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Research Article

Tolerance of lonkida (*Nauclea orientalis* L.) seedlings inoculated with mycorrhizae against drought and waterlogging stress

Faisal Danu Tuheteru[1*], Asrianti Arif[1], Husna[1], Basrudin[1], Albasri[1], Irdika Mansur[2], Maman Turjaman[3], Miranda Hadiyanti Hadijah[4], Agnitje Rumambi[5], Budi Prasetya[6], Armila R. Male[1]

[1] Department of Forestry, Faculty of Forestry and Environmental Science, Universitas Halu Oleo, Kendari, 93121, Indonesia.
[2] Department of Silviculture, Faculty of Forestry, IPB University, Dramaga, Bogor 16680, Indonesia
[3] Forest Research and Development Center, Jl. Raya Gn. Batu No.5, Bogor 16119, Indonesia
[4] Department of Forestry, Faculty of Agriculture, Universitas Pattimura, Jl. Ir. Putuhena, Poka, Ambon 97233, Indonesia
[5] Department of Animal Nutrition, Faculty of Animal Science, Sam Ratulangi University, Bahu-Manado, 95115, Indonesia
[6] Departement of Soil, Faculty of Agriculture, Universitas Brawijaya, Jl. Veteran No. 1, Malang 65145, Indonesia

[*]corresponding author: faisal.danu.tuheteru_fhut@uho.ac.id

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Abstract

Abiotic stress is a limiting factor for plant growth and development. The use of arbuscular mycorrhizal fungi can reduce the negative effects of abiotic stress. This study aimed to determine the tolerance of *Nauclea orientalis* inoculated with mycorrhizae to drought and waterlogging stresses. This research was carried out at the Indonesian Mycorrhizal Association's greenhouse and Forestry laboratory the University of Halu Oleo in Kendari City, Southeast Sulawesi Province, Indonesia, from March to June 2019. The study used a factorial, completely randomized design consisting of two factors. The first factor was Arbuscular Mycorrhizal Fungi (AMF) inoculations (A) consisting of control, AMF types of *Acaulospora* sp.1, and *Claroideoglomus etunicatum.* The second factor was environmental stress treatments (B) consisting of a control, soil moisture 25% of field capacity, 50% of field capacity, inundated as high as the polybag (9 cm high) and inundated over the polybag. The results showed that local AMF was effective in improving plant growth. Interaction between inoculation of *Acaulospora* sp.1 and environmental stress significantly increased AMF colonization on the *N. orientalis* roots. Inoculation of *C.etunicatum* significantly improved the *N. orientalis* growth. The treatment of drought stress with a field of 50% field capacity negatively influenced plant dry weight and the relative growth of the *N. orientalis*.

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Introduction

Environmental stresses (such as abiotic and biotic) are factors influencing physiological processes, development and function, as well as causing damage to plant systems (Ashraf et al., 2018; Kumar and Verma, 2018). Abiotic stresses such as drought, waterlogging, salinity, high temperature, heavy metal toxicity, and oxidative are dangerous threats to plants and the environment (Nadeem et al., 2019). Drought is a limiting factor in physiological aspects, growth and production (Symanczik et al., 2018; Seleiman et al., 2021). Plants respond to drought stress at morphological, anatomical and cellular levels with modifications that allow plants to avoid stress or increase their tolerance (Nadeem et al., 2019).

Whereas waterlogged is one of the environmental factors that are unfavorable to plants and mycorrhizal fungi (Tuheteru et al., 2015). Plant tolerance to environmental stress varies greatly and is determined by the plant species and its symbiosis with soil microbes (Tuheteru et al., 2015). Arbuscular mycorrhizal fungi (AMF) are considered an efficient and environment-friendly biotechnology approach and can be used to reduce the negative effects of environmental stress (Begum et al., 2019**;** Evelin et al., 2019).

AMF are soil microorganisms that are in symbiosis with 80-90% of woody plant species (Smith and Read, 2008). AMF is classified as a member of the phylum Glomeromycota including three classes, 11 families, 25 genera and nearly 250 species (Schüßler et al., 2001; Spatafora et al., 2016). AMF forms symbioses with plant roots to grow in adverse ecosystems (Husna et al., 2016; Tedersoo et al., 2018). The results of a review of various publications show that the application of AMF can increase plant growth and tolerance under biotic and abiotic stress conditions (Tuheteru and Wu, 2017**;** Bahadur et al., 2019; Begum et al., 2019**;** Evelin et al., 2019). AMF increases plant tolerance through a series of mechanisms, including increasing water and nutrient uptake of host plants, increasing osmotic adjustment and protection against damage and increasing stomatal conductance and transpiration and photosynthesis. The symbioses have been reported to increase tolerance of *Leucaena leucocephala* (Fagbola et al., 2001), *Triticum aestivum* (Al-Karaki et al., 2003), *Populus* spp. (Liu et al., 2015), *Zenia insignis* (Zhang et al., 2019, *Cirus* sp. (Wu et al., 2019), *Acacia seyal* Del. (Abdelmalik et al., 2020), *Cenostigma microphyllum* (Pereira et al., 2021) and *Ceratonia siliqua*'s (Jadrane et al., 2021) to drought conditions. They also increase the tolerance of *Pterocarpus officinalis* (Fougnies et al., 2007), *Citrus junos* (Wu et al., 2013) and *Poncirus trifoliata* (Zou et al., 2014) to waterlogged conditions.

Lonkida (*Nauclea orientalis*) is a species of tree that has a wide ecological range. Lonkida is a tropical tree species from the family Rubiaceae (Raghavamma et al., 2010) and grows naturally in Indonesia in permanent swamps, temporal swamps, savanna, and dry land (Tuheteru et al., 2014). This species is a multipurpose tree species. This tree species has a medicinal function (Lim, 2013), such as anti-malaria (Sichaem et al., 2010). and bleeding drugs (Collins et al., 2007). The wood of this type can be used for various needs, such as flooring, furniture, moulding, veneer and plywood (Dayan et al., 2007; Van Sanh and Duy, 2009). This tree can grow to up to 35-50 m in height and 80-100 cm in diameter (Dayan et al., 2007). Lonkida forms a mutual symbiosis with AMF (Tuheteru et al., 2015). AMF increased plant growth, biomass and nitrogen accumulation in seedling roots under waterlogged conditions (Tuheteru et al., 2015). AMF also increased *N. orientalis* L. growth on gold tailing medium (Tuheteru et al., 2020) and serpentine soil (Tuheteru et al., 2017). However, the effect of local AMF on the growth of lonkida under drought and waterlogged conditions has not been reported. Therefore, the objective of this research was to assess the capacity of local AMF species *Acaulospora* sp. 1 and *C. etunicatum* to improve the growth of lonkida under drought and waterlogged stress.

Materials and Methods

Plant material and treatment

This research was conducted at the Mycorrhizal Association greenhouse and the Laboratory of the Faculty of Forestry and Environmental Sciences, Halu Oleo University, Kendari City, Southeast Sulawesi Province, Indonesia, from March to June 2019. The experimental design was a factorial completely randomized design consisting of two factors. The first factor was AMF inoculation (A) consisting of control (non-inoculated treatment), inoculated with *Acaulospora* sp.1, and inoculated with *C. etunicatum.* The second factor was environmental stresses (B), consisting of control (no-drought stress, watered daily), drought treatment with 25% field capacity, drought treatment with 50% field capacity, inundated as high as the polybag (9 cm high), and inundated over the polybag. Each treatment consisted of three replications, and each replication consisted of three seedling units.

Nauclea orientalis seeds were collected from mature trees around the campus area of Halu Oleo University. Brownish ripen fruits were collected from the parent trees. Seeds were extracted by crushing the fruits in a bucket filled with water. The seeds were separated using a strainer under running water. The seeds were germinated in a germination box size of 20 x 20 x 5 cm that has been perforated at the bottom to drain excess water, and filled with sterile sand media, for 40 days. The AMF inoculums used were *Acaulospora* sp.-1 and *C. etunicatum* culture results using zeolite medium and *Pueraria javanica* as host plant with the 5 g of inoculum. Before AMF inoculation, a polybag of8 cm width x 9 cm height was filled with sterile soil, sand, and rice husk charcoal with a proportion of 6:1:3 by weight media. Inoculation of AMF was conducted as the designed treatment. The inoculum was placed near the roots of the 40-day-old *N. orientalis* seedlings with a minimum height of 2 cm and 4 leaves. Non-inoculated seedlings were used as controls.

Determination of field capacity

The moisture content at field capacity was determined by watering the media excessively and then letting it stand for 24 hours. The moisture content of the media at field capacity was determined by taking and weighing samples with three replications. The samples were then dried in an oven at 60 °C for 24 hours and re-weighted. The moisture contents at 25 and 50% field capacity were calculated as follows:

$$\text{FC } 25\% = \frac{25}{100} \times (\text{FC} - (\text{FC} \times \text{MC}))$$

$$\text{FC } 50\% = \frac{50}{100} \times (\text{FC} - (\text{FC} \times \text{MC}))$$

where: FC = Moisture content at field capacity, MC = Moisture content

Seedling Maintenance

Seedling maintenance of *N.orientalis* was carried out by watering and controlling weeds. Watering was done to maintain 25% field capacity and 50% field capacity (by weight). Weeds control was done manually and inundated over the polybag.

Data collection

Plant height (cm) and diameter (mm) measurements, and leaves number counting were conducted at the end of the experiment (240 days after transplanting), seedlings were harvested and separated from the media. Fresh roots samples were also taken to observe AMF colonization. Shoot and root were divided, put in envelopes, oven-dried at 70°C for two times 24 hours, then allowed to stand for a while in the desiccator and then determined shoot, root, and total plant dry weights. Shoot, root, and total plant dry weights were used to calculate Sensitivity Index and Relative Growth. The Sensitivity Index of all plant variables measured was calculated according to Tuheteru et al. (2015) with the following formula:

SI = 1 - the magnitude of the ratio between water stress plants and plants without water stress.

where: SI = Sensitivity Index; SI is positive when the water stress treatment decreases the measured value compared to the control or negative when it is applied otherwise.

Relative growth of plants, average relative growth of plants for total plant dry weight (RGTt), shoots (RGTs) and roots (RGTr) under standing conditions and without water stress (control), was calculated using the formula of Garcia et al. (2008) as follows:

RGRi = (ln Wtf-ln Wto) / (tf-to)

where:

RGR	=	relative growth of plants
i	=	total dry weight, shoots and roots;
Wtf	=	total dry weight, shoots and roots at the end of the study period (240 days);
Wto	=	dry weight in normal periods (150 days)
(tf-to)	=	difference between normal conditions and total growth periods (90 days).

AM colonization was observed by a staining technique using a number of root samples, then the roots were washed thoroughly and immersed in the 10% KOH solution for 24 hours. The roots sample were washed in running water to clean and remove KOH. Then the roots were soaked in a 2% HCl solution for 30 minutes and HCl solution was removed. The root sample was then immersed in a staining solution (trypan blue 0.05% + glycerol 70% + distilled water 30%) for 24 hours. After that, the staining solution was removed, then the roots were put in a 50% glycerol solution and AMF colonization activities were measured. Calculation of AMF colonization was made using the method of infected root length (Brundrett et al., 1996). According to O'Connor et al. (2001), the percentage of root colonization was divided into three categories, i.e. 1-10% (low category), 10-30% (medium category), and >30% (high category).

Data analysis

Data from observation were subjected to analysis of variance (F test), followed by the Duncan Multiple Range Test (DMRT) at a 95% significant level.

Results and Discussion

AMF colonization

The results of the analysis of AMF colonization of *N. orientalis* roots are presented in Table 1. Data presented in Table 1 show that the highest AMF colonization was found in the interaction of *Acaulospora* sp.1 in drought treatment with 25% field capacity and inundated as high as the polybag compared to control and AMF type of *C. etunicatum.* The AMF inoculation in this study showed that AMF increased the growth of *N. orientalis* in all observed variables except the number of leaves and root-shoot ratio. The highest colonization of AMF in *N. orientalis* roots was observed in the treatment of *Acaulospora* sp. 1 with a drought condition of 25% field capacity and inundated as high as the polybag. AMF colonies were high in drought conditions because *N. orientalis* can grow well in unsuitable conditions. According to Smith and Read (2008), the percentage of root colonization is influenced by the type of AMF, pH, temperature, humidity, heavy metals, and nutrient content. In drought conditions, plants experience stress, so plants will come into contact with AMF because AMF can infect plant roots in dry conditions (Bahadur et al., 2019).

Growth of lonkida (N. orientalis)

The results of the analysis of the effect of AMF inoculation treatment and water stress on the growth of *N. orientalis* plants are presented in Table 2. Data presented in Table 2 show that the inoculation of *C.*

etunicatum significantly increased the height of *N. orientalis* compared with other treatments. The inoculation of *Acaulospora* sp. 1 increased height compared to controls. Treatments of environmental stress under inundation conditions were not significantly different but significantly different in drought conditions and control with an index of sensitivity negatively affected by the drought treatment with 25% field capacity and inversely proportional to other stress conditions.

Table 1. Effect of AMF inoculation interaction and environmental stress treatment on AMF colonization.

Treatments		AMF Colonization (%)
AMF Inoculation	**Environmental Stress**	
Control	Control (100% field capacity)	8.37 e
	Drought treatment with 25% field capacity	18.84 b
	Drought treatment with 50% field capacity	15.37 d
	Inundated as high as the polybag (9 cm high)	11.61 d
	Inundated s of over the polybag	15.31 c
Acaulospora sp. 1	Control (100% field capacity)	16.65 b
	Drought treatment with 25% field capacity	28.50 a
	Drought treatment with 50% field capacity	22.94 b
	Inundated as high as the polybag (9 cm high)	28.16 a
	Inundated over the polybag	21.21 b
Claroideoglomus etunicatum	Control (100% field capacity)	16.84 b
	Drought treatment with 25% field capacity	23.19 b
	Drought treatment with 50% field capacity	12.33 c
	Inundated as high as the polybag (9 cm high)	21.21 b
	Inundated over the polybag	29.44 a
Coefficient of variation		6.79%

Note: Numbers followed by the same letters in the same column show no significant difference according to the Duncan Multiple Range Test (α = 0.5%).

Table 2. Effect of AMF inoculation and environmental stress on the growth of *N. orientalis*.

Treatments	Height (cm)	Diameter (mm)	Leaf number
AMF Inoculum (A)			
Control	12.20 c	3.92 b	7.57
Acaulospora sp. 1	14.87 b	4.88 a	7.33
C. etunicatum	16.27 a	4.89 a	7.40
Environmental Stress (B)			
Control	15.21 b	4.45 b	7.67 ab
Drought treatment with 25% field capacity	12.31 c$^{(-)}$	4.33 b$^{(-)}$	6.44 b$^{(-)}$
Drought treatment with 50% field capacity	13.65 bc$^{(-)}$	4.25 b$^{(-)}$	7.11 b$^{(-)}$
Inundated as high as a polybag (9 cm high)	15.28 ab$^{(+)}$	4.91 a$^{(+)}$	7.06 b$^{(-)}$
Inundated over polybags	15.79 a$^{(+)}$	4.88 a$^{(+)}$	8.89 a$^{(+)}$
Coefficient of Variation	12.34%	8.93%	17.11%

Note: Numbers followed by the same letters in the same column show no significant difference according to the Duncan Multiple Range Test (α = 0.5%), Coefficient of Variation. The (+) sign indicates that the susceptibility index influences when inundation treatment decreases the measured value compared to the control, and vice versa (-)

Inoculation of *Acaulospora* sp.1 and *C. etunicatum* significantly increased the diameter of *N. orientalis*. The treatment of environmental stress under inundated conditions was significantly different from drought and control conditions. The sensitivity index on the diameter variable had a positive effect on inundated conditions as high as the polybags and was inversely proportional to other stress conditions. AMF inoculation did not affect the leaf number variables of the plant. In environmental stress treatments, the leaf number in the inundated over the polybag treatment and control were significantly different from that in other stress conditions. The sensitivity index on the variable number of leaves was positively affected by the dry conditions of drought treatment with 50% field capacity and inundated over the polybag, and inversely proportional to the dry conditions of drought treatment with 25% field capacity and inundated as high as the polybag. The inoculation of *C. etunicatum* significantly increased the height and relative growth of *N. orientalis* compared to *Acaulospora* sp.1. However, the inoculation of *Acaulospora* sp. 1 significantly increased the percentage of AMF colonization. The inoculation of *C. etunicatum* and *Acaulospora* sp. 1 was also effective in increasing plant diameter, plant dry weight and seed quality index. The increased growth of *N. orientalis* is thought to be the role of AMF in supplying water (Zhang et al., 2018) and increasing P nutrient uptake (Zhang et al., 2019). This study is in line with research (Wu et al., 2013; Tuheteru et al., 2015) that AMF can increase growth in inundated conditions. *C. etunicatum* is one type of AMF that has a wide distribution (Husna et al., 2015). The distribution of AMF is strongly influenced by environmental factors, such as soil type and texture, land damage, humidity and temperature and nutrient availability (Kivlin et al., 2011). According to Rillig et al. (2002), abiotic factors are important factors in determining the extent of AMF distribution.

Plant dry weight

The results of the analysis of the effect of AMF inoculation and environmental stress treatments on the dry weight of *N. orientalis* are presented in Table 3. Data presented in Table 3 show that inoculation of *Acaulospora* sp.1 and *C. etunicatum* significantly increased shoot dry weight, root dry weight, and total dry weight of *N. orientalis*. The environmental stress treatment did not have significant different effects on shoot dry weight and total dry weight of the plant. However, in the root dry weight variable, the stagnant condition was not significantly different in the drought conditions and significantly different in the control condition. Data of shoot, root, and total dry weight of the plant showed that in drought treatment with 50% field capacity, the sensitivity index had a negative effect and was inversely proportional to other environmental stress conditions.

The increased dry weight of the plant is thought to be influenced by high nutrient uptake by the plant inoculated with AMF (Symanczik et al., 2018; Zhang et al., 2019). AMF inoculation and environmental stress increased the dry weight of *N. orientalis* roots. This is because of the direct role of AMF that helps roots in increasing water absorption from the soil into the roots (Zhang et al., 2019). *N. orientalis* inoculated with AMF also had seed quality index values greater than 0.09. According to (Duryea and Dougherty, 1991), plants can grow well with good seed quality if they have a seedling quality index value greater than 0.09. The results of this study are in line with the research of Husna et al. (2016) that AMF is able to increase the seed quality index in *Pericopsis mooniana* seedlings.

Table 3. Effect of AMF inoculation treatment and environmental stress on dry weight of *N. orientalis*.

Treatments	**Dry Weight of the Plant (g)**		
	Shoots	**Root**	**Total**
AMF Inoculum (A)			
Control	1.09 b	0.37 b	1.46 b
Acaulospora sp. 1	1.72 a	0.49 a	2.21 a
C. etunicatum	1.79 a	0.57 a	2.36 a
Environmental Stress (B)			
Control	1.49	0.42 bc	1.91
Drought treatment with 25% field capacity	1.59 (+)	0.43 bc(+)	2.02 (+)
Drought treatment with 50% field capacity	1.31 (-)	0.40 c(-)	1.71 (-)
Inundated as high as the polybag (9 cm high)	1.65 (+)	0.60 a(+)	2.25 (+)
Inundated over the polybag	1.64 (+)	0.53 ab(+)	2.17 (+)
Coefficient of Variation	34.65%	22.83%	28.95%

Note: Numbers followed by the same letters in the same column show no significant difference according to the Duncan Multiple Range Test (α = 0.5%), Coefficient of Variation. The (+) sign indicates that the susceptibility index influences when inundation treatment decreases the measured value compared to the control, and vice versa (-)

Relative growth of the plant

The results of the analysis of the effect of AMF inoculation treatment and environmental stress on the dry weight of *N. orientalis* plants are presented in Table 4. The inoculation of *C. etunicatum* significantly increased the relative growth of *N. orientalis* compared to *Acaulospora* sp.1 inoculation. The inoculation of *Acaulospora* sp.1 significantly increased the relative growth of the plant compared to control. The environmental stress treatment was not significantly different in the shoot and total plant relative growth. However, the relative growth variables of the roots under flooded conditions were not significantly different but were significantly different in drought conditions and control treatments. The effect of environmental stress treatments on the shoot, root, and total dry weight variables of the plant showed that in the dry conditions of drought treatment with 50% field capacity, the susceptibility index had a negative effect and was inversely proportional to other environmental stress conditions. The results showed that the environmental stress reduced shoot dry weight and total dry weight of *N. orientalis*. This is presumably because *N. orientalis* is very sensitive to water deficits. The results of the study are in line with Pebriansyah (2012) research that AMF can increase plant dry weight, but environmental stress treatment can increase root dry weight and reduce shoot dry weight on tropical grass. In addition, the low RGRs in environmental stress is thought to be a mechanism for protecting plants from dehydration (transpiration) (Lambers and Oliveira, 2019). So that what is possible for dry habitat seedlings is to translocate C more to root organs than shoots (Martínez-Alcántara et al., 2012).

Table 4. Effect of AMF inoculation treatment and environmental stress on the relative growth of *N. orientalis* plants.

Treatments	RGRs	RGRr	RGRt
AMF Inoculum (A)			
Control	0.010027 c	0.006580 c	0.009407 c
Acaulospora sp. 1	0.017687 b	0.012540 b	0.016667 b
C. etunicatum	0.023367 a	0.015340 a	0.021247 a
Environmental Stress (B)			
Control	0.0165	0.0097 bc	0.015011
Drought treatment with 25% field capacity	0.0183 (+)	0.0101 bc(+)	0.016367 (+)
Drought treatment with 50% field capacity	0.0152 (-)	0.0089 c(-)	0.013611 (-)
Inundated as high as the polybag (9 cm high)	0.0175 (+)	0.0154 a(+)	0.017278 (+)
Inundated over the polybag	0.0176 (+)	0.0132 ab(+)	0.016600 (+)
Coefficient of Variation	34.07%	30.75%	30.05%

Note: Numbers followed by the same letters in the same column show no significant difference according to the Duncan Multiple Range Test (α = 0.5%), Coefficient of Variation. The (+) sign indicates that the susceptibility index influences when inundation treatment decreases the measured value compared to the control, and vice versa (-)

Results of this study also showed that the drought treatment with 50% field capacity had a negative impact on plant dry weight and relative growth of *N. orientalis* compared to other treatments. This is presumably because *N. orientalis* could not adapt to drought conditions with 50% field capacity. Conversely, *N. orientalis* was able to grow at drought treatment with 25% field capacity, inundated as high as polybags and inundated over polybags. This is because the original habitat of *N. orientalis* in permanent swamps, temporal swamps, savanna, and dry land (Tuheteru et al., 2014) so that tolerance to inundation. Tolerance to inundation has a form of adaptation of *N. orientalis* in the form of wild roots, lenticels and aerenchyma. This is in line with the research of Tuheteru et al. (2015), which shows that AMF shows the response of *N. orientalis* growth under inundation conditions. Results of this study also showed that environmental stresses in puddle conditions affected plant growth. Whereas drought stress did not affect the growth of *N. orientalis*, because *N. orientalis* is tolerant to inundation. Therefore, *N. orientalis* can survive in conditions of low water availability.

Conclusion

The interaction of AMF type of *Acaulospora* sp. 1 inoculation and environmental stress significantly increased AMF colonization in *N. orientalis*. The inoculation of AMF type of *C. etunicatum* significantly increased the growth of *N. orientalis*. Inoculation of AMF type of *Acaulospora* sp. 1 and *C. etunicatum* are effective in increasing growth in diameter, plant dry weight, and seed quality index. Environmental stresses in inundated conditions effectively increased plant growth. However, there was a tendency for daily watering to increase the growth of the number of leaves of the plant.

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