Effect of *Rhizophagus irregularis* on Morphological and Biochemical Characteristics of *Pako* (*Diplazium esculentum)* Under Drought Stress

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ABSTRACT

This study presents the first documented report on the successful colonization of the indigenous and underutilized fern species *Diplazium esculentum* by the fungi *Rhizophagus irregularis* in the Philippines. The beneficial role of the microorganism was evaluated through its morphological characteristics, with improved growth parameters of the plant, and biochemical characteristics revealed a significant increase in relative water content, membrane stability index, total soluble sugar, amino acids, and sucrose content. Hydrogen peroxide and malondialdehyde were recorded to have high levels under drought stress conditions. This study showed a decline in concentrations due to the inoculation of *R. irregularis*, highlighting its potential to alleviate stress. These results feature the potential of this microorganism to minimize the impact of drought stress on indigenous fern plants and allow a better adaptation to dry conditions.

Keywords: *Rhizophagus irregularis*, drought stress, root colonization, *Diplazium esculentum*

INTRODUCTION

The impact of climate change was observed in recent decades, and it was an alarming issue to humankind, leading to a devastating increase in the severity of drought events (Silva et al., 2023). Drought events highlight severe constraints on agriculture, which debilitate plants' cellular functions, structure, and activity, such as photosynthetic efficiency, reduced assimilation of nutrients, and low plant productivity (Begum et al., 2019; Kaushal, 2019). Plants' flexibility to respond to their environment has evolved various strategies enabling them to withstand the effects of water scarcity (Leventis et al., 2021). Notably, He et al. (2020) described the arbuscular mycorrhizal fungi (AMF) as highly evolved microorganisms and as symbionts with most terrestrial plants, with an exceptional contribution to the plant's resilience and tenacity to withstand environmental constraints such as drought (Cosme, 2023).

AMF are soil microbes that colonize most plant roots and establish a connection between the plant and AMF, helping produce plant growth hormones, increasing nutrient availability, resistance to pathogens, and resilience to water scarcity (Kameoka et al., 2018; Pozo et al., 2015). Concurrently, several studies from Pozo et al. (2015), Yang et al. (2021), and Zhang et al. (2018) emphasized that the improved defense response to pathogens and increased resilience to salinity are due to arbuscular mycorrhiza (AM) symbiosis.

In the Philippines, *Diplazium esculentum* is an indigenous plant locally known as *pako* and is often served as an ingredient for preparing vegetable salads and pickles (Amoroso et al., 2014). Additionally, it is an excellent source of food nutrients and phytochemicals (Junejo et al., 2015; Koniyo et al., 2021), which have the potential to be developed into pharmaceutical products (Koniyo et al., 2021). In the marginal farming environment, agricultural sustainability depends on many lesser known crops, such as underutilized, typically low-yielding crops (Pattanayak et al., 2019). Introducing neglected and underutilized species (NUS) into the current cropping systems could help reduce food scarcity and malnutrition (Katoch et al., 2023). Sustainable agriculture depends on crops that are tolerant to biotic and abiotic stresses, requires minimal input of water and nutrients, and can be cultivated with a minimal carbon footprint (Luo et al., 2022). These underutilized indigenous plants are neglected or undervalued “minor” crops having low production and sale (Gupta et al., 2022). As research on drought tolerance has mainly focused on major staple crops, the potential of some NUS to be naturally more drought tolerant than most staple crops has been overlooked (Rosero et al., 2020). In fact, due to their nutrient-dense nature, they have the potential to help overcome malnutrition (Sanmartín et al., 2020).

Many studies have focused on the association between plants, mainly staple crops, and AMF and have shown improvements in growth and other biological aspects, including physiological and biochemical traits, with the acquisition of water and mineral elements from the host plant, inducing tolerance against drought conditions. To date, the understanding and knowledge of the nature and interaction of mycorrhizal species *(R. irregularis*) and *Diplazium esculentum* need to be better analyzed. Hence, no reports on the symbiosis of *D. esculentum* and mycorrhizal species, specifically the *Rhizophagus irregularis*, exist. **This study aims to evaluate the morphological and biochemical characteristics of *D. esculentum* under drought stress and to determine the presence of AMF *Rhizophagus irregularis* on the roots of the plant sample *D. esculentum.* The information from this study will provide additional knowledge on the cultivation and symbiosis of AMF and fern species in the Philippines.**

MATERIALS AND METHODS

Experimental Design

The current research was conducted using a descriptive-experimental design. The factorial experiment was set up in a 2 × 2 randomized complete block design. The first factor consists of two water levels (non-drought and drought stressed), while the second factor involves two AMF treatments (with and without AMF inoculation). The plants were then grown for a period of one month. To experimentally induce drought stress, the plants were withheld from water for 7 days (Li et al., 2022).

Plant and Fungal Materials

Pako (*Diplazium esculentum*) seedlings purchased at Pako Farm, Brgy. 395, Santa Rita, Pampanga, were used as experimental plant material. **The plant sample was authenticated at the Natural Science Research Unit (NSRU), School of Advanced Studies, Saint Louis University, Baguio City, Philippines, and verified by Dr. Teodora Balangcod, botanist from**

the Northern Luzon Herbarium at the University of the Philippines, Baguio City. The stipe of the fern propagules was cut and sterilized in 1.25% sodium hypochlorite for 30 min to remove other microorganisms, and plant samples were washed thoroughly thrice with distilled water. *D. esculentum* seedlings were sown, distributed in 50-ml plastic pot trays, and allowed to grow. During the drought stress condition, the temperature was set at 26°C–28°C inside the NSRU, Saint Louis University, with 81%–85% humidity. The AMF pure culture inoculum *Rhizophagus irregularis* with ID number Dn201_R used in this study was purchased at the International Collection of (Vesicular) Arbuscular Mycorrhizal Fungi, University of Kansas, US (https://invam.ku.edu/).

Growth Measurement

On the 30th day of harvest after the treatment, the growth of the plant was determined using the following parameters: the plant height, frond length, number of fronds, number of leaves, frond fresh weight (FW), and frond dry weight (DW). The FWs of the fronds were measured immediately after the plants were harvested and washed thrice with distilled water and air dried for 30 min. The DWs of the plant samples were recorded after constant drying at 85°C for 48 hr. **Thirty-six plant samples were used in this study in three replicates. And a total of four samples were analyzed for each treatment.**

Leaf Relative Water Content Measurement

Leaf relative water content (RWC) was measured using the method described by Sade et al. (2015) and the formula described by Leventis et al. (2021): $RWC = 100 \times (FW - DW / TW - DW)$, in which TW represents the turgid weight. Leaf samples were collected in the morning, and FW was immediately recorded. The TW was determined after placing the samples fully submerged in $CaCl_2$ in the dark for 8 hr at 20°C. Finally, the leaf samples were oven dried at 85°C for 48 hr, and DW was recorded.

Membrane Stability Index Measurement

The membrane stability index (MSI) was determined according to the method developed by Alam et al. (2021). Two grams of fresh *D. esculentum* leaf samples was divided into two groups, 10 ml of distilled water was used to rinse the samples, and the samples were dried independently. The leaf samples were placed in a test tube containing 10 ml of distilled water. Two groups of leaf samples were hot bathed at 40°C for 30 min, and the electrical conductivity of the first set of samples (EC1) was immediately measured with a conductivity meter (Waterproof EC-PhTEST30, Eutech). The electrical conductivity of the second set of samples (EC2) was measured after boiling at 100°C in a water bath for 10 min. The MSI was then calculated based on the following formula: $MSI = [1 - (EC1 / EC2)] \times 100$.

Chlorophyll Content Measurement

The chlorophyll content of the samples was determined according to the method described by Jadrane et al. (2021). One gram of fresh *D. esculentum* leaf samples was ground and mixed with 15 ml of 80% acetone, then centrifuged (Biofuge Pico Centrifuge, Heraeus) at 5,000 rpm for 10 min. The supernatant was read using an ultraviolet-visible (UV-VIS) spectrophotometer (PD-3000UV, APEL) at 663, 645, and 750 nm. Chlorophyll a, chlorophyll b, and total chlorophyll content were calculated according to the formula developed by Jadrane et al. (2021): chlorophyll a = Absorbance (Abs) 663 − Abs 645; chlorophyll b = Abs 645 − Abs 643; total chlorophyll = chlorophyll a + chlorophyll b.

Hydrogen Peroxide Content Measurement

A method described by Junglee et al. (2014) was used to measure the content hydrogen peroxide (H_2O_2). Briefly, 0.5 g of leaf samples was digested with 5 ml of trichloroacetic acid (TCA) for 10 min and centrifuged at 12,000 rpm for 15 min. Five hundred microliters of potassium phosphate buffer and 2 ml of potassium iodide were mixed with 500 µl of supernatant. The samples were vortexed, and the absorbance was read at 390 nm (PD-3000UV UV-VIS spectrophotometer, APEL) to measure the H_2O_2 using the standard curve of H_2O_2 and expressed as micromoles per gram FW (Sergiev et al., 1997).

Malondialdehyde Content Measurement

D. esculentum leaves were used to investigate the malondialdehyde (MDA) using the method adapted from Rasouli et al. (2023) and Kamali and Mehraban (2020) with minimal modifications. Briefly, 0.5 g of leaf samples was homogenized by adding 5 ml of 5% TCA and allowed to stand for 10 min and centrifuged at 10,000 rpm for 15 min. Then, 1 ml of the supernatant was mixed with 2 ml of thiobarbituric acid; at 95°C, the mixtures were heated for 30 min. To stop the reaction of the solution, it was allowed to cool down at room temperature. After centrifugation at 10,000 rpm for 10 min, the supernatant was collected, and the absorbance of the supernatant was recorded at 440-, 532-, and 600-nm wavelengths (PD-3000UV UV-VIS spectrophotometer, APEL). The concentration of MDA was calculated following the method of Hashem et al. (2018): MDA (µmol/g FW) = $[(A532 - A600) / 156] \times 10 \times$ dilution factor.

Total Soluble Sugar, Sucrose, and Free Amino Acid Measurement

The samples were oven dried for 30 min and had another drying at 85°C for 48 hr. Then, the samples were powdered with a mortar and pestle and immersed in 8 ml of 90% ethanol. The supernatant was collected and centrifuged at 5,000 rpm for 30 min. To ensure the complete extraction of sugars and amino acids, the extraction procedures were repeated twice. The total soluble sugar content was carefully assessed using the anthrone method, and the absorbance was recorded at 620 nm following the method of Somani et al. (1987). Sucrose content was measured following the resorcinol method adapted from Buysse and Merckx (1993) with minimal modifications, and the absorbance was recorded at 480 nm. The method of Liu et al. (2023) was used to analyze the content of free amino acids following the ninhydrin reaction at 570 nm with minor modifications.

Root Staining and Mycorrhizal Colonization Observation

Four root samples per treatment were used for the observation of AM fungi colonization. According to the method of Sakya et al. (2018), root samples were cut into 1 cm in length and thoroughly rinsed with distilled water and cleared with 10% KOH. Then, the samples were immersed in 2% HCl for 15–20 min and stained with trypan blue in 0.05% lactoglycerol. For the microscopic observation (Olympus model CX21FS1, Tokyo, Japan), one to two root samples were transferred to a glass slide containing one to two drops of glycerin.

Statistical Analysis

All statistical analyses were performed using SPSS version 25.0. All available data were tested for normality and homogeneity of variance before analysis. Data were subjected to a two-way analysis of variance to determine the main and interaction effects of drought stress and inoculation treatments. For the comparisons between means, Tukey's test range test was employed ($p < 0.05$).

RESULTS AND DISCUSSION

This study confirmed the presence of *Rhizophagus irregularis* on the underutilized indigenous roots of fern species *Diplazium esculentum* in the Philippines. In addition, this study also provided evidence of the active colonization of the AMF *R. irregularis*.

Figure 1. Growth promotion of *R. irregularis* from nondrought without AMF (first image), nondrought with AMF (second image), drought stressed with AMF (third image), and drought stressed with AMF (last image).

Presence of Mycorrhiza in *D. esculentum* Roots

Microscopic observation reveals the presence of *R. irregularis* colonizing the roots of *D. esculentum* with visible structures such as hyphae, vesicles, and arbuscules (Figure 1), while no colonization was found in samples without AMF. Notably, the study's findings demonstrated the first report on the successful colonization of the AMF *R. irregularis* within the root tissues of the underutilized indigenous *D. esculentum* fern species in the Philippines.

Morpho-Anatomical Characteristics of *D. esculentum* Inoculated With and Without AMF Under Well-Watered and Water-Stressed Treatments

Inoculated *D. esculentum* showed a significant increase in plant growth (Figure 2) and root and frond FWs among the different treatments, and a dramatic reduction in these growth parameters was observed in noninoculated samples (Table 1). Results also showed the significant role of AMF in the parameters mentioned above, even in the absence of drought stress.

Physiological Characteristics of *D. esculentum* Inoculated With and Without AMF Under Well-Watered and Water-Stressed Treatments

The results of the study presented in Table 2 showed a significant increase in RWC and MSI under drought-stressed conditions, which may be due to the presence of AMF compared to the other treatments, with a decline in physiological results. In addition, due to water scarcity, a decrease in chlorophyll a, chlorophyll b, and total chlorophyll content was observed due to the inoculation of AMF with 27.50%, 30.70%, and 33.43%, respectively.

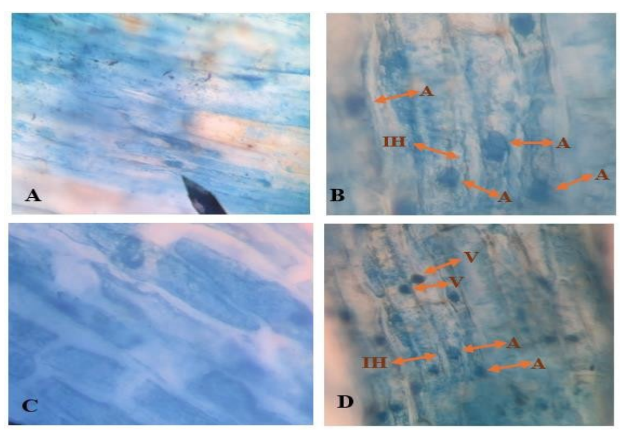

Figure 2. Lactoglycerol staining of *D. esculentum* roots in nondrought treatments and drought-stressed treatments. Panel A: Nondrought without AMF, not colonized. Panel B: Nondrought with AMF, colonized with intraradical hyphae structures (IH) and arbuscules (A). Panel C: Drought stressed without AMF, not colonized. Panel D: Drought stressed, colonized with AMF structures vesicles (V), intraradical hyphae, and arbuscules.

Table 1. Effect of Drought and AMF *Rhizophagus irregularis* on Growth Parameters of *Diplazium esculentum*

Treatments		Plant Height (cm)	Frond Length (cm)	Number of Leaves	Frond Fresh Weight (g)	Frond Dry Weight (g)	Root Fresh Weight (g)	Root Dry Weight (g)
Drought	AMF							
Nondrought	+ AMF	45.07 ± 3.34^{a}	32.45 ± 3.18^{a}	28.50 ± 1.05^{a}	3.74 ± 0.77^{a}	0.72 ± 0.27^{a}	15.97 ± 0.71^{a}	4.94 ± 0.79^{a}
stress	− AMF	40.53 ± 0.41^{b}	28.33 ± 1.27^{b}	25.83 ± 0.98^{ab}	2.69 ± 0.21^{b}	0.37 ± 0.07^{b}	13.67 ± 0.38^{b}	3.39 ± 0.34^{b}
Drought	+ AMF	34.34 ± 1.88^{c}	22.67 ± 2.17^{c}	23.18 ± 0.87^{b}	1.62 ± 0.30^{c}	0.16 ± 0.04^{bc}	11.31 ± 1.26^{c}	2.58 ± 0.21^{bc}
stress	− AMF	25.87 ± 3.01^{d}	12.33 ± 3.76^{d}	17.43 ± 4.65^{c}	0.73 ± 0.33^{d}	0.07 ± 0.03^{c}	7.84 ± 1.59^{d}	1.92 ± 0.40^{c}
Drought		202.41***	159.80***	58.46***	159.66***	45.51***	146.65***	77.68***
AMF		53.04***	50.27***	22.01***	36.11***	12.03**	44.52***	25.68***
Drought × AMF		4.85*	9.32**	2.96^{ns}	0.23^{ns}	4.60*	1.86^{ns}	4.19*

Note. Mean values ± SD in the same column followed by the same lowercase letters are not significantly different at $p \leq 0.05$ by Tukey test. AMF = arbuscular mycorrhizal fungi; ^{ns} = nonsignificant.
*$p \leq 0.05$. **$p \leq 0.01$. ***$p \leq 0.001$. ^{ns}Nonsignificant.

Table 2. Effect of Drought and AMF *R. irregularis* on Physiological Parameters of *D. esculentum*

Treatments		RWC (%)	MSI (%)	Chl a (mg/g FW)	Chl b (mg/g FW)	Chl a + b (mg/g FW)
Drought	AMF					
Nondrought	+ AMF	90.99 ± 3.13^{a}	46.60 ± 6.11^{a}	0.56 ± 0.03^{a}	1.00 ± 0.05^{a}	1.55 ± 0.06^{a}
stress	− AMF	83.55 ± 2.18^{ab}	33.88 ± 4.02^{b}	0.55 ± 0.02^{a}	0.97 ± 0.10^{a}	1.52 ± 0.10^{a}
Drought	+ AMF	64.13 ± 0.10^{b}	23.22 ± 1.60^{c}	0.55 ± 0.03^{a}	0.93 ± 0.09^{a}	1.49 ± 0.10^{a}
stress	− AMF	39.78 ± 13.69^{c}	5.73 ± 0.48^{d}	0.51 ± 0.03^{b}	0.59 ± 0.08^{b}	1.10 ± 0.08^{b}
Drought		64.24***	148.41***	10.51**	37.49***	45.51***
AMF		13.02*	51.05***	7.89**	79.76***	12.03**
Drought × AMF		3.68^{ns}	1.27^{ns}	4.67*	57.93***	4.60

Note. Mean values ± SD in the same column followed by the same lowercase letters are not significantly different at $p \leq 0.05$ by Tukey test. AMF = arbuscular mycorrhizal fungi; RWC = relative water content; MSI = membrane stability index; FW = fresh weight; Chl a = chlorophyll a; Chl b = chlorophyll b.
*$p \leq 0.05$. **$p \leq 0.01$. ***$p \leq 0.001$. ^{ns}Nonsignificant.

Biochemical Characteristics of *D. esculentum* Inoculated With and Without AMF Under Well-Watered and Water-Stressed Treatments

This study's initial report on *R. irregularis* (Dn201_R) successfully colonized the roots of *D. esculentum* with well-defined structures under microscopic observation. The result of this study suggests the beneficial role of the AMF *R. irregularis*. Therefore, the morphological and biochemical positive results (Table 3) highlight this organism's potential role in colonizing indigenous underutilized species of ferns in the Philippines. **Introducing the *R. irregularis* into the native Philippine plant species reveals the adaptive mechanism of this microorganism to thrive under stressed conditions. Moreover, it infects indigenous plants, influencing the interaction between the fungi and the fern species through its observed beneficial role in plant growth and significant increase in biochemical** parameters. This underlies the results of Verbruggen et al. (2013) showing the effectiveness of AMF inoculation in agricultural soils by growth improvement, similar to Essahibi et al. (2017) and Hashem et al. (2019), revealing the improved growth of chickpeas under drought and normal conditions. Reports from this study emphasized the essential role of RWC, which reflects the endurance of *D. esculentum* under drought conditions inoculated with *R. irregularis* and served as an indicator of the plant's water status and level of hydration similar to the reports of Tang et al. (2022) demonstrating the role of mycorrhizal fungi in maintaining optimal water content with improved RWC, in response to drought stress. In addition, the increase in MSI showed a protective effect corroborating the findings of Tas and Tas (2007) demonstrating that the high MSI is associated with better plant survival and resilience to severe drought.

Table 3. Effect of Drought and AMF *R. irregularis* on Biochemical Parameters of *D. esculentum*

Treatments		H_2O_2	MDA (μmol/g FW)	Soluble Sugar (mg/g FW)	Sucrose (mg/g FW)	Amino Acid (mg/g FW)
Drought	AMF					
Nondrought stress	+ AMF	2.41 ± 0.27^{ab}	17.21 ± 3.45^{b}	173.45 ± 8.93^{a}	19.58 ± 1.88^{a}	6.60 ± 2.38^{a}
	− AMF	2.92 ± 0.06^{a}	20.32 ± 1.80^{a}	134.37 ± 25.04^{a}	14.02 ± 2.37^{ab}	3.36 ± 0.36^{b}
Drought stress	+ AMF	1.65 ± 0.49^{bc}	6.28 ± 0.35^{d}	67.48 ± 23.88^{b}	11.17 ± 1.69^{bc}	2.03 ± 0.73^{b}
	− AMF	2.02 ± 0.56^{b}	11.22 ± 1.79^{c}	49.16 ± 13.45^{c}	6.97 ± 1.28^{c}	1.08 ± 0.06^{b}
Drought		12.53**	71.87***	74.28***	44.71***	26.31**
AMF		3.46^{ns}	11.61**	6.70*	17.81**	9.92*
Drought × AMF		0.10^{ns}	0.60^{ns}	0.88^{ns}	0.35^{ns}	2.99^{ns}

Note. Mean values ± SD in the same column followed by the same lowercase letters are not significantly different at $p \leq 0.05$ by Tukey test. AMF = arbuscular mycorrhizal fungi; H_2O_2 = hydrogen peroxide; MDA = malondialdehyde; FW = fresh weight.
*$p \leq 0.05$. **$p \leq 0.01$. ***$p \leq 0.001$. ^{ns}Nonsignificant.

Consistently, a total reduction in the chlorophyll content was also reported in this study; however, in the presence of *R. irregularis,* an improved chlorophyll content was recorded with a positive correlation that enhances mineral uptake and provides structural stabilization of proteins and cell tissues (Cosme, 2023). Furthermore, the biochemical findings of this study indicated low concentrations of H_2O_2 and MDA revealing the benefits of AMF inoculation, leading to a reliable marker for assessing the extent of stress-induced damage in plants (Zhang et al., 2021). **Consistent with the results of this study, drought stress alters the physiology of plants. However, literature reports indicate that sucrose regulates "crosstalk" or "interactions" between plant hormones and sugars (Yusuf et al., 2016). Additionally, drought stress triggers sugar accumulation in plants, providing energy and aiding in protection against oxidative damage (Du et al., 2020). Also, according to Dien et al., (2019), total soluble sugars and amino acids are known to maintain the overall structure and growth of the plants, which was observed in this study**.

CONCLUSION

In conclusion, through morphological and biochemical characterization of *D. esculentum,* the AMF *R. irregularis* showed a promising role in mitigating the harmful effects in *D. esculentum* in response to drought. The results of this study present novel findings on the symbiotic relationship between *R. irregularis* and the roots of *D. esculentum.* **Furthermore, additional research on the role and mechanism of AM fungi on their host range in the Philippines is necessary to evaluate and confirm their pivotal role.**

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