agronomy

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Secondary Metabolism and Plant Growth of *Piper divaricatum* (Piperaceae) Inoculated with Arbuscular Mycorrhizal Fungi and Phosphorus Supplementation

Joyce Solange de Oliveira [1], Nayara P. Ramos [2], Jair Leão Júnior [2], Luciana P. Xavier [2], Eloisa Helena Andrade [3], Andréa H. Mello [4], William N. Setzer [5] and Joyce Kelly R. Da Silva [1,*]

1 Programa de Pós-Graduação em Biotecnologia, Universidade Federal do Pará, Belém 66075-110, Brazil; joycesolange@ufpa.br
2 Laboratório de Biotecnologia de Enzimas e Biotransformações, Universidade Federal do Pará, Belém 66075-110, Brazil; nayara1955paranhos@gmail.com (N.P.R.); junior.quimica.ind@gmail.com (J.L.J.); lpxavier@ufpa.br (L.P.X.)
3 Coordenação de Botânica, Museu Paraense Emílio Goeldi, Belém 66040-170, Brazil; eloisa@museu-goeldi.br
4 Instituto de Estudo do Desenvolvimento Agrário e Regional, Universidade Federal do Sul e Sudeste do Pará, Marabá 68507-590, Brazil; andreahentz@unifesspa.edu.br
5 Aromatic Plant Research Center, 230 N 1200 E, Suite 100, Lehi, UT 84043, USA; wsetzer@aromaticplant.org
* Correspondence: joycekellys@ufpa.br; Tel.: +55-91-3201-7297

Abstract: Arbuscular mycorrhizal fungi (AMF) species and/or phosphorus addition in the soil can affect secondary metabolism production and growth in plants. This study evaluated these effects on *Piper divaricatum* culture, which presents an essential oil rich in phenylpropanoids. Growth parameters, leaf volatile composition, total phenolic content, and the enzymatic activity of lipoxygenase (LOX) and phenylalanine ammonia-lyase (PAL) were monitored. At 90 days post inoculation (dpi), the treatments with AMF and AMF + P were more effective in terms of the number and biomass of leaves. In addition, the AMF group had increased plant height and root length. Phenylpropanoid and methyl eugenol contents were higher at 30 dpi in the P and AMF + P association treatments. However, at 90 dpi, the plants with P addition at 20 mg·dm^{-3} and AMF + 200 mg of P·dm^{-3} showed higher phenolic contents and PAL activity, respectively. All treatments increased LOX activity, especially with P addition at 200 mg·dm^{-3}. These results demonstrate that P and AMF can be applied to optimize leaf biomass and volatile compound production in *P. divaricatum*.

Keywords: phenylpropanoids; *Claroideoglomus etunicatum*; *Rhizophagus clarus*; volatile compounds

Citation: Oliveira, J.S.d.; Ramos, N.P.; Júnior, J.L.; Xavier, L.P.; Andrade, E.H.; Mello, A.H.; Setzer, W.N.; Da Silva, J.K.R. Secondary Metabolism and Plant Growth of *Piper divaricatum* (Piperaceae) Inoculated with Arbuscular Mycorrhizal Fungi and Phosphorus Supplementation. *Agronomy* **2022**, *12*, 596. https://doi.org/10.3390/agronomy12030596

Academic Editor: Cinzia Margherita Bertea

Received: 15 January 2022
Accepted: 22 February 2022
Published: 27 February 2022

1. Introduction

Piper divaricatum G. Meyer is a native shrub and has a wide distribution in the Brazilian Amazon, mainly in Pará and Amazonas states, as well as other Brazilian states [1]. Popularly known as pau-de-angola, jaborandi-manso, and "bettle", the root is aromatic, with a strong flavor, similar to ginger [2,3]. The *Piper* genus has an important prominence in folk medicine for the treatment of numerous symptoms, such as pain, convulsions, insomnia, hemorrhages, stomach problems, etc. [4,5]. The leaves and roots of *P. divaricatum* are used in folk medicine, when cooked, for anti-rheumatic baths and, in infusion, against rheumatic pain and abdominal cramps [3].

The most common chemotype of *P. divaricatum* essential oil (EO) is rich in phenylpropanoids, such as methyl eugenol and eugenol, to which several biological activities are attributed. In the literature, this EO is described as antioxidant [6,7], antibacterial against Gram-negative and Gram-positive bacteria [8], insecticidal [9], and antifungal against phytopathogens [6,10].

The production of secondary metabolites in plants is susceptible to genetic, environmental, and morphological factors [11]. Under natural conditions, plant growth is

associated with various soil microorganisms [12]. AMFs (arbuscular mycorrhizal fungi) are beneficial microorganisms that can develop a symbiotic association with more than 80% of plant species [13,14]. Root colonization by AMFs is established by a mycelial network of fungi that spread under plant roots, facilitating the uptake of nutrients that are generally inaccessible to plants [15].

Previous studies have reported that AMFs can enhance plant growth by promoting nutrient uptake, especially in sparse conditions [16,17]. They can also increase the absorption of nutrients, especially phosphorus (P), and the tolerance of plants to various types of stresses, influencing their growth and reproduction [18,19]. P is an essential macronutrient for plants, influencing their productivity [20]. Thus, the use of P by plants is a significant factor in determining the final total yield [21] due to its high affinity in the P uptake mechanism by the host plant [22].

Inoculation of AMF and P in soil can increase the content and modify the composition of EO in plants. It can interfere with the biological potential of the EO produced for use in the agricultural, food, pharmaceutical, and cosmetic industries [23]. *Mentha arvensis* plants inoculated with *Rhizophagus clarus* under the addition of P increased EO content by 89% [23]. Similarly, oregano and mint plants inoculated with the AMFs *Glomus etunicatum* and *G. lamellosum* exhibited increased growth and EO yield [24]. The association of AMF *Glomus etunicatum* and concentrations of 21 and 200 mg of P·dm^{-3} added to the soil and fumigated for 3 days was considered a viable alternative for increasing the essential oil content in *Mentha crispa* and obtaining 80% carvone, its main component [25]. Thus, the aim of this study was the evaluation of the growth and production of secondary metabolites in *P. divaricatum* under the influence of P contents in the soil during the plant × AMF interaction.

2. Materials and Methods

2.1. Plant Material and Cultivation

Piper divaricatum plants were collected in the city of Ananindeua (Pará State, Brazil), and for botanical identification, an exsiccate was deposited in the Herbarium of the Emílio Goeldi Museum (MPEG) under register MG 224386. Cuttings containing 1 to 2 nodes and vermiculite-expanded type-B substrate (Urimamã Mineração Ltd., Santa Maria da Boa Vista, Brazil) and 70% shading were propagated in a greenhouse. A commercial nutrient solution containing N, $Ca(H_2PO_4)_2$, K, S, B, Cl, Cu, Fe, Mn, Mo, and Zn was applied and reapplied after 15 days to promote root development. The cuttings were moistened daily according to the soil field capacity. After 21 days of roots development, the plants were transplanted to a sterilized commercial substrate. Soil chemical analysis showed the following parameters: pH (H_2O): 5.4; P: 3.0 mg·dm^{-3} (Mehlich I) [26]; K: 0.02 cmolc·dm^{-3} (Mehlich I) [26], Na: 0.01 cmolc·dm^{-3} (Mehlich I) [26]; Ca: 1.1 cmolc·dm^{-3} (extracted by KCl 0.01 mol L^{-1}); Mg: 1.0 cmolc·dm^{-3} (extracted by KCl 0.01 mol L^{-1}); Al: 0.0 cmolc·dm^{-3} (extracted by KCl 0.01 mol L^{-1}); H + Al: 2.5 cmolc·dm^{-3}; sum of bases (SB): 2.13 cmolc·dm^{-3}; cation-exchange capacity (CEC): 4.63 cmolc·dm^{-3}; base saturation (V): 46%; and C: 5.13 g·dm^{-3} (dichromate/colorimetric).

2.2. Addition of Phosphorus and Arbuscular Mycorrhizal Fungal Inoculation

After 30 days of transplanting, the source of P applied in the substrate containing *P. divaricatum* plants was the granular simple superphosphate ($Ca(H_2PO_4)_2$) at concentrations of 20 and 200 mg of P·dm^{-3} [25].

Spore extraction was performed by wet sieving and decanting [27], as well as 40% sucrose centrifugation [28] from a soil rhizosphere collected in the southeast of Pará state (Brazil). Based on its morphological characteristics [29,30], the AMF was identified and propagated using *Brachiaria brizantha* as trap culture [31], as well as sterile sand. A mixture of spores (density of 90 spores/g soil), hyphae, root fragments, and sterile sand containing a proportion of 50% of each fungal species (*Rhizophagus clarus* and *Claroideoglomus etunicatum*) was used during inoculation.

After 30 days of P addition, the seedlings were removed from the planting bags, and pits approximately 2.0 cm deep were opened. Then, 6.0 g of inoculum containing AMF spores was dispersed superficially on the roots and covered by the substrate.

2.3. Experimental Design and Material Collection

The experiment was conducted in a randomized block design, containing six blocks with twelve replications each, totaling 72 plants. The six groups consisted of: Control (*P. divaricatum* Control), AMF (*P. divaricatum* inoculated with arbuscular mycorrhizal fungi), P20 (*P. divaricatum* with 20 mg P·dm^{-3} of soil), P200 (*P. divaricatum* with 200 mg P·dm^{-3} of soil), P20 + AMF (*P. divaricatum* with 20 mg P·dm^{-3} of soil and inoculated with arbuscular mycorrhizal fungi), and P200 + AMF (*P. divaricatum* with 200 mg P·dm^{-3} of soil and inoculated with arbuscular mycorrhizal fungi). Leaf samples of each treatment were collected at 30, 60, and 90 days post-inoculation (dpi).

2.4. Evaluation of Plant Development

Plant height (cm), leaf number, basal plant stem (mm), number of nodes, root length (cm), and the biomass of leaves and roots (g) were evaluated for each plant per replicate. Fresh-leaf biomass production was based on the total weight per plant, and the fresh-root biomass was based on the total root weight per plant.

2.5. Extraction and Chemical Analysis of Volatile Compounds

The essential oils were extracted from fresh leaves of *P. divaricatum* (2.0 g) using a Likens–Nickerson apparatus with simultaneous distillation extraction with *n*-pentane (3.0 mL) over a 2 h period. The samples were injected (1.0 µL) in the gas chromatography–mass spectrometry (GC–MS) apparatus (Shimadzu QP2010 Ultra, Shimadzu Corporation, Tokyo, Japan). The parameters for the analysis were: Rtx-5MS silica capillary column (30 m and 0.25 mm internal diameter and 0.25 µm film thickness; Restek Corporation, Bellefonte, PA, USA); temperature, 60–240 °C (3 °C/min); injector temperature, 200 °C; carrier gas, helium, adjusted to a linear velocity of 1.2 mL/min; injection type, split (the split flow was adjusted to yield a 20:1 ratio); septum sweep, constant 10 mL/min; EIMS, electron energy, 70 eV; and temperature of the ion source and connection parts, 200 °C. Compound identification was achieved by comparing the retention indices, determined using a homologous series of *n*-alkanes (C8-C32, Sigma-Aldrich, St. Louis, MO, USA) [32] and mass spectral fragmentation patterns with data present in the libraries of Adams [33] and the National Institute of Standards and Technology (NIST) [34]. The component percentages are based on peak integrations without standardization.

2.6. Lipoxygenase (LOX) Activity

The leaves of each sample were pulverized in liquid nitrogen. Then, 1.0 g samples were mixed with 3 mL of sodium phosphate buffer (50 mM, pH 6.5) and centrifuged, and the supernatant was used to obtain the enzyme source. Linoleic acid (78 µL, Sigma-Aldrich, St. Louis, MO, USA) and Tween 20 (90 µL, Sigma-Aldrich) were mixed in boiling water (10 mL) with a few drops of sodium hydroxide (0.5 N). The final concentration was adjusted to 10 mM sodium linoleate solution and stored at −20 °C. LOX activity was determined using 5 µL of the enzyme solution and 50 µL of sodium linoleate (10 mM) solution diluted with 1945 µL of sodium phosphate buffer. The formation of the final product was monitored at 234 nm for 60 s using a UV–visible spectrophotometer [35,36].

2.7. Phenylalanine Ammonia Lyase (PAL) Activity

A homogenous mixture was prepared containing pulverized leaves (1.0 g), 2.0 mL sodium borate buffer (0.3 mM, pH 8.8), 1 mM dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA), and 5% polyvinylpolypyrrolidone (PVP). After centrifugation, an aliquot of the supernatant (0.5 mL) was added to 1.0 mL of reaction buffer that contained sodium borate (0.3 mM, pH 8.8) and L-phenylalanine (0.03 mM). PAL activity was assessed

by measuring the concentration of (E)-cinnamic acid produced from the L-phenylalanine substrate at an absorbance of 290 nm using a UV–visible spectrophotometer after incubation of the reaction mixture for 15 min at room temperature [37].

2.8. Determination of Total Phenolics

Fresh leaves (2.0 g) were extracted with ethyl acetate (50 mL) by percolation at room temperature for 96 h and concentrated with a rotary evaporator. The total phenolic content of the samples was determined by the Folin–Ciocalteu method [38]. Sample solutions were prepared in methanol at an initial concentration of 20 mg/mL and then diluted in water according to their reactivity. A 500-µL aliquot of the plant extract reacted with 250 µL of Folin-Ciocalteu reagent (1 N) and 1250 µL of sodium carbonate (75 g/L) for 30 min in the dark. The absorbance of the reaction was measured at 760 nm using a UV–visible spectrophotometer. The total phenolic content in the extracts was expressed as gallic acid equivalents (GAEs) in milligrams per gram of extract (mg GAE g^{-1}) based on the standard curve prepared using gallic acid at concentrations of 0.5 to 10.0 mg/mL.

2.9. Statistical Analyses

All measurements were carried out in quadruplicate and compared with the control group. The data are expressed as means ± standard deviation. Analysis of variance was conducted by two-way ANOVA, followed by the Bonferroni test using GraphPad (GraphPad Software Inc.: San Diego, CA, USA) 7.0 software. Differences at $p < 0.05$ were considered significant. The analysis was carried out using the data from volatile composition, phenolic compounds, LOX, and PAL classes for leaves.

3. Results

3.1. Growth and Development of Seedlings

The results indicated a significant influence of P addition on the development of *P. divaricatum* plants ($p < 0.05$) (Table 1). P20-group plants showed an increase of 30% and 35% in the basal stem diameter at 60 and 90 dpi, respectively. Regarding the number of leaves and leaf biomass, an increase was observed only at 30 dpi. However, the P200-group plants displayed higher leaf numbers during evaluation, especially at 30 dpi (50%). In addition, root length showed an increase of 30% and 19% in both treatments at 90 dpi.

Table 1. Growth parameters of *Piper divaricatum* at different concentrations of P.

	Groups	Evaluation Parameters [a]						
		Basal Stem (mm)	**Leaf Number**	**Node Number**	**Height (cm)**	**Root (cm)**	**Fresh Biomass (Leaves)**	**Fresh Biomass (Root)**
30 dpi	Control	2.9 ± 0.4	4.0 ± 0.0	5.0 ± 0.0	27.2 ± 1.2	25.7 ± 0.8	53.9 ± 4.7	47.8 ± 9.1
	P20	3.2 ± 0.3	5.0 ± 0.0 *	6.0 ± 0.0	29.1 ± 1.8	28.7 ± 1.2 *	65.8 ± 2.1 ***	65.4 ± 9.5
	P200	3.3 ± 0.2	6.0 ± 0.6 ***	6.0 ± 0.6	25.8 ± 0.8	25.9 ± 2.0	76.0 ± 1.0	54.8 ± 2.7
60 dpi	Control	3.0 ± 0.2	5.3 ± 0.6	6.0 ± 0.0	28.4 ± 1.3	33.0 ± 1.0	64.9 ± 1.4	73.5 ± 4.7
	P20	3.9 ± 0.6 *	5.0 ± 0.5	6.0 ± 0.0	32.2 ± 2.7	34.5 ± 0.4	54.6 ± 15.9 ***	59.9 ± 6.7
	P200	3.4 ± 0.4	7.0 ± 0.0 ***	6.7 ± 1.2	31.9 ± 5.4	33.3 ± 0.6	67.19 ± 1.5	83.5 ± 17.9 *
90 dpi	Control	3.7 ± 0.2	6.0 ± 0.0	7.8 ± 1.0	29.0 ± 2.5	36.2 ± 0.4	72.3 ± 14.5	84.5 ± 17.1
	P20	5.0 ± 0.4 ***	5.6 ± 1.2	5.5 ± 1.0 ***	32.3 ± 1.9	43.0 ± 2.6 ****	73.2 ± 3.6	117.3 ± 22.5 ***
	P200	4.0 ± 0.6	7.0 ± 0.0 *	7.0 ± 0.0	31.8 ± 2.0	47.0 ± 0.9 ****	70.6 ± 15.3	93.5 ± 35.2 *

dpi: days post-inoculation; [a] means ± standard deviation (n = 4). Control: (control *P. divaricatum*); P20: (*P. divaricatum* with 20 mg of P·dm^{-3} of soil); P200: (*P. divaricatum* with 200 mg of P·dm^{-3} of soil). Statistical difference according to Bonferroni test ($p < 0.05$). Significance level: **** extremely significant ($p < 0.0001$), *** very significant ($p < 0.001$), * low significance ($p < 0.1$).

In the *P. divaricatum* plants, inoculation with AMF increased the basal stem diameter at 60 dpi. Leaf numbers also increased by 25% and 22% at 30 and 90 dpi, respectively, whereas biomass increased by around 22% and node number increase by 40% at 30 dpi. Height

increased by 14% at 60 and 90 dpi compared to plants in the control group. AMF increased root length at 30, 60, and 90 dpi (19%, 8%, and 12%, respectively) and root biomass by around 40% at 30 and 90 dpi in relation to the plants of the control groups (Table 2).

Table 2. Growth parameters of *Piper divaricatum* inoculated with AMF.

	Groups	Evaluation Parameters [a]						
		Basal Stem (mm)	Leaf Number	Node Number	Height (cm)	Root (cm)	Fresh Biomass (Leaves)	Fresh Biomass (Root)
30 dpi	Control	2.9 ± 0.4	4.0 ± 0.0	5.0 ± 0.0	27.2 ± 1.2	25.7 ± 0.8	53.9 ± 4.7	47.8 ± 9.1
	AMF	3.5 ± 0.4	5.0 ± 0.0 *	7.0 ± 1.2 *	26.3 ± 0.7	30.5 ± 1.5 ****	65.8 ± 4.2 *	67.9 ± 2.5 *
60 dpi	Control	3.0 ± 0.2	5.3 ± 0.6	6.0 ± 0.0	29.1 ± 1.3	33.0 ± 1.0	54.6 ± 1.4	73.5 ± 4.7
	AMF	4.0 ± 0.6 **	5.3 ± 0.6	6.5 ± 0.6	33.2 ± 1.5 *	35.5 ± 0.9 *	60.6 ± 1.9	77.2 ± 16.2
90 dpi	Control	3.7 ± 0.2	6.0 ± 0.0	7.8 ± 1.0	29.0 ± 2.5	36.2 ± 0.4	72.3 ± 14.5	84.5 ± 17.10
	AMF	4.0 ± 0.0	7.3 ± 0.6 **	7.3 ± 0.5	33.0 ± 0.8 *	40.7 ± 0.2 ***	88.8 ± 2.4	119.2 ± 4.0 ****

dpi: days post-inoculation; [a] means ± standard deviation (n = 4). Control: (control *P. divaricatum*); AMF: (*P. divaricatum* inoculated with AMF). Statistical difference according to Bonferroni test ($p < 0.05$). Significance level: **** extremely significant ($p < 0.0001$), *** very significant ($p < 0.001$), ** significant ($p < 0.01$), * low significance ($p < 0.1$).

The addition of P at concentrations of 20 and 200 mg of P·dm^{-3} and inoculation with AMFs promoted an increase in different developmental parameters in *P. divaricatum* plants compared to the control group. Both treatments, P200 + AMF and P20 + AMF, displayed an increase of 20% in basal stem diameter at 60 dpi and a significant increase of around 30% in leaf number at 90 dpi. However, only the P20 + AMF group showed greater leaf biomass, with an increase of 47% at 60 dpi. This group also exhibited significant increases in root length on all days of analysis (Table 3).

Table 3. Growth parameters of *Piper divaricatum* inoculated with AMF at different P concentrations.

	Groups	Evaluation Parameters [a]						
		Basal Stem (mm)	Leaf Number	Node Number	Height (cm)	Root (cm)	Fresh Biomass (Leaves)	Fresh Biomass (Root)
30 dpi	Control	2.9 ± 0.4	4.0 ± 0.0	5.0 ± 0.0	27.2 ± 1.2	25.7 ± 0.8	53.9 ± 4.7	47.8 ± 9.1
	P20 + AMF	3.1 ± 0.4	5.0 ± 0.0 ***	6.0 ± 0.0 *	29.1 ± 0.5	30.8 ± 1.4 **	64.9 ± 2.2	78.8 ± 0.9 **
	P200 + AMF	3.7 ± 0.5 **	4.0 ± 0.0	4.0 ± 0.0 *	24.7 ± 1.8	29.2 ± 3.3 *	45.4 ± 5.5	48.7 ± 10.4
60 dpi	Control	3.0 ± 0.2	5.3 ± 0.6	6.0 ± 0.0	29.1 ± 1.3	33.0 ± 1.0	54.6 ± 1.4	73.5 ± 4.7
	P20 + AMF	3.6 ± 0.3 *	6.0 ± 0.0 *	7.0 ± 0.0 *	30.0 ± 1.4	36.7 ± 0.8 *	80.0 ± 0.6 *	87.0 ± 10.9
	P200 + AMF	3.6 ± 0.1 *	5.0 ± 0.0	5.8 ± 0.6	29.8 ± 2.3	30.5 ± 0.5	53.9 ± 3.9	64.0 ± 8.1
90 dpi	Control	3.7 ± 0.2	6.0 ± 0.0	7.8 ± 1.0	29.0 ± 2.5	36.2 ± 0.4	72.3 ± 14.5	84.5 ± 17.10
	P20 + AMF	4.1 ± 0.1	8.0 ± 0.0 ****	7.3 ± 1.0	31.1 ± 2.3	43.7 ± 2.2 ****	84.0 ± 6.6	102.3 ± 25.4
	P200 + AMF	4.1 ± 0.2	7.6 ± 0.6 ****	7.4 ± 1.0	30.7 ± 3.6	35.7 ± 2.5	84.2 ± 21.7 *	102.4 ± 36.7

dpi: days post-inoculation; [a] means ± standard deviation (n = 4). Control: (control *P. divaricatum*); P20 + AMF: (*P. divaricatum* inoculated with AMF and 20 mg of P·dm^{-3} of soil); P200 + AMF: (*P. divaricatum* inoculated with AMF and 200 mg of P·dm^{-3} of soil). Statistical difference according to Bonferroni test ($p < 0.05$). Significance level: **** extremely significant ($p < 0.0001$), *** very significant ($p < 0.001$), ** significant ($p < 0.01$), * low significance ($p < 0.1$).

3.2. Volatile Compounds Profile

The total percentage of volatile compounds identified in essential oil from the leaves was around 99.0%. The most predominant compound class was phenylpropanoids, which was higher in all treatments, P20, P200, P20 + AMF, and P200 + AMF at 30 dpi (88.7%, 87.2%, 86.8%, and 87.5% respectively) in comparison to the control group (78.2%). However, at 90 dpi, only the AMF and P200 + AMF groups showed an increase in phenylpropanoid content (71.3% and 72.0%, respectively) compared to the control group (62.4%) (Figure 1, Table 4).

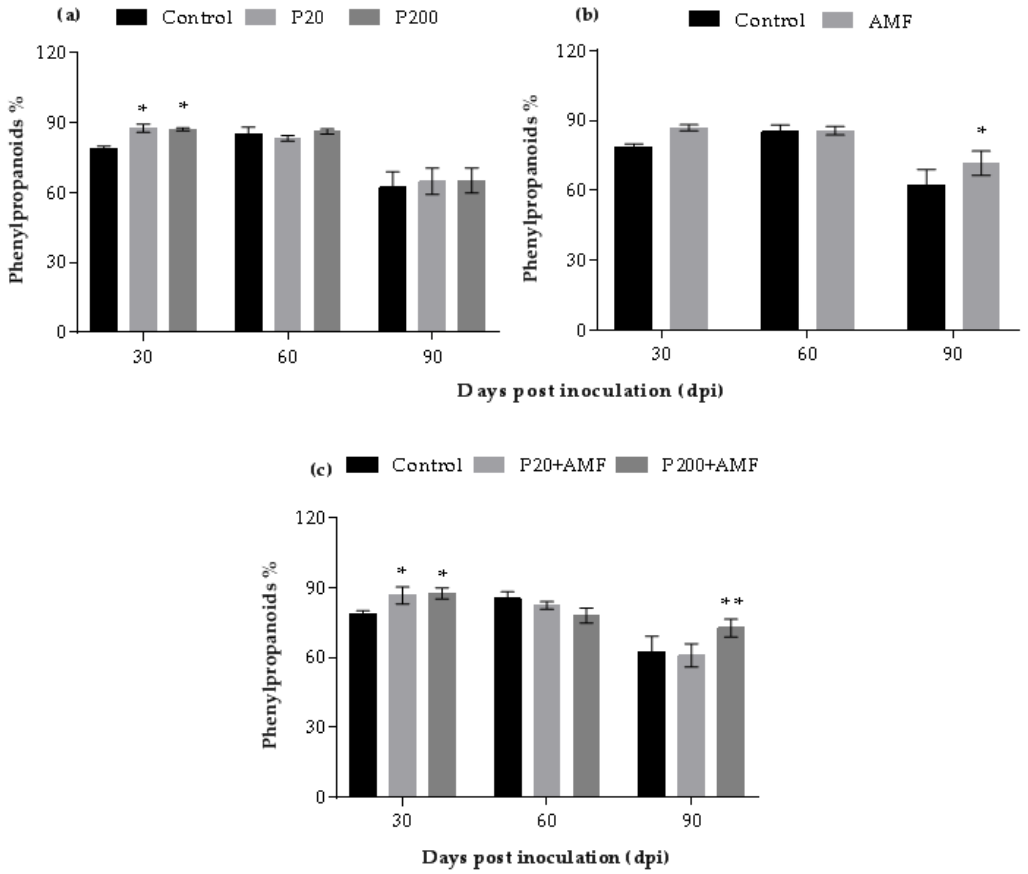

Figure 1. Variation of the phenylpropanoid content of *Piper divaricatum*: (**a**) plants treated with 20 and 200 mg of P·dm^{-3} of soil (P20 and P200), (**b**) plants inoculated with AMFs (AMF), and (**c**) plants inoculated with AMFs and treated with 20 and 200 mg of P·dm^{-3} of soil (P20 + AMF and P200 + AMF). Statistical difference according to Bonferroni test ($p < 0.05$). Significance level: ** significant ($p < 0.01$), * low significance ($p < 0.1$).

Mycorrhizal association and the addition of P at different concentrations influenced the production of the major compound, methyl eugenol (53.9–87.5%), a phenylpropanoid, followed by the terpenoids β-elemene (5.5–12.7%) and (*E*)-β-ocimene (1.6–8.0%) in *P. divaricatum* plants (Table 4).

At 30 dpi, the P20 and P200 groups showed a greater variation in the amount of methyl eugenol, which was higher in the P20 (76.1–87.5%) and P200 (76.1–87.0%) groups in relation to the control group (Figure 2a, Table 4). Plants in the P20 + AMF (76.1–84.9%) and P200 + AMF (76.1–84.3%) groups had the highest concentration of this compound (Figure 2c, Table 4). The amount of the major compound, methyl eugenol, in plants inoculated with AMFs was not affected by colonization (Figure 2b).

3.3. Total Phenolic Content

Changes in the content of phenolic compounds were observed after P addition and inoculation by AMF (Figure 3) in comparison to the control group. The levels in the plants from the P20 group were 32% higher at 90 dpi (22.50–29.76 mg GAE g^{-1}). However, the P200 group (24.26–33.60 mg GAE g^{-1}) showed a similar result at only 30 dpi, with an increase of 39%. At 60 dpi, both AMF (21.06–40.09 mg GAE g^{-1}) and P200 + AMF (21.06–23.83 mg GAE g^{-1}) groups produced a higher amount of phenolic content than the control group. On the other hand, the total phenolic content decreased, on average, by 22% in the P20 + AMF group during the experimental period.

Table 4. Chemical composition of essential oil of *Piper divaricatum* leaves with addition of P and inoculated with AMF (means ± standard deviation).

	Compound	RI [a]	RI [b]	Control	AMF	P20	P200	P20 + AMF	P200 + AMF
30 dpi	**(*E*)-β-Ocimene**	**1042**	**1044**	**1.7 ± 0.9**	**3.9 ± 1.2**	**2.8 ± 0.5**	**2.6 ± 0.5**	**3.4 ± 0.6**	**3.1 ± 0.8**
	β-Elemene	**1396**	**1389**	**6.3 ± 1.1**	**5.5 ± 0.5**	**5.6 ± 0.9**	**6.4 ± 0.2**	**6.1 ± 1.8**	**5.6 ± 0.7**
	Methyl eugenol	**1410**	**1403**	**76.1 ± 2.6**	**85.1 ± 3.6**	**87.5 ± 3.0 ****	**87.0 ± 0.6 ***	**84.9 ± 4.2 ***	**84.3 ± 4.8 ***
	Monoterpene hydrocarbons			7.7 ± 8.4	4.2 ± 1.5	2.9 ± 0.6	2.6 ± 0.5	3.6 ± 0.7	3.2 ± 0.9
	Sesquiterpene hydrocarbons			9.7 ± 2.5	8.1 ± 0.9	8.2 ± 1.86	9.4 ± 0.5	8.8 ± 2.9	8.8 ± 1.1
	Phenylpropanoids			**78.2 ± 6.1**	**86.9 ± 6.5**	**88.7 ± 5.0 ***	**87.2 ± 0.6 ***	**86.8 ± 7.7 ***	**87.5 ± 8.7 ***
	Others			0.2 ± 0.3	0.6 ± 0.2	0.1 ± 0.1	0.0 ± 0.0	0.32 ± 0.2	0.1 ± 0.1
	Total			**95.8 ± 17.3**	**99.9 ± 9.1**	**99.9 ± 7.6**	**99.2 ± 1.7**	**99.6 ± 11.5**	**99.6 ± 10.7**
	Compound	**RI [a]**	**RI [b]**	**Control**	**AMF**	**P20**	**P200**	**P20 + AMF**	**P200 + AMF**
60 dpi	**(*E*)-β-Ocimene**	**1042**	**1044**	**1.62 ± 1.3**	**3.3 ± 0.3**	**3.3 ± 0.6**	**2.7 ± 0.2**	**4.1 ± 0.3**	**2.7 ± 1.6**
	β-Elemene	**1396**	**1389**	**5.7 ± 0.7**	**6.9 ± 1.3**	**7.8 ± 0.7**	**7.1 ± 0.7**	**7.8 ± 1.0**	**7.4 ± 0.7**
	Methyl eugenol	**1413**	**1403**	**84.6 ± 4.4**	**85.4 ± 1.8**	**82.2 ± 2.6**	**85.8 ± 0.7**	**81.8 ± 1.7**	**78.0 ± 5.9**
	Monoterpene hydrocarbons			5.0 ± 6.0	3.3 ± 0.4	3.4 ± 0.7	2.8 ± 0.3	4.4 ± 0.4	6.3 ± 8.2
	Sesquiterpene hydrocarbons			8.5 ± 1.6	10.5 ± 2.2	12.8 ± 1.1	10.7 ± 1.3	12.6 ± 1.8	12.9 ± 1.9
	Phenylpropanoids			**85.2 ± 4.7**	**85.7 ± 1.9**	**83.1 ± 3.7**	**86.3 ± 1.0**	**82.2 ± 1.7**	**79.9 ± 7.1**
	Others			0.6 ± 0.1	0.3 ± 0.3	0.3 ± 0.1	0.2 ± 0.2	0.5 ± 0.2	0.4 ± 0.2
	Total			**99.2 ± 12.5**	**99.8 ± 4.8**	**99.6 ± 5.8**	**99.9 ± 2.8**	**99.7 ± 4.0**	**99.5 ± 17.4**
	Compound	**RI [a]**	**RI [b]**	**Control**	**AMF**	**P20**	**P200**	**P20 + AMF**	**P200 + AMF**
90 dpi	**(*E*)-β-Ocimene**	**1040**	**1044**	**6.1 ± 2.2**	**7.6 ± 1.9**	**5.9 ± 2.3**	**4.2 ± 2.2**	**8.0 ± 1.6**	**4.8 ± 1.8**
	β-Elemene	**1381**	**1389**	**12.7 ± 0.9**	**7.4 ± 1.6**	**8.9 ± 2.4**	**11.8 ± 5.5**	**11.5 ± 2.0**	**10.2 ± 1.8**
	Methyl eugenol	**1409**	**1403**	**60.2 ± 11.0**	**67.5 ± 10.0**	**56.1 ± 7.3**	**58.415.7**	**53.9 ± 1.9**	**66.9 ± 2.7**
	Monoterpene hydrocarbons			7.5 ± 3.9	9.5 ± 3.8	7.1 ± 3.2	8.6 ± 9.2	10.1 ± 2.6	5.6 ± 2.4
	Sesquiterpene hydrocarbons			24.2 ± 9.1	13.5 ± 4.5	16.3 ± 5.5	21.9 ± 11.4	23.7 ± 6.6	19.0 ± 6.5
	Phenylpropanoids			**62.4 ± 13.21**	**71.3 ± 12.1 ***	**64.9 ± 19.8**	**65.0 ± 21.5**	**61.1 ± 9.3**	**72.0 ± 6.0 ****
	Others			1.2 ± 1.5	0.8 ± 1.3	0.9 ± 1.1	1.2 ± 0.7	1.2 ± 0.7	1.1 ± 0.3
	Total			**95.3 ± 27.6**	**95.1 ± 21.7**	**89.2 ± 29.5**	**96.6 ± 42.8**	**96.1 ± 19.2**	**97.6 ± 15.2**

RI [a]: calculated retention index; RI [b]: retention index of library; Control: (control *P. divaricatum*); AMF: (*P. divaricatum* inoculated with AMF); P20: (*P. divaricatum* with 20 mg of P·dm^{-3} of soil); P200: (*P. divaricatum* with 200 mg of P·dm^{-3} of soil); P20 + AMF: (*P. divaricatum* inoculated with AMF and 20 mg of P·dm^{-3} of soil); P200 + AMF: (*P divaricatum* inoculated with AMF and 200 mg of P·dm^{-3} of soil). Statistical difference according to Bonferroni test ($p < 0.05$). ** significant ($p < 0.01$), * low significance ($p < 0.1$).

3.4. Lipoxygenase (LOX) Activity

The addition of P was efficient in the two concentrations, especially at 90 dpi. The P200 and P20 groups exhibited a significant increase in LOX enzyme activity from 3.8 to 15.3 × 10^{-4} M·s $^{-1}$ and 13.2 × 10^{-4} M·s $^{-1}$, respectively. These results corresponded to an increase in activity of more than 240% compared to the control group (Figure 4a).

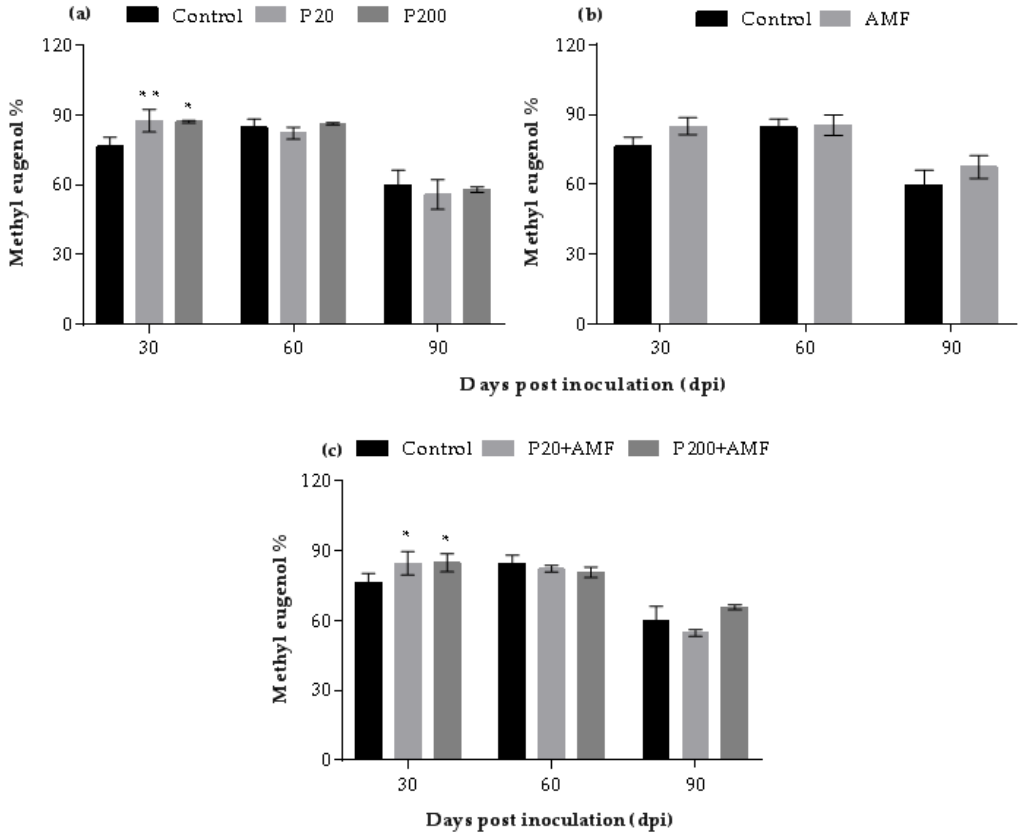

Figure 2. Methyl eugenol variation in *Piper divaricatum*: (**a**) plants treated with 20 and 200 mg of P·dm^{-3} of soil (P20 and P200); (**b**) plants inoculated with AMFs (AMF); (**c**) plants inoculated with AMFs and treated with 20 and 200 mg of P·dm^{-3} of soil (P20 + AMF and P200 + AMF). Statistical difference according to Bonferroni test ($p < 0.05$). ** significant ($p < 0.01$), * low significance ($p < 0.1$).

AMF inoculation increased the enzymatic activity of LOX in plants by about 129% (7.2–16.5 × 10^{-4} M·s $^{-1}$) at 30 dpi (Figure 4b). Similarly, the combination with P addition in the P20 + AMF and P200 + AMF treatments induced the highest production of LOX at 30 (7.2–12.7, 7.2–16.3 × 10^{-4} M·s $^{-1}$) and 90 dpi (3.8–8.3, 3.8–13.7 × 10^{-4} M·s $^{-1}$), respectively (Figure 4c).

3.5. Phenylalanine Ammonia Lyase (PAL)

The influence of P addition and inoculation by AMF on enzymatic activity of phenylalanine ammonia-lyase was quantified. Plants treated only with P or AMF did not display variation in PAL activity (Figure 5a,b). However, at 90 dpi in the P20 + AMF group, there was a decrease of 24% (28.25–21.35 µU mL^{-1}) compared to the control group. On the other hand, the P200 + AMF group had 15% increased PAL activity (28.25–32.49 µU mL^{-1}) compared to the control group (Figure 5c).

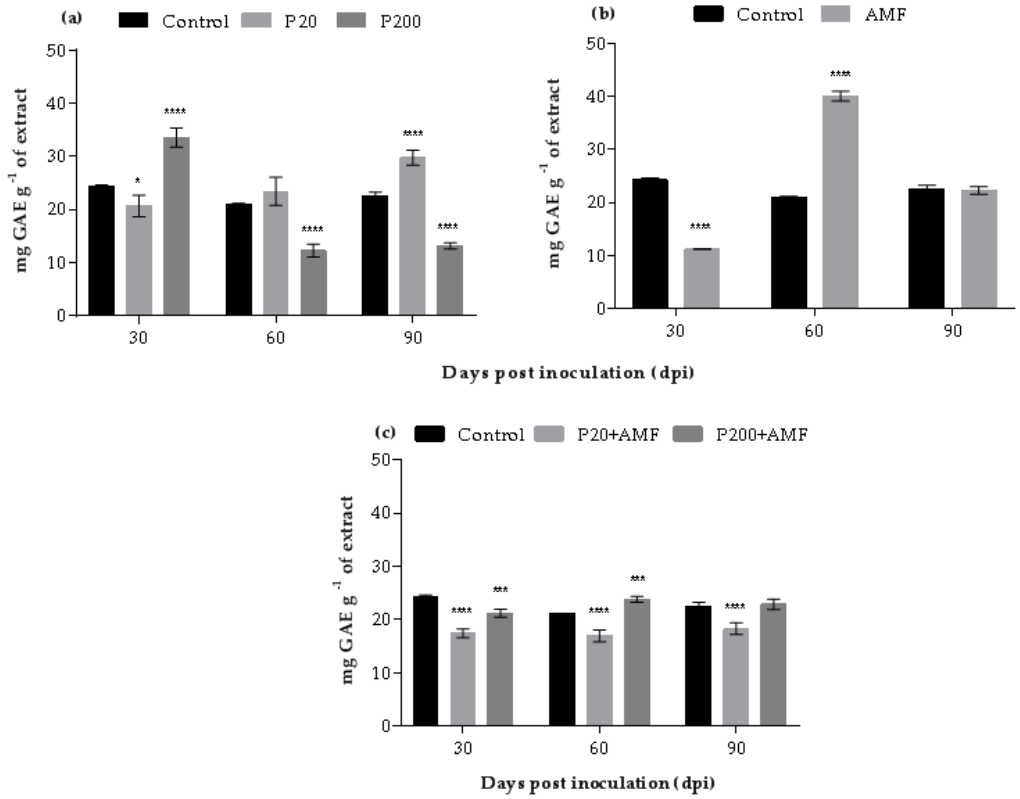

Figure 3. Variation of phenolic compounds produced by *Piper divaricatum*: (**a**) plants treated with 20 and 200 mg of P·dm^{-3} of soil (P20 and P200); (**b**) plants inoculated with AMFs (AMF); (**c**) plants inoculated with AMFs and treated with 20 and 200 mg of P·dm^{-3} of soil (P20 + AMF and P200 + AMF). Statistical difference according to Bonferroni test ($p < 0.05$). Significance level: **** extremely significant ($p < 0.0001$), *** very significant ($p < 0.001$), * low significance ($p < 0.1$).

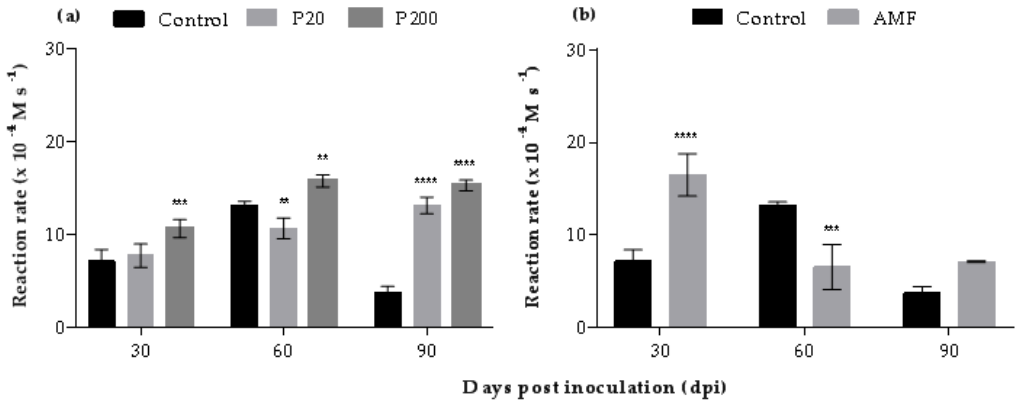

Figure 4. *Cont.*

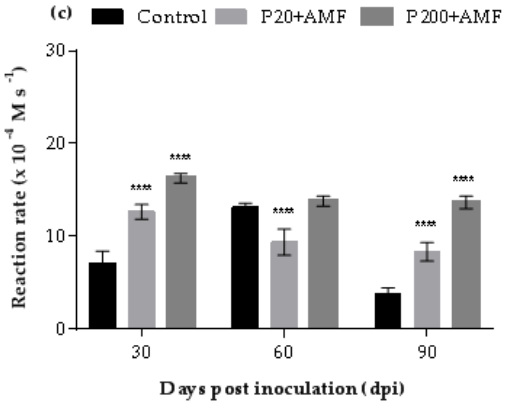

Figure 4. Variation in lipoxygenase activity in *P. divaricatum*: (**a**) plants treated with 20 and 200 mg of P·dm^{-3} of soil (P20 and P200); (**b**) plants inoculated with AMFs (AMF); (**c**) plants inoculated with AMFs and treated with 20 and 200 mg of P·dm^{-3} of soil (P20 + AMF and P200 + AMF). Statistical difference according to Bonferroni test ($p < 0.05$). Significance level: **** extremely significant ($p < 0.0001$), *** very significant ($p < 0.001$), ** low significant ($p < 0.01$).

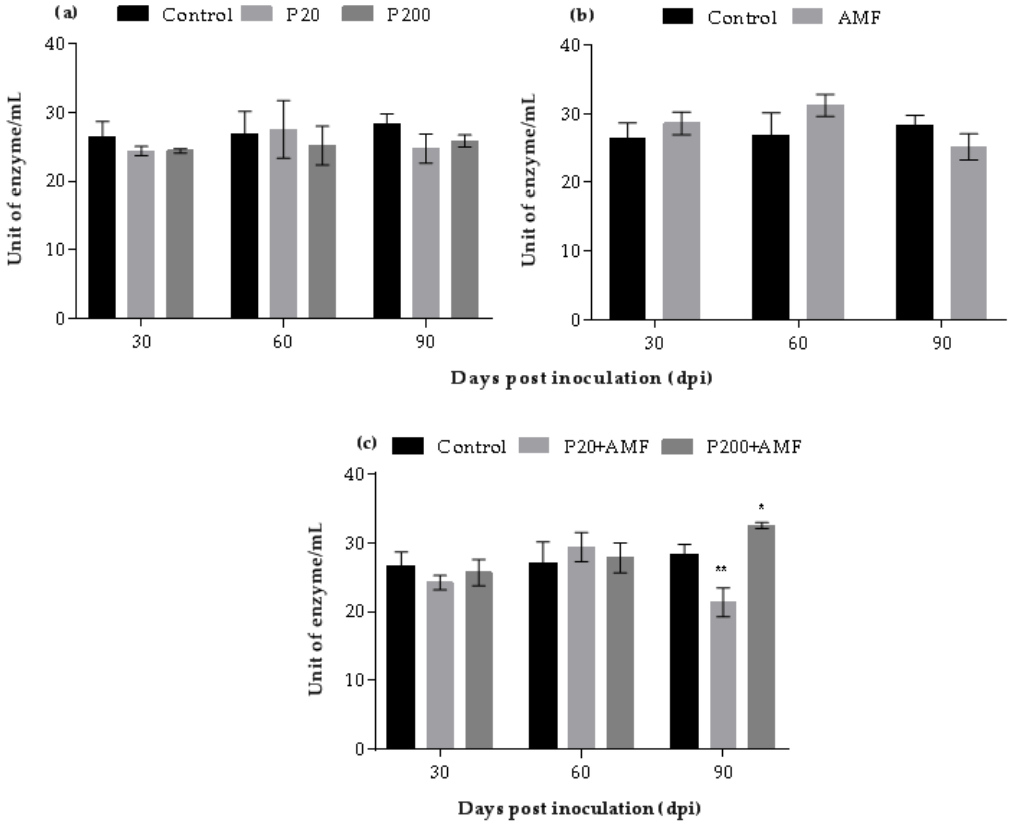

Figure 5. Phenylalanine ammonia-lyase enzyme activity in *Piper divaricatum*: (**a**) plants treated with 20 and 200 mg of P·dm^{-3} of soil (P20 and P200); (**b**) plants inoculated with AMFs (AMF); (**c**) plants inoculated with AMFs and treated with 20 and 200 mg of P·dm^{-3} of soil (P20 + AMF and P200 + AMF). Statistical difference according to Bonferroni test ($p < 0.05$). Significance level: ** significant ($p < 0.01$), * low significance ($p < 0.1$).

4. Discussion

The association of arbuscular mycorrhizal fungi and the addition of P at different concentrations in *P. divaricatum* plants influenced the growth, volatile composition, phenolic compounds, and enzymatic activity of enzymes related to plant defense. Developmental parameters such as basal stem, leaf number, number of nodes, root length, and root biomass

showed significant variation after the addition of P at concentrations of 20 and 200 mg of P/kg, inoculation with AMF, as well as the association of both treatments. Supplementation with P and association with AMF improved growth and biomass accumulation in *P. divaricatum* plants. The absorption of nutrients, such as P, can be considered the main factor of nutritional benefit, as the impact of AMF on plant growth can be replaced by the application of nutrients, especially P [22,39].

Similar results were observed in *Plectranthus amboinicus* seedlings inoculated with *Rhizophagus clarus* and associated to a high dose of P (200 mg P·dm^{-3} of soil), which exhibited a significant increase in root biomass (174%) in relation to the control treatment [40]. On the other hand, *Piper longum* plants inoculated with various AMF species (*Glomus fasiculatum*, *Acaulospora fovata*, and *Gigaspora margirata*) showed a decrease in root development, especially for plants inoculated with AMF of the species *Glomus fasiculatum* [41]. Our results corroborate a study of of *Poncirus trifoliata* L. plants inoculated with *Paenibacillus mucilaginosus* and *Rhizophagus intraradices* and cultivated with P concentrations of 0 mg (P0), 73.41 mg (P1), and 220.23 mg (P2), indicating increases in stem diameter of 44.73%, 20.86%, and 16.14%, respectively [42]. Basal stem circumference is an important parameter, as it shows the survival and development capacity of the plant in the greenhouse and in the field [43]. The number of leaves did not differ significantly in groups with added P and associated with P and AMF. An increase in the number of leaves was observed in seedlings of *Poincianella pyramidalis* inoculated with the AMFs *Acaulospora longula* and *Claroideoglomus etunicatum* using concentrations of 9, 15, 27 and 33 mg P·dm^{-3} of soil [44]. The increase in height but not in the number of leaves indicates a possible production of photoassimilates directed to the plant's needs [45].

In this study, *P. divaricatum* essential oil showed a predominance of phenylpropanoids, and the Plant×P×AMF triphasic interaction promoted an increase at 30 and 90 dpi (Figure 1). *Piper aduncum* inoculated with the AMF species *R. clarus* and *C. etunicatum* showed a reduction in the concentration of phenylpropanoids, especially at 60 dpi, as well as gradual increases in the production of monoterpenes and sesquiterpenes [46]. Methyl eugenol has been reported as the main volatile compound produced in *P. divaricatum* leaves [35]. In addition, the monoterpene (*E*)-β-ocimene showed a gradual increase during the triphasic interaction (Table 4). Volatile organic compounds (VOCs), including low-molecular-weight monoterpenes, such as myrcene, and compounds of mixtures of (*E*)-β-ocimene, (*Z*)-β-ocimene, and *allo*-ocimene isomers are involved in plant interactions [47]. VOCs are emitted by all plants, both constitutively and in response to biotic and abiotic stress, and are considered mediators in communication between plants and other organisms [48].

Phenolic compounds are secondary metabolites produced by the shikimate and pentose phosphate pathways through the metabolization of phenylpropanoids [49,50]. They are essential in the symbiotic interaction between microorganisms and plants. In addition, they act as signaling molecules for the recognition of symbionts and as defense compounds in plants [51]. The quantification of phenolic compounds in this study showed significant variations among the groups and over the days analyzed (Figure 3). Phenolic compound production was higher (around 90%) in plants inoculated with AMF (21.06–40.09 mg gallic acid equivalent (GAE) g^{-1}) at 60 dpi. AMF inoculation can induce changes in plants in the accumulation of secondary metabolites, including phenolic compounds that metabolize phenylpropanoids [52].

The highest LOX activities were observed at 90 dpi in plants supplemented with P and associated with AMF and P (Figure 4). The increase in LOX activity indicates a possible response mechanism of the plant's defense through the LOX pathway [53]. LOX activity is considered one of the main criteria for measuring stress intensity [54], and its changes depend on plant genotype and physiological conditions [55]. LOX enzymes participate in volatile compound production from leaves and roots of plants, such as alcohols, ethers, and aldehydes, which are considered important for growth and defense signaling [56]. The highest LOX activity observed in the present study, at 90 dpi, coincides with the

results observed in *P. aduncum* seedlings inoculated with the AMFs *Rhizophagus clarus* and *Claroideoglomus etunicatum*, which showed an increase in LOX activity at 60 and 90 dpi, respectively [46]. LOX also showed increased activity in previous studies under biotic and abiotic stimuli [36,57,58].

The PAL enzyme is a key enzyme in the phenylpropanoid biosynthetic pathway and plays an important role in the production of metabolites in response to plant defense [59]. The results showed that *P. divaricatum* increased PAL activity in the plants when they were inoculated with AMF and associated with AMF and P (Figure 5). The increase in PAL activity may be related to the induction of defense responses in plants [60], as colonization by symbiotic microorganisms may initially be recognized as an infection by pathogens, causing biotic stress [60,61]. *Cucumis sativus* L. cv. Jincun 2 inoculated with AMF under different temperature conditions showed an increase in PAL enzyme activity. Thus, under normal temperature conditions, the PAL activity of seedlings inoculated with AMF was 77.45% higher than in non-inoculated seedlings. Under low-temperature conditions, the inoculated seedlings presented increases of 44.44% in enzyme activity [62]. These results indicate that metabolic activity was induced by additional P, as well as association of AMF and different concentrations of P in *P. divaricatum* (Tables S1–S3). Thus, the results suggest that the production of secondary metabolites can be optimized, taking into account factors such as selected microorganisms, nutrients available in the soil, and host plant.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12030596/s1, Table S1: Chemical composition at 30 dpi in *Piper divaricatum* leaves inoculated with AMFs and concentrations of P (mean ± standard deviation), Table S2: Chemical composition at 60 dpi in *Piper divaricatum* leaves inoculated with AMFs and concentrations of P (means ± standard deviation), Table S3: Chemical composition at 90 dpi in *Piper divaricatum* leaves inoculated with AMFs and concentrations of P (means ± standard deviation).

Author Contributions: J.K.R.D.S. participated in study design; J.S.d.O., N.P.R., J.L.J. and L.P.X. conducted the experiments; E.H.A. contributed to GC–MS analyses; A.H.M. provided and identified the AMF species; W.N.S. and J.K.R.D.S. edited and approved the final version of the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research received financial support from the Conselho Nacional de Desenvolvimento de Científico e Tecnológico (National Council for Scientific and Technological Development, CNPq).

Data Availability Statement: Data supporting reported results are shown in the present article and in the Supplementary Materials.

Acknowledgments: We are grateful for CNPQ and CAPES (Coordination for the Improvement of Higher Education Personnel, CAPES) for providing scholarships to J.K.R.d.S. and J.S.d.O., respectively.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Guimarães, E.F.; Medeiros, E.V.S.S.; Queiroz, G.A. *Piper* in Flora Do Brasil. 2020. Jardim Botânico do Rio de Janeiro. Available online: http://floradobrasil.jbrj.gov.br/reflora/floradobrasil/FB32156 (accessed on 22 April 2021).
2. Peckolt, T.; Peckolt, G. *História das Plantas Medicinais e Úteis do Brazil*; Laemmert: Rio de Janeiro, Brazil, 1888; Volume 1, p. 1369.
3. Ferreira, G.K.S.; Margalho, J.F.; Almeida, L.Q.; Dos Anjos, T.O.; Cascaes, M.M.; Do Nascimento, L.D. Seasonal and circadian evaluation of the essential oil *P. divaricatum* G. Mey (Piperaceae) Leaves. *Braz. J. Develop.* **2020**, *6*, 41356–41369. [CrossRef]
4. Blumenthal, M.; Sing, Y.N. Pharmacology of kava and its constituents. *Herbal Gram.* **1997**, *39*, 50–56.
5. Sousa, P.J.C.; Sousa, P.J.C.; Barros, C.A.L.; Rocha, J.C.S.; Lira, D.S.; Monteiro, G.M.; Maia, J.G.S. Toxicological evaluation of the essential *Piper* L. *Rev. Bras. Farmacogn.* **2008**, *18*, 217–221. [CrossRef]
6. Da Silva, J.K.R.; Andrade, E.H.A.; Guimarães, E.F.; Maia, J.G.S. Essential oil composition, antioxidant capacity and antifungal activity of *Piper divaricatum*. *Nat. Prod. Commun.* **2010**, *5*, 477–480. [CrossRef]
7. Corpes, R.; Monteiro, L.; Andrade, E.H.; Maia, J.G.; Menezes, I.; Setzer, W.N.; Ramos, A.R.; Da Silva, J.K.R. Comparison of volatile profile and antioxidant activity of *Piper divaricatum* G. Meyer (Piperaceae) using cuttings and cell tissue. *J. Braz. Chem. Soc.* **2019**, *30*, 2291–2298. [CrossRef]

8. Barbosa, Q.P.S.; Da Câmara, C.A.G.; Ramos, C.S.; Nascimento, D.C.O.; Lima-Filho, J.V.; Guimaraes, E.F. Chemical composition, circadian rhythm and antibacterial activity of essential oils of *Piper divaricatum*: A new source of safrole. *Quím. Nova* **2012**, *35*, 1806–1808. [CrossRef]
9. Souto, R.N.P.; Harada, A.Y.; Andrade, E.H.A.; Maia, J.G.S. Insecticidal activity of *Piper* essential oils from the Amazon against the fire ant *Solenopis saevissima* (Smith) (Hymenoptera: Formicidae). *Neotrop. Entomol.* **2012**, *41*, 510–517. [CrossRef]
10. Da Silva, J.K.R.; Silva, J.R.A.; Nascimento, S.B.; Da Luz, S.F.M.; Meireles, E.N.; Alves, C.N.; Ramos, A.R.; Maia, J.G.S. Antifungal activity and computational study of constituents from *Piper divaricatum* essential oil against *Fusarium* infection in black pepper. *Molecules* **2014**, *19*, 17926–17942. [CrossRef] [PubMed]
11. Simões, C.M.O.; Schenkel, E.P.; Mello, J.C.P.; Mentz, L.A.; Petrovick, P.R. *Farmacognosia: Do Produto Natural ao Medicamento*, 1st ed.; Artmed: Guelph, ON, Canada, 2017; p. 486.
12. Bárzana, G.; Aroca, R.; Paz, J.A.; Chaumont, F.; Martinez-Ballesta, M.C.; Carvajal, M.; Ruiz-Lozano, J.M. Arbuscular mycorrhizal symbiosis increases relative apoplastic water flow in roots of the host plant under both well-watered and drought stress conditions. *Ann. Bot.* **2012**, *109*, 1009–1017. [CrossRef] [PubMed]
13. Toussaint, J.P.; Smith, F.A.; Smith, S.E. Arbuscular mycorrhizal fungi can induce the production of phytochemicals in sweet basil irrespective of phosphorus nutrition. *Mycorrhiza* **2007**, *17*, 291–297. [CrossRef] [PubMed]
14. Ahanger, M.A.; Hashem, A.; Abd Allah, E.F.; Ahmad, P. Arbuscular Mycorrhiza in Crop Improvement Under Environmental Stress. In *Emerging Technologies and Management of Crop Stress Tolerance*; Ahmad, P., Rasool, S., Eds.; Elsevier: Amsterdam, The Netherlands, 2014; pp. 69–95.
15. Begum, N.; Qin, C.; Ahanger, M.A.; Raza, S.; Khan, M.I.; Ashraf, M.; Ahmed, N.; Zhang, L. Role of arbuscular mycorrhizal fungi in plant growth regulation: Implications in abiotic stress tolerance. *Front. Plant Sci.* **2019**, *10*, 1068. [CrossRef] [PubMed]
16. Meng, L.; Zhang, A.; Wang, F.; Han, X.; Wang, D.; Li, S. Arbuscular mycorrhizal fungi and rhizobium facilitate nitrogen uptake and transfer in soybean/maize intercropping system. *Front. Plant Sci.* **2015**, *6*, 339. [CrossRef] [PubMed]
17. Tarraf, W.; Ruta, C.; Tagarelli, A.; De Cillis, F.; De Mastro, G. Influence of arbuscular mycorrhizae on plant growth, essential oil production and phosphorus uptake of *Salvia officinalis* L. *Ind. Crops Prod.* **2017**, *102*, 144–153. [CrossRef]
18. Jeffries, P.; Gianinazzi, S.; Perotto, S.; Turnau, K.; Barea, J.M. The contribution of arbuscular mycorrhizal fungi in sustainable maintenance of plant health and soil fertility. *Biol. Fert. Soils.* **2003**, *37*, 1–16. [CrossRef]
19. Moreira, F.M.S.; Siqueira, J.O. *Microbiologia e Bioquímica do Solo*, 2nd ed.; UFLA: Lavras, Brazil, 2006; p. 626.
20. Becquer, A.; Trap, J.; Irshad, U.; Ali, M.A.; Claude, P. From soil to plant, the journey of P through trophic relationships and ectomycorrhizal association. *Front. Plant Sci.* **2014**, *5*, 548. [CrossRef] [PubMed]
21. Shen, J.; Yuan, L.; Zhang, J.; Li, H.; Bai, Z.; Chen, X.; Zhang, W.; Zhang, F. Phosphorus dynamics: From soil to plant. *Plant Physiol.* **2011**, *156*, 997–1005. [CrossRef]
22. Smith, S.E.; Smith, F.A. Fresh perspectives on the roles of arbuscular mycorrhizal fungi in plant nutrition and growth. *Mycologia* **2012**, *104*, 1–13. [CrossRef] [PubMed]
23. Freitas, M.S.M.; Martins, M.A.; VIEIRA, I.J.C. Yield and quality of essential oils of *Mentha arvensis* in response to inoculation with arbuscular mycorrhizal fungi. *Pesqui. Agropecu. Bras.* **2004**, *39*, 887–894. [CrossRef]
24. Karagiannidis, N.; Thomidis, T.; Lazari, D.; Panou-Filotheou, E.; Karagiannidou, C. Effect of three Greek arbuscular mycorrhizal fungi in improving the growth, nutrient concentration, and production of essential oils of oregano and mint plants. *Sci. Hortic.* **2011**, *129*, 329–334. [CrossRef]
25. Urcoviche, R.C.; Gazim, Z.C.; Dragunski, D.C.; Barcellos, F.G.; Alberton, O. Plant growth and essential oil content of *Mentha crispa* inoculated with arbuscular mycorrhizal fungi under different levels of phosphorus. *Ind. Crop. Prod.* **2015**, *67*, 103–107. [CrossRef]
26. Mehlich, A. New extractant for soil test evaluation of phosphorus, potassium, magnesium, calcium, sodium, manganese and zinc. *Commun. Soil Sci. Plant Anal.* **1978**, *9*, 477–492. [CrossRef]
27. Gerdemann, J.W.; Nicolson, T.H. Spores of mycorrhizal Endogone species extracted from soil by wet sieving and decanting. *Trans. Br. Mycol. Soc.* **1963**, *46*, 235–244. [CrossRef]
28. Jenkins, W.R. A rapid centrifugal-flotation technique for separating nematodes from soil. *Plant Dis. Report* **1964**, *48*, 692. [CrossRef]
29. Schenck, N.; Pérez, Y. *Manual for the Idenfication of VA Mycorrhizal Fungi*, 3rd ed.; Synergistic Publications: Gainesville, FL, USA, 1987; p. 286.
30. International Culture Collection of Arbuscular and Vesicular-Arbuscular Mycorrhizal Fungi—INVAM 2018. Available online: http://invam.wvu.edu/methods/cultures/single-species-cultures (accessed on 15 June 2019).
31. Da Luz, S.F.M.; Reis, L.A.; Lemos, O.F.; Maia, J.G.S.; Mello, A.H.; Ramos, A.R.; Da Silva, J.K.R. Effect of arbuscular mycorrhizal fungi on the essential oil composition and antioxidant activity of black pepper (*Piper nigrum* L.). *Int. J. Appl. Res. Nat. Prod.* **2016**, *9*, 10–17.
32. Van Den Dool, H.; Kratz, P.D. A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. *J. Chromatogr. A* **1963**, *11*, 463–471. [CrossRef]
33. Adams, R. *Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry*, 4th ed.; Allured Publishing Corporation: Carol Stream, IL, USA, 2007; p. 804.
34. NIST. National Institute of Standard and Technology. NIST Standard Reference Database Number 69. Available online: http://webbook.nist.gov/ (accessed on 25 March 2020).

35. Axelrod, B.; Cheesbrough, T.M.; Laakso, S. Lipoxygenases in soybean. *Methods Enzymol.* **1981**, *71*, 441–451. [CrossRef]
36. Meireles, E.M.; Xavier, L.P.; Ramos, A.R.; Maia, J.G.S.; Setzer, W.N.; Da Silva, J.K.R. Phenylpropanoids produced by *Piper divaricatum*, a resistant species to infection by *Fusarium solani* f. sp. piperis, the pathogenic agent of fusariosis in black pepper. *J. Plant Pathol. Microbiol.* **2016**, *7*, 333–338. [CrossRef]
37. Vaganan, M.M.; Ravi, I.; Nandakumar, A.; Sarumathi, S.; Sundararaju, P.; Mustaffa, M.M. Phenylpropanoid enzymes, phenolic polymers and metabolites as chemical defenses to infection of *Pratylenchus coffeae* in roots of resistant and susceptible bananas (*Musa* spp.). *Indian J. Exp. Biol.* **2014**, *52*, 252–260.
38. Singleton, V.L.; Rossi, J.A. Colorimetric of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* **1965**, *16*, 144–158.
39. Clark, R.B.; Zeto, S.K. Mineral acquisition by arbuscular mycorrhizal plants. *J. Plant Nutr.* **2000**, *23*, 867–902. [CrossRef]
40. Merlin, E.; Melato, E.; Lourenço, E.L.B.; Jacomassi, E.; Gasparotto Junior, A.; Da Cruz, R.M.S.; Otênio, J.K.; Da Silva, C.; Alberton, O. Inoculation of arbuscular mycorrhizal fungi and phosphorus addition increase coarse mint (*Plectranthus amboinicus* Lour.) plant growth and essential oil content. *Rhizosphere* **2020**, *15*, 100217–100222. [CrossRef]
41. Seema, H.S.; Garampalli, R.H. Effect of Arbuscular Mycorrhizal fungi on growth and biomass enhancement in *Piper longum* L. (Piperaceae). *Int. J. Curr. Microbiol. Appl. Sci.* **2015**, *5*, 11–18.
42. Wang, P.; Wu, S.H.; Wen, M.X.; Wang, Y.; Wu, Q.S. Effects of combined inoculation *Rhizophagus intraradices* and *Paenibacillus mucilaginosus* on plant growth, root morphology, and physiological status of trifoliate orange (*Poncirus trifoliata L. Raf.*) seedlings under different levels of phosphorus. *Sci. Hortic.* **2016**, *205*, 97–105. [CrossRef]
43. Daniel, O.; Vitorino, A.C.T.; Alovisi, A.A.; Mazzochin, L.; Tokura, A.M.; Pinheiro, E.R.P.; Souza, E.F. Aplicação de fósforo em mudas de *Acacia mangium* Willd. *Rev. Árvore* **1997**, *21*, 163–168.
44. Frosi, G.; Barros, V.A.; Oliveira, U.M.T.; Cavalcante, L.C.; Santos, M.G. Increase in biomass of two woody species from a seasonal dry tropical forest in association with AMF with different phosphorus levels. *Appl. Soil Ecol.* **2016**, *102*, 46–52. [CrossRef]
45. Neumann, E.; Schmid, B.; Römheld, V.; George, E. Extraradical development and contribution to plant performance of an arbuscular mycorrhizal symbiosis exposed to complete or partial roots one drying. *Mycorrhiza* **2009**, *20*, 13–23. [CrossRef] [PubMed]
46. De Oliveira, J.S.F.; Xavier, L.P.; Lins, A.; Andrade, E.H.A.; Maia, J.G.S.; De Mello, A.H.; Setzer, W.N.; Ramos, A.R.; Da Silva, J.K.R. Effects of inoculation by arbuscular mycorrhizal fungi on the composition of the essential oil, plant growth, and lipoxygenase activity of *Piper aduncum* L. *AMB Express* **2019**, *9*, 29. [CrossRef] [PubMed]
47. Godard, K.; White, R.; Bohlmann, J. Monoterpene-induced molecular responses in *Arabidopsis thaliana*. *Phytochemistry* **2008**, *69*, 1838–1849. [CrossRef] [PubMed]
48. Effah, E.; Holopainen, J.K.; McCormick, A.C. Potential roles of volatile organic compounds in plant competition. *Perspect. Plant Ecol. Syst.* **2019**, *38*, 58–63. [CrossRef]
49. Michalak, A. Phenolic compounds and their antioxidant activity in plants growing under heavy metal stress. *Pol. J. Environ. Stud.* **2006**, *15*, 523–530.
50. Zaynab, M.; Fatima, M.; Abbas, S.; Sharif, Y.; Umair, M.; Zafar, M.H.; Bahadar, K. Role of secondary metabolites in plant defense against pathogens. *Microb. Pathog.* **2018**, *124*, 198–202. [CrossRef]
51. Mandal, S.M.; Chakraborty, D.; Dey, S. Phenolic acids act as signaling molecules in plant-microbe symbioses. *Plant Signal. Behav.* **2010**, *5*, 359–368. [CrossRef]
52. Devi, M.C.; Reddy, M.N. Phenolic acid metabolism of groundnut (*Arachis hypogaea* L.) plants inoculated with VAM fungus and Rhizobium. *Plant Growth Regul.* **2002**, *37*, 151–156. [CrossRef]
53. Baysal, T.; Demirdoven, A. Lipoxygenase in fruits and vegetables: A review. *Enzyme Microb. Technol.* **2007**, *40*, 491–496. [CrossRef]
54. Begum, N.; Ahanger, M.A.; Zhang, L. AMF inoculation and phosphorus supplementation alleviates drought induced growth and photosynthetic decline in *Nicotiana tabacum* by upregulating antioxidant metabolism and osmolyte accumulation. *Environ. Exp. Bot.* **2020**, *176*, 104088–104103. [CrossRef]
55. Silva, H.S.A.; Romeiro, R.S.; Macagnan, D.; Halfeld-Vieira, B.A.; Pereira, M.C.B.; Mounteer, A. Rhizobacterial induction of systemic resistance in tomato plants: Non-specific protection and increase in enzyme activities. *Biol. Control* **2004**, *29*, 288–295. [CrossRef]
56. Liavonchanka, A.; Feussner, I. Lipoxygenases: Occurrence, functions and catalysis. *J. Plant Physiol.* **2006**, *163*, 348–357. [CrossRef]
57. Nahar, K.; Hasanuzzaman, M.; Suzuki, T.; Fujita, M. Polyamines-induced aluminum tolerance in mung bean: A study on antioxidant defense and methylglyoxal detoxification systems. *Ecotoxicology* **2017**, *26*, 58–73. [CrossRef]
58. Ahanger, M.A.; Qin, C.; Begum, N.; Maodong, Q.; Dong, X.X.; El-Esawi, M.; El-Sheikh, M.A.; Alatar, A.A.; Zhang, L. Nitrogen availability prevents oxidative effects of salinity on wheat growth and photosynthesis by up-regulating the antioxidants and osmolytes metabolism, and secondary metabolite accumulation. *BMC Plant Biol.* **2019**, *19*, 479. [CrossRef]
59. Sharma, A.; Shahzad, B.; Rehman, A.; Bhardwaj, R.; Landi, M.; Zheng, B. Response of phenylpropanoid pathway and the role of polyphenols in plants under abiotic stress. *Molecules* **2019**, *24*, 2452. [CrossRef]
60. Compant, S.; Duffy, B.; Nowak, J.; Clément, C.; Barka, E.A. Use of plant growth-promoting bacteria for biocontrol of plant diseases Principles, mechanisms of action, and future prospects. *Appl. Environ. Microbiol.* **2005**, *71*, 4951–4959. [CrossRef]

61. Ngadze, E.; Icishahayo, D.; Coutinho, T.A.; Van Der Waals, J.E. Role of polyphenol oxidase, peroxidase, phenylalanine ammonia lyase, chlorogenic acid, and total soluble phenols in resistance of potatoes to soft rot. *Plant Dis.* **2012**, *96*, 186–192. [CrossRef] [PubMed]
62. Chen, S.; Jin, W.; Liu, A.; Zhang, S.; Liu, D.; Wang, F.; Lin, X.; He, C. Arbuscular mycorrhizal fungi (AMF) increase growth and secondary metabolism in cucumber subjected to low temperature stress. *Sci. Hortic.* **2013**, *160*, 222–229. [CrossRef]