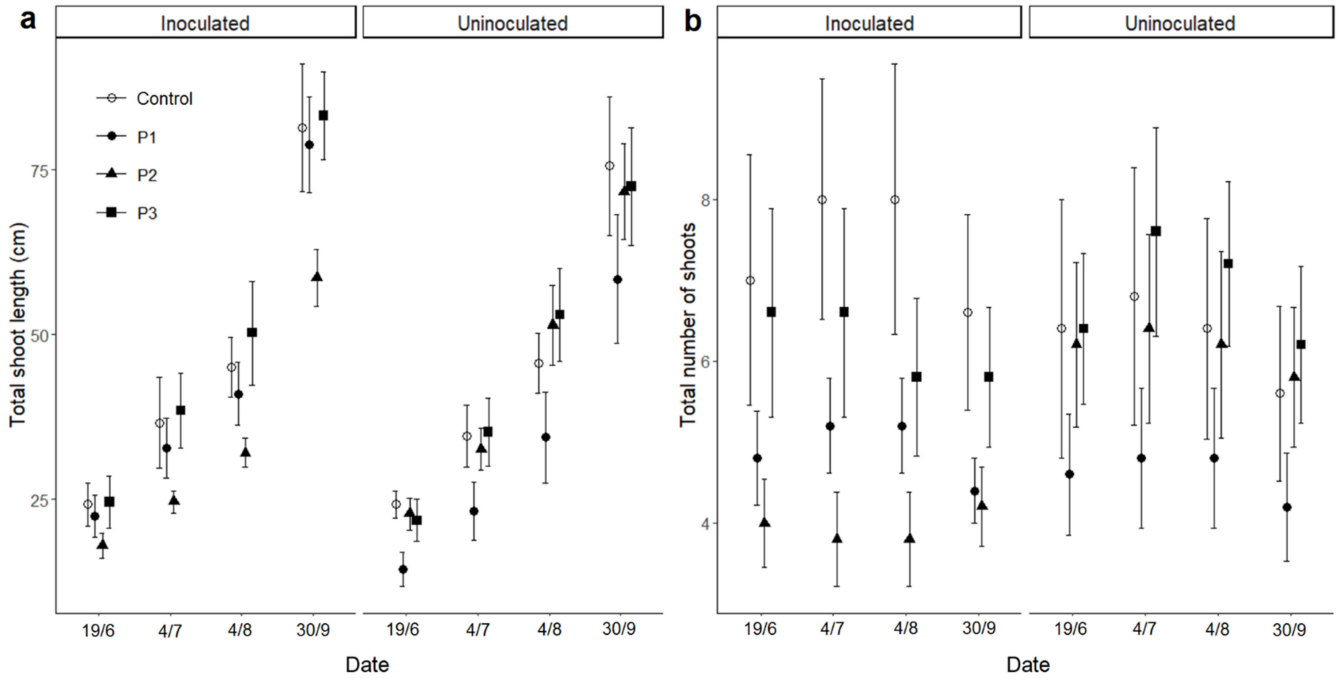

Figure 1. The average total shoot length (cm, (**a**)) and total number of shoots (**b**) of inoculated and uninoculated Ajania plants treated with different phosphorus levels (P1, P2 and P3) and without phosphorus (control) in 2015. The vertical bars represent ± SE of the mean value (n = 5).

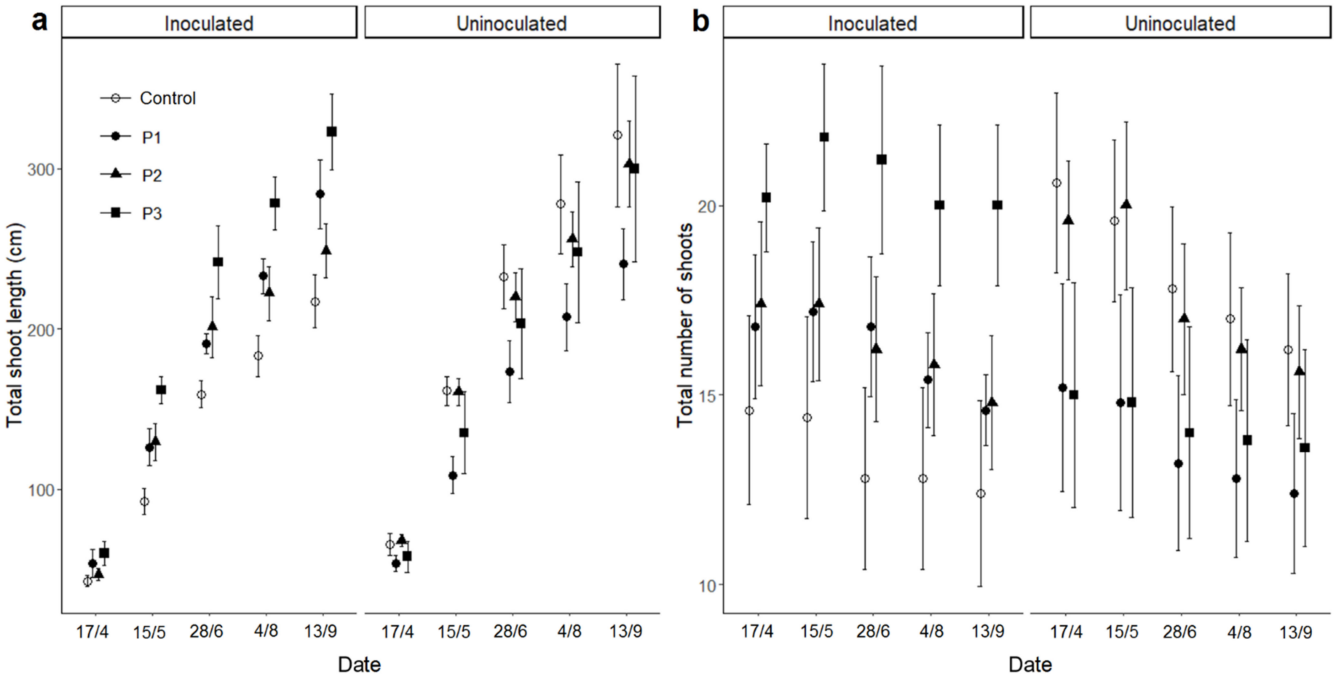

Figure 2. The average total shoot length (cm, (**a**)) and total number of shoots (**b**) of inoculated and uninoculated Ajania plants treated with different phosphorus levels (P1, P2 and P3) and without phosphorus (control) in 2016. The vertical bars represent ± SE of the mean value (n = 5).

In addition, the results showed statistically significant effect of triple interaction time × inoculum × phosphorus treatment ($p < 0.05$) on total shoot length in 2016 (Table S2). Inoculated control plants had statistically significant lower ($p < 0.05$) shoot length than inoculated plants of P3-phosphorus treatment. Only inoculated Ajania plants of the P3

phosphorus treatment had a statistically significant increase in shoot length (on average 1.67 ± 0.12 cm per day) compared to inoculated control plants (on average 1.15 ± 0.12 cm per day).

In 2015, time had a statistically significant effect on the total number of shoots in 2015 (Table S1). In 2016, however, analysis of the data showed a statistically significant effect of time, and the two-factor interactions inoculum×time and phosphorus treatment × time on the number of shoots (Table S2). In 2015, the analysis showed a statistically significant negative trend over time at the 95% confidence level and the number of shoots significantly decreased over time in traces (on average for 0.007 ± 0.0 shoots per day). In 2016, the number of shoots increased statistically more over time in plants with added inoculum than in plants without inoculum (Figure 2).

Flowering of the Ajania plants in our study was weak, which contributed to greater variability in the data. Analysis of variance showed statistically significant effect of inoculum on the flowering parameters evaluated ($p < 0.01$) in our study in both years. Inoculated plants developed on average statistically significantly more inflorescences, a greater number of flowers, and had a longer flowering time than uninoculated plants (Table 4).

Table 4. The average number of inflorescences, number of flowers and days of flowering (± standard error) (n = 20) of inoculated and uninoculated Ajania plants.

Main Effect	Number of Inflorescences	Number of Flowers	Days of Flowering
Mycorrhizal inoculum			
Inoculated plants	1.0 ± 0.2 a	29.8 ± 6.5 a	26.2 ± 5.4 a
Uninoculated plants	0.2 ± 0.1 b	6.4 ± 2.4 b	8.0 ± 2.8 b
ANOVA			
Inoculum	**	**	**
Phosphorus treatment	NS	NS	NS
Time	NS	NS	NS
Inoculum × Phosphorus treatment	NS	NS	NS
Inoculum × Time	NS	NS	NS
Phosphorus treatment × Time	NS	NS	NS
Inoculum × Phosphorus treatment × Time	NS	NS	NS

Means followed by different letters in a column indicate statistically significant differences (Duncan test, $p < 0.05$). **, statistically significant differences at $p < 0.01$; NS, not significant.

4. Discussion

The influence of mycorrhizal inoculum in combination with different phosphorus treatments on mycorrhizal colonization, shoot length and flowering of Ajania plants was investigated in the present study by evaluating the frequency, intensity of mycorrhizal colonization, abundance of different mycorrhizal structures, measurement of shoot length, number of shoots and evaluation of flowering parameters of the plants.

Our results show that mycorrhization of the inoculated plants was successful. Among the fungal structures, hyphae, vesicles, coils, arbuscules and microsclerotia were observed, indicating the presence of arbuscular mycorrhizal (AM) fungi and dark septate endophytic (DSE) fungi. AM fungi form different structures that can be present both in the substrate in which the plant grows and in the roots of the host plant [1]. In our study, hyphae that are non-septate and extend into the primary root tissue were observed mainly in root segments. Arbuscules were observed only in 2016. Arbuscules are short-lived branched hyphal structures that decay after a few days [5]. Due to their large surface area, they represent the most important site for metabolic nutrient exchange between the fungus and the plant, in addition to the hyphae [1,4]. The absence of arbuscules can be influenced by the needs of the plant, the season or the stress to which plants are exposed in the environment (physicochemical properties of the soil, the availability of water and biogenic elements, agricultural practices, and weather conditions) [32], or by the developmental cycle of the plant [4]. If the mycorrhiza is functional, a large turnover of these structures can be expected due to their short-lived nature. Vesicles were observed in the roots in

both years, but their density was higher in 2016. These are long-lived structures, similar to hyphae, and can remain in the root for several months [5,6]. They are normally formed in the period from mid to late vegetative growth, in the period after the development of the first arbuscules, as well as after the decay of the arbuscules [5]. They have a storage function, especially for lipids [5]. The coils were present in both years, but their density was higher in 2016. The coils are in contact with the cell membrane and may serve as an exchange site for nutrients [3] during the flowering phase, when the nutrient demand of the plant is highest [33]. In addition, the presence of DSE fungi and their microsclerotial structures were detected in both years, with a higher density in 2016. The effects of DSE on the host plant have not been well studied. Their effect on plant growth and mineral nutrition can be positive or negative depending on the plant species and environment [11]. DSE fungi increase the uptake of nitrogen and phosphorus into the plant [34] and improve plant growth, especially under controlled conditions, especially when nitrogen is present in organic form [9]. In our study, less or no mycorrhizal colonization was observed on root segments with many root hairs. The intensity of mycorrhizal colonization in our study might be related to the morphology of Ajania, which has a stronger root system, with more root hairs. It has been found that plants with a simple root system, with few or no root hairs, are generally more susceptible to mycorrhizal colonization than plants with a stronger root system, such as grasses [35].

As Azcon-Aguilar and Barea [36] reported, several factors are important for successful mycorrhizal inoculation. In addition to agronomic practices, these are sufficient doses of inoculum and timing of inoculation or developmental stage of the plant at the time of addition of inoculum. Barea et al. [5] found that the earlier the inoculation is carried out, the greater are the beneficial effects on the plant.

In our experiment, a substrate with low phosphorus content (control) could not be provided, because the substrate had high phosphorus content due to excessive release of phosphorus by the added meat bone meal. The additional fertilization during the growing season and the addition of meat bone meal increased the phosphorus content in the substrate from two to five times the recommended levels for growing chrysanthemums (500 mg L^{-1} substrate) [26] by the end of the experiment. Phosphorus concentrations and varying inorganic phosphate supply in our experiment far exceeded this level, so that effective differences between treatments P1, P2 and P3, in terms of available phosphorus in the substrate, were not achieved. High phosphorus concentrations most likely affected mycorrhization in our study. Shukla et al. [37] reported phosphorus thresholds for AM colonization of *Phaseolus mungo* Roxb. and *Triticum aestivum* L. They found that phosphorus thresholds for maximum benefit from AM symbiosis varied from 5 to 20 µg g^{-1} in the observed plant species. Additionally, Habte and Manjunath [38] reported that when the phosphorus concentration in the soil solution is at or near 0.002 mg L^{-1}, most plant species respond dramatically to mycorrhizal colonization, but when the phosphorus concentration increases from this level to 0.02 mg L^{-1}, the dependence of plants on mycorrhizal fungi for phosphorus uptake gradually decreases, and at 0.2 mg L^{-1}, only the very highly mycorrhizal-dependent species respond significantly to mycorrhizal colonization. In the control pots, the substrate was not fertilized with superphosphate, but only with meat bone meal, but the amount of phosphorus available to plants increased. The increase was mainly due to the addition of meat bone meal, from which the phosphorus was apparently released slowly over the duration of the experiment, which was not foreseen in the experimental design. The meat bone meal we used in the experiment is a by-product of the meat industry and an important route for nitrogen and phosphorus recycling [39]. The added meat bone meal served as an initial source of phosphorus for better fungal growth, and plants generally have difficult access to the phosphorus in meat bone meal. Phosphorus in meat bone meal is present as hydroxyapatite ($Ca_5(PO_4)_3OH$) in the bone fraction and in organic form in the meat fraction [40–42].

The release of phosphorus from meat bone meal depends on the product and the properties of the substrate [43]. Since H^+ ions are important for the solubility of apatite

in the bone fraction, pH is a key factor affecting the release of phosphorus from the bone fraction [41]. We hypothesize that the greater release of phosphorus from meat bone meal was most likely due to the favorable pH of the substrate, which has a positive effect on phosphorus release. The pH of the substrate in which the plants were planted was 6.05. As reported by Boen and Haraldsen [43], Chen et al. [40] and Jeng et al. [41], phosphorus from meat bone meal is released more rapidly at acidic pH, resulting in higher availability, and less at basic pH. Organically bound phosphorus in meat and bone meal is converted to inorganic phosphate by mineralization, which is more soluble and accessible to plants than phosphorus in the inorganic (bone) fraction [41]. In addition, it has been reported that the release of phosphorus from meat bone meal is time dependent. In the first year after the addition of meat bone meal, only 19% of phosphorus from meat bone meal is available to plants, but in the second and third year, this availability increases to 63% [40,43]. Inorganically bound phosphorus in meat bone meal is in principle more difficult to access for plants, but also in our case, it has been shown that phosphorus is rapidly released into the substrate solution mainly due to the pH value and was thus readily available for plants [40,41,43].

The success of mycorrhizal colonization depends on the nutrient conditions in the substrate, especially the availability of nitrogen and phosphorus, which are considered the most restrictive nutrients for plant growth [6,44]. It has been reported that AM fungi play little or no role in substrates where there is high availability and supply of nutrients [8]. Both plants and fungi can access phosphorus, albeit with varying degrees of success, through specific mechanisms such as phosphatases released into the rhizosphere. AM fungi secrete phosphatase enzymes that hydrolyze phosphate from organically bound phosphorus, which improves plant productivity and increases the uptake of ammonium ions, non-mobile trace elements such as copper and zinc, and other cations (K^+, Ca^{2+}, Mg^{2+}) [2,6]. Higher phosphorus concentrations in the substrate usually inhibit the development of mycorrhizal colonization of roots [45] and reduce the ability of the host plant to develop symbiosis with AM fungi [46]. In addition, higher phosphorus concentrations, as observed in our study, are expected to result in slower and poorer infection, reduced ability to form spores, arbuscules, vesicles and other fungal structures (or these are smaller and less numerous), poorer germination of AM spores, slower hyphal growth and changes in root secretion content [47].

Although due to the higher levels of phosphorus available to plants in our study, mycorrhizal colonization was successful but weaker. Nevertheless, some significant differences in growth and flowering parameters were observed between inoculated and uninoculated plants. Mycorrhizal plants had a higher number of shoots than uninoculated plants in 2016. Increased growth and development in mycorrhizal plants, compared to non-mycorrhizal, has been reported for many different species (reviewed in Smith and Read [3]). The growth of the Ajania plants used in our study was greater in 2016 than in 2015. Herbaceous perennials, like Ajania, do not die after the end of the growing season, but sprout more vigorously from the strengthened underground root parts the next season. The number of shoots decreased slightly over time in our study, which was due to shoot breakage and was not influenced by the factors studied. Ajania plants were more elongated in the experiment, probably due to fertilization and favorable conditions in the greenhouse, which may influence shoot breakage at the end of the growing season. A similar phenomenon has been reported for some other cultivated herbaceous perennials [48,49].

Many plants in our experiment did not flower because they grew under natural conditions. Since Ajania is a short-day plant, its flowering depends on the length of the day, with a short day being crucial. Better, more uniform flowering and better flower quality could be achieved by darkening or artificially shortening the day [26,50], but this is not normally applied to perennials [26]. Such a technique is only possible when the plants are cultivated indoors, which is avoided in perennials. In practice, Ajania as a perennial plant is usually cultivated outdoors where the plants are exposed to natural day shortening. Therefore, we did not artificially darken the plants in the experiment but left

them to natural conditions. Under such conditions, the plants flower unevenly and of inferior quality.

The inoculum had a significant effect on the observed flowering parameters Mycorrhizal plants in our study had a higher number of inflorescences, higher number of flowers, and plants flowered longer than uninoculated plants. In accordance with our results, higher yield was reported for mycorrhizal *Tagetes erecta* L. [51] and *Gazania rigens* L. [52] compared to non-mycorrhizal plants. According to studies by Garmendia and Mangas [53] on roses and Scagel and Schreiner [54] on Zantedeschia plants, AM fungi affect flowering by shortening the time to flower formation, increasing the number of flowers per plant, and/or extending the flowering time of the plant. Aboul-Nasr [55] reported earlier and longer flowering with higher number of flowers and inflorescences of mycorrhizal Zinnias and Tagetes than non-mycorrhizal plants.

In our study, the inflorescences of the mycorrhizal Ajania plants developed gradually and not all at once, so that the plants with more developed inflorescences had a longer flowering period. Similarly, McGraw and Schenck [18] reported a positive effect of AM fungi on flowering time, number of flowers and inflorescences of chrysanthemum. Similar results on the positive effect of AM fungi were also reported in *Callistephus chinensis* (L.) Nees [21], *Petunia hybrida* Vilm., *Callistephus chinensis* (L.) Nees and *Impatiens balsamina* L. [20], and *Zantedeschia* Sprengel. [54]. Mycorrhizal plants accumulate nutrients in a shorter time so that they are adequately supplied with the nutrients they need to begin flower development and prolong flowering at an early stage of development [55].

5. Conclusions

Our results show that inoculated Ajania plants were successfully colonized by arbuscular mycorrhizal fungi and dark septate endophytic fungi, as specific fungal structures were observed in all root segments examined. Mainly hyphae were observed in the root segments, as well as vesicles, coils, arbuscules and microsclerotia, but their density was lower, probably due to the higher concentrations of phosphorus available to the plants. Higher density of fungal structures was observed in 2016. Mycorrhizal plants had higher number of shoots in 2016, higher number of inflorescences, higher number of flowers and longer flowering time than uninoculated non-mycorrhizal plants. The study of the positive effects of mycorrhizal fungi on the growth and flowering of Ajania plants provides useful information for the cultivation of Ajania plants and other related herbaceous perennial species.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/horticulturae7070178/s1, Table S1: Statistically significant differences (*lme* model) for main effects and interactions for the total shoot length and total number of shoots of inoculated and uninoculated Ajania plants treated with different phosphorus levels in 2015, Table S2: Statistically significant differences (*lme* model) for main effects and interactions for the total shoot length and total number of shoots of inoculated and uninoculated Ajania plants treated with different phosphorus levels in 2016.

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